Advances in Shrimp Biotechnology

Proceedings to the
Special Session on Shrimp Biotechnology
5th Asian Fisheries Forum
Chiengmai, Thailand
11-14 November 1998

Edited by

T.W. Flegel

Department of Biotechnology
Faculty of Science, Mahidol University
Rama 6 Road, Bangkok 10400, Thailand

BIOTEC
The National Center for
Genetic Engineering and Biotechnology
Thailand

Advances in Shrimp Biotechnology
Proceedings to the special session on shrimp biotechnology
5th Asian Fisheries Forum, 11-14 November 1998
Chiengmai, Thailand

ISBN 974-7578-02-6

National Center for Genetic Engineering and Biotechnology
National Science and Technology Development Agency
National Science and Technology Agency (NSTDA) Building
73/1 Rama 6 Road, Bangkok 10400, Thailand

Tel. (66-2) 644-8150 to -8154
Fax. (66-2) 644-8107
www.biotec.or.th

Produced by Multimedia Asia Co. Ltd.
979/37-41 Phaholyothin Rd., 18/F SM Tower
Bangkok 10400, Thailand

Tel. (66-2) 298-0646 to -0651
Fax.(66-2) 298-0679
e-mail: john@Multimedia-Asia.com

Message from the Editor

This is an exciting time for the study of biotechnology related to shrimp rearing. Up to now, basic knowledge regarding the major cultivated shrimp species has lagged far behind the technical innovations that have led to successful intensification of culture and to ever increasing world production. However, rearing problems and sometimes catastrophic farm losses have forced the realization that the lack of basic knowledge must be redressed if the causes of lost production are to be determined and rectified so that high levels of production can be maintained. Furthermore, since shrimp farming is a potentially self-polluting industry, it is well understood that sustaining high production levels will also require further innovation to minimize its adverse environmental impacts. Biotechnology will play a central role in helping us to understand the shrimp and to improve all aspects of rearing practice. The contributions in this volume show that an international effort in the field is already yielding beneficial returns to the industry. The papers cover broad topics from environmental issues, to shrimp domestication and larval rearing. Much of what is reported is new and in the early stages of development and I am certain that many readers will be excited by the results and their implications. The National Center for Genetic Engineering and Biotechnology (BIOTEC), an implementing arm of the National Science and Technology Development Agency (NSTDA) of Thailand, was pleased to accept the invitation of the Asian Fisheries Society to organize this special session on shrimp biotechnology at the 5th Asian Fisheries Forum. Given the importance of shrimp exports to the Thai economy, BIOTEC has had an active program to support and stimulate research on shrimp, and this 5th AFF activity was considered an appropriate extension of that program. I am grateful to everyone for their enthusiastic support for the session and to the many colleagues who prepared presentations for it. I was especially pleased that many were able to prepare full papers in advance of the meeting for publication in this volume. I would like to thank Dr. M. Shariff, President of the Asian Fisheries Society, Dr. Piamsak Menasveta, the Chairman of the local organizing committee and the other members of the local organizing committee for their help in organizing the special shrimp session. I would also like to thank the staff of BIOTEC and especially Khun Roongthip Rojjananavin whose assistance was so vitally important in making all the arrangements and handling all of the correspondence related to the session and to this publication. I am grateful to the staff at Multimedia Asia Co. Ltd. who introduced me to the intricacies of electronic publishing and who were so patient and helpful in getting all the necessary work done to have the CD ROM ready in time for the meeting. Finally, I would like to thank Mr. Ian H. MacRae for his vital assistance in overcoming the inevitable unexpected problems that were encountered in generating the ultimate portable document format (PDF) file for the CD ROM.

T.W. Flegel
Director, Shrimp Biotechnology Program
BIOTEC

Contents

Overview

Sustainable Shrimp Culture Development: Biotechnological Issues and Challenges 13
Rohana P. Subasinghe, Devin M. Bartley, Sharon McGladdery, Uwe Barg

Alternatives in Shrimp Biowaste Processing 19
Willem F. Stevens, Preiradda Cheypratub, Sjeng Haiqing, Pranee Lertsutthiwong, Ng Chuen How, Suwalee Chandrkrachang

Maturation and Genetics

Hormonal Control of Vitellogenesis in Penaeid Shrimp 29
I. Yano

Effect of Methyltestosterone and 17α-hydroxyprogesterone on Spermatogenesis in the Black Tiger Shrimp, *Penaeus monodon* Fab. 33
R. Yashiro, P. Na-anant, V. Dumchum

The Effects of Cryoprotectants, Chilling and Freezing on *Penaeus esculentus* Embryos and Nauplii 37
N.P. Preston, F.E. Coman

Advances in Gene Mapping in Penaeid Shrimp 45
S.S. Moore, S.A. Lehnert

Development of Simple Sequence Length Polymorphisms in the Black Tiger Shrimp (*Penaeus monodon*) 49
A. Vanavichit, S. Wuthimaethavee, D. Taechayinkphibool, P. S. Kruthoo, P. Lumubol, S. Tragoonrung

Genetic Variation, Population Differentiation and Gene Flow of the Giant Tiger Shrimp (*Penaeus monodon*) Inferred from mtDNA-RFLP Data 51
Sirawut Klinbunga, David J. Penman, Brendan J. McAndrew, Anchalee Tassanakajon, Padermsak Jarayabhand

Quantitative Genetics and Genetic Transformation for the Selection of Pathogen-Resistant Shrimp 61
Virna Cedeño, Evelyne Bachère, Jenny Rodriguez, Edouard Bédier, Emmanuel Goyard, Viviane Boulo, Jean-Paul Cadoret, Eric Mialhe

Estimated Heritabilities for Early Growth Rate of the Black Tiger Prawn, *Penaeus monodon*, Fabricius 67
P. Jarayabhand, S. Uraiwan, S. Klinbunga, A. Tassanakajon, P. Srimukda, P. Pattanachan, R. Panakulchaiwit, P. Menasveta

Triploidy induction in chinese shrimp (*Penaeus chinensis*) with special reference to a new chemical inducer 6-DMAP 71
Jianhai Xiang, Linghua Zhou, Fuhua Li, Changgong Wu

Domestication and Selective Breeding of *Penaeus monodon* in Thailand 73
Boonsirm Withyachumnarnkul, Vichai Boonsaeng, T.W. Flegel, Sakol Panyim, Chainarong Wongteerasupaya

Shrimp Rearing

Study on the Impact of Intensive Marine Shrimp Farm Effluent on Sediment Quality in Kung Krabaen Bay, Eastern Thailand **81**
Siri Tookwinas, Chanin Sangrungruang, Osamu Matsuda

Some Recent Innovations in Marine Shrimp Pond Recycling Systems **87**
Arlo W. Fast, Piamsak Menasveta

Preservation of Water Quality in Shrimp Ponds by Ozone **93**
M. Matsumura, V.P. Migo, D. Balobalo, H.K. Young, J.D. Albaladejo

Use of Probiotics for Improving Soil and Water Quality in Aquaculture Ponds **101**
Claude E. Boyd, Amit Gross

Application of Phytoplankters as Live Diet and Biocontrol in Improving the Hatchery Production of Commercially Important Penaeid Shrimp in Asia **107**
Jesse Dapon Ronquillo, Toshio Saisho, Shigehisa Yamasaki

Manipulation of Bacterial Populations in Shrimp Larval Cultures Fed Artificial Diets ... **109**
N. Misciattelli, D.A. Jones, N. Simoes, J.W. Latchford, P. Bridson

Effects of Astaxanthin on Larval Growth and Survival of the Giant Tiger Prawn, *Penaeus monodon* **117**
Jintana Darachai, Somkiat Piyatiratitivorakul, Prasat Kittakoop, Charoen Nitithamyong, Piamsak Menasveta

Low Salinity Culture of *Penaeus monodon* Fabricius and Its Effect on the Environment **123**
Yont Musig, Sathit Boonnom

A Closed Recycle System for Sustainable Black Tiger Shrimp Culture in Freshwater Areas **125**
Chalor Limsuwan, Pornlerd Chanratchakool

The Shrimp Defence System

Shrimp Immunity and Disease Control: An Integrated Approach **129**
Evelyne Bachère

Review of Crustacean Immunity **135**
Kenneth Söderhäll

Evidence for the haemocytic origin of lymphoidal spheroids in *Penaeus monodon* **137**
Margaretha Anggraeni, Leigh Owens

Immuno-physiology of the Black Tiger Shrimp (*Penaeus monodon*) **139**
Kidchakan Supamattaya

Studies on IgY for Passive Immunization of Shrimp Against White Spot Syndrome Virus **141**
Victoria Alday-Sanz, Sarayut Thaikua, A.N. Yousif, L.J. Albright, T.W. Flegel

Effect of Oxygen Depletion on Some Parameters of the Immune System in Black Tiger Shrimp (*Penaeus monodon*) **147**
Sataporn Direkbusarakom, Yaowanit Danayadol

Molecular Cloning and Characterization of Prophenoloxidase in the Black Tiger Shrimp, *Penaeus monodon* 151
Kallaya Sritunyalucksana, Lage Cerenius, Kenneth Söderhäll

Comparison of Shrimp High Density Lipoprotein and Beta Glucan Binding Protein 153
Gloria Yepiz-Plascencia, Francisco Vargas-Albores, Flor Jimenez-Vega, Lydia M. Ruiz-Verdugo, Gabriela Romo-Figueroa

Probiotics and Immunostimulants

Activation of shrimp cellular defence functions by microbial products 161
Francisco Vargas-Albores, Jorge Hernández-López, Teresa Gollas-Galván, Karla Montaño-Pérez, Flor Jiménez-Vega, Gloria Yepiz-Plascencia

Immunostimulation of Shrimp Through Oral Administration of *Vibrio* Bacterin and Yeast Glucan 167
T.N. Devaraja, S.K. Otta, G. Shubha, Indrani Karunasagar, P. Tauro, Iddya Karunasagar

Efficacy of Oral Administration of Fucoidan, a Sulfated Polysaccharide, in Controlling White Spot Syndrome in Kuruma Shrimp in Japan 171
Yukinori Takahashi, Kaori Uehara, Rikio Watanabe, Takekazu Okumura, Tetsuro Yamashita, Hiroshi Omura, Toshikatsu Yomo, Toshiro Kawano, Akio Kanemitsu, Hideo Narasaka, Nobutaka Suzuki, Toshiaki Itami1

Selection of Probiotic Bacteria for Use in Aquaculture 175
Bruno Gomez-Gil, Ana Roque

Probiotics in Aquaculture: A Case Study of Probiotics for Larvae of the Black Tiger Shrimp (*Penaeus monodon*) 177
Sirirat Rengpipat, Sombat Rukpratanporn, Somkiat Piyatiratitivorakul, Piamsak Menasveta

Use of By-9 as a Probiotic Agent in the Larval Rearing of *Penaeus monodon* 183
Ketut Sugama Haryanti, S. Tsumura

Will Microbial Manipulation Sustain the Ecological Balance in Shrimp (*Penaeus monodon*) Hatcheries? 185
C.R. Lavilla-Pitogo, L.J. Albright, M.G. Paner

Influence of LPS-Injection on Morphological, Antigenic and Functional Aspects of Hemocytes from *Penaeus monodon* 193
C.B.T. van de Braak, W.P.W. van der Knaap

Evaluation of a Booster Diet for the Nursery of Penaeid Shrimp 195
Greet Merchie, Marleen Dehasque, Piet Verstraete, Pete Bridson, David Jones

Bacterial Diseases and Toxins

Standardisation of Three Techniques for Experimental *Vibrio* Infections in the Marine Shrimp *Penaeus vannamei* 199
Ana Roque, Bruno Gomez-Gil, Ana Luisa Guerra Flores

Luminous Bacteria Associated with Shrimp Mortality **205**
Lila Ruangpan

Luminous *Vibrio harveyi* Associated with Tea Brown Gill Syndrome in Black Tiger Shrimp **213**
Tirasak Pasharawipas, Siriporn Sriurairatana, Sataporn Direkbusarakom, Yaowanit Donayadol, Surayut Thaikua, Lila Ruangpan, T.W. Flegel

Genetic Diversity of Luminous *Vibrio* Isolated from Shrimp Larvae **217**
Antonius Suwanto, Munti Yuhana, Emi Herawaty, Sri Lestari Angka

Vibrionaceae Associated with the Larvae and Larval Rearing System of *Macrobrachium rosenbergii* : Systematics and Pathogenicity **225**
Sarita G. Bhat, I.S. Bright Singh

Detection of *Vibrio parahaemolyticus* in Shrimp Haemolymph by DNA Hybridization and PCR Amplification **227**
Prasert Rojlorsakul, Vichai Boonsaeng, Watanalai Panbangred, Orasa Suthienkul, Tirasak Pasharawipas, T.W. Flegel

Successful use of Bacterial Bioremediation to Control *Vibrio* Populations in Shrimp Ponds **235**
Jason J. David, C.T. Chu, S. Santhana Krishnan

Ultrastructure of a Mollicute Associated, Gut-node Disease of Penaeid Shrimp (*Penaeus chinensis*) **237**
Yang Jifang

Toxiciological Studies with Juveniles of the Marine Shrimp *Penaeus vannamei* Using a Flow-Through System **241**
Miguel Betancourt-Lozano, Donald J Baird, Luz Maria Garcia-de al Parra, Ferdenando Gonzalez-Farias, Sylvia Morales-Gonzalez

Viral Diseases

Active Viral Accommodation: A New Concept for Crustacean Response to Viral Pathogens **245**
T.W. Flegel, T. Pasharawipas

Screening for Shrimp Viruses in the Philippines **251**
J.D. Albaladejo, L.M. Tapay, V.P. Migo, C.G. Alfafara, J.R. Somga, S.L. Mayo, R.C. Miranda, K. Natividad, F.O. Magbanua, T.Itami, M. Matsumura, E.C.B. Nadala, Jr., P.C. Loh

Recent Developments in Immunologically-Based and Cell Culture Protocols for the Specific Detection of Shrimp Viral Pathogens **255**
Philip C. Loh, E. Cesar, B. Nadala Jr., Lourdes M. Tapay, Yuanan Lu

Genome Organization and Detection of Hepatopancreatic Parvovirus (HPV) from *Penaeus monodon* in Thailand **261**
Wasana Sukhumsirichart, Chainarong Wongteerasupaya, Vichai Boonsaeng, Sakol Panyim, Siriporn Sriurairatana, Boonsirm Withyachumnarnkul, T.W. Flegel

The Emergence of Yellow Head-Related Viruses in Australia **263**
Peter J. Walker, Jeff A. Cowley, Kirsten M. Spann, Christine M. Dimmock

Multiplex PCR for Detection of Yellow-Head Virus and White Spot Syndrome Virus in *Penaeus monodon* 265
Chainarong Wongteerasupaya, Vichai Boonsaeng, Wansika Tongchuea, Nusra Sitidilokratana, Panan Kanchanaphum, Ratchanee Klinputsorn, Sakol Panyim

Tissue distribution of white spot syndrome virus (WSSV) in shrimp and crabs 267
Guang-Hsiung Kou, Shao-En Peng, Ya-Lin Chiu, Chu-Fang Lo

Primary Shrimp Cell Culture: Applications for Studying White Spot Syndrome Virus (WSSV) 273
J. Kasornchandra, S. Boonyaratpalin

White Spot Virus Infective Properties Determined by a Single Tube Nested and Competitive PCR 277
Lee Kok Leong, Samson Soon, Tan Lee Tung, Mohd. Shariff Mohd. Din

One-step Nested PCR for Grading the Severity of White Spot Syndrome Virus Infections in *Penaeus monodon* 279
Wansika Tongchuea, Vichai Boonsaeng, Chainarong Wongteerasupaya, Karnyupha Jittivadhna, Anchalee Tassanakajon, Sakol Panyim

PCR Monitoring of Cultured Shrimp for White Spot Syndrome Virus (WSSV) Infection in Growout Ponds 281
Chu-Fang Lo, Yun-Shiang Chang, Chin-Te Cheng, Guang-Hsiung Kou

Application of PCR and Formalin Treatment to Prevent White Spot Disease in Shrimp 287
Pornlerd Chanratchakool, Chalor Limsuwan

Possible Prevention of White Spot Syndrome (WSS) in Kuruma Shrimp*, Penaeus japonicus*, in Japan 291
Toshiaki Itami, Minoru Maeda, Nobutaka Suzuki, Kazuo Tokushige, Atsushi Nakagawa, Oscar Hennig, Masakazu Kondo, Jiraporn Kasornchandra, Ikuo Hirono, Takashi Aoki, Riichi Kusuda, Yukinori Takahashi

Overview

Sustainable Shrimp Culture Development: Biotechnological Issues and Challenges

[1]Rohana P. Subasinghe, [1]Devin M. Bartley, [2]Sharon McGladdery, [1]Uwe Barg

[1]Fisheries Department, Food and Agriculture Organization of the United Nations (FAO)
Viale delle Terme di Caracalla, 00100 Rome, Italy
[2]Department of Fisheries and Oceans, Canada, Gulf Fisheries Centre
P.O. Box 5030, Moncton, N.B., E1C 9B6 Canada

ABSTRACT: The shrimp farming sector has been growing at a compounded annual rate of about 16% over the past decade, with much originating from low-income, food deficit, countries (LIFDCs). Continued development of the shrimp aquaculture sector, however, is constrained by i) recurrent disease epizootics with limited prevention and control measures, ii) a lack of consistent broodstock and post-larvae quality iii) inconsistent quality and limited choices of feed and iv) inadequate water control of water quality. Biotechnological advances are now opening new opportunities for ameliorating these developmental constraints. Improved sensitivity and accuracy of disease diagnosis is being developed using molecular detection techniques such as immunoassays, PCR gene amplification and molecular probes. Such molecular techniques may also play a role in the eventual development of cell-lines for aquatic invertebrates. Definition of general health parameters for shrimp are also being investigated using haematograms, enzyme analyses and quantification methods for serum proteins and non-protein defense molecules. Controlled breeding programs and genetic markers are being co-developed to enhance selection of genetic lines which are free of specific pathogens (specific pathogen free - SPF) or resistant to the disease(s) they cause (specific pathogen resistant - SPR). Advances in understanding of shrimp immunity are also assisting development of immunostimulants and vaccines. Broodstock and post-larval quality are being improved through application of hormonal control of reproduction and development of genetic tags to identify and produce pedigrees with optimal health and productivity. Feed enhancement using microencapsulation of nutrient supplements and probiotics may also play a role in enhancing quality of post-larvae, as well as reducing the need for antibiotic intervention. Bioremediation, recirculation and biofiltration technology all show promise for improving water quality control. The present paper attempts to outline the recent advamces in these fields and the potential contribution of modern biotechnology to the sustainable development of shrimp aquaculture.

KEY WORDS: Biotechnology, bioremediation, bioencapsulation, probiotics, immunostimulants, vaccines, gene probes, PCR, microsatellite markers, SFP, SPR, shrimp, aquaculture, disease diagnosis, disease control, broodstock, larvae, quality and control.

INTRODUCTION

At present, aquaculture is the world's fastest growing food-production sector, providing an acceptable, protein rich supplement to, and substitute for, wild aquatic animals and plants. Over the last decade, aquatic production from capture fisheries and aquaculture has increased steadily, reaching 120.7 million mt in 1995, an increase of around 15.6 million mt since 1989. Much of this increase is attributable to aquaculture. The proportion of total aquatic production attributable to aquaculture (including plants), increased from 14.4% in 1989 to 23.0% in 1995. By 1995, the total production of cultured finfish, shellfish and aquatic plants reached a record 27.8 million mt, valued at US$ 42.3 thousand million. Much of this increase originated from low-income food deficit countries (LIFDCs), in particular China, and reflects the continuing trend in these countries for increased use of aquatic resources to increase food production (FAO 1997).

The cultured shrimp and prawn subsector grew at an annual percent rate (APR) of 16.8 between 1984 and 1995. This increase was principally due to culture of penaeid shrimp species, which in 1995 accounted for 96.3% of all cultured shrimp and prawns. Penaeid production, notably of giant tiger prawn (*Penaeus monodon*) and other *Penaeus* species, increased from 31% or 54,000 mt and 12% or 21,000 mt respectively, in 1984, to 54% or 503,000 mt and 18% or 165,000 mt in 1995. However, production of the fleshy prawn (*Penaeus chinensis*), 99.5% of which was produced in China in 1995, has decreased substantially in terms of tonnage since 1993. A decrease in the expansion rate of shrimp farming during 1990-1995 was also evident at a global level for each of the top five penaeid species and species groups. This decrease in expansion and production tonnage has been attributed to environmental degradation, farm mismanagement and losses due to disease, making disease and environmental health the critical constraints to continued development of

Subasinghe RP, Bartley DM, McGladdery S, Barg U (1998) Sustainable shrimp culture development: biotechnological issues and challenges. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

sustainable shrimp aquaculture (FAO 1997). In addition, inadequate domestication (i.e., poor closed-recirculation system performance of broodstock with the resultant inadequate supply of quality post-larvae), limited choice of efficient and economical live feed, and inadequate water quality management have been identified as further significant constraints to the development of shrimp culture. There is, therefore, considerable scope for application of a wide range of the newly developing biotechnologies in an effort to ameliorate these problems.

APPLICATIONS TO SHRIMP HEALTH AND DISEASE MANAGEMENT

Infectious disease is considered to be the single most devastating problem in shrimp culture. Conventional methods for controlling aquatic animal pathogens, such as chemotherapy, appear less effective in managing newly emerging pathogens. Thus, molecular biotechnology has an increasingly important role for application in screening and detection of pathogens, elucidation of pathogenicity, development of effective control and preventive measures, and treatment of diseases. The improvement of diagnostic methodologies for detection and identification of pathogens using immunological and/or nucleic acid probes for prevention, management, and control of disease in cultured shrimp is one of the most important applications of biotechnology. Unlike traditional chemotherapeutic methods which have been plagued by development of pathogen resistance, these new technologies provide an opportunity for prophylactic intervention to minimise disease outbreaks.

Enhanced pathogen detection sensitivity

Early, rapid and accurate diagnostics

A very promising and immediate use of molecular genetic technology in shrimp aquaculture is its application to disease diagnosis and screening. Ultra-sensitive DNA probes can detect the presence of minute quantities of DNA, amplified by PCR, from pathogens such as viruses, fungi and bacteria, before clinical symptoms of infection become evident. Commercially available molecular probes have already been developed for IHHNV (Durand *et. al*. 1996), and probes for other viral pathogens, such as white spot syndrome virus (WSSV), MBV, TSV, HPV, YHV are in the process of being developed. PCR amplification of DNA sequences specific to the WSSV (DNA "fingerprints") is being utilised with related DNA probes for early diagnosis of white spot disease (Wongteerasupaya *et. al*. 1996). DNA probes and PCR are extremely sensitive diagnostic tools that can detect cryptic infections before they produce symptoms. In addition, these probes label DNA sequences which are highly sensitive to individual pathogen species. This greatly enhances the accuracy of diagnostic identifications and differentiation between opportunistic (usually managed by improved husbandry) and significant (contagious organisms which cannot be controlled easily through husbandry adjustments) infectious agents. Further development of biomolecular tools for screening and rapid detection of shrimp pathogens undoubtedly will also reduce the current need for, and use of, antibiotics and antibacterials in shrimp aquaculture.

Diagnosis and research on intracellular pathogens

Since the majority of severe pathogens of shrimp are viruses, the lack of self-replicating cell-line cultures for their i*n vitro* detection and isolation is a significant disease diagnostic constraint. Considerable research has gone into the development and maintenance of crustacean cell-lines but, to date, success has been marginal. Some researchers have managed to develop primary cell cultures, but most of these have failed to achieve cell-division for subculture and continuity of consistent cell characteristics (Le Groumellec *et. al*. 1995). Thus, the need for viable crustacean (and other aquatic invertebrate) cell-lines continues to be a significant challenge for disease research and management. Interestingly, molecular biotechnology may also assist this area of research. Molecular intervention, at the genetic level, has the potential to promote independent cell-division, overcoming the current cell-line development barrier of limited longevity. Should this occur, it would have significant positive consequences for economic application to shrimp aquaculture and disease control. Furthermore, cell-line based diagnostics have a well-established history and these techniques fall within the current capability of many aquatic pathology laboratories.

Disease risk assessment for transboundary transfers of live shrimp

Transboundary movement of shrimp broodstock and post-larvae has been perceived as one of the main reasons for the spread of epizootic viral diseases of shrimp. Development of reliable and sensitive diagnostic tools which can be used to screen shrimp for known, significant, pathogens, prior to movement from one zone or country to another, will greatly reduce the risk of inadvertent pathogen introduction to susceptible populations. This is of particular importance, bearing in mind the difficulty in detecting sub-clinical ("healthy") carriers with low levels of significant pathogens using some routine diagnostics. Increased diagnostic sensitivity will, in turn, promote greater confidence in the shrimp culture industry, which has been plagued by the shadow of viral proliferation and spread. It will also facilitate access to a wider international market.

Human pathogen detection

The ultimate goal of all shrimp culture is optimal marketing of the final product. Growing concerns about human health aspects of aquatic animal products, as well as the emerging number of international trade and quality agreements and understandings, mean that improved diagnostic technologies (i.e., DNA probes and PCR) to identify human pathogens in aquaculture products is imperative. The same technologies now being applied to shrimp pathogens, are equally applicable to human gastro-intestinal pathogens, and more research and development in this area is clearly needed to enhance consumer confidence and meet tightening international trade requirements.

Assessment of general health status

Besides screening for pathogens, one of the most urgent needs for health management in shrimp aquaculture is to establish standard quantitative methods for accurate assessment of shrimp health. Biotechnological methods used for ascertaining the health status of many aquatic organisms include, haematocrit, leucocrit, blood cell differential counts,

neutrophil counts, oxidative radical production, myeloperoxidase activity, phagocytic functions, etc.. Plasma samples also show total protein, immunoglobulin, lysozyme, cortisol and ceruloplasmin levels. Methods such as agglutination of precipitin gel tests used to assay antibody after immunisation can now be supplemented with FAT and ELISA immunoassay techniques. Also samples of blood, or immunopoietic organs, can be taken to determine which cells are producing antibody (plaque-forming cells), e.g., by the haemolytic plaque assay or by an enzyme labelled tag (ELISPOT). The latter is quantifiable and can show numbers of immunoglobulin or non-specific antibody-secreting cells (Anderson 1995). Monoclonal antibodies have also been prepared and are routinely used in immunodiagnosis, e.g., immunofluorescence detection. Such diagnostic tools are relatively new to aquaculture, but have enormous potential (Austin 1998). Although these may not all be applicable to shrimp culture, further research is clearly needed to ascertain their potential uses, especially in shrimp health management.

Such techniques could also found the basis for development of simple and rapid diagnostic tests for use under field conditions by field health technicians or farmers themselves. There is also ample scope for developing methods to assess the health status of shrimp using haemolymph parameters, such as cell counts, cell activities, enzyme levels, etc. Such tests could be used as early warning indicators of impending disease outbreaks and, thus, enhance the early application of preventative measures (Flegel 1996).

Immunostimulants and vaccines

Harnessing the hosts' specific and non-specific defence mechanisms for controlling diseases has considerable potential for health management in shrimp aquaculture. This will help reduce stress from handling (grading, manipulating stocking densities, removing mortalities, etc.) and environmental manipulation (application of chemical treatments, pond drainage, etc.) in order to control disease expression under intensive culture conditions. Important biotechnological interventions are being developed in the field of immunostimulants and modulators in an effort to reduce shrimp susceptibility to disease. Immunostimulants and non-specific immune-enhancers are being incorporated into diets (see Feed Quality) to provide added protection to the animals, even though our knowledge of shrimp immunity is limited at present, The large number of commercial "immunostimulants" available on the market reflects the interest of the industry in broadening the scope of tools available to manage shrimp diseases However, the effectiveness of many of these products has yet to be established. Preliminary results from biological trials appear highly variable. Further research and field trials are clearly essential to determine the precise mechanisms of the action of these products and to evaluate their efficacy in commercial shrimp production (Flegel 1996, Subasinghe *et. al.* 1998).

Specific pathogen free (SPF) and specifc pathogen resistant (SPR) shrimp

Production of specific pathogen free (SPF) animals and the development of specific pathogen resistant (SPR) strains are two complementary approaches which are currently possible using broodstock management programmes (see Broodstock Development). SPF animals are produced by selecting animals free of known and detectable pathogens and raising them under controlled and strict sanitary conditions. The SPR animals are developed through selective breeding of animals known to be less susceptible to specific pathogens. These concepts are now being used in countries like the USA, Venezuela and French Polynesia with *P. vannamei* and *P. stylirostris* (Bedier 1998). The main benefit of this concept is production of high health (HH) post-larvae, free of, or resistant to, known pathogens. It should be noted, however, that many SPF stocks which have not been exposed to pathogens (specific or general), perform poorly when pathogens are present (Browdy 1998). As with most organisms, a lack of exposure to infectious organisms increases clinical susceptibility. If the immune or physiological traits are heritable, this translates into performance improvement at the farm level. The selection of lines with reinforced non-specific defence or enhanced tolerance of external infection challenges, appears to be a promising option (Bedier 1998). Considering the major contribution of *P. monodon* to the global shrimp production and the economic losses encountered due to disease outbreaks, it is appropriate and timely to concentrate further research on the development of SPF and SPR broodstock of this species. Research in this area, through collaboration between scientists and producers from different regions, should, therefore, be given high priority.

BROODSTOCK DEVELOPMENT

Hormonal control of reproduction

The successful expansion and sustainable development of shrimp culture depends largely on the consistent availability of quality broodstock and post-larvae. Endocrine regulation of reproduction has been effectively applied in fish culture but is not yet at a practical stage of use in shrimp culture. Recent research has shown that inhibition of shrimp gonad inhibiting neurohormone (GIH) by chemical treatment can promote reproduction without the negative side effects of eye stalk ablation. Shrimp GIH isolation techniques are still under development but, by elucidating the structure and function of GIH, it should be possible to devise strategies to counter the inhibitory effects of GIH. This will significantly enhance consistent hatchery production of quality shrimp post-larvae. Further research in this direction is needed and collaboration between researchers, shrimp aquaculturists and resource providers from different regions is essential in order to achieve this.

Genetic improvement, selective breeding and engineering techniques

The application of genetic principles to increase production from aquatic animals lags far behind that of the plant and livestock sectors. Only a small percentage of farmed aquatic animals have been subjected to genetic improvement programmes (Gjedrem 1997), and the application of genetic techniques to marine shrimp aquaculture is at an extremely early stage. Biotechnology and genetics have great potential

to increase production from shrimp farming and to help make shrimp farming sustainable. However, Benzie (1998) notes that there are still gaps in our basic knowledge of shrimp genetics and physiology that must be addressed before 'super-prawn biotechnology' can be produced.

Genetic improvement

To date, several standard techniques for genetic improvement have not yielded good results with marine shrimp. For example, additive genetic variance and hybridisation has been complicated by pre-zygotic and post-zygotic reproductive isolation problems. When accomplished it has not consistently produced heterosis in the F1 generation for either increased growth rate or disease resistance. Nor has hybridisation been an effective means of combining desirable traits from different species (Benzie *et al*. 1995). Chromosome manipulation (polyploidy) has not been practical, and selective breeding programmes have been hindered by difficulties with reproduction of key species, such as *P. monodon*, marking individuals, low heritability of growth-related traits (i.e., environmental factors greatly influence growth) (Benzie *et al*. 1997), and by the generally low priority afforded to genetic research by private industry (Wang 1998). However, it is interesting to note that more and more collaborations are being established between scientists, researchers, laboratories, regional and international organisations, and the donor community to further research efforts on shrimp genetics.

Selective breeding

In order to minimise environmental impacts on wild populations and to fully realise the value of genetic selection and diversity, shrimp aquaculture must break its reliance on wild post-larvae (Wang 1998). Although wild-caught post-larvae may currently perform better than some hatchery produced post-larvae, it inevitably involves the risk of introducing pathogens into the culture environment, and may pose a threat to aquatic biodiversity through removal of egg and larval stages of other species as a by-catch. Although, there is no direct evidence to strengthen the latter, hatchery production of post-larvae will facilitate a year-round uninterrupted supply of healthy post-larvae.

Tracking genetic lines and heritability

Modern molecular techniques also show promise for shrimp hatchery production, in that they can provide accurate information on the genetic diversity of natural stocks, enhance selective breeding programmes, and allow genetic tagging of shrimp to facilitate selection of individuals with the characteristics which meet various market needs (size, coloration, rate of growth, time of spawning, etc.). Molecular markers have already been developed to identify genetically discrete natural populations and to provide family identification and pedigree information in some selective breeding programmes. Identification of individual animals allows tracking of their reproductive performance and the traits expressed in their offspring, which, in turn produces a broodstock line with a defined pedigree. Physical tagging of early life-history stages of many aquatic species is difficult, especially with marine shrimp. Genetic markers using microsatellite DNA, and AFLP's (amplified fragment length polymorphisms) are beginning to be produced which provide a non-invasive mechanism of tracking broodstock and their offspring, in order to produce a well-documented pedigree. Molecular genetic markers are also being used to map the genome of commercially important shrimp species such as *P. japonicus* and *P. monodon*. This will improve identification of loci, genes, and gene complexes, that improve culture performance, product quality and profit. These markers can also be used to map genetically linked characteristics and identify loci with quantifiable traits (Garcia *et. al.* 1996, Benzie 1998, S. Moore, pers. comm). These advances will make shrimp breeding programmes more efficient and profitable.

Genetic engineering

Production of transgenic (genetically modified) shrimp has been reported (Mialhe *et al.* 1995). However, this has not yet been achieved at a commercial scale (Benzie 1998). At present, the use of transgenic animals for aquaculture is controversial and it is not well accepted by the industry or consumers for any shrimp species

LARVAL CULTURE AND LIVE FEEDS

Dependable availability of quality fry to stock grow-out production systems has been one of the most critical factors in the commercial success of industrial production of fish and shellfish (Sorgeloos 1995). More attention to the "quality" and the "competence" of hatchery-produced post-larvae is needed, to ensure better performance under grow-out conditions, with particular focus on bioaugmentation and microbial management of the various culture steps in the hatchery. Although nutritional and dietary requirements of many fish and shellfish species have been identified, large-scale hatchery production of most aquatic invertebrates still depends on live feed, such as selected species of microalgae, the rotifer *Brachionus* and the brine shrimp *Artemia*.

Consistency and quality of shrimp feed

More than 15 species of diatoms and green algae are used in first-feeding of hatchery produced shrimp larvae (zoea stage). Selection of these species has been based on their digestibility, as determined by trial and error rather than by scientific analysis. The systems used in most developing countries are still labour intensive. This is not cost effective and poses many problems for mass production, including inconsistent nutritional quality and microbial contamination leading to decreased or lost production. This has created a whole new area of biotechnological research to find cost effective and efficient supplements to live microalgae, This includes research into commercial production of freeze-dried algae, microencapsulated diets, and manipulated yeasts. This area of biotechnology, however, requires further research in an effort to reduce current reliance on mass production of live feed in shrimp hatcheries, with all the handling complications and costs this entails.

Artemia nauplii are the most widely used live feed in shrimp aquaculture (Sorgeloos & Leger 1992). Over the years considerable progress has been made in improving their dietary value through batch selection of traits including efficient cyst disinfection and decapsulation, success of nauplius hatching, and cold storage (Sorgeloos 1995). In addition,

improvement of the nutritional quality of *Artemia* through bioencapsulation (enrichment), especially with highly unsaturated fatty acids and vitamins, has improved larviculture outputs in terms of quality, survival, growth, and stress resistance.

Dietary nutrients and medical supplementation

Bioencapsulation has also been applied for oral delivery of vaccines, vitamins, and chemotherapeutants (Lavens *et. al.* 1995). These positive results mean that continued research into bioencapsulation and use of live feed as a means of oral delivery of dietary supplements and/or medication to shrimp, and other crustaceans, should be given high priority.

AQUATIC ENVIRONMENTAL QUALITY MANAGEMENT

Probiotic application to shrimp culture

Probiotics are microbes (usually bacteria) selected for their ability to outcompete and displace potentially harmful sympatric species. They are usually administered as feed supplements to improve the intestinal microbial balance. A stable gut microflora helps digestion efficiency and the ability of animals to resist pathogenic infections, particularly of the gastro-intestinal tract. Use of antibiotics, by definition, reduces the level of the gut microflora. Restoration of this essential microflora can be accelerated through the use of probiotic-enhanced feeds designed to boost colonisation by the most efficient digestive bacterial species. Probiotics are widely used in terrestrial animal husbandry, but their use in aquaculture is still in its infancy. Reports on the potential of probiotics in shrimp aquaculture are, however, on the increase. In some countries it is reported that probiotic use has significantly reduced antibiotic use in shrimp hatcheries. Probiotics have been used to suppress the growth of pathogenic *Vibrio* spp. in many shrimp hatcheries by introducing (inoculating) non-pathogenic strains or species. This procedure appears effective and economical and demonstrates a clear need for further research into identifying potential probiotic strains of micro-organisms and evaluating their efficacy under field/farm conditions.

Bioremediation techniques

Bioremediation is another promising biotechnological approach, involving degradation of hazardous waste to environmentally safe levels by use of selected micro-organisms, bivalves, algae, etc. (Srinivasa Rao & Sudha 1996). Although bioremediation has been well-used in situations such as sewage treatment, its application to shrimp aquaculture is fairly novel. Many commercial products are available in the market, mainly bacterial preparations, although, as with immunostimulants, their mode of action and efficacy has yet to be scientifically ascertained. In addition to microbes, bivalves, seaweed, holothurians, etc., have been tested and used to reduce organic loading and excess nutrients in shrimp ponds (polyculture systems or reservoir ponds or "bio ponds"). Various preparations have also been developed with the aim of removal of nitrogenous and other organic waste from the water and bottom sludge. This improves the pond environment and reduces physiological stress on shrimp. More products will, undoubtedly, emerge as research continues However, controlled field trials are urgently needed to determine the cost benefits and effectiveness of these under commercial culture conditions.

Recirculation techniques for water quality control

Improved water management techniques such as partial and complete recirculation of water, with the view to avoid introduction of sub-clinically infected wild carriers (e.g., WSSV carriers), has been effective in controlling the recent viral disease epizootics which have spread throughout Asia. In addition, this technique has also proven effective in reducing proliferation of opportunistic pathogens. As a large number of aquatic organisms have been identified as potential carries of WSSV, avoidance of contamination through water exchange is now considered as one of the most effective preventive measures against this viral epizootic. Creating an ecologically sound environment, which is less stressful to shrimp, through water recirculation and avoidance of frequent water exchange, is a challenge for the future. Development of water management options through the increased use of biological agents and biotechnological means, to reduce excess nutrients and to maintain optimal physico-chemical and microbial quality of water, will continue to be a challenge for environmental scientists, and will certainly contribute to the success of shrimp aquaculture.

CONCLUSIONS

Aquaculture biotechnology can be described as the scientific application of biological concepts that enhance the productivity and economic viability of its industrial sectors (Liao & Chao 1997). Such technology is certainly emerging as one of the most rapidly growing "new frontiers" in research, using a knowledge-intensive and advantage-oriented approach. These developments are helping to diversify aquaculture studies, potential investment, and international exchange. Continued development of biotechnology in shrimp aquaculture has the potential to provide a means of producing "super shrimp", healthy and fast growing, through environmentally friendly means. This development, however, depends on the desire and willingness of the producers to work hand-in-hand with scientists and the international donor community to assist all countries, and especially LIFDC's, in this research. This applies particularly to capacity building and infrastructure development. Improved exchange of information and discussion of problems and achievements between scientists, researchers, and producers from different regions is also essential to help this important food production sector further develop globally sustainable production of healthy shrimp. It should be noted that immediate and preliminary use of most of these new biotechnologies will likely be restricted to specialised users and laboratories. However, this is normal for all technological advancements, as has been previously demonstrated in this field with fluorescence and electron microscopy, molecular electrophoresis and polyclonal antibody immunodiagnostics. It is, therefore, expected that industry and diagnostic demands will urge rapid refinement of these newer technologies for more general use within the next decade. The initiative is clearly established, it just requires maintenance of the developmental momentum.

LITERATURE CITED

Anderson DP (1995) Novel techniques in fish disease diagnosis. In: Shariff M, Arthur, JR, Subasinghe RP (eds) Diseases in Asian Aquaculture, Vol II, Fish Health Section of the Asian Fisheries Society, Manila, Philippines, p 27-42

Austin, B (1998) Biotechnology and diagnosis and control of fish pathogens. J Mar Biotech 6:1-2

FAO (1997) FAO Fisheries Circular No 886, Rev 1, Rome, FAO. 1997

Bedier E, Cochard JC, Le Moullac G, Patrois J, Aquacop (1998) Selective breeding and pathology in penaeid shrimp culture: the genetic approach to pathogen resistance. World Aquaculture 29(2):46-51

Benzie JAH (1998) Penaeid genetics and biotechnology. Aquaculture 164: 23-47

Browdy, CL (1998) Recent developments in penaeid broodstock and seed production technologies: improving the outlook for superior captive stocks. Aquaculture 164: 3-21

Durand -S, Lightner DV, Nunan LM, Redman RM, Mari -J, Bonami J-R (1996) Application of gene probes as diagnostic tools for white spot baculovirus (WSBV) of penaeid shrimp. Dis aquat Org 27(1):59-66

Flegal TW (1996) A turning point for sustainable aquaculture: the white spot virus crisis in Asian shrimp culture. Aquaculture Asia, July-September 1996. NACA, Bangkok, Thailand, p 29-34

Garcia DK, Dhar AK, Alciva-Warren A (1996) Molecular analysis of PAPD marker (B20) reveals two microsatellite and differential mRNA expression in *Penaeus vannemei*. Mol Mar Biol Biotech 5:1-83

Gjedrem T (1997) Selective breeding to improve aquaculture production. World Aquaculture 28:33-45

Gjerde B, Rye M (1998) Design of breeding programmes in aquaculture species: possibilities and constraints. In: Bartley D, Basurco B (eds) Genetics and breeding of Mediterranean aquaculture species. Cahiers OPTIONS Mèditerranèennes, Vol 34. Zaragoza, Spain. p 181-192

Le Groumellec M, Martin C, Haffner P, Martin B, Aquacop (1995) Cell culture from tropical shrimp. J Aquacult Trop 10: 277-286

Lavens P, Sorgeloos P, Dhert P, Devresse,D (1995) Larval foods In: Bromage NR, Roberts JR (eds) Broodstock management and egg and larval quality. Blackwell Science Limited, Oxford, p 373

Liao IC, Chao NH (1997) Developments in aquaculture biotechnology in Taiwan. J Mar Biotech 5:16-23

Mialhe E, Bachere E, Boulo V, Cadoret JP, Rousseau C, Cedeno V, Saraiva E, Carrera L, Colwell RR (1995) Future of biotechnology-based control of disease in marine invertebrates. Mol Mar Biotechnol 4: 275-283

Sorgeloos P (1995) Bioengineering of hatcheries for marine fish and shellfish. J Mar Biotech 3:42-45

Sorgeloos P, Leger P (1992) Improved larviculture outputs of marine fish, shrimp, and prawn. J World Aquacult Soc 23(4): 251-264

Sriniwasa Rao PS, Sudha PM (1996) Emerging trends in shrimp farming. Fishing Chimes 16(3):25-26

Subasinghe RP, Barg U, Phillips MJ, Bartley D, Tacon A (In Press) Aquatic animal health management: investment opportunities within developing countries. J Appl Ichth 14(3)

Wang Y (1998) Utilization of genetic resources in aquaculture: a farmer's view for sustainable development. Bellagio Conference, Towards Policies for Conservation and Sustainable Use of Aquatic Genetic Resources. FAO/ICLARM, Bellagio, Italy, 14-18 April, 1998

Wongteerasupaya C, Wongwisansri S, Boonsaeng V, Panyim S, Pratanpipat P, Nash GL, Withyachumnarnkul B, Flegel TW (1996) DNA fragment of *Penaeus monodon* baculovirus PmNOBII gives positive *in situ* hybridization with white spot viral infections in six penaeid shrimp species. Aquaculture 143: 23-32

Alternatives in Shrimp Biowaste Processing

Willem F. Stevens, Preiradda Cheypratub, Sjeng Haiqing Pranee Lertsutthiwong, Ng Chuen How, Suwalee Chandrkrachang

Bioprocess Technology Program, Asian Institute of Technology, Bangkok, Thailand

ABSTRACT: In marine food processing, about half of shrimp weight is biowaste. Without further processing, industrial quantities of this waste are a severe burden for the environment. Decay starts in a few hours leading to a bad smell and, after disposal, to a high load in terms of COD and BOD. Shrimp biowaste can be valorized by drying and mixing with other raw materials to compose animal feed. As an alternative, shrimp biowaste can be treated to isolate chitin, the aminocellulose component in the exoskeleton, and its valuable derivative chitosan. Steamed biowaste is applied in large quantities in animal feed. The process is highly efficient and automated. All components of the waste including the pigment contribute to the nutritional value of the end product. The disadvantage of the process is the low added value. Moreover, in small scale operations there is the risk of insufficient inactivation of shrimp pathogens present in the waste that can lead to a feed born reinfection in shrimp aquaculture. Experimental data will be discussed to estimate the significance of this argument. For separation of shrimp protein and chitin components, usually chemical extraction is applied. The common industrial process is efficient but uses chemicals including hot 50% alkali to produce deacetylated chitin or chitosan. However, the chemical treatment causes hydrolysis of the chitosan product and corrosion of the equipment. In addition it leads to generation of a hazardous high alkali, high protein waste. Systematic research of the chemical processing has resulted in an improved system for production of chitin with a high degree of purity and with a 10-30 fold higher viscosity. Also the quality of the waste water has been improved considerably. On the basis of the recent technology, high quality chitin and chitosan can be produced in a sustainable way. Biotechnology has opened other alternatives to isolate chitin and chitosan. In this approach the waste is stabilized firstly by an ensilage process using partial fermentation by *Lactobacillus* bacteria. The fermentation leads to production of lactic acid. This will decrease the pH of the medium (to pH 4-5) and inhibit the growth of other bacteria. It also prevents the formation of off-flavor amines. After fermentation, most of the shrimp protein is detached from the solid component and can be isolated easily and turned into a protein preparation suitable for human food. For the isolation of chitin, only simple, further chemical treatment is required. Chitin can be deacetylated into chitosan using a fungal enzyme, chitin deacetylase (CDA). The CDA enzyme has been isolated from the fungus *Absidia cocruleus* and characterized. Although with excess solid chitin as a substrate the enzyme activity is high, only a small percentage of the substrate is converted. Due to the high crystallinity of the natural substrate, only the chitin acetyl groups on the outside of chitin particles might be accessible to the enzyme. Therefore, larger scale production of chitosan using enzymes will only be successful if the chitin can be decrystallized and the acetyl groups exposed to the enzyme. In this report a number of experiments with decrystallized amorphous chitin will be discussed. Either chemically or biotechnologically or in mixed procedures, cleaner and more intact chitosan can be produced. The high quality chitosan has many industrial applications including manufacture of pharmaceutical and medical products, drugs and food additives. It is also used in paper and textile manufacturing and in other industrial processing. Progress in chitin applications in a few selected areas will be summarized. The biotechnological methods have the advantage of the production of human food protein. The separation of shrimp waste into its components leads not only to high value products but also to safe food product cycles with regard to the chances of shrimp reinfection through feed containing shrimp waste.

KEY WORDS: shrimp waste, chitin, chitosan, *Lactobacillus, Absidia cocruleus*, chitin deacetylase, fermentation

INTRODUCTION

The major product of the shrimp marine food industry is shrimp meat. However, the meat constitutes only about 50% to the shrimp wet body weight. The other half, the residual biomaterial or biowaste, contains many valuable compounds that after appropriate processing can add substantially to overall profitability. This shrimp biomateral can be valorized without fractionation. Usually it is applied as such for feeding in veterinary practice and aquaculture. Medium and large scale processing has been developed to dry the waste and to mix it with other agricultural raw materials to produce animal feed.

The shrimp biomaterial can be valorized further by fractionation into its major components: protein, minerals and chitin. The protein has high potential as a human food additive and food supplement while the chitin is usually converted into its deacetylated product, chitosan. Chitin and chitosan are N containing polysaccharides which have a structure similar to cellulose. Chitosan, in particular, has many

Stevens WF, Cheypratub P, Haiqing S, Lertsutthiwong P, How NC, Chandrkrachang S (1998) Alternatives in shrimp biowaste processing. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

applications in foods, medicines, cosmetics and water purification and as an ionic biopolymer for paper and textile finishing and for flocculation processes. Various other valuable products can be extracted from the biomaterial. These include shrimp flavor components and the natural dye asthaxanthin that have many applications in the food and feed industry.

A major problem with shrimp biomaterial valorization is the high perishability of the material. Under tropical climatic conditions, decay starts within an hour after processing and leads to the production of biogenic amines with a very offensive smell. If this decay is unavoidable or not prevented, the biomaterial turns into real waste and due to its high protein content, it becomes a real threat to the environment and a financial burden if discarded properly.

It is obvious for both environmental and economic reasons that, wherever possible, appropriate technology should be applied to prevent decay and to convert the biomaterial into valuable products. Technology should provide systems for the delay or prevention of decay and procedures for fractionation. In this paper alternative technologies for conservation and valorization of shrimp biomaterial are discussed and evaluated.

USE OF NON FRACTIONATED BIOMATERIAL

In areas with a low technical infrastructure, where fishermen individually catch and clean small amounts, most of the biowaste is used as feed for livestock without any treatment. In these cases, decay of a part of the waste will be unavoidable, leading to pollution of land and water. At locations where large amounts of shrimp are landed or produced in aquaculture, processing infrastructure to handle the waste is usually developed. The waste is collected at certain places and dried. At most times of the year, solar drying is not sufficient to prevent decay and odor formation. Heat, usually steam, is used to preserve the waste and to dry it quickly into dry shell material and nearly dry protein. Due to the heat, many valuable compounds in the waste are lost. The dried material is ground and sold as shrimp meal, to be used as a protein rich component in fish feed.

In areas where massive amounts of shrimp biowaste are generated, large industrial facilities process it in mixtures with soy products, with cheap carbohydrate products like cassava and potato and with minerals. After drying and grinding into a dry meal it is sold in large quantities in the international market. These forms of direct treatment do not require fractionation and can be carried out in a straightforward manner. However the added value of approximately 10 cents US (4 Thai baht) per kg of non fractionated waste is low.

CHEMICAL FRACTIONATION

A much higher economic value can be added to the biowaste through fractionation. The most common procedure is chemically very simple: treatment of the biowaste with 4% alkali to separate the protein and treatment with 4% acid to remove the calcium carbonate. The resulting chitin product can be further deacetylated by concentrated 50% alkali to produce chitosan. The individual steps are outlined in the following paragraphs.

Deproteination

In the first step, the waste is treated with 4% sodium hydroxide (NaOH) at elevated temperatures (70-120°C). Under these conditions the protein becomes detached from the solid component in the shrimp biowaste. To prevent oxidation of the products, the process is usually carried out in a nitrogen atmosphere and in the presence of sodium borohydride ($NaBH_4$). After completion of the deproteination step, the protein hydrolysate is removed easily by separation of the solids from the protein slurry by filtration. The protein hydrolysate can be dried and used in the form of a cake or powder as a protein supplement in feed. This protein hydrolysate also contains most of the shrimp flavor. The solid fraction consists mainly of chitin and calcium carbonate. It also contains most of the pigment.

Demineralization

In the next step, the solid fraction is treated with 4% hydrochloric acid (HCl) which converts the insoluble calcium carbonate into soluble calcium chloride that can subsequently be removed by washing. With appropriate deproteination and demineralization, the remaining product consists mainly of chitin with minor amounts of protein and calcium, that can be judged from a weak Biuret reaction and a low weight after ashing, respectively. The chitin product should be white. It is insoluble in alkali and in most acids and organic solvents. Due to its low reactivity, chitin is usually deacetylated to chitosan.

Deacetylation.

The chemical deacetylation of chitin into chitosan requires strong chemical conditions: 50% NaOH and elevated temperatures as high as 70-90°C. The highest degree of deacetylation possible is desired and several treatments are usually required to reach a sufficient degree to obtain a marketable product (see Fig. 1). Chitin deacetylated by 70-90% (also referred to as 30-10% acetylated chitosan) is considered to be a good end product. The material should be low in protein and ash. Chitosan can be dissolved in 1-2% acetic acid and high viscosity of this solution is indicative of a well prepared chitosan. If too rigorous conditions are applied during deacetylation, the main chain of the chitin breaks and this results in low viscosity of chitosan dissolved in acetic acid. In addition the broken molecules cause discoloration and condensation, resulting in reduced transparency and solubility. A good chitosan preparation has a low ash content (< 1%) and dissolves well in acetic acid giving high transparency (>90% Transmission) and a viscosity of at least 300 centipoise.

One might wonder why chitin cannot be deacetylated by 90% in one step since the reagent, NaOH, is in excess, the acetate formed does not inhibit the deacetylation and the conditions are quite aggressive. It seems that there is a transport problem. The chitosan formed at the outside of the chitin particle seems to inhibit entry of NaOH into the particle. After washing with water this barrier is removed and the particle is again open so that the process of deacetylation

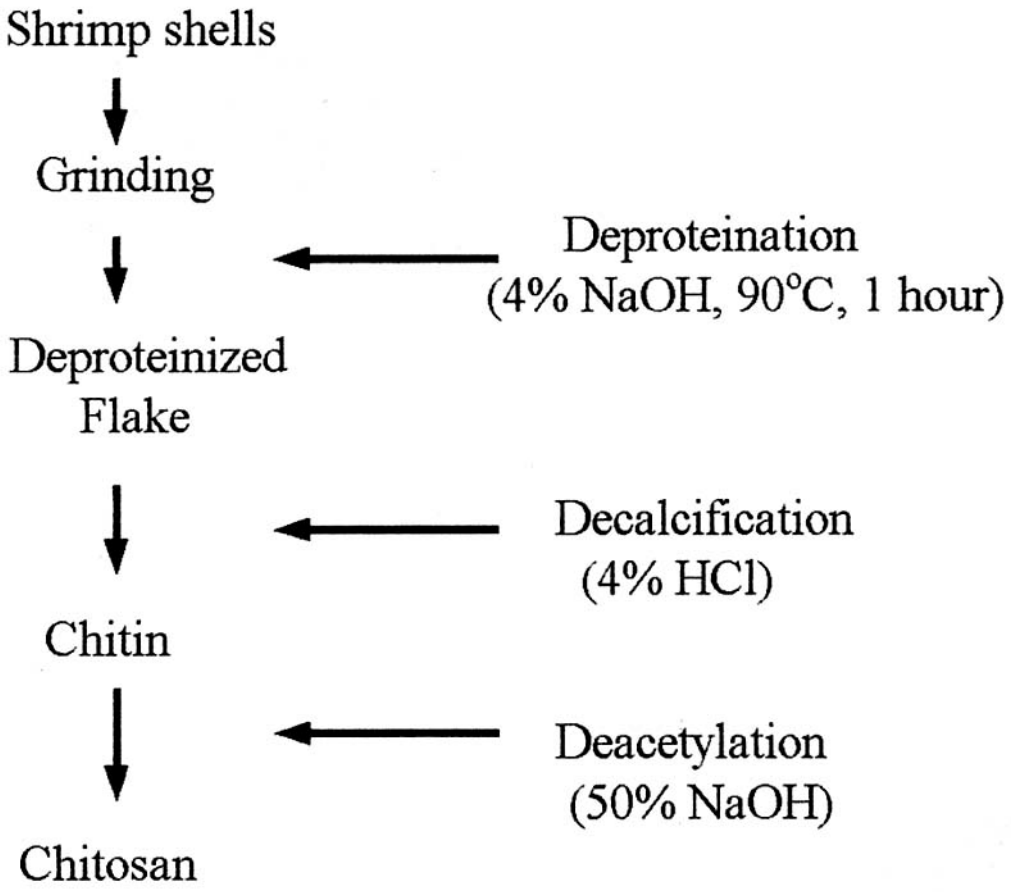


Figure 1. Production of chitin and chitosan by the chemical method.

can continue. Systematic research on its chemical processing at AIT has resulted in an improved system for production of chitin with a high degree of purity and chitosan with a 10-30 fold higher viscosity than that of standard commercial chitosans.

BIOTECHNOLOGICAL PROCESSING

Fermentative deproteination and demineralization

Biotechnology is providing new alternatives for the isolation of chitin and chitosan. Shrimp biowaste can be stabilized by an ensilage process using partial fermentation by lactobacillus bacteria. The fermentation leads to production of lactic acid. This decreases the pH of the medium (to pH 4-5) and inhibits the growth of spoilage bacteria. It also prevents the formation of off-flavor amines. After fermentation, most of the shrimp protein is detached from the solid component and can be isolated easily. For the isolation of chitin, a simple, further chemical treatment only is required.

The partial fermentation can be carried out by food grade (GRAS) lactobacilli. This process therefore can lead to a protein product that is suitable for use in human food, either as a protein component or as a nutritional supplement. The protein product has the same richness in amino acids as the shrimp meat itself. It has a very strong flavor that makes it very suitable as a flavor enhancer. Depending on the conditions for the fermentation, a part of the demineralization can also be carried out simultaneously since the condition of pH 5.0 and the presence of lactic acid can result in conversion of at least a part of the calcium carbonate into soluble Ca and to Ca lactate.

Both deproteination and demineralization of chitin are not completely achieved during 16 hours of fermentation. In order to arrive at maximal deproteination and demineralization, treatments with NaOH and HCl at low concentration and for short duration have been adopted to complement the fermentation. This removes remaining traces of protein and minerals, particularly calcium.

Enzymatic deacetylation.

Chitin can be deacetylated into chitosan using the fungal enzyme, chitin deacetylase (CDA). CDA has been isolated from the fungus *Absidia coerulea* and characterized. Although with excess solid chitin as a substrate the enzyme activity is high, only a small percentage of the substrate is converted. This is due to the high crystallinity of the natural substrate. As a result, only the chitin acetyl groups at the outside of the chitin particle are accessible to the enzyme.

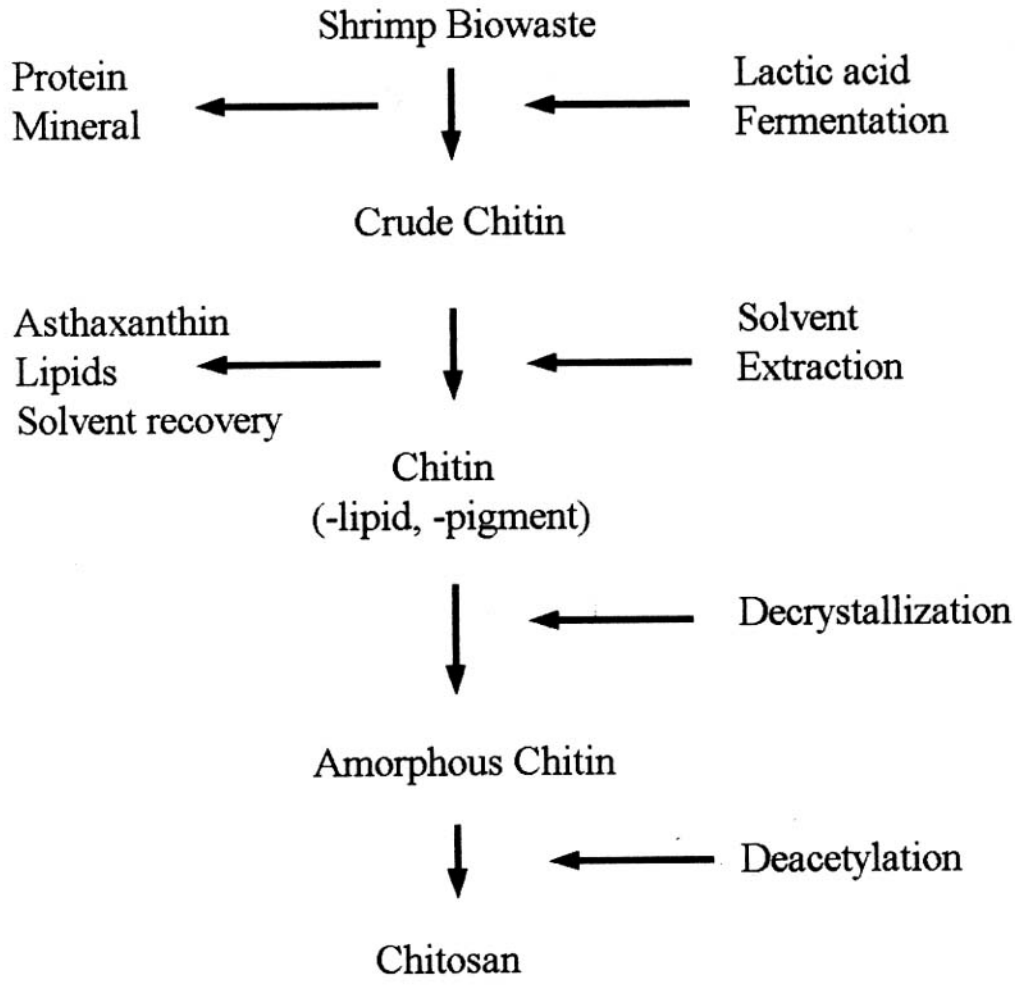


Figure 2. Total biochemical conversion of shrimp biowaste into chitin and chitosan.

Therefore larger scale production of chitosan using enzymes will only be successful if the chitin can be decrystallized and the acetyl groups exposed to the enzyme.

A number of experiments have been carried out in order to decrease the crystallinity of the chitin and make it a more accessible CDA substrate. Physical methods like heating and ultrasonic treatment did not improve the chitin as a substrate for CDA. Limited steam explosion (i.e., heating to 120°C at high pressure followed by sudden pressure release) did not alter its properties. However, chemical treatment using strong acids did create a chitin with a more open structure. This problem of substrate accessibility has not been solved.

EVALUATION OF CHEMICAL AND BIOCHEMICAL METHODS

In cases where proper conditions are applied and the temperature is kept below 70°C, chitin and chitosan can be prepared very well by chemical methods. Chitin is resistant to alkali and quite resistant to 4% hydrochloric acid. Chitosan is very resistant to 50% alkali. The major drawback of the chemical method is the non sustainability of the process. The alkaline deproteination leads to a voluminous 4% alkaline waste containing a high amount of protein per liter. The 50% alkaline condition for deacetylation is also highly corrosive for the equipment used, especially at elevated temperatures. The waste after this step is highly alkaline and must be discarded properly, not released into the environment. The alkaline treatment has also a drawback for the protein and the pigment. The protein once treated at elevated temperature with 4% alkali or acid is not considered to be food grade. The carotenoid pigment also disintegrates under conditions of high temperature and light.

Processing of the shrimp protein by partial fermentation and (in future) by enzymatic deacetylation (Fig. 2) has two advantages. The protein hydrolysate remains a valuable product with a high nutritional value for human purposes. Furthermore, the process does not result in an alkaline proteineous waste water stream that is difficult to clean. On the other hand, the biotechnological process also has disadvantages. It is slightly more complicated as the chemical process and the product quality might decrease if chitin and chitosan degrading enzymes are present under the procedures used. However, on the basis of our experience up to now, this has not been a problem.

Nearly all chitin and chitosan is currently produced through chemical methods. The apparent ease of making chitin and chitosan has encouraged many small firms to try, but the resistance of the biomaterial to treatment and problems of scale have caused many unexpected problems. In

Figure 3. Model for a chitin and chitosan quality assurance sheet.

CHITIN AND CHITOSAN QUALITY ASSURANCE FORM

	Chitin
✓	Chitosan

(Tick whatever appropriate) Grade GRAND

Parameters	Mean Value	Standard Deviation
% Deacetylation	85	0.5
Apparent Viscosity (cps)	1500	50
Intrinsic (dL/g)	14.5	1.0
Viscosity Average Molecular Weight (10^6)	1.8	0.2
% Ash	0.45	0.05
% Moisture	10.0	0.1
% Protein	0.1	0.03
Turbidity (NTU)	2	0.1
% Insoluble	0	
Solid (Qualitative Observation)		
- Sample size (mesh)	14~20	
- Color	Milky white	
Heavy Metals		
- Cu (ppm)	0.02	0.00
- Fe (ppm)	0.08	0.00
- Cd (ppm)	0.02	0.00
- Ni (ppm)	0.01	0.00
- Cr (ppm)	0.02	0.00
- Zn (ppm)	0.02	0.00
- Hg (ppb)	< 2 -	
- As (ppb)	< 2 -	
Coliform groups	None	

Comments :
Prepared by : Ng Chuen How Date : 25 Sept 1998
Approved by : Prof. Willem Stevens Date : 26 Sept 1998

some cases, the use of high temperatures has caused much chemical damage and resulted in end products of low quality. Since the physico chemical qualitative assessment of biopolymers requires specific expertise, many low quality chitosans have entered the market. There is a need for international criteria for chitin quality and for a reference centre that can check for compliance with these criteria. (see Fig. 3 for an example of a possible quality report). The Bioprocess Technology Program at the Asian Institute of Technology has the objective to realize such a center for the S. E. Asian region.

Research on the prevention of proteineous waste water during chemical treatment has lead to a significant reduction in released BOD. On the basis of the present technology, high quality chitin and chitosan can be produced chemically in a sustainable way. In the biotechnological method, there is actually no waste left, the protein hydrolysate is collected as a human food supplement and the chitin/calcium carbonate is used to make chitosan. If in future a technical process becomes available for biocatalytic deacetylation, the whole process would be truly sustainable.

AVAILABILITY OF BIOWASTE

The amounts of chitin in the world are immense. It has been estimated that the total amount of chitin equals or even exceeds the amount of wood cellulose. Chitin is an important component of fungal cell walls, it is the building material for insect exoskeletons and together with calcium carbonate, it forms the rigid structure of the crustacean shell. However, in comparison to cellulose, the possibilities for collection of chitin are much more limited. The fungi and insects are highly dispersed in nature and most of the crustaceans are in the deep sea. One source for chitin might be the mycelium of industrially produced fungi, but recovery from that source does not seem very promising at this time.

The most attractive sources are the crab and shrimp sea food industries. At places where large volumes of crab and shrimp meat are processed, nearly equal amounts of biowaste are generated. For the time being only a small fraction of total crab and shrimp biowaste is being processed into chitin. Most of it is discarded if not used for food. The logical places to produce chitin and chitosan are areas where the biowaste

Table 1. Estimated consumption of chitin, chitosan and their derivatives in the Japanese market in 1994.

Uses	Consumption[a] (tons/year)
Cationic flocculating agents	350
Living wastewater treatment (200)	
Food manufacturing wastewater treatment (100)	
Sugar manufacturing (50)	
Food additives	125
Food processing (45)	
Functional health foods (80)	
Agricultural materials	120
(e.g., plant seed coating, fertilizers)	
Feed additives for pets, fishes and animals, etc	60
Textiles and fabrics	50
Cosmetic ingredient for hair and skin cares	40
D-glucosamine and oligosaccharides	13
Biomedical materials	20
(e.g., adsorbable suture, wound dressings)	
Paint and dyeing	10
Thickeners	10
Membranes	1
Chromatographic media and reagents	1
(e.g., colloid titration, enzyme substrates, etc)	

[a]Estimated as chitosan

is being produced in large quantities. Examples are along the coastal zones in Thailand and India and the landing harbors for crabs and shrimp in northern Europe and America. Seafood and shrimp factories that do not presently valorize their chitin containing wastes might consider ways of getting more value from the waste by extracting chitin, protein and pigment.

APPLICATIONS

Processing the biowaste cannot be discussed apart from the applications of the chitin, chitosan and shrimp waste products. In this paragraph only a few examples of application are presented (Table 1). The application of non fractionated biowaste in aquaculture was mentioned at the start of this paper. In addition, preparations of the pigment asthaxanthin are used.

Chitin itself has only limited applications. This is due to its insolubility in water and common organic solvents and its limited chemical reactivity. Chitin is being applied successfully in cultivation of mushrooms for instance. In soil it is broken down by chitinases and acts as a slow release system to provide organic nitrogen. The same amount of nitrogen supplied as urea is toxic.

Chitosan has a free amino group and therefore has much higher chemical reactivity and solubility than chitin. It can be produced as a powder, beads, flakes or as membranes. It can also be re-acetylated to chitin to regain the insolubility and chemical resistance of chitin. Thus, chitin can be converted to chitosan, cast into a membrane and transformed back into a chitin membrane.

Table 2. Shrimp mortality from injected WSSV after various treatments. The table shows daily morality and cumulative mortality after injection of heat treated viral preparations, an untreated viral preparation (positive control) and carrier solution (negative control).

	Day post injection of viral preparations										
Treatment	1	2	3	4	5	6	7	8	9	10	Cumulative % Mortality
Neg. control	0	0	0	0	0	0	0	0	0	0	0
100°c	0	0	3	0	0	0	0	0	0	0	25
80°c	0	0	0	0	0	0	0	0	0	0	0
60°c	0	0	1	2	2	1	0	1	0	0	58.3
40°c	0	0	8	4	0	0	0	0	0	0	100
Pos. control	0	1	6	5	0	0	0	0	0	0	100

The most important applications of technical grade chitosan are in environmental applications concerned mainly with water and waste water purification. Chitosan is an efficient aid in flocculation and a very effective binding material for metals like copper and chromium.

Technical chitosan is also being used on a large scale as an antifungicide and as a binder in the textile industry. Paper treated with chitosan becomes stronger and non wettable.

Pure chitosan is used in food, cosmetics, drugs and medical products. The human consumption of chitosan has boomed during last years as a dietary health food that binds bile acids and so reduces uptake of cholesterol and lipids in the body. In cosmetics, chitosan has been used for many years to enhance binding of cosmetics to negatively charged skin and hair. In pharmacy, chitosan can be applied as slow release matrix for a variety of drugs and peptides. Medical products include chitin surgical threads and transparent bandages for wound healing.

At present there is a large imbalance between scale of possible applications of chitin and chitosan and the limited amounts actually being used. The reason for this imbalance concerns the low quality of some of the chitins offered on the world market and the problems in establishing clear criteria for chitin/chitosan quality. In the next decade, the demand for chitosan will rise further and so the valorization of shrimp and crab biowaste should be of major interest of the shrimp and crab industries.

CONSTRAINTS ON THE USE OF SHRIMP BIOMATERIAL IN AQUACULTURE

The use of shrimp biowaste in shrimp meal and fish meal needs to be considered further due to the high incidence of virus infections in shrimp aquaculture. Several viruses highly pathogenic for shrimp have been identified and studied in detail. Advanced methods for virus diagnosis have been developed. However, the mechanism of infection, distribution and spread of these viruses is still poorly understood. It is supposed that shrimp viruses have a complicated structure and are rather labile, but in most cases, this has not been scientifically confirmed.

Shrimp infected with viral pathogens cannot be excluded from processing plants. In the large amounts of biowaste processed, the chances are high that some processed shrimp shell may still contain viable shrimp viruses. Therefore, the processing of shrimp biowaste for shrimp meal should be carried out in a manner that can guarantee that the resulting feed is free of shrimp pathogens. Inspection of large and a medium size production plants for shrimp and fish meal in Thailand revealed that the chances for shrimp viruses to survive the drying process are extremely small. In the large scale operations, the shrimp waste was heated together with other ingredients in a fluidized bed reactor to140^{o}C for 30 min. In the medium size operations, the shrimp biowaste was steamed and then dried with constant mixing over a period of about one hour. Presumably labile viruses would not survive either treatment, even when somewhat protected inside shrimp tissues. However, some viruses may have high heat stability and have more chance to survive.

The heat inactivation of white spot syndrome virus (WSSV) has been investigated in order to check the efficiency of heat in killing. The system designed for the tests could be used to study the inactivation of other viruses as well. WSSV was subjected to a temperature treatment (40 to 100^{o}C for 1 hour) and then the infectivity of the heated material was tested by injection into live shrimp. To check the cause of any mortality, PCR tests were performed on the shrimp haemolymph after injection. As may be concluded from preliminary data shown in Table 2, WSSV survived treatment for 1 hour at 40 and 60^{o}C but was inactivated at temperatures of 80^{o}C or higher. PCR data confirmed that death for the low temperature treatments was caused by WSSV. Some shrimp did die after receiving virus treated at 100^{o}C, but they were negative for WSSV by PCR and must have died from some other cause. In this preliminary study, heat treatment was carried out on extracted virus and not on virus in shrimp tissues. Whether virus in tissue would have better survival at high temperatures is presently under investigation. Other pathogens, including a *Vibrio* bacteriophage implicated in shrimp mortality, will be studied using this system.

In general, one may comment that it is risky to feed an animal species with biowaste of the same species. Feeding chicks with residuals from chicken processing enhances the chance for chicken disease. Shrimp should not be fed with shrimp, especially when other economic alternatives are available. The production of chitosan, protein and pigment from shrimp should also be considered in this perspective.

OUTLOOK AND RECOMMENDATIONS

The demand for high quality chitosan, preferentially decrystallized will increase in the coming decade. As mentioned, sharp growth is expected for application of chitin compounds in food, natural fungicides, waste water treatment, textile coating and cosmetics and medicines. Chitin and chitosan should be produced in various grades of purity and consistency depending on the requirements for a particular type of application. Due to process improvements, the defined quality will go up and the price will change depending on the use and the quality. Overall profitability will increase. Emphasis should be given to local systems for treatment of biowaste and to the organization of a multi tier system for chitin valorization. In the interim, shrimp drying procedures should be monitored for their efficiency in killing shrimp pathogens.

Maturation and Genetics

Hormonal Control of Vitellogenesis in Penaeid Shrimp

I. Yano

Department of Marine Bioscience, Fukui Prefectural University, Gakuen-cho 1-1, Obama, 917 Fukui, Japan

ABSTRACT: Control of maturation is a major problem in the development of commercial aquaculture programs for penaeid shrimp. In penaeid shrimp, maturation involves two main processes, vitellogenesis and final maturation of oocytes. In vitellogenesis of penaeid shrimp, two phases are involved: vitellogenin (Vg, a precursor of egg yolk protein) synthesis in the ovary and accumulation of vitellin in oocytes. Vg synthesis in ovarian tissue pieces can be stimulated by thoracic ganglion extract prepared from vitellogenic females. Vitellogenin synthesis in the ovary is stimulated by vitellogenesis-stimulating hormone (VSH), a neuropeptide of approximately 10000 Da, secreted from the thoracic ganglion in the kuruma prawn, *P. japonicus*. Vg synthesis in incubated previtellogenic ovarian pieces can be stimulated by estradiol-17ß. Oil globule stage oocytes, which are the oocytes at the initial stage of primary vitellognesis, were found in incubated previtellogenic ovarian pieces after treatment with estradiol-17ß *in vitro*. These results suggested that estradiol-17ß, probably secreted from the ovary, stimulated Vg synthesis in the ovary as a Vg-stimuating ovarian hormone in penaeid shrimp. Unilateral eyestalk ablation is effective in increasing serum Vg in the kuruma prawn. Vg synthesis can be induced slightly in isolated previtellogenic ovarian pieces, which are not affected directly by hormones from eyestalks *in vitro* in the kuruma prawn. This suggests that Vg synthesis in the ovary may be inhibited directly by vitellogenesis-inhibiting hormone (VIH) secreted from eyestalks. On the other hand, Vg synthesis in ovarian pieces incubated with thoracic ganglion pieces prepared from vitellogenic females could be stimulated by serotonin. However, serotonin was not effective in stimulating Vg synthesis in ovarian pieces incubated without thoracic ganglion pieces. These results suggested that secretion of vitellogenesis-stimulating hormone from the thoracic ganglion may be induced by serotonin.

KEY WORDS: neurohormone, estradiol-17ß, vitellogenin synthesis, penaeid shrimp

INTRODUCTION

Control of maturation and spawning is a major problem in the development of commercial aquaculture programs for penaeid shrimp. Controlling maturation and spawning in captivity could help to provide a reliable year-round supply of juveniles, serve in developing selective breeding programs, and be generally useful for obtaining disease-free spawners. Eyestalk ablation has been used to mature and spawn female shrimp in captivity in conjunction with management of water temperature, photoperiod, light intensity, density, sex ratio, and nutrition (Yano 1993). Knowledge of hormonal control of maturation and spawning in crustaceans, however, is fragmentary. Since the pioneering work of the 1940s (Panouse 1943, 1944), many observations of endocrine systems have been made with respect to the inhibition of maturation and spawning by eyestalk hormone(s). However, recent research has focused mostly on organs (brain, thoracic ganglion and ovary) and their functions which are closely related to the release of vitellogenesis-stimulalting hormones and ovarian hormone (Yano 1992,1993). This paper summarizes our present knowledge of the endocrine systems of penaeid shrimp and how they control vitellogenesis.

THORACIC GANGLION HORMONE EFFECTS ON VITELLOGENESIS

In penaeid shrimp, maturation involves two main processes, vitellogenesis (Yano et al. 1996) and final maturation with germinal vesicle breakdown (GVBD) and ovulation in oocytes (Yano 1988, 1995). Vitellogenesis occurs in hard-shelled shrimp at the intermolt stage C_4, shortly after molting and continues until immediately before final maturation in penaeid shrimp. Smaller size *P. vannamei* did not initiate vitellogenesis even after eyestalk ablation (Yano, unpublished data). Thus, vitellogenesis is controlled by two factors, one which inhibits and the other which stimulates. In previtellogenic (immature) females, the vitellogenesis-stimulating principle is absent or not yet functioning. Yano et al. (1988) demonstrated that vitellogenesis in shrimp can be stimulated by implantation of pieces of thoracic ganglion tissue prepared from the female lobster, *Homarus americanus*, with vitellogenic ovaries. This result indicated that vitellogenesis could be stimulated by a vitellogenesis-stimulating hormone (VSH) secreted by neurosecretory cells of the thoracic ganglion of vitellogenic females and that lobster VSH was not species-specific in activity. Injection of thoracic ganglion extract prepared from vitellogenic females is effective in increasing serum Vg in the kuruma prawn, *P. japonicus*. On the other hand, Vg (a necessary prerequisite for ovarian oocytes to reach full vitellogenesis) is synthesized in the ovary in penaeid shrimp (Yano & Chinzei, 1987). Yano and Itakura found that Vg synthesis in incubated ovarian pieces can be stimulated by thoracic ganglion extract in kuruma prawn (unpublished data). Thoracic ganglion extract, prepared from vitellogenic kuruma prawn females, was fractioned by gel filtration high-performance liquid chromatography, and high Vg-stimulating activity was detected in

Yano I (1998) Hormonal control of vitellogenesis in penaeid shrimp. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

e-mail: yano@fpu.ac.jp

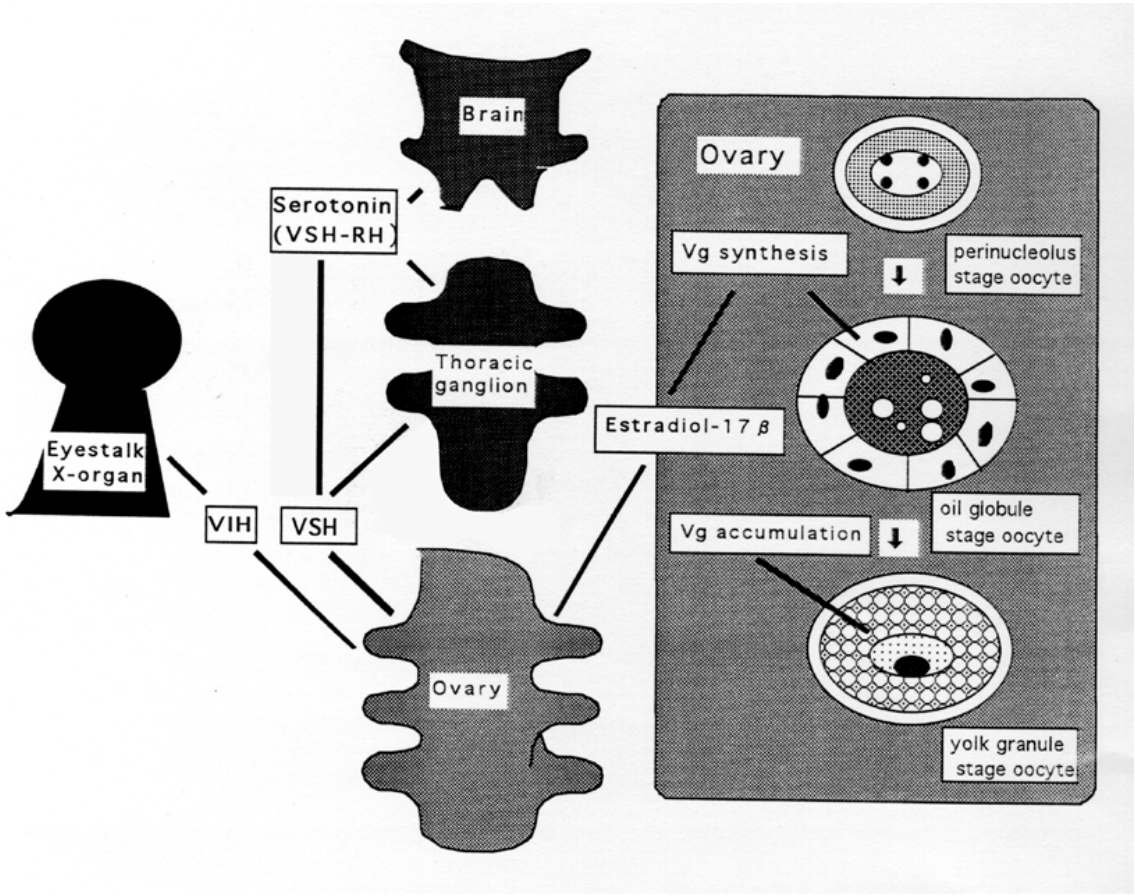


Figure 1. Proposed model for the hormonal control of vitellogenesis in penaeid shrimp. VSH-RH, vitellogenesis-stimulating hormone-releasing hormone; VSH, vitellogenesis-stimulating hormone;VIH, vitellogenesis-inhibiting hormone.

the fraction corresponding to a molecular weight of 10,000 (Yano, unpublished data). This fraction was inactivated by trypsin. Therefore, the bioactive factor, VSH, could be characterized as a peptide hormone. The results indicated that VSH secreted from the thoracic ganglion stimulated Vg synthesis in the ovary and vitellin (egg yolk protein) accumulation in ovarian oocytes in penaeid shrimp (Fig. 1).

EYESTALK HORMONE DIRECTLY INHIBITS VITELLOGENIN SYNTHESIS

Unilateral eyestalk ablation is effective in increasing serum Vg in the kuruma prawn (Yano 1992). Also for this species, Vg synthesis can be induced in isolated previtellogenic ovarian pieces *in vitro*. However, they are not affected directly by hormone from eyestalks *in vitro*. These results suggest that Vg synthesis in the ovary may be inhibited directly by vitellogenesis-inhibiting hormone (VIH) secreted from eyestalks (Fig. 1). Unilateral eyestalk ablation is not effective in increasing serum Vg in vitellogenic female kuruma prawn. This suggests that VIH levels decrease quickly immediately before the initiation of vitellogenesis and stay at a low level until after vitellogenesisis is completed. Therefore, eyestalk ablation may not be effective in regulating the production of VIH after vitellogenesis has been initiated.

INDUCTION OF VITELLOGENIN SYNTHESIS IN OVARY BY ESTRADIOL-17ß

Injection of estradiol-17ß is effective in increasing serum Vg in the kuruma prawn, (Yano, unpublished data). Yano and Hoshino found that Vg synthesis in incubated ovarian pieces can be stimulated by estradiol-17ß (unpublished data). In addition, oil globule stage oocytes (i.e., the oocytes at initial stages of primary vitellognesis) were found in incubated previtellogenic ovarian pieces after treatment of estradiol-17ß *in vitro*. Evidence has been presented to show that 17α-hydroxy-progesterone is effective in increasing serum Vg in kuruma prawn (Yano, 1987). The hormone estradiol-17ß or 17α-hydroxy-progesterone is generally distributed in the ovary of crustaceans (Kanazawa & Teshima 1971, Teshima & kanazawa 1971, Jeng et al. 1978, Couch et al. 1987, Faires et al. 1989). Considering these observations, 17α-hydroxy-progesterone may be worked into the Vg synthesis mechanism in kuruma prawn as a precursor of estradiol-17ß. It is suggested that estradiol-17ß, probably secreted from ovarian follicle cells, induces Vg synthesis in the ovary as a Vg-stimuating ovarian hormone in penaeid shrimp (Fig. 1).

SEROTONIN STIMULATES THE RELEASE OF VITELLOGENESIS-STIMULATING HORMONE SECRETED FROM THE THORACIC GANGLION

Vg synthesis in ovarian pieces incubated with thoracic ganglion pieces prepared from vitellogenic females can be stimulated by serotonin (Yano & Itakura, unpublished data). However, serotonin was not effective in stimulating Vg synthesis in ovarian pieces incubated without thoracic ganglion pieces. Serotonin is found widely distributed throughout the nervous system in decapod crustaceans (Fingerman et al. 1994). These facts suggest that the release of vitellogenesis-stimulating hormone from the thoracic ganglion may be induced by serotonin present in the central nervous system. Therefore, neurohormonal serotonin is nominated as a vitellogenesis-stimulating hormone-releasing hormone (VSH-RH) responsible for ovarian Vg synthesis in penaeid shrimp.

LITERATURE CITED

Couch EF, Hagino N, Lee JW (1987) Changes in estradiol and progesterone immunoreactivity in tissues of the lobster (*Homarus americanus*) with developing and immature ovaries. Comp Biochem Physiol 87:765-770

Faires NJ, Evershed RP, Quinlan PT, Goad LJ (1989) Detection of unconjugated and conjugated steroids in the ovary, egg, and hemolymph of the Decapod crustacean, *Nephrops norvegicus*. Gen Comp Endocrinol 4:199-208

Fingerman M, Nagabhushanam R, Sarojini R, Reddy PS (1994) Biogenic amines in crustaceans: Identification, localization, and roles. J Crust Biol 14: 413-437

Jeng SS, Wan WCM, Chang CF (1978) Existence of an estrogen-like compound in the ovary of the shrimp *Parapenaeus fissurus*. Gen Comp Endocrinol 36:211-214

Kanazawa A, Teshima S (1971) In vivo conversion of cholesterol to steroid hormones in the spiny lobster, *Panulirus japonicus*. Bull Jpn Soc Sci Fish 37:891-898

Panouse JB (1943) Influence de l'ablation du predoncule sur la croissance de l'ovaire chez la crevette Leander serratus. C R Acad Sci , Paris 217: 553-555.

Panouse JB (1944) L'action de la glande du sinus sur l'ovaire chez la crevette Leander. C R Acad Sci, Paris 218: 293-294.

Teshima S, kanazawa A (1971) Bioconversion of progesterone by the ovaries of crab, *Portunus trituberculatus*. Gen Comp Endocrinol 17:152-157

Yano I (1987) Effect of 17b-hydroxy-progesterone on vitellogenin secretion in kuruma prawn, *Penaeus japonicus*. Aquaculture 61:49-57.

Yano I (1988) Oocyte development in the kuruma prawn *Penaeus japonicus*. Mar Biol 99:547-553

Yano I (1992) Hormonal control of reproductive maturation in penaeid shrimp. NOAA Tech Rep NMFS 106:7-14

Yano I (1993) Ultraintensive culture and maturation in captivity of penaeid shrimp. In: McVey JP (ed). Mariculture, vol 1. CRC Press, Boca Raton, pp 289-313

Yano I (1995) Final oocyte maturation, spawning and mating in penaeid shrimp. J Exp Mar Biol Ecol 193: 113-118

Yano I, Chinzei Y (1987) Ovary is the site of vitellogenin synthesis in kuruma prawn, *Penaeus japonicus*. Comp Biochem Physiol 86:213-218

Yano I, Tsukimura B, Sweeney JN, Wyban JA (1988) Induced ovarian maturation of *Penaeus vannamei* by implantation of lobster ganglion. J World Aquacult Soc 19:204-209

Yano I, Krol RM, Overstreet RM, Hawkins WE (1996) Route of egg yolk protein uptake in the oocytes of kuruma prawn, *Penaeus japonicus*. Mar Biol 125: 773-781

Effect of Methyltestosterone and 17α-hydroxyprogesterone on Spermatogenesis in the Black Tiger Shrimp, *Penaeus monodon* Fab.

***R. Yashiro, P. Na-anant, V. Dumchum**

Shrimp Culture Research Institute, Tasa-annt, Songkhla, Thailand

ABSTRACT: Methyltestosterone (MT) and 17α-hydroxyprogesterone(17α-HP) were injected into the abdominal muscle of male *Penaeus monodon* in two experiments at various concentrations (100 and 200ng/g BW in the first and 50 and 100ng/g BW in the second). The shrimp used were earthen pond reared (260 days old) and had an average body weight of 95.6 ±11.6 g. The sperm sacs at the bases of the 5th walking legs were checked at the beginning and removed prior to hormone injection. After injection, they were checked every 7-10 days for a total period of 40 days. Three developmental definitions were set:- 1) Productive : both sperm sacs with full sperm masses (whitish) or one sperm sac full and one half-full. 2) Semi-productive: both sperm sacs half-filled with sperm mass or one full and one empty (transparent). 3) Non-productive: both sperm sacs empty or one side half-filled and one side empty. The results from the first experiment showed that 100% productive sperm sacs were obtained by day 25 after injection with MT 100ng/g BW. By contrast, 100% productive sperm sacs were obtained by day 33 after injection with MT 200ng/g BW, 17α-HP 200ng/g BW and control solution (a mixture of distilled water and ethanol). The results from the second experiment showed that 100% productive sperm sacs were obtained by day 22 after injection with MT 100ng/g BW. On the same day, the other treatments (MT 50ng/g BW, 17α-HP 50, 100ng/gBW and control solution) gave 75%, 50%, 50% and 20% productive sperm sacs, respectively. Thus, MT 100ng/g BW was the best among all treatments for enhancing spermatogenesis of male black tiger shrimp, yielding full sacs from empty sacs within 22-25 days. The sperm sacs in the productive group had an average weight of 45.8 ±17.8 mg and contained 8.17 ± 13.62 x 10^6 cells/ml.

KEY WORDS: *Penaeus monodon*, spermatogenesis, steroid hormone

INTRODUCTION

Steroid hormone levels in crustaceans have been studied and reported for many species such as the American lobster *Homarus americanus* (Burns *et al*. 1984, Couch *et al*. 1987) brine shrimp *Artemia spp*. (Van Beek & De Loof 1988), the crab *Cacinus mianus* (Hazel 1986), the crayfish *Astacus leptodactylus* (Ollevier *et al.* 1986) and the black tiger shrimp *Penaeus monodon* (Fairs *et al*. 1990, Yashiro & Chaisriha 1991). Testosterone levels were significantly higher in mature male *P. monodon* than in females from all maturation stages. Progesterone was also reported to play an important role in ovarian maturation of female *P. monodon* (Yashiro & Chaisriha 1991). 17α-hydroxyprogesterone (17α-HP) has been shown to promote ovarian maturation in penaeid shrimp (Kulkani *et al.* 1979, Nagabhushanum *et al.* 1980, Yano 1985, 1987, Yashiro 1992). Therefore, there is a possibility for use of methyltestosterone (MT) and 17α-HP for enhancing spermatogenesis in mature male penaeid shrimp. These experiments aimed to test the effect of MT and 17α-HP on spermatogenesis of *P. monodon* from earthen pond culture by examination of the degree of development of external male reproductive structures. This can be achieved by observing the enlarged distal vas deferens or terminal ampoules located at the bases of the fifth pair of periopods.

MATERIALS AND METHODS

Experimental shrimp and facilities

Male *P. monodon* about 9 months or 260 days old from earthen culture ponds were used for the experiments. Their average body weight was 95.6±11.6g and average body length was 18.6±0.73cm. Two experiments were carried out. The first experiment tested 50 and 100ng/g-body weight over a total period of 53 days. The second experiment tested 100 and 200ng/g-body weight over a total period of 40 days. The sperm sacs at the bases of the 5th periopods (Motoh 1979) were checked at the beginning of experiment and extracted prior injection of the hormone. They were then checked every 7-10 days after injection. There were 5-10 shrimp in each treatment and control group. The percentage productivity from each group was recorded and compared statistically. Weight of extracted sperm sacs was also recorded and compared.

Criteria for examination of spermatogenesis

Three criteria for were set examination of sperm sacs at the terminal ampoule (Fig. 1). These were 1) productive with both sperm sacs full (i.e., with a full, whitish sperm mass) or one sperm sac full and the other half-full, 2) semi-productive with both sperm sacs half-full or one full and the other empty (transparent), 3) non-productive with both sperm sacs empty or one half-full and the other empty.

Yashiro R, Na-anant P, Dumchum V (1998) Effect of methyltestosterone and 17α-hydroxy-progesterone on spermatogenesis in the black tiger shrimp, *Penaeus monodon* Fab.*In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: nicas@t-rex.hatyai.inet.co.th

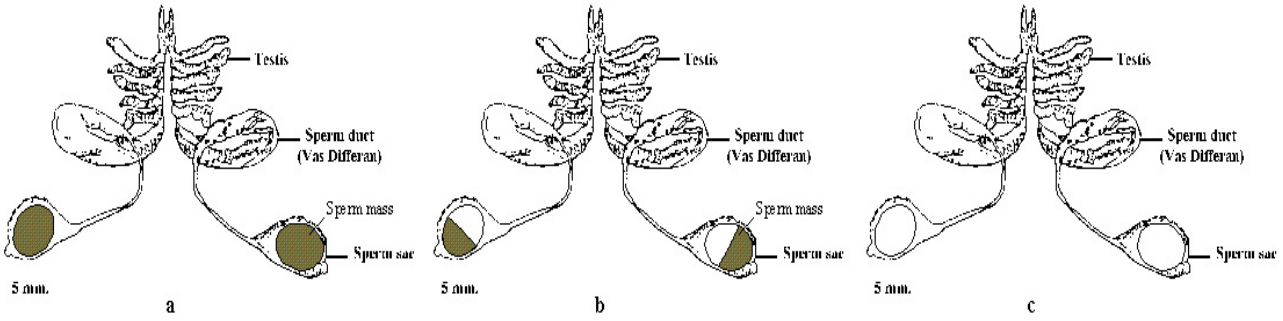


Figure 1. Male reproductive system of *P. monodon.* The sperm mass in the sacs was used for determination of productivity degree. a. productive, b. semi-productive and c. non-productive (Modified from Motoh 1979).

Hormone preparation and administration

MT and 17-aHP were prepared in the same way by dissolving in ethanol first followed by dilution with distilled water. The ratio of ethanol: distilled water was 1:4 in the first experiment and 1:8 in the second experiment. Hormone was prepared immediately prior to use and injected intramuscularly between the first and the second abdominal segments using a G23 needle. The needle had to be carefully inserted under the exoskeleton before injection into the muscle and the hormone mixture had to be slowly released.

Experimental design

The experiment employed a complete randomized design (CRD) to compare the effect of MT and 17α-HP at 50, 100, 200ng/g body weight to the untreated control shrimp for spermatogenesis of male *P. monodon* broodstock reared in earthen ponds. The percentages of productivity from each experiment was recorded and means were compared using a one way analysis of variance (ANOVA). Mean percentages of sperm sac productivity from groups that showed significant differences ($P<0.05$) were compared using Duncan's new multiple range tests.

RESULTS

Effect of hormones on sperm sac productivity

The results from the first experiment showed that 100% productive sperm sacs were obtained by the 25th day after injection of MT at 100ng/g BW. With MT 200ng/g BW, 17α-HP 200ng/g BW and control treatment (i.e., injected with a mixture of distilled water and ethanol), 100% productive sperm sacs were obtained by the 33rd day after injection. The results from injection of MT 100ng and MT and 17α-HP 200ng/g BW were not significantly different ($P>0.05$), but the treatment with MT 100ng/g BW gave 100% productivity sooner. Comparison of productivity over time for each group revealed that productivity tended to increase with increasing time regardless of the treatment. The percentage of productivity before treatment was comparable to 19, 25 and 33 days for the respective treatments. The productivity on day eight (Table 1) was significantly low due to extraction of sperm sacs before treatment.

The results from the second experiment showed that, 100% productive sperm sacs were obtained on the 22nd day after injection with MT 100ng/g BW. The other treatments

Table 1. Percentage productivity for each group from 2 experiments on the effect of methyltestosterone (MT) and 17α-hydroxyprogesterone (17α-HP) on spermatogenesis in male *Penaeus monodon* (nd = no data, day –1 = day before extraction of sperm sacs) Numbers in the same row with the same superscript letter were not significantly different ($P>0.05$).

EXPERIMENT 1	**Period (Days post injection)**						
Treatment	-1	0	8	19	25	33	40
MT-100 ng/gBW	80.0 ^{ab}	0	44.4 ^{b}	75.0 ^{ab}	100.0 ^{a}	85.7 ^{a}	100.0 ^{a}
MT-200 ng/gBW	44.4 ^{c}	0	50.0 ^{b}	66.7 ^{ab}	83.3 ^{a}	100.0 ^{a}	100.0 ^{a}
17α-HP 200 ng/gBW	88.9 ^{a}	0	12.5 ^{d}	83.3 ^{ab}	83.3 ^{a}	100.0 ^{a}	100.0 ^{a}
Ethanol+distilled water	100.0 ^{a}	0	28.6 ^{bd}	83.3 ^{ab}	80.0 ^{a}	100.0 ^{a}	66.7 ^{a}
EXPERIMENT 2	**Period (Days post injection)**						
Treatment	-1	0	10	22	36	53	
MT-50 ng/gBW	50.0	0	25.0	75.0	66.7	0.0	
MT-100 ng/gBW	75.0	0	25.0	100.0	50.0	100.0	
17α-HP 50 ng/gBW	75.0	0	0.0	50.0	33.3	nd	
17α-HP 100 ng/gBW	75.0	0	50.0	50.0	75.0	66.7	
Distilled water	50.0	0	25.0	20.0	nd	nd	

Table 2. Percentage of semi-productive shrimp from each group in 2 experiments on the effect of methyltestosterone (MT) and 17α-hydroxyprogesterone (17α-HP) on spermatogenesis of male *Penaeus monodon* (nd = no data, day –1 = before extraction of sperm sacs).

EXPERIMENT 1	**Period (Days post injection)**						
Treatment	-1	0	8	19	25	33	40
MT-100 ng/gBW	10.0	0	33.3	25.0	0.0	14.3	0.0
MT-200 ng/gBW	55.6	0	50.0	33.3	0.0	0.0	0.0
17α-HP 200 ng/gBW	11.1	0	37.5	16.7	16.7	0.0	0.0
Ethanol+distilled water	0.0	0	0.0	0.0	20.0	0.0	33.3
EXPERIMENT 2	**Period (Days post injection)**						
Treatment	-1	0	10	22	36	53	
MT-50 ng/gBW	50.0	0	25.0	25.0	33.3	0.0	
MT-100 ng/gBW	25.0	0	0.0	0.0	25.0	0.0	
17α-HP 50 ng/gBW	25.0	0	0.0	50.0	66.7	0.0	
17α-HP 100 ng/gBW	25.0	0	25.0	50.0	25.0	0.0	
Distilled water	50.0	0	0.0	40.0	0.0	0.0	

Table 3. Percentage of non-productive shrimp from each group in 2 experiments on the effect of methyltestosterone (MT) and 17α-hydroxyprogesterone (17α-HP) on spermatogenesis of male *Penaeus monodon* (nd = no data, day –1 = before extraction of sperm sacs).

EXPERIMENT 1	**Period (Days post injection)**						
Treatment	-1	0	8	19	25	33	40
MT-100 ng/gBW	10.0	0	22.2	0.0	0.0	0.0	0.0
MT-200 ng/gBW	0.0	0	0.0	0.0	16.7	0.0	0.0
17α-HP 200 ng/gBW	0.0	0	50.0	0.0	0.0	0.0	0.0
Ethanol+distilled water	0.0	0	71.4	0.0	0.0	0.0	0.0
EXPERIMENT 2	**Period (Days post injection)**						
Treatment	-1	0	10	22	36	53	
MT-50 ng/gBW	0.0	0	50.0	0.0	0.0	100.0	
MT-100 ng/gBW	0.0	0	75.0	0.0	25.0	0.0	
17α-HP 50 ng/gBW	0.0	0	100.0	0.0	0.0	0.0	
17α-HP 100 ng/gBW	0.0	0	25.0	0.0	0.0	33.3	
Distilled water	0.0	0	75.0	40.0	0.0	0.0	

(i.e., MT 50ng/g BW, 17α-HP 50 and 100ng/g BW and the control without hormone) gave 75%, 50%, 50% and 20% productivity, respectively, on the same checking date. The percentage of semi-productive shrimp before hormone injection seemed to be higher than it was after the treatment had begun (Table 2) which opposite to the non-productive group (Table 3).

Weight of sperm sacs and number of sperm contained

The sperm sacs in the productive group had an average weight of 45.8 ±17.8 mg and 8.17 ± 13.62 x 10^6 cells/ml and over 90% was alive cell.

DISCUSSION AND CONCLUSIONS

The steroid hormones MT at 100ng/g and 200ng/g BW and 17α-HP at 200ng/g BW gave no significantly different percentages of productive shrimp at any date post injection. However, the best result for enhancing spermatogenesis of male black tiger shrimp from empty to full sacs within 22-25 days seemed to be MT at 100ng/g BW. In female *P. monodon* and other penaeid shrimp, the steroid hormones progesterone and 17α-hydroxyprogesterone were reported to play important roles in promotion of ovarian maturation (Kulkani *et al.* 1979, Nagabhushanum *et al.* 1980, Yano 1985, 1987, Yashiro & Chaisriha 1991, Yashiro 1992). The results from the experiments here showed that MT has some effect for enhancing spermatogenesis in male *P. monodon*. Suitable doses of MT for this stimulation should be between 50-100 ng/g BW. The effect of MT seems to be more positive than that of 17-aHP. However, 17α-HP at 200 ng/g BW seemed to be more effective than lower doses. MT has also been reported to alter the ratio of female: male *P. monodon* from 1:1 to 1:1.5 when feed to PL_{15} for 126 days (Yashiro *et al.* 1995). The average weight of sperm sacs in the productive

group was 45.8±17.8 mg with an average sperm count of 8.17±13.62 x 10^6 cells/ml. This did not differ much from 13 month old and wild males (Yashiro & Charoensri 1996).

Primavera (1978) reported the presence of mature sperm in 10 month old, 40 g males from both ponds and the wild. Male shrimp in our experiments were cultured in earthen ponds for a total of 260 days or about 9 months. Gonadal maturation in male penaeid shrimp is indicated by the presence of fully developed spermatozoa with spikes (Clark *et al. 1*973), as observed with a microscope. In our observation of the sperm sacs at the base of the fifth pair of periopods, shrimp in the productive and semi-productive groups (two of our defined stages) had mature spermatozoa with spikes.

LITERATURE CITED

Burns BG, Saugalang GB, Freeman HC, Mc Menemy MB (1984) Isolation and identification of testosterone from the serum and testes of the American lobster (*Homarus america- nus)*. Gen Comp Endocrinol 54:429-432

Couch ER, Hagino N, Lee JW (1987) Changes in estradiol and progesterone immunoreactivity of the lobster *Homarus americanus* with developing and immature ovaries. Comp Biochem Physiol 85A: 765-770

Fairs NJ, Quinlan PT, Goad LJ (1990) Changes in ovarian unconjugated and conjugated steroid titers during vitellogenesis in *Penaeus monodon* Aquaculture 89:83-99

Fingerman M (1987) The Endocrine Mechanisms of Crustaceans J Crust Biol 7(1):1-24

Hazel CM (1986) Steroidogenesis in the female crab *Cacinus maenas* Ph.D. Thesis University of Liverpool. pp 81-121

Kulkarni GK, Nagabhushanam R, Joshi PK (1979) Effect of progesterone on ovarian maturation in a marine penaeid prawn *Parapenaeopsis hardwickii.* Ind J Exp Biol 17:986-987

Motoh H (1981) Studies on the fisheries biology of the giant tiger prawn *Penaeus monodon* in the Philippines. Tech Rep No 7, SEAFDEC Aquaculture Dept., 128 pp

Motoh H (1979) Preliminary study on the internal genital organ of the giant tiger prawn, *Penaeus monodon.* SEAFDEC, Quarterly Res Rpt 3(1):15-18

Nagabhushanam R, Joshi PK, Kulkarni GK (1980) Induced spawning in the prawn *Parapenaeopsis stylifera* (H Milne Edwards) using a steroid hormone 1-hydroxyprogesterone. Ind J Mar Sci 9:227

Primavera JH (1978) Induced maturation and spawning in five-month-old *Penaeus monodon* Fabricius by eyestalk ablation. Aquaculture 13: 355-359

Primavera JH (1984) A review of maturation and rematuration in closed thelycum penaeids. In: Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps. Iloilo City Philippines. P. 47-64

Sarojini R, Jayalakshimi K, Sambashivarao S (1986) Effect of external steroids on ovarian development in freshwater prawn *Macrobrachium lamerrii.* J Adv Zool 7:50-53.

Van Beek E, De Loof A (1988) Radioimmunological determination of concentration of sex C_{12}, C_{19} and C_{18} steroids during the reproductive cycle of *Artemia* sp Comp Biochem Physilo. 89A:595-599

Yano I (1985) Induced ovarian Maturation and spawning in greasyback shrimp *Metapenaeus ensis* by progesterone. Aquaculture. 47:223-229

Yano I (1987) Effect of 17α-hydroxyprogesterone on Vitellogenin secretion in kuruma prawn *Penaeus japonicus*. Aquaculture. 61:49-57

Yashiro R, Chaisriha U (1991) Effect of Injection Steroid Hormones on Gonad Development of *Penaeus monodon* I. Sex Steroid Levels in Broodstock of *Penaeus monodon*. Technical Paper No. 9/1991 National Institute of Coastal Aquaculture Songkhla Thailand. (In Thai).

Yashiro R (1992) Effect of Injection Steroid Hormones on Gonad Development of *Penaeus monodon* II. Effects of Some Exogenous Hormones and Thoracic Ganglion on Ovarian Maturation of *Penaeus monodon.* Technical Paper No. 15/1992 National Institute of Coastal Aquaculture Songkhla Thailand. (in Thai).

Yashiro R, Onlamai P, Charoensri C (1995) Effects of sex sterrid on variation o f sex ratio in *P . monodon*. P.568–570 In : Proceeding of the seminar on fisheries 1995. Department of Fisheries, Thailand

Yashiro R, Charoensri C (1996) Morphology, weight, number and development of spermatozoa and sperm sac in *Penaeus monodon* Fab. In: Book of Abstract, Proceeding of the seminar on fisheries 1995. Department of Fisheries, Thailand

The Effects of Cryoprotectants, Chilling and Freezing on *Penaeus esculentus* Embryos and Nauplii

***N.P. Preston, F.E. Coman**

CSIRO Division of Marine Research, P.O. Box 120, Cleveland, Qld 4163, Australia.

ABSTRACT: The responses of *Penaeus esculentus* embryos to chilling, to a range of penetrating and non-penetrating cryoprotectants and various freeze-thaw protocols were investigated. The results revealed several barriers to the successful cryopreservation of penaeid prawn embryos. Both early-stage and late-stage embryos were very sensitive to chilling and to osmotic change: their tolerance of -1°C was seldom more than 20 minutes. Rapid exposure to hyperosmotic or hypo-osmotic conditions was lethal to both stages. Tolerance to cryoprotectants varied according to the molecular weight and concentration of the cryoprotectant. All the cryoprotectants tested were non-toxic at low concentrations, but none entirely prevented the formation of ice. Our results have confirmed previous observations that, in the absence of ice, prawn embryos and nauplii can temporarily withstand sub-zero temperatures. However, the formation of intracellular or extracellular ice was lethal to embryos at every cryoprotectant/freeze-thaw protocol tested. We suggest that the sensitivity of prawn embryos to ice formation is partly due to their high yolk content.

KEY WORDS: penaeid prawn embryos, cryopreservation, cryoprotectants, chilling, freeze-thaw protocols.

INTRODUCTION

Cryopreservation of the embryos of penaeid prawns would be of considerable value to the prawn farming industry and to researchers. The ability to stockpile and store embryos for extended periods would make them available on demand. Cryopreservation would also provide the means to conserve natural gene pools and to store, indefinitely, such desirable traits as rapid growth and disease resistance. Because of the poor viability of prawn tissue cultures, cryopreserved embryos could provide an alternative source of cells for testing responses to toxins or pathogens.

Compared to the long-established success with the cryopreservation of mammalian embryos (e.g. Whittingham et al. 1972) and the more recent ones for insect embryos (Mazur et al. 1992), efforts to cryopreserve the embryos of marine invertebrates have been less successful. Limited tolerance to freezing has been demonstrated in late-stage embryos of mussels (Toledo et al. 1989) and early-stage embryos of oysters (Renard 1991). While late-stage embryos of sea urchins can survive freezing, in the presence of suitable cryoprotectants, their post-thaw development is often abnormal (Asahina & Takahashi 1978).

Cryopreservation has been achieved for some aquatic invertebrates, such as rotifers, which are naturally able to withstand desiccation (King et al. 1983; Toledo & Kurokura 1990; Toledo et al. 1991). Of the marine arthropods, only *Artemia* larvae have been reported to tolerate cryopreservation (Baust & Lawrence 1980), but *Artemia* cysts provide a simpler method of storing embryos.

Previous studies have shown that penaeid prawn embryos can tolerate exposure to some cryoprotectants (Robertson & Lawrence 1987). It has also been demonstrated that, in the presence of suitable cryprotectants, embryos of *Penaeus semisulcatus* can tolerate short term exposure (up to 6 h) to temperatures as low as –10 °C (Diwan & Kandasami 1997) and nauplli of *P. indicus* can briefly tolerate exposure to –40 °C (Subramoiam & Newton 1993, Subramoniam 1994). However, the capacity to successfully cryopreserve penaeid embryos or nauplii for subsequent use in research or commerce has yet to be demonstrated.

The objective of our study was to examine the potential for successful cryopreservation techniques that would permit storage of penaeid prawn embryos or larvae for extended periods for subsequent use in research or commerce. In order to do this we followed the general research protocol suggested by Leopold (1992) and examined responses to chilling, to different solute concentrations, and to exposure to various cryoprotectants and freeze-thaw protocols for embryos and nauplii of the subtropical prawn *P. esculentus*.

MATERIALS AND METHODS

Shrimp and broodstock

Adult *P. esculentus* were trawled from Albatross Bay, Gulf of Carpentaria, Australia and air-freighted to the Marine Laboratory in Cleveland, Brisbane. They completed their ovarian development in 10,000 L maturation tanks (temperature range 25°C to 28°C and salinity range 30 to 32 ppt),

Preston NP, Coman FE (1998) The effects of cryoprotectants, chilling and freezing on *Penaeus esculentus* embryos and nauplii. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: nigel.preston@marine.csiro.au

and were then transferred to individual 90 L spawning tanks maintained at the same temperature and salinity as the maturation tanks. After spawning, embryos or nauplii were collected from the spawning tanks by siphoning into 2 L glass beakers.

Chill tolerance

The effects of chill tolerance were examined at two stages of embryo development: early embryos (1 to 2 h after spawning, at the two-cell stage) and late embryos (6 to 8 h after spawning, at the multicellular stage with limb development well advanced). The effects of chilling on hatching success was determined by exposing embryos from three separate single spawnings to seawater pre-cooled to 16°C, 11°C, 6°C and 1°C. At each of these temperatures the periods of exposure tested were: 0.5, 1, 1.5, 2, 2.5 and 3 h. For each temperature, three replicates of approximately 100 embryos were maintained in separate beakers of 500 mL of seawater. After the required exposure time, the embryos were returned directly to 28°C and incubated through to hatching. Tolerance of sub-zero chilling was examined by pipetting embryos directly into seawater pre-chilled to -1°C. The embryos were incubated for 10, 20, 30, 40 or 50 min and then warmed rapidly (80°C /min) by returning them directly to ambient spawning temperatures.

Changes in solute concentration

The effects of hyperosmotic and hypo-osmotic solutions were determined by measuring the changes in the diameter of the hatching membrane of two-celled embryos and the hatch rates of two-cell and multicell embryos in response to changes in salinity. The diameters were measured using an ocular micrometer and camera lucida. The salinity treatments were: 0, 16, 32, 48, 63 and 126 ppt corresponding to 0, 0.25, 0.5, 0.75, 1.0 and 2.0 molar solutions of filtered (1 mm) natural seawater. For each treatment there were three replicates of approximately 100 embryos each in 500 mL of seawater. After exposure to one of the salinities for 20 min, subsets of 10 embryos from each treatment were removed for measurement; the remainder were returned to their pre-treatment salinity (30.5 ppt). Twenty minutes later a further subset of 10 embryos were removed from each treatment

Table 1. The response of embryos (E) and nauplii (N) of *Penaeus esculentus* to cryoprotectants and temperature. + denotes successful hatching of embryos or successful development of nauplii to protozoea 1. 0 denotes zero hatching or development of nauplii. n denotes no sample.

Cryoprotectant	M.W.	Ambient 28°C		Chilled -1°C		Super cooled -30°C		Frozen -196°C	
		E	N	E	N	E	N	E	N
Penetrating compounds									
Methanol	32	+	+	+	+	+	+	0	0
Ethylene glycol	62	+	+	+	+	+	+	0	0
Propylene glycol (P.G.)	76	+	+	+	+	+	+	0	0
Dimethyl sulphoxide (DMSO)	78	+	+	+	+	+	+	0	0
Glycerol	92	+	+	+	+	+	+	0	0
Acetamide	59	0	0	n	n	n	n	n	n
Non-penetrating compounds									
L- Proline	115	+	+	+	+	0	0	0	0
Galactose	180	+	+	+	+	0	0	0	0
Glucose	181	+	+	+	+	0	0	0	0
Sucrose	342	+	+	+	+	0	0	0	0
Trehalose	342	+	+	+	+	0	0	0	0
Polyethylene glycol (P.E.G.)	6000	+	+	+	+	0	0	0	0
Polyvinylpyrrolidone (P.V.P.)	1000	+	+	+	+	0	0	0	0
Combined compounds									
D.M.S.O. & P.V.P.	78+1000	+	+	+	+	0	0	0	0
D.M.S.O. & P.E.G.	78+6000	+	+	+	+	0	0	0	0
D.M.S.O. & Sucrose	78+342	+	+	+	+	0	0	0	0
Glycerol & Glucose	92+181	+	+	+	+	0	0	0	0
Glycerol & P.V.P.	92+1000	+	+	+	+	0	0	0	0
Glycerol & P.E.G.	92+6000	+	+	+	+	0	0	0	0
P.G. & P.V.P.	76+1000	+	+	+	+	0	0	0	0
P.G. & P.E.G.	76+6000	+	+	+	+	0	0	0	0
P.G. & Sucrose	76+342	+	+	+	+	0	0	0	0
D.M.S.O. & Proline	78+115	+	+	+	+	0	0	0	0
Vitrification compounds									
VSI (Steponkus et al., 1990)		0	0	0	0	0	0	0	0

and the diameter of their hatching membranes measured. The percentage of embryos that hatched successfully in each treatment was recorded.

Cryopreservation protocols

Initially we tested the tolerance of embryos and nauplii to a total of 6 low molecular weight (penetrating) cryoprotectants and 6 high molecular weight (non-penetrating) cryoprotectants, plus ten combinations each comprised of one penetrating and one non-penetrating cryoprotectant (Table 1). We also tested the tolerance of embryos and nauplii to one vitrification compound. The concentrations of penetrating compounds tested were 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 M. The concentrations of the non-penetrating compounds tested were 0.1 and 0.2 M for those with molecular weights less than 350, and 5% by volume for those with molecular weights greater than 350 (Table 1).

Cryoprotectants at concentrations that were not lethal to embryos or nauplii were then used in a second stage of screening in which embryos and nauplii were subjected to three different low temperature regimes; chilling to -1°C, supercooling (-30°C without ice formation) and freezing (-196°C with ice formed). The protocol used for these was the relatively slow freezing method of Toledo et al. (1989). Embryos or nauplii were added to cryoprotectants at ambient temperature (28°C). A programmable, controlled-rate freezing chamber (AGTEC - Embryo Freezer) was then used to lower the temperature using the method of Toledo et al. (1989). In this protocol temperature was reduced to -1°C at 5°C /min; held for 5 minutes; cooled to -80°C at 0.5 °C / min; held for 5 min. The embryos were then plunged into liquid nitrogen (-196°C) for 30 min. Subsets (vials) of embryos or nauplii were removed when the temperature in the freezer reached –1°C, -30°C and –196°C. The vials were then warmed rapidly by immersing them in seawater at 28°C. The embryos or nauplii were then flushed with seawater at 28°C to remove any cryoprotectant and the subsequent hatch rates/survival recorded.

Based on the results of this initial screening (see results section) we selected subsets of cryoprotectants and concentrations together with two freeze/thaw protocols (slow and rapid) for further investigation.

Cryoprotectants

The subset of the cryoprotectants selected for further testing are listed in Table 2 in the results section. In these experiments early stage embryos (1 to 2 hours after spawning) and late stage embryos (6 to 8 hours after spawning) were exposed for 20 minutes to the various concentrations of cryoprotectant at 28°C. There were three replicates for each treatment, each with 100 embryos in 100 mL of cryoprotectant solution. After exposure, the embryos were flushed immediately with seawater at 28°C and their subsequent hatch rates recorded in relation to the hatch rates of controls.

The rate of cooling during freezing

The response of embryos to variations in freeze/thaw protocols was tested in a programmable, controlled-rate freezing chamber (AGTEC - Embryo Freezer). Two freeze-thaw protocols were tested for each type and concentration of cryoprotectant. The experiments at each cooling rate were repeated three times.

The first protocol was the relatively slow freezing method of Toledo et al. (1989) in which the temperature was reduced to -5°C at 5°C /min; held for 5 minutes; cooled to -80°C at 0.5 °C /min; held for 5 min. The embryos are then plunged into liquid nitrogen (-196°C) for 30 min and thawed rapidly by immersing the cryovials in seawater at 28°C. The thawed samples are then flushed with seawater at 28°C to remove any cryoprotectant.

The second protocol was the relatively rapid freezing method described by Asahina and Takahashi (1978). With this protocol the cooling rate was 8°C /min from 28°C to -80°C. The embryos were then held at -80°C for 5 min before being plunged into liquid nitrogen for 5 min. After freezing, the embryos were thawed by warming to 28°C at 35°C /min. The cryovials were then flushed with seawater at 28°C.

The temperature and time at which freezing occurred were determined with a cryomicroscope which permitted direct observations of embryos or nauplii during the experiments (details about the construction of the cryomicroscope are available from the authors). Damage to embryos was recorded on photomicrographs. Post-thaw hatch rates were recorded for each freeze-thaw protocol.

Statistical analyses

The results of the experiments were analysed using analysis of variance (ANOVA). Cochran's test was used to determine the homogeneity of variances in these analyses. Where significant interaction terms were detected, further analysis was done using the Student-Newman-Keuls (SNK) procedure (Winer 1971).

RESULTS

Chill tolerance

The results of chill tolerance experiments demonstrated that *P. esculentus* embryos were very sensitive to chilling. Both early-stage and late-stage embryos withstood exposure

Table 2. Molecular weight (MW) and concentration (Molarity) of cryoprotectants tested. The chemicals were diluted with seawater (35 ppt).

Compounds	MW	Concentration (M)				
Penetrating compounds						
Methanol	32	0.1	-	0.5	1.0	2.0
Ethylene glycol	62	0.1	-	0.5	1.0	2.0
Dimethyl Sulphoxide (DMSO)	78	0.1	-	0.5	1.0	2.0
Non-penetrating compounds						
L-Proline	115	0.1	0.2	0.5	-	-
Sucrose	342	0.1	0.2	0.5	-	-
Trehalose		0.1	0.2	0.5	-	-

to 16°C for 2 h; longer exposure resulted in poor hatching success (Fig. 1A,B). With each 5°C reduction in temperature below 16°C there was a significant reduction in hatching success. Exposure to any temperature below 16°C for 2 h was lethal to most embryos. At the lowest temperature tested (-1°C) 20 min was the limit for most embryos, irrespective of stage of development. Longer exposure resulted in a pronounced decline in hatch rates. However, late-stage embryos were significantly more tolerant to this temperature than early embryos (Fig. 1C, D).

Changes in solute concentration

Early-stage and late-stage embryos were very sensitive to changes in solute concentration (Table 3). There was no significant difference in the response of the two stages. Rapid exposure of embryos to concentrations above or below 31.6 ppt (0.5 M) were lethal. Exposure to these hyperosmotic or hypo-osmotic conditions produced rapid changes in the diameter of the hatching envelope, which was most pronounced when the embryos swelled in fresh water. On return to ambient salinity, the diameters all returned to approximately the same size except for those exposed to 31.6 ppt, which were larger.

Cryopreservation protocols

Among the penetrating cryoprotectants tested, embryos and nauplii did not tolerate acetamide at any temperature (Table 1). Otherwise embryos and nauplii survived chilling to –30°C in the presence of at least one tested concentration of methanol, ethylene glycol, propylene glycol, glycerol and dimethyl sulphoxide. However, at lower temperatures, when ice formation occurred, none of the embryos or nauplii survived.

Embryos and nauplii tolerated exposure to -1°C in the presence of at least one concentration of every non-penetrating compounds tested (Table 1). However no embryos or nauplii tolerated exposure to -30°C in the presence of any of these compounds. Similar results were obtained with the combinations of penetrating and non-penetrating compounds tested. Embryos and nauplii did not tolerate exposure to -30°C in presence of any combination of penetrating and non-penetrating compounds.

The vitrification compounds of Steponkus et al. (1990) were lethal to embryos and nauplii.

Cryoprotectants

Based on the results of this initial screening, we selected the cryoprotectants and concentrations shown in Table 2 for further testing. None of these cryoprotectants were lethal to embryos at the lower concentrations tested (Fig. 2). At 0.1

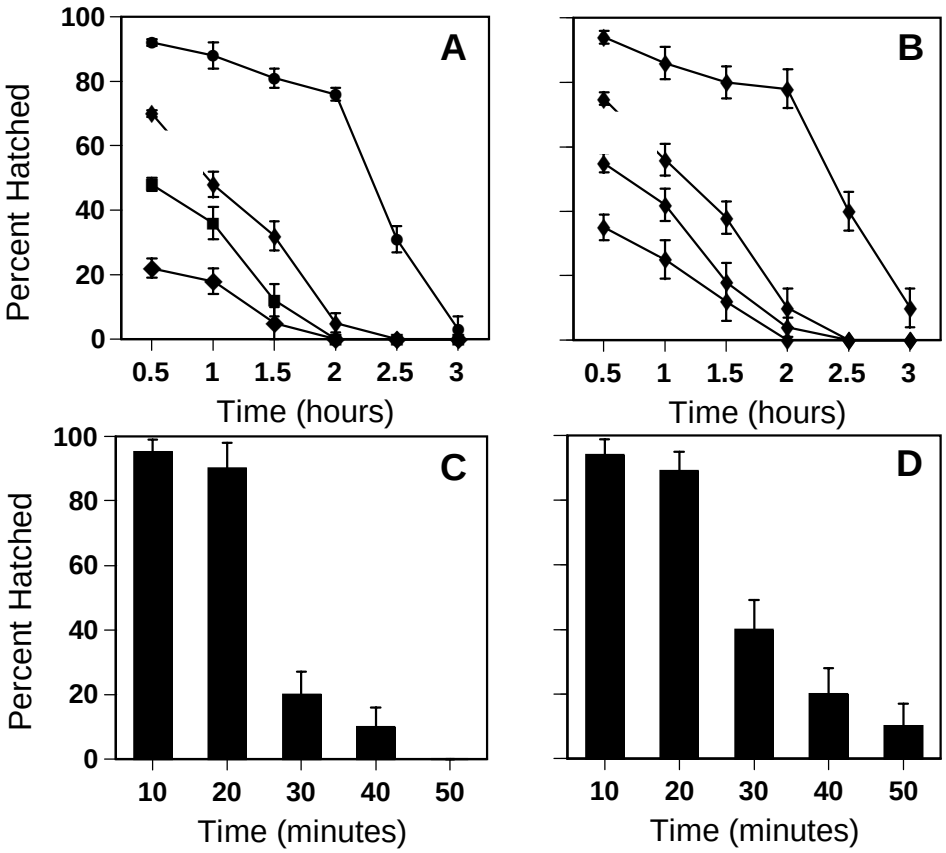


Figure 1. The effects of temperature and length of exposure on the percent hatching success of embryos of *Penaeus esculentus*. Hatch rates are shown for (A) early-stage and (B) late-stage embryos exposed to seawater pre-chilled to 16°C, 11°C, 6°C and 1°C; and (C) early-stage and (D) late-stage embryos exposed to seawater pre-chilled to -1°C.

and 0.5 M of the three penetrating cryoprotectants, the hatching success of both stages was not significantly different from seawater controls (mean hatch rate = 82%). However, at 1 or 2 M ethylene glycol and dimethyl sulphoxide gave significantly lower hatching success. Both stages of embryos were significantly more tolerant to methanol at 1 and 2 M, although late-stage embryos were significantly more tolerant to 2 M methanol than early-stage embryos.

Table 3. Changes in the mean diameter (mm ± s.e.) and hatch rates of two-celled and late-stage embryos of *Penaeus esculentus* in response to changes in salinity. The initial mean diameter of embryos at the ambient salinity (30.5 ppt) was 0.320 (± 0.002) mm. The results are shown following exposure to each treatment and following the return of embryos to ambient salinity.

Salinity (ppt)	Molarity	Diameter after 20 minutes exposure	Diameter on return to ambient salinity	% hatched (s.e.)
0.0	0.0	0.361 (0.012)	0.317 (0.006)	0
15.8	0.25	0.336 (0.013)	0.316 (0.007)	0
31.6	0.50	0.319 (0.003)	0.320 (0.004)	85 (6.2)
47.4	0.75	0.313 (0.009)	0.316 (0007)	0
63.2	1.00	0.310 (0.016)	0.316 (0.006)	0
126.4	2.00	0.310 (0.020)	0.316 (0.008)	0

Tolerance to non-penetrating cryoprotectants varied with stage of embryonic development as well as the type and concentration of cryoprotectant (Fig. 2C, D). At the lowest concentration tested (0.1 M) early-stage embryos showed significantly greater tolerance to L-proline and sucrose than to trehalose. At higher concentrations (0.2, 0.5 M) early-stage embryos were significantly more tolerant of proline than either trehalose or sucrose. Late-stage embryos were significantly more tolerant than early-stage embryos to medium concentrations (0.2 M) of each of the penetrating cryoprotectants. At the highest concentration tested (0.5 M) very few early-stage or late-stage embryos survived exposure to sucrose or trehalose and there was no significant difference in the tolerance of the two stages. Both early- and late-stage embryos showed significantly higher tolerance to L-proline.

The rate of cooling (during freezing)

Neither of the freeze-thaw protocols, nor any of the types or concentrations of cryoprotectants used entirely prevented the formation of either intracellular or extracellular ice. With or without cryoprotectants, the relatively slow freezing protocol of Toledo et al. (1989) resulted in the formation of extracellular ice. The results were similar at the relatively higher cooling rate of Asahina and Takahashi (1978), but intracellular ice was also formed particularly at low cryoprotectant concentrations. In all cases the formation of extracellular or intracellular ice was lethal to embryos.

DISCUSSION

The results of this study support previous observations that penaeid prawn embryos and nauplii can tolerate exposure sub-zero temperatures. Subramoiam and Newton (1993) and Subramoniam (1994) observed motility of *P. indicus* nauplii after exposure to –40°C, although they did not report the duration of exposure, or whether nauplii were subsequently able to complete normal larval development. More recently, Diwan and Kandasami (1997) found that nauplii of *P. semisulcatus* tolerated exposure to -10°C for 6 h in the presence of dual cryoprotectants (DMSO + glycerol). However, it has yet to be demonstrated that embryos or nauplii can tolerate conditions that permit cryopreservation for any significant length of time beyond a few hours.

The results of our study showed that *P. esculentus* embryos only tolerated sub-zero temperatures in the absence of ice. The formation of intracellular or extracellular ice was lethal to embryos and nauplii at every cryoprotectant/freeze-thaw protocol that we tested. Our results indicate that the principal barriers to the successful cryopreservation of prawn embryos are similar to those encountered with *Drosophila* embryos (Mazur et al. 1992).

Early- and late-stage prawn embryos proved sensitive to chilling and extremely sensitive to osmotic change. This confirms previous studies demonstrating their low tolerance to changes in salinity (Preston 1985 a,b). Their sensitivity to chilling and osmotic change are major barriers the use of slow freezing techniques, although there is evidence that prawn embryos might have some cellular capacity to adapt to the latter (Preston 1985 b). Tolerance to changes in osmotic concentration might be improved if the changes were slow. Likewise, it is possible that the sensitivity of prawn embryos to chilling could be improved by acclimation. It would also be of interest to determine whether prawns have relatively chill-tolerant stages during their embryonic development, as do *Drosophila* embryos (Mazur et al. 1992).

One of the conventional approaches to cryopreservation is to cool cells sufficiently slowly for intracellular water to be removed by osmosis, thus reducing the amount of intracellular ice formed when the cells freeze (Ashwood-Smith 1986). But these slow cooling rates (<1°C/min) are lethal to early *Drosophila* embryos (Mazur et al. 1992) and to *P. esculentus* embryos, which appear to be more sensitive to chilling than *Drosophila* embryos.

One successful strategy to overcome chilling injury, at least in some cell types, is to cool and warm very rapidly (Fahy et al. 1984). This requires high concentrations of permeating cryoprotectants (5 to 9 molar) to induce vitrification and thus avoid the formation of intracellular ice (Fahy et al. 1984). We found that prawn embryos could not tolerate such high concentrations of cryoprotectants. For example, exposure of prawn embryos to a 2 molar concentration of ethylene glycol reduced hatching rates to <5% at the one-cell stage and <20% at the late embryo stage. By compari-

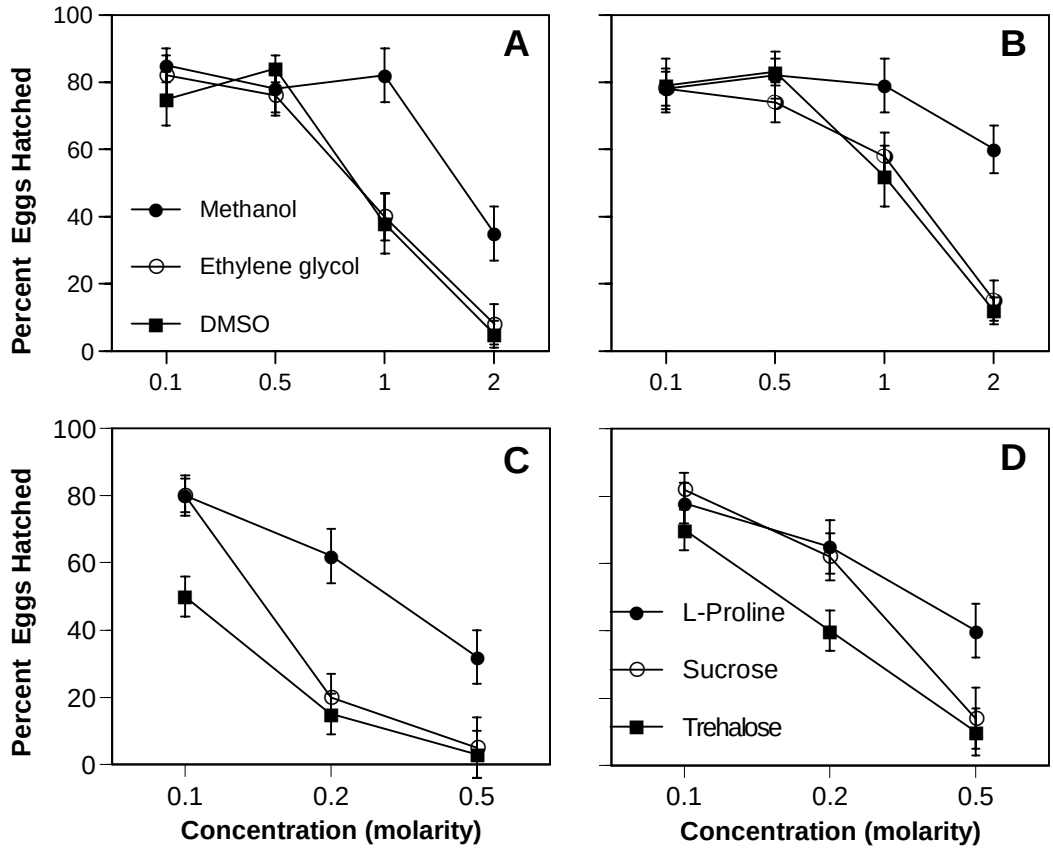


Figure 2. Percent hatching success for *Penaeus esculentus*. (A) early-stage and (B) late-stage embryos exposed to different concentrations of the penetrating cryoprotectants methanol, ethylene glycol and dimethylsulfhoxide (DMSO); and hatch rates of (C) early-stage and (D) late-stage embryos exposed to different concentrations of the non-penetrating cryoprotectants L-proline, sucrose and trehalose.

son, successful vitrification of *Drosophila* embryos requires a concentration of 8.5 molar ethylene glycol (Steponkus et al. 1990). Future attempts to develop a vitrification technique for prawn embryos will have to overcome their sensitivity to changes in solute concentration. It also remains to be determined whether the harmful effects of changes in solute concentration are due to electrolytes reaching critical levels (Lovelock 1953), osmotic shrinkage (Meryman 1974) or loss of plasma membrane integrity (Steponkus 1984).

In this study we tested a variety of penetrating and non-penetrating cryoprotectants. Penetrating compounds are generally used to protect cells during slow cooling and freezing. They act by reducing cell dehydration, maintaining electrolyte balance and increasing the amount of water remaining in extracellular spaces during freezing (Meryman 1971). Non-penetrating cryoprotectants such as sugars and amino acids are generally used to protect cells during rapid cryopreservation; they appear to protect plasma membrane structure under the stress of freezing and thawing (Rudolph & Crowe 1985).

Prawn embryos were able to tolerate up to a 2 molar level of penetrating cryoprotectants and submolar levels of non-penetrating cryoprotectants. However, we were unable to find any combination of cryoprotectant(s) and freeze/thaw regime that reduced the formation of intracellular or extracellular ice to sub-lethal levels.

The acute sensitivity of prawn embryos to the formation of ice may be due, in part, to their high content of yolk. The yolk provides nutrients for the growth and development of the non-feeding nauplius larvae. Lipovitellin, a major component of prawn egg yolk disassociates upon freezing (Lea & Hawke 1952). Lipid-protein complexes are thought to be particularly vulnerable to chilling and freezing because they are held together by weak associations rather than covalent bonding (Lovelock 1953).

There is some evidence that prawn embryos are less tolerant to chilling than nauplii (Subramoniam 1994; Diwan & Kandasami 1997) which supports the suggestion by Subramoniam (1994) that problems associated with the yolk content of embryos could be partly alleviated as yolk reserves are used up. In this respect the potential for the cryopreservation of post-hatch nauplii warrants further investigation.

In summary, we have demonstrated that the sensitivity of prawn embryos to the effects of chilling, changes in solute concentration, cryoprotectants and the formation ice present

formidable obstacles that will have to be overcome if cryopreservation is to be achieved. Whilst it is encouraging that similar problems have been overcome in *Drosophila*, the results of our study suggest that prawn embryos present an even more challenging task to cryobiologists.

Acknowledgments. This project was funded by the Rural Bank of Australia, the Australian Fisheries Research and Development Corporation (project 89/50) and a CSIRO postdoctoral fellowship. Bob Pendry constructed the cryomicroscope. Anne Leitch and Adrian Just provided technical assistance. Dr R.A. Leopold assisted in revising this manuscript. Our thanks to all.

LITERATURE CITED

Asahina E, Takahashi T (1978) Freezing tolerance in embryos and spermatozoa of the sea urchin. Cryobiol 15: 122-127

Ashwood-Smith MJ (1986) The cryopreservation of human embryos. Human Repro 1: 319-332

Baust JG, Lawrence AL (1980) Freezing tolerance in larval *Artemia*. In: Persoone G , Sorgeloos P, Roels O, Jaspers E (eds) The brine shrimp *Artemia*. Physiology, Biochemistry and Molecular Biology. Universal Press, Wetteren, Belgium. Vol 2: 115-122

Diwan AD, Kandasami K (1997) Freezing of viable embryos and larvae of marine shrimp, *Penaeus semisulcatus* de Haan. Aquaculture research 28: 947-950

Fahy GM, MacFarlane DR, Angell CA, Meryman HT (1984) Vitrification as an approach to cryopreservation. Cryobiol 21: 407-426

King CE, Berkeley Bayne H, Cannon TK, King AE (1983) Cryopreservation of monogonont rotifers. Hydrobiol 104: 85-88

Lea CH, Hawke JC (1952) The influence of water on the stability of lipovitellin and the effects of freezing and drying. Biochem J 52:105

Leopold RA (1992) Cryopreservation of Insect germplasm: cells, tissues and organisms. In: Lee RE, Denlinger D (eds), Insects at Low Temperature. Chapman and Hall, New York, pp. 379-407

Lovelock JE (1953) The mechanism of the cryoprotective action of glycerol against haemolysis by freezing and thawing. Biochim Biophys Acta 11: 28-36

Mazur P, Cole KW, Hall JW, Schreuders PD, Mahowald AP (1992) Cryobiological preservation of *Drosophila* embryos. Science 258: 1932-1935

Meryman HT (1971) Cryoprotective agents. Cryobiol 8: 173-183

Meryman HT (1974) Freezing injury and its prevention in living cells. Annu Rev Biophys 3: 341-363

Preston NP (1985a) The combined effects of temperature and salinity on hatching success and the survival, growth and development of the larval stages of *Metapenaeus bennettae* (Racek & Dall). J Exp Mar Biol Ecol 85: 57-74

Preston NP (1985b) The combined effects of temperature and salinity on survival and growth of larval *Penaeus plebejus*, *Metapenaeus macleayi* and *M. bennettae*. In: Rothlisberg PC, Hill BJ, Staples DJ (eds) Second Australian Prawn Seminar. NPS2, Cleveland, Queensland, Australia, pp. 125-136

Renard P (1991) Cooling and freezing tolerances in embryos of the Pacific oyster, *Crassostrea gigas*: methanol and sucrose effects. Aquaculture 92: 43-57

Rudolph AS, Crowe JH (1985) Membrane stabilisation during freezing: the role of two natural cryoprotectants, trehalose and proline. Cryobiol 22: 367-377

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Ann Rev Plant Physiol 35: 534-584

Steponkus PL, Myers SP, Lynch DV, Gardner L, Bronshteyn V, Leibo SP, Rall WF, Pitts RE, Linn TT, MacIntyre RJ (1990) Cryopreservation of *Drosophila melanogaster* embryos. Nature 345: 170-172

Subramoniam T (1994) Cryopreservation of crustacean gametes and embryos. Proceedings Indian National Science Academy B 60(3) 229-236

Subramoniam T, Newton SS (1993) Cryopreservation of the embryos of the penaeid prawn *Penaeus indicus*. Current Science 65: 176-177

Toledo JD, Kurokura H, Nakagawa H (1991) Cryopreservation of different strains of the euryhaline rotifer *Brachionus plicatilis*. Nippon Suisan Gakkaishi 57: 1347-1350

Toledo JD, Kurokura H (1990) Cryopreservation of the euryhaline rotifer *Brachionus plicatilis*. Aquaculture 91: 385-394

Toledo JD, Kurokura H, Kasahara S (1989) Preliminary studies on the cryopreservation of blue mussels. Nippon Suisan Gakkaishi 55: 1661

Whittingham DG, Leibo SP, Mazur P (1972) Survival of mouse embryos frozen to -196°C and -269°C. Science 178: 414

Winer BJ (1971) Statistical principles in experimental design, 2nd edn. McGraw-Hill, Kogskusha, Tokyo

Advances in Gene Mapping in Penaeid Shrimp

***S.S. Moore, S.A. Lehnert**

CSIRO, Tropical Agriculture, Molecular Animal Genetics Centre
Brisbane, Australia

ABSTRACT: PCR-based gene mapping technologies are being applied to *P. japonicus* and *P. monodon*. Genetic linkage maps based on amplified fragment length polymorphisms (AFLP) will enable quantitative trait loci (QTL) influencing economically important traits to be identified and marker assisted breeding programs developed. However, maps based on Type II anonymous markers will be specific to each species of shrimp. Type I anchor loci are being developed allowing integration of genetic maps. Such markers will facilitate transfer of information between species and open the possibility of comparative mapping with model organisms such as *Drosophila melanogaster*.

KEY WORDS: Penaeid shrimp, gene mapping, AFLP, microsatellite, comparative mapping

INTRODUCTION

Gene mapping technologies have developed rapidly over the past decade, to the point where gene maps are now available in many species where this was not previously considered feasible. The first human genetic linkage map (Donnis-Keller et al. 1987) based on restriction fragment length polymorphisms (RFLPs), was followed by the development of much less expensive technologies. These are based on the polymerase chain reaction (PCR) (Saiki et al. 1985) and hypervariable loci such as variable number of tandem repeat (VNTR) (Nakamura et al. 1987) and the simple tandem repeat (STR) in microsatellite loci (Weber & May, 1989). These techniques reduce cost and increase throughput to the stage that most livestock species such as cattle (Barendse et al. 1997, Kappes et al. 1997) and sheep (Crawford et al. 1995) now have moderately dense genetic maps available.

Initial efforts to develop genetic maps in penaeid shrimp have been hampered by the difficulty in characterising microsatellite markers from these species (Moore et al. 1998, GIS report 1997). Application of new technology based on identification and PCR amplification of restriction site length polymorphisms (Vos et al. 1995) has meant that genetic linkage maps can now be developed rapidly and inexpensively for all species of penaeid shrimp.

In order to optimise the information resulting from such mapping studies, integrated genetic maps will be required which incorporate information from multiple species. To this end, Type I markers (O'Brien et al. 1993) are being developed to "anchor" maps from different penaeid species with each other and with maps from model organisms such as *Drosophila melanogaster*.

TECHNIQUES

Gene mapping technologies include genetic, physical and comparative mapping. Together these assign location, order and spacing of genes or genetic markers on chromosomes. Such knowledge can be used to identify genes influencing phenotypic variation, either directly through genetic mapping or indirectly, using information from other species through comparative mapping approaches.

Genetic maps

Genetic maps assign order and spacing of genes or genetic markers based on recombination units. The predominant markers on such maps are usually Type II (e.g., microsatellites or more recently AFLPs). These maps are particularly useful in identifying genes influencing traits which exhibit continuous variaton (QTL). Such information can be used in selective breeding programs where markers associated with economically important traits can be assessed in broodstock (Davis & DeNise, 1998).

Physical maps

Physical maps assign order and spacing of genes on chromosomes, chromosomal regions or syntenic groups (groups of genes residing on a chromosome of unknown identity). The development of physical maps relies on a number of techniques. These including somatic hybrid cell line analysis (Womack & Moll, 1986), fluorescent *in situ* hybridisation (Trask, 1991) and large insert-size genomic libraries such as yeast artificial chromosome (YAC) (Burke et al. 1987) and bacterial artificial chromosome (BAC) (Shizuya et al. 1992) libraries. The first two techniques rely on availability of cell lines in the organisms of interest.

Comparative maps

Comparative gene mapping aims to map homologous genes in multiple species (O'Brien et al. 1993). This allows an understanding of chromosomal evolution which accompanies speciation. It also allows gene mapping information from one species to be extrapolated to other species. When one species has been extensively studied (e.g., as with model organisms in the human genome project), it can provide large

Moore SS, Lehnert SA (1998) Advances in gene mapping in penaeid shrimp. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: Steve.Moore@tag.csiro.au

amounts of useful information economically for less characterised species.

Genetic markers

Genetic mapping efforts have benefited greatly from technological developments in DNA marker identification and analysis. Early genetic maps were based on RFLP markers (Donnis-Keller et al. 1987). This technique is expensive and requires relatively large quantities of DNA. More recent maps are based on hypervariable sequences such as variable number of tandem tepeat (VNTR) (Nakamura et al. 1987) and microsatellites (Weber & May 1989). These markers can be analysed utilising PCR-based methods, significantly reducing the effort and expense of processing. These techniques have allowed the rapid development of genetic maps for most livestock species.

More recently PCR based methods have been developed to identify multiple restriction fragment length variation. The AFLP technique (Vos et al. 1995) reduces the effort required for genetic mapping further, to the extent where it is now feasible to construct maps for particular resource populations *de novo* . The disadvantage is that although polymorphic AFLP bands are conserved across families of the same species, the resulting maps may still be difficult in practice to transfer across families. However, the inclusion of other markers such as microsatellites or Type I markers will resolve this issue.

Recently a genetic linkage map for Tilapia (*Oreochromis niloticus*) has been reported using a combination of microsatellite and AFLP markers (Kocher et al. 1998). The first genetic linkage map for a penaeid species (Moore et al. 1998) has also been developed using AFLP markers.

THE STATUS OF GENE MAPPING IN PENAEID SHRIMP

International efforts are now underway to develop gene maps in a number of penaeid species (Alcivar-Warren 1997), including *P. vanameii* , *P. monodon* and *P. japonicus*. Early efforts in DNA marker development were based on DNA microsatellites, and this work continues. Such markers have successfully been isolated in small numbers from the abovementioned species of prawn (GIS report 1997, Tiptawonnukul et al. 1997, Wolfus et al. 1997, Moore et al. 1998).

DNA marker isolation

Type II loci

Efforts to isolate the large numbers of markers necessary for genetic mappings have been hampered by large numbers of very long simple repeat arrays present in the penaeid genome. Although simple repeat sequences are abundant, the length and complexity of these repeats makes unique flanking sequences suitable for PCR primer design difficult to obtain (Moore et al. 1998).

Microsatellites developed from *P. japonicus* and *P. esculentus* have been shown to be species specific (Moore et al. 1998). Combined with the difficulty in characterising usable microsatellites, this makes these markers less attractive as a primary tool for genetic mapping. However, microsatellites are highly effective when used for pedigree verification or to monitor inbreeding in selection lines (Moore et al. 1998). Levels of variation in wild populations of penaeid shrimp are such that heterozygosities at microsatellite marker loci approach 100% in some instances (Moore et al. 1998, Alcivar-Warren personal communication).

AFLP markers (Vos et al. 1995) have been developed for *P. japonicus* (Moore et al. 1998). Although this methodology reveals RFLPs with individually low levels of polymorphism throughout a population, PCR primer combinations can be used to rapidly define large numbers of polymorphisms. A total of 570 polymorphic loci were defined using combinations of 5 forward and 8 reverse primers.

Type I loci

As most anonymous markers such as the hypervariable microsatellites or AFLPs will be species-specific, strategies need to be put into place to develop Type I anchor loci (O'Brien et al. 1993) for use in penaeid shrimp. These loci can integrate genetic maps both within and across species. Furthermore the use of such markers has the potential to allow comparative maps to be developed between penaeids and arthropods. Thus, the wealth of information developed for model organisms such as *D. melanogaseter* could be utilised in penaeids. One limiting factor is the dearth of information available in DNA databases for any penaeid species. Our laboratory has begun random sequencing from a *P. monodon* cephalothorax cDNA library in order to establish a modest expressed sequence tag (EST) database. Sequencing of 115 clones resulted in characterisation of 87 unique sequences, 35 of which could be putatively identified through BLAST homology searches of the Genbank database (Table 1). Polymorphisms are being identified using single stranded conformational analysis (SSCP) or exon primed intron centred (EPIC) PCR (Slade et al. 1993, Palumbi et al. 1996). Presently 10 polymorphic loci have been defined using these methods.

Linkage maps

A genetic linkage map for *P. japonicus* has been constructed using AFLPs (Moore et al. 1998). Using 15 selective primer pairs, 246 polymorphic loci were analysed in a single family of 42 F2 progeny. Genome coverage was estimated at 57% with 129 loci linked to at least one other. This map has recently been extended by use of four families each with 46 F2 progeny genotyped using the same 15 primer combinations. Four hundred polymorphic loci were analysed, of which 191 were linked to at least one other locus in 51 linkage groups. This genetic map is being utilised to map QTL for growth and morphology traits in these families.

Three generation family DNA is being made available in a joint CSIRO/AIMS initiative aimed at developing a genetic map for *P. monodon*. The map will form a reference resource for other gene mapping studies in this species. International participation is invited in the development of the map. To date, ten of a proposed 15 AFLP primer pairs have been run across these families at CSIRO, Tropical Agriculture. Further to these proposed 15 primer pairs, it is envisaged that microsatellite and Type I markers will be added to the map as they become available.

Table 1. Homologous genes identified for randomly sequenced *P. monodon* cDNA clones.

Clone ID	Seq. length	Gene	GenBank accession	Species with closest homology	Probability	Score
SAL109	825	acidic ribosomal phosphoprotein	M17885	human	1.0e-39	394
SAL006	300	actin (cytoplasmic)	U09635	sea urchin	3.0e-70	775
SAL114	787	aldose reductase	M59754	cow	5.0e-55	787
SAL048	677	arginine kinase	X68703	lobster: *H.vulgaris*	1.1e-36	513
SAL040	509	assembly protein 180 (AP 180)	X68878	rat	9.3e-16	313
SAL099	754	cathepsin L-like cysteine proteinase	X85127	shrimp: *P.vannamei*	5.5e-163	1274
SAL068	674	cysteine string protein	S81917	rat	7.8e-42	296
SAL078	785	elongation factor (EF2)	M86959	nematode	5.1e-84	449
SAL117	845	GTP binding protein	M33141	cow	8.6e-86	1106
SAL081	854	GTP binding protein	D10715	mouse	8.6e-77	797
SAL010	295	heat shock protein hsp 60	X99341	fruit fly	1.4e-13	283
SAL046	644	hemocyanin	X82502	shrimp: *P.vannamei*	2.4e-117	1148
SAL042	541	mitochondrial ATP synthase	Y07894	fruit fly	3.2e-34	535
SAL067	570	muscle actin	D87740	fish	1.3e-96	1285
SAL053	817	muscle LIM protein	X81192	fruit fly	1.9e-41	624
SAL033	697	myosin heavy chain	M61229	fruit fly	3.0e-108	1427
SAL096	890	myosin light chain	L08051	fruit fly	8.3e-51	737
SAL026	693	opsin	X71665	mantis	1.9e-54	780
SAL043	684	putative transcriptional regulator (CON7)	AF015771	fungus	3.1e-08	224
SAL028	707	pyruvate kinase	X15800	rat	1.1e-11	266
SAL005	414	receptor for activated kinase (RACK1)	AF025331	fish	9.7e-25	194
SAL037	500	ribosomal protein L7a	M36072	human	7.8e-08	127
SAL111	861	ribosomal protein L7a	X62640	chicken	1.3e-71	597
SAL012	538	ribosomal protein L27a	U66358	fruit fly	9.1e-58	782
SAL039	505	ribosomal protein S1a	X57322	frog	1.1e-65	913
SAL084	857	ribosomal protein S2	U01334	fruit fly	2.5e-120	1386
SAL063	631	ribosomal protein S7	L20096	tobacco hornworm	9.8e-33	442
SAL088	845	sarco/endoplasmic reticulum Ca2+ ATPase (SERCA)	AF025848	crayfish: *Procambarus clarkii*	2.2e-107	1417
SAL072	631	thrombospondin-1	U76994	chicken	1.4e-13	289
SAL082	834	thrombospondin-4	Z19585	human	1.5e-37	369
SAL097	894	tropomyosin, fast isoform	AF034954	lobster: *H.americanus*	1.4e-117	1540
SAL110	878	tropomyosin, slow isoform	U08008	shrimp: *Metapenaeus ensis*	4.8e-106	818
SAL107	809	trypsin	X86369	shrimp: *P.vannamei*	2.8e-122	875
SAL008	311	U2 snRNP-specific A'protein...	X13482	human	8.8e-36	539
SAL087	873	ubiquitin carbohydroxyl-terminal reductase	M30496	human	4.1e-15	132

Physical maps

Until such time as cell lines become available (Tong & Miao 1996), physical mapping strategies based on *in situ* hybridisation techniques or using hybrid somatic cell lines are not feasible for penaeids.

Large insert sized DNA libraries such as YAC (Burke et al. 1987) and BAC (Shizuya et al. 1992) libraries offer the ability to physically map genes, at least at a regional level. A *P. monodon* BAC library is being developed at CSIRO, Tropical Agriculture. This library will be utilised to characterise chromosomal segments around important genes.

Comparative maps

Comparative maps will depend on the characterisation of large numbers of specific gene sequences in penaeids.

Random cDNA sequencing has already yielded sequences homologous to genes in a number of species including arthropods and vertebrates. PCR primers generated in the 3'UTR and EPIC primers developed in coding sequence have both been shown to work across various species of penaeid. The challenge will be to develop the number of polymorphic loci required to link the various penaeid species maps.

CONCLUSIONS

Gene mapping strategies are developing rapidly. These developments allow medium density genetic maps to be constructed for all economically important species of farmed penaeids. To maximise returns for efforts in map construction, an international effort is required to develop and map markers that link the various species maps. Further tools such as BAC libraries and EST databases are under development at CSIRO, Tropical Agriculture to achieve this end.

LITERATURE CITED:

Alcivar-Warren A, Dunham R, Gaffney P, Kocher T, Thorgaard, G (1997) First aquaculture species genome mapping workshop. Animal Genetics 28: 451-452

Barendse W, Vaiman D, Kemp SJ, Sugimoto Y, Armitage SM (1997) A medium-density genetic linkage map of the bovine genome. Mammalian Genome 8: 21-28

Burke DT, Carle GF, Olson MV (1987) Cloning large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236: 806-812

Crawford AM, Dodds KG, Ede AJ, Pierson CA, Montgomery GW (1995) An autosomal genetic linkage map of the sheep genome. Genetics 140, 703-724

Davis GP, DeNise SK (1998) A review of the impact of genetic markers on selection and how to evaluate their economic impact. J Animal Science *in press*

Donis-Keller H, Green P, Helms C, Cartinhour S, Weiffenbach B (1987) A genetic linkage map of the human genome. Cell 51: 319-337

GIS (1997) Developing genetic markers in *Penaeus vanameii* for use in shrimp aquaculture. Final report submitted to United States Department of Agriculture Small Business Innovative Research Program. USDA grant 97-33610-4084. Genetic Identification Inc. 9552 Topanga Canyon Blvd. Chatsworth, California 91311

Kappes SM, Keele JW, McGraw RA, Sonstegard TS, Smith TPL, Lopex-Corrales NL, Beattie CW (1997) A second-generation linkage map of the bovine genome. Genome Research 7: 235-249

Kocher TD, Lee W-J, Sobolewska H, Penman D, McAndrew B (1998) A genetic linkage map of a cichlid fish, the tilapia (*Oreochromis niloticus*). Genetics 148: 1225-1232

Moore SS, Whan V, Davis GP, Byrne K, Hetzel DJS, Preston N (1998) The development and application of genetic markers for the Kuruma prawn *Penaeus japonicus*. Aquaculture *in press*

Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T Culver, M Martin C, Fujimoto E, Hoff M, Kumlin E, White R (1987) Variable number of tandem repeat (VNTR) makrers for human gene mapping. Science 235, 1616-1622

O'Brien SJ, Womack JE, Lyons LA, Moore KJ, Jenkins NA, Copeland NG (1993) Anchored reference loci for comparative genome mapping in mammals. Natute Genet 3: 103-112

Palumbi S, Hillis D, Moritz C (1996) Molecular Systematics. Sinauer MS. USA

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of b-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia Science 230: 1350-1354

Shizuya H, Birren B, Kim U-J, Mancino V, Slepak T, Tachiiri Y, Simon, M (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using F-factor-based vector. Proc Natl Acad Sci USA 89:8794-8797

Slade R, Moritz C, Heideman A, Hale PT (1993) Rapid assessment of single-copy nuclear DNA variation in diverse species. Molecular Ecology: 359-373

Tiptawonnukul A, Cook D, Maunprasitporn C, Rimphanitchayakit V, Jarayabhand P, Boonsaeng V, Tassanakajon A (1997) Microsatellite sequences in the black tiger prawn *(P. monodon)*. Proceeding of the Sixth International Symposium on Genetics in Aquaculture. Sterling, Scotland

Tong S-l, Miao H-Z (1996) Attempts to initiate cell cultures from *Penaeus chinensis* tissues. Aquaculture 147: 151-157

Trask BJ (1991) Fluorescent *in situ* hybridisation : applications in cytogenetics and gene mapping. Trends in Genetics 7:149-154

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research, 23, 4407-4414

Weber JL, May PE (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44: 388-396

Womack JE, Moll YD (1986) Gene map of the cow: conservation of linkage with mouse and man. J Hered 77: 2-7

Wolfus GM, Garcia DK, Alcivar-Warren A (1997) Application of the microsatellite technique for analysing genetic diversity in shrimp breeding programs. Aquaculture 152: 35-47

Development of Simple Sequence Length Polymorphisms in the Black Tiger Shrimp (*Penaeus monodon*)

[1]A. Vanavichit, [1]S. Wuthimaethavee, [1]D. Taechayinkphibool, [1]P. S. Kruthoo [2]P. Lumubol, [1]S. Tragoonrung

[1]DNA Fingerprinting Unit, National Center for Genetic Engineering and Biotechnology Rama 6 Road, Bangkok, Thailand
[2]Faculty of Fisheries, Kasetsart Univesity, Bangkaen, Bangkok, Thailand

ABSTRACT: Microsatellite markers are ideal for shrimp genome study and broodstock management. Genomic libraries containing di, tri and tetra nucleotide repeats were enriched by different methods. Enriched libraries contained 34% more clones containing microsatellite repeats than controls. The dinucleotide repeats, TG, TC, TA and GC, represent the most abundant microsatellites in the shrimp genome. Several clones contained mixtures of di, tri, and tetra nucleotide repeats. Of 52 dinucleotide-containing clones, primers could be designed for only half. These primers were polymorphic, and PCR products were generated ranging from 5-25 alleles per primer. These loci provided sufficient resolution to distinguish parentage of half-sib progenies raised in the same pond and, thus, facilitated broodstock evaluation. However, a high degree of artefactual bands made allele identification difficult. Moreover, some primers were not specific to *P. monodon*. Several genomic libraries enriched for the trinucleotide repeats, GAT, ATC, GAA, CAG, and the tetranucleotide repeats, CATA, GATA, and TCAG were contructed and nucleotide sequences of flanking repeat motifs of positive clones have now been determined. These primers generated unique banding patterns and could be used in genome mapping of *P. monodon*.

KEY WORDS: *Penaeus monodon*, microsatellites, genomic libraries, broodstock evaluation

Vanavichit A, Wuthimaethavee S, Taechayinkphibool D, Kruthoo PS, Lumubol P, Tragoonrung S (1998) Development of simple sequence length polymorphisms in the black tiger shrimp (*Penaeus monodon*). *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Genetic Variation, Population Differentiation and Gene Flow of the Giant Tiger Shrimp (*Penaeus monodon*) Inferred from mtDNA-RFLP Data

***[1]Sirawut Klinbunga, [2]David J. Penman, [2]Brendan J. McAndrew**
[3]Anchalee Tassanakajon, [4,5]Padermsak Jarayabhand

[1]Marine Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Development Agency, Bangkok, 10400, Thailand
[2]Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK
[3]Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[4]Aquatic Resources Research Institute, Chulalongkorn University, Bangkok, 10330, Thailand
[5]Angsila Marine Biological Research Station, Department of Marine Science Chulalongkorn University, Chonburi, 20210, Thailand

ABSTRACT: Genetic diversity, population differentiation, and gene flow level of *Penaeus monodon* were examined using restriction analysis of the entire mitochondrial DNA (mtDNA) of two hundred and six individuals collected from 10 geographic locales (Dungun, Kedah, Lamu, Lingayen, Medan, North Java, South Java, Satun, Surat and Trat) using 5 restriction endonucleases (*Bam* HI, *Sac* I, *Eco* RV, *Pvu* II and *Hind* III). Nineteen composite haplotypes generated from twenty-two restriction profiles were observed from all investigated individuals. The mitochondrial genome size of *P. monodon* was estimated to be 15.858 ± 0.138. The average haplotype diversity of *P. monodon* mtDNA was 0.537 ± 0.006. The average nucleotide diversity within and between samples were 3.341 ± 0.001% and 4.676 ± 0.000%, respectively, whereas the average nucleotide divergence between all pairs of samples was 1.335 ± 0.000%. The overall gene flow level was 2.6 female migrants per generation whereas at the microgeographic level (within the Southeast Asian region), the level of gene flow was greater (4.8 female migrants). Based on analysis of geographic heterogeneity, phylogenetic reconstruction and the variance of allele frequencies among samples, the wild *P. monodon* samples in the Southeast Asia could be allocated to 3 different stocks: A (Trat, Surat, Dungun, North and South Java from the east of the Malaysian peninsula), B (Kedah Medan and Satun from the Andaman Sea), and C (Lingayen from the Philippines). Additionally, *P. monodon* from Lamu (Kenya) should be recognised as a different stock from those of Southeast Asia.

KEY WORDS: mtDNA-RFLP, shrimp genetics, populations, *Penaeus monodon*, gene flow

INTRODUCTION

Giant tiger shrimp, *Penaeus monodon*, a member of Penaeidae, is an economically important species. The culture of this species has shown rapid development during the last decade (Suraswadi 1995). However, the farming of this species relies entirely on wild females for the supply of juveniles. This may result in overexpliotation of the female broodstock in natural populations. One of the primary objectives of fishery management is to understand the population structure of any exploited species. The recognition of reproductively isolated and genetically differentiated populations within a species is of importance for their management. In the long term, the most important goal of fishery management is to conserve the existing resources of exploited species to ensure sustainable yields (Allendorf *et al*. 1987, Ryman 1991). However, relatively little is known about the population structure and dynamics of *P. monodon*, information which is extremely important for management of the wild broodstock.

At present, mtDNA RFLP is commonly used for determination of intraspecific population structure. The reason for this is that two properties of the mitochondrial genome, rapid evolution without major rearrangement and predominantly maternal inheritance in most species, enable female lineages to be tracked over relatively short time periods (Avise 1994). Due to its haploid nature and mode of inheritance, the effective population size estimated from mtDNA is smaller than that of nuclear genes, unless there is extensive heteroplasmy or the sex ratio is heavily biased in favour of females. This increases its susceptibility to genetic drift, inbreeding and bottleneck events (Birky *et al*. 1989, Ward & Grewe 1994).

Several factors are responsible for intraspecific differentiation: migration or gene flow, random genetic drift, modes of natural selection, mutation and genetic recombination through mating systems. In addition to such general categories, biological factors related to individual groups of organ-

Klinbunga S, Penman DJ, McAndrew BJ, Tassanakajon A, Jarayabhand P (1998) Genetic variation, population differentiation and gene flow of the giant tiger shrimp (*Penaeus monodon*) inferred from mtDNA-RFLP data. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: sirawut@biotec.or.th

isms, for instance ecological factors and life history, also play a partitioning role in population differentiation (Avise 1994). Based on a comparative summary of literature from more than 300 animal species, it was concluded that mobility is the most important factor reflecting the apparent magnitude of intraspecific subdivisions. Therefore, it is not surprising that vagile organisms (high degree of gene flow) for example insects and birds, have lower levels of population differentiation than do relatively sedentary species (Ward *et al.* 1992).

Gene flow is the exchange of genetic material between populations caused by movement of individuals or their successfully fertilising gametes. Basically, it is possible to estimate only the parameter N_em. This is interpreted as the absolute number of successfully reproducing individuals exchanged between populations per generation (Ovenden 1990, Avise 1994). Generally, a long-duration planktonic larval stage influences the opportunity for a high degree of gene flow as evidenced by a near absence or lack of genetic differentiation over vast geographic areas in several taxa (e.g., the sea urchins, *S. purpuratus* and *Hediocedaris tuberculata* and the red rock lobster, *Jasus edwardsii*) (Palumbi & Wilson 1990, McMillan et al. 1992, Ovenden *et al*. 1992).

The genetic structure of penaeid shrimps inferred from allozyme analysis has been reported from various geographic locales in Australia. Population structure was illustrated in *M. bennettae, M. endeavouri* and *P. latisulcatus*. On the other hand, a lack of geographical subdivision was shown in *M. ensis, P. esculentus, P. merguiensis, P. plebejus* and *P. semisulcatus* (Mulley & Latter 1981a, 1981b).

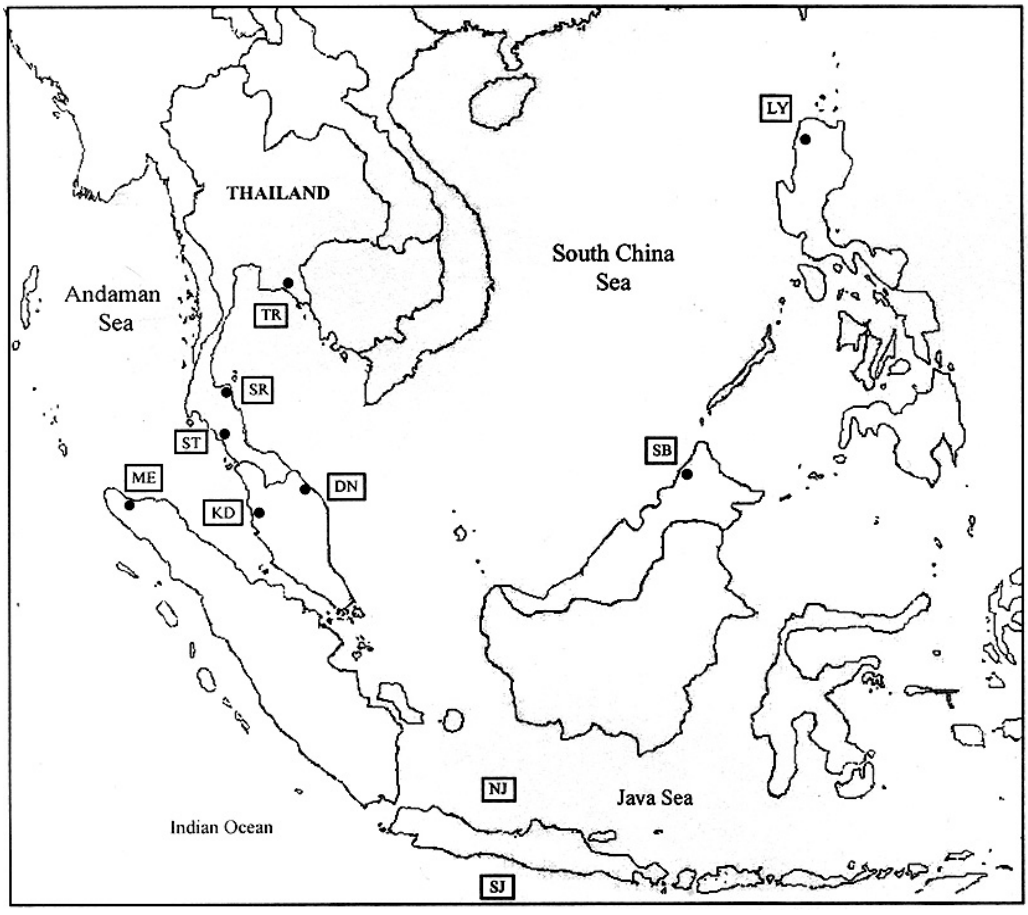
There have been a few publications concerning genetic structure based on mtDNA variability of the tiger shrimp, *P. monodon*. The first publication on this species was by Benzie *et al.* (1993) who determined population structure of *P. monodon* in Australia collected from Cairns (n=6) and Townsville (n=6) from the east and De Grey River (n=3) from the west coasts. The entire mitochondrial DNA was isolated and analysed with *Bam* HI, *Eco* RV, *Sac* I and *Eco* O109. The mtDNA genotype frequencies were significantly different between the eastern and western populations ($P < 0.05$). Subsequently, a publication on genetic variation in laboratory strains originating from Fiji and Australia/Malaysia of this species was reported (Bouchon *et al.* 1994). However, the mtDNA RFLP data from both studies were obtained from rather small sample sizes. The objective of this study was to investigate genetic variation, the population genetic structure and the gene flow level of *P. monodon* in the South East Asian region with a large sample size using mtDNA-RFLPs.

MATERIALS AND METHODS

Sampling

Two hundred and six wild individuals of *P. monodon* were

Figure 1. Map of South East Asia showing collection sites for *P. monodon* samples used in this study. (I = Indonesia; M = Malaysia; P = Philippines, T = Thailand; DN = Dungun; LY = Lingayen, KD = Kedah; ME = Medan, NJ = northern Java, SJ = southern Java; SR = Surat, ST = Satun, TR = Trat) Note that Lamu (Kenya) is not on this map.



captured alive from ten geographic locations (Fig. 1). These compirsed three locations from Indonesia (Medan, southern and northern Java), one site each from Lamu (Kenya) and Lingayen (Philippines), two locations from Malaysia (Kedah and Dungun) and three locations from Thailand (Trat, Surat and Satun). The specimens were transported back to a laboratory at the Institute of Aquaculture, the University of Stirling, UK on dry ice and stored in a -70° C freezer until required.

DNA extraction and preparation

Total DNA was extracted from the largest proximal section of frozen pleopods of *P. monodon* according to Klinbunga *et al*. (1996b). MtDNA was isolated from abdominal pleopods of *P. monodon* using a modification of the method of Chapman and Powers (1984) as described in Klinbunga (1996a). The mtDNA probe was prepared using the hexanucleotide random primed method (Feinberg & Vogelstein 1983).

Restriction endonuclease analysis, Southern transfer and hybridisation

Two µg of total DNA isolated from each individual was singly restricted with a battery of five restriction enzymes; *Bam* HI (G/GATCC), *Eco* RV (GAT/ATC), *Hind* III (A/AGCTT), *Pvu* II (CAG/CTG) and *Sac* I (GAGC/TC) using the condition recommended by the manufacturer (Promega). The restricted DNA was electrophoresed through a 0.8% agarose gel before alkaline-transfer onto a nylon membrane (Hybond N^+, Amersham). The membrane was prehybridised and hybridised using the method described by Reed and Mann (1985). Each membrane was then subjected to autoradiography detection for 16-24 h at -70°C with an intensifying screen before developing and fixing of the film. Size estimations of fragments were calculated by comparing their electrophoretic mobility with those of λ-*Hind* III and/or KB markers.

Data analysis

Restriction patterns obtained with each enzyme were alphabetically assigned as A, B, C etc, in order of frequency. The composite haplotype of each *P. monodon* individual was generated as a five letter code arranged according to the enzyme generating the lowest number of restriction patterns (*Bam* HI) to that generating the highest number of restriction patterns (*Hind* III).

The genetic distance (percent sequence divergence) between mtDNA composite haplotypes was estimated following Nei and Li (1979). The haplotype diversity was calculated after Nei and Tajima (1981). The nucleotide diversity per sample (the average number of nucleotide substitutions within a geographic sample), nucleotide diversity between samples (the average number of nucleotide substitutions between DNA haplotypes from samples X and Y) and nucleotide divergence (the average number of nucleotide substitutions per site where the effect of within sample polymorphism has been subtracted) were calculated according to Nei and Tajima (1983). Phenograms based on interpopulation nucleotide divergence (d_A) were constructed using unweighted pair-group method with arithmetic mean (UPGMA) implemented in Phylip 3.5c (Felsenstein 1993).

To determine whether there was significant heterogeneity in the distribution of mtDNA composite haplotypes among different geographic samples, a χ^2 test based on Monte Carlo simulations was carried out ten thousand times (Roff & Bentzen 1989) using REAP (McElroy *et al.* 1991). The results were expressed as the probability of homogeneity between compared samples or regions. The degrees of population subdivision at the haplotype and nucleotide levels were estimated using F_{ST} and N_{ST} (Weir & Cockerham 1984, Lynch & Crease 1990). The F_{ST} and N_{ST} estimates of 0 and 1 indicate zero and complete population subdivision, respectively. Levels of female gene flow ($N_{e(f)}m$) among pairs of samples or regions were estimated using the following equation:

$$F_{ST} \cong 1/(1+2\ N_{e(f)}m)$$

RESULTS

Digestion of the entire *P. monodon* mtDNA with *Bam* HI, *Sac* I, *Eco* RV, *Pvu* II and *Hind* III generated 2, 3, 5, 6 and 6 restriction profiles, respectively (Fig. 2 and Table 1). A total of 47 restriction fragments were observed from all restriction enzyme digestions with an average of 9.4 investigated fragments per enzyme. Nineteen composite haplotypes were generated (Table 2). Nevertheless, only three composite haplotypes (I, AAAAA; II, ABBAC and V, BBBBB) were

Table 1. Restriction fragment sizes (indicated in kilobases) of the digestion patterns observed from digestion of *P. monodon* mtDNA with each of the restriction endonucleases. Numbers in parenthesis indicate missing bands for which a single band of their sum was inferred.

Enzyme	Restriction patterns observed
Bam HI	A: 9.25, 6.9
	B: 16.0
Sac I	A: 15.8
	B: 13.0, 2.675
	C: 12.0, 3.9
Eco RV	A: 16.0
	B: 9.4, 6.3
	C: 11.25, 4.6
	D: 9.4, 5.4, (0.9)
	E: 9.4, 4.9, (1.4)
Pvu II	A: 8.2, 7.6
	B: 15.75
	C: 8.2, 4.9, 2.6
	D: 7.6, 6.6, 1.575
	E: 13.25, 2.6
	F: 9.4, 6.6
Hind III	A: 3.8, 3.2, 2.65, 1.875, 1.6, 1.5, 0.93, 0.365
	B: 4.9, 4.9, 3.2, 2.45, 0.365
	C: 3.8, 2.725, 2.65, 1.875, 1.6, 1.5, 0.93, 0.46, 0.365
	D: 4.15, 3.8, 3.2, 1.8, 1.5, 0.93, 0.365, (0.075)
	E: 4.9, 4.9, 2.45, 1.7, 1.475, 0.365
	F: 3.8, 3.2, 2.65, 1.6, 1.5, 1.25, 0.93, 0.66, 0.365
	Average mitochondrial genome size = 15.858 ± 0.138

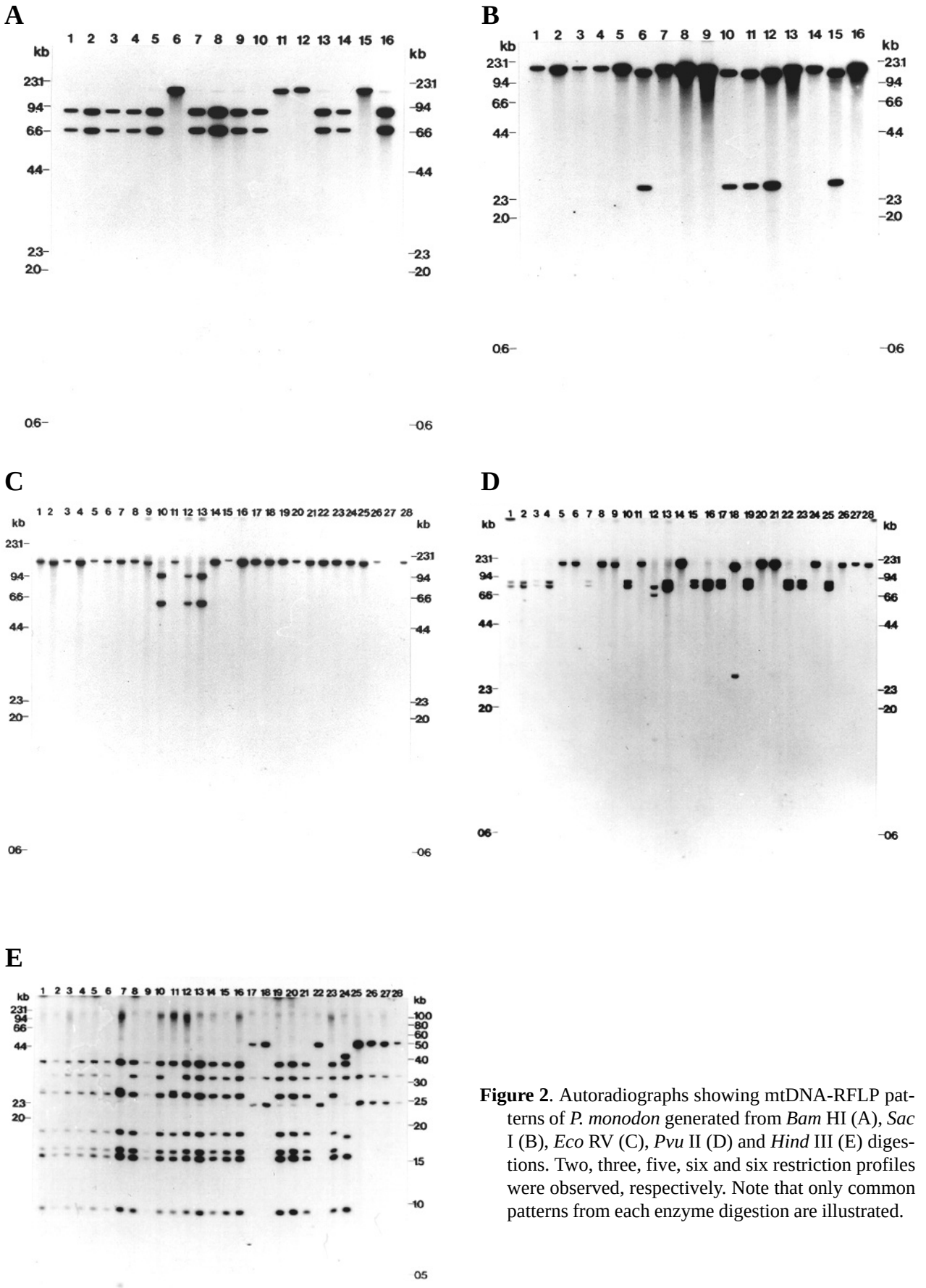


Figure 2. Autoradiographs showing mtDNA-RFLP patterns of *P. monodon* generated from *Bam* HI (A), *Sac* I (B), *Eco* RV (C), *Pvu* II (D) and *Hind* III (E) digestions. Two, three, five, six and six restriction profiles were observed, respectively. Note that only common patterns from each enzyme digestion are illustrated.

Table 2. Geographic distribution of 19 composite haplotypes from 10 samples of *P. monodon* based on 5 restriction enzymes (*Bam* HI, *Sac* I, *Eco* RV, *Pvu* II and *Hind* III).

Haplotype	Geographic distribution										Total
	LY	SR	ST	TR	KD	DN	NJ	SJ	ME	LM	
I AAAAA	20	11	9	12	9	11	7	14	6	0	99
II ABBAC	2	6	0	5	1	0	0	5	1	0	20
III BBCCD	1	0	0	2	0	0	1	0	0	0	4
IV AABAA	1	0	0	0	0	0	0	0	0	0	1
V BBABB	0	3	11	4	7	1	4	3	14	12	59
VI ACAAA	0	0	1	0	2	0	0	0	0	0	3
VII ABAAA	0	0	0	0	1	0	0	0	0	0	1
VIII ABBDC	0	0	0	0	0	0	2	1	0	0	3
IX BBECD	0	0	0	0	0	0	0	1	0	0	1
X BBDBB	0	0	0	1	0	0	0	0	0	0	1
XI BBAEB	0	0	1	0	0	0	0	0	0	0	1
XII BBABE	0	0	2	0	0	1	1	0	0	0	4
XIII AACAA	0	0	0	0	0	0	1	0	0	0	1
XIV BBAFB	0	0	0	0	0	0	0	0	1	0	1
XV BBCBB	0	1	0	0	0	0	0	0	1	0	2
XVI AABAC	0	0	0	0	0	0	0	0	1	0	1
XVII BBBBB	0	2	0	0	0	0	0	0	0	0	2
XVIII ABBAA	0	1	0	0	0	0	0	0	0	0	1
XIX AAAAF	0	0	0	0	0	1	0	0	0	0	1
Total	24	24	24	24	20	14	16	24	24	12	206

possessed by at least 2% of investigated individuals. These common haplotypes were not population-specific but overlappingly distributed across vast geographic regions. The most common haplotype I (AAAAA) was found in all Southeast Asian samples with geographic distribution frequencies between 25.00% (Medan) to 83.33% (Lingayen). Generally, the frequencies of this haplotype in each of the east coast samples were higher than that of the Andaman Sea . The other common haplotype V (BBABB) was observed in all but Lingayen. The distribution frequencies of this haplotype were in the opposite direction to that of haplotype I. Haplotype VIII (ABBDC) was found only in specimens originating from the Java Sea. A total of eleven composite haplotypes were private (observed in only one geographic sample).

Haplotype diversity was high in all samples except Lamu (0.000 ± 0.000), Lingayen (0.301 ± 0.083) and Dungun (0.381 ± 0.112). The average haplotype diversity for overall samples was 0.537 ± 0.006. The nucleotide diversity within a geographic sample of the Southeast Asian *P. monodon* varied from 0.788 (Lingayen) to 5.090 (N. Java), whereas the diversity of Lamu was 0.000. The average nucleotide diversity within samples was 3.341 ± 0.003% (Table 3). The nucleotide diversity between a pair of samples ranged from 1.819 (Dungun - Lingayen) to 8.538 (Lamu - Lingayen). The amount of nucleotide divergence between pairs of samples was less than zero for seven out of forty-five possible pairwise comparisons. The mean nucleotide divergence for all pairwise combinations was 1.335 ± 0.000% providing an indication of genetic structuring between different geographic *P. monodon* (Table 4).

Table 3. Genetic variability of *P. monodon* mtDNA indicated by haplotype and percent nucleotide diversity within samples.

Geographic location	No. of individuals	Haplotype diversity (± SE)	Nucleotide diversity x100
Lingayen	24	0.301 ± 0.083	0.788
Surat	24	0.716 ± 0.048	4.163
Satun	24	0.653 ± 0.042	4.670
Trat	24	0.684 ± 0.052	4.102
Kedah	20	0.677 ± 0.047	4.208
Dungun	14	0.381 ± 0.112	2.716
North Java	16	0.742 ± 0.056	5.090
South Java	24	0.610 ± 0.064	2.987
Medan	24	0.603 ± 0.063	4.681
Lamu	12	0.000± 0.000	0.000
Average	20.6	0.537 ± 0.006	3.341 ± 0.003

Additionally, the nucleotide divergence between all pairs of samples was subjected to phylogenetic reconstruction using UPGMA (Fig. 3). Apparently, there were at least three phylogenetically different *P. monodon* lineages in all the samples from the east of the peninsula and Kedah, Satun and Medan from the Andaman Sea and Lamu from Kenya.

Based on geographic heterogeneity analysis using Monte Carlo simulations, the distributions of haplotype frequencies for overall samples and overall Southeast Asian samples were significantly different (P = 0.0000). This indicated that population differentiation among *P. monodon* originating from different geographic locales does exist. This was further supported by hierarchical analysis of population structure using F_{st} and N_{st} (Table 5). Moreover, *P. monodon* from the Andaman Sea had significantly different haplotype frequencies to that from the east of the Malaysian Pe-

Table 4. Percent nucleotide divergence (below diagonal) and percent nucleotide diversity (above diagonal) estimates between pairs of *P. monodon* samples.

-	LY	SR	ST	TR	KD	DN	NJ	SJ	ME	LM
LY	-	3.125	5.419	2.918	3.409	1.819	3.733	2.035	6.047	8.538
SR	0.650	-	5.290	4.005	4.273	3.706	4.493	3.581	5.540	6.595
ST	2.690	0.873	-	5.232	4.677	5.143	5.181	5.330	4.522	3.647
TR	0.474	-0.128	0.846	-	4.165	3.492	4.423	3.498	5.551	6.679
KD	0.911	0.088	0.238	0.011	-	3.658	4.444	3.871	4.965	5.417
DN	0.067	0.266	1.450	0.083	0.196	-	4.075	2.811	5.659	7.337
NJ	0.794	-0.134	0.301	-0.169	-0.205	0.172	-	4.140	5.402	6.014
SJ	0.147	0.005	1.501	-0.047	0.273	-0.041	0.101	-	5.786	7.505
ME	3.312	1.118	-0.153	1.160	0.521	1.961	0.516	1.952	-	3.263
LM	8.144	4.514	1.311	4.628	3.313	5.979	3.469	6.012	0.923	-
Average percent nucleotide diversity between all pairs of samples = 4.676 ± 0.000										
Average percent nucleotide divergence between all pairs of samples = 1.335 ± 0.001										

ninsula (P = 0.0001 to Lingayen, P = 0.0000 to the South China and P = 0.0008 to Java Seas). Within the east coast samples, pairwise comparisons for distribution of haplotype frequencies among Lingayen, the South China and Java Seas were not significantly different. In contrast, statistically significant differences of the F_{st} estimates between Lingayen-the South China Sea, Lingayen-the Java Sea were, however, observed (P < 0.05) but not between the South China and Java Seas (P > 0.05).

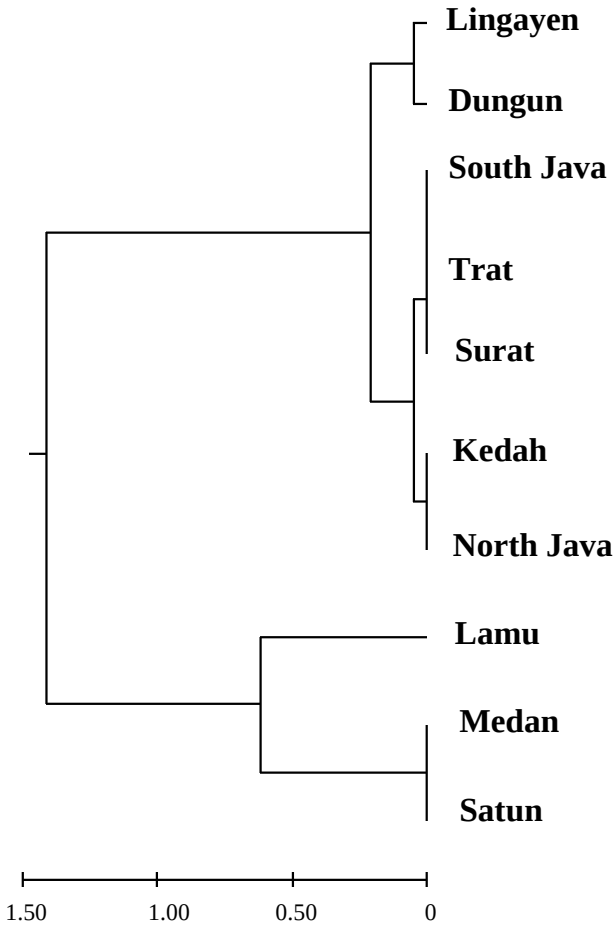


Figure 3. A UPGMA phenogram illustrating the phylogenetic relationships among 10 geographic samples of *P. monodon* based on percent nucleotide divergence.

Estimation of population structure at haplotype (F_{ST}) and at nucleotide levels (N_{ST}) was generally in agreement. The overall F_{ST} and N_{ST} were 0.160 and 0.286, respectively. These values indicated that 84.0% and 71.4% of the mtDNA variation was from within samples. Gene flow levels between Lamu and each of the Southeast Asian samples were low (0.1 - 1.6 female migrants per generation). The mean gene flow level for all samples was 2.6 female individuals. This value was, however, approximately twice greater (4.8 female individuals) when Lamu was excluded from the analysis. Based on all analyses described above, *P. monodon* in the Southeast Asian region could be regarded as 3 different populations (stocks) composing of A (Trat Surat, Dungun, North and South Java), B (Kedah Medan and Satun), and C (Lingayen). Although a geographic heterogeneity test did not indicate significant differences in haplotype frequencies between *P. monodon* from Lamu (Kenya) and the Andaman samples (P = 0.2723), F_{ST} and N_{ST} showed population differentiation between these conspecific samples (F_{ST} = 0.238, P < 0.01 and N_{ST} = 0.233). Further investigation using six additional restriction enzymes (*Ava* II, *Bgl* II, *Cla I*, *Dra* I, *Sca* I and *Xba* I) showed that the Lamu *P. monodon* significantly differed from the Southeast Asian *P. monodon* (P = 0.0000). Therefore, it should be recognised as a different population.

DISCUSSION

The mitochondrial genome size of *P. monodon* was estimated to be 15.858 ± 0.138 kb (average from all restriction patterns observed), This estimate generally agrees with those from other invertebrates. For example, sizes are 17.7 ± 0.3 kb and 18.7 ± 0.2 kb in the Japanese oyster, *Crassostrea gigas* (Oohara & Mori 1989, Boom *et al. 1*994), 16.68 ± 0.44 kb in *P. japonicus* (Bouchon et al. 1994), and 16.920 ±

Table 5. Estimates of geographic heterogeneity (χ^2), population differentiation at haplotype (F_{ST}) and nucleotide levels (N_{ST}) and female gene flow ($N_{e(f)}$m) of *P. monodon*.

Geographic location	F_{ST}	N_{ST}	χ^2	$N_{e(f)}$m[a]
Overall samples	0.160***	0.286	P = 0.0000	2.6
Overall South-East Asian samples	0.095***	0.115	P = 0.0000	4.8
The Andaman v Lingayen	0.274***	0.259	P = 0.0001	1.3
The Andaman v the South China Seas	0.110***	0.122	P = 0.0000	4.0
The Andaman v the Java Seas	0.076**	0.104	P = 0.0008	6.0
The Andaman v Lamu	0.238**	0.233	P = 0.2723	1.6
Lingayen v The South China Sea	0.065*	0.074	P = 0.2913	7.2
Lingayen v the Java Sea	0.084*	0.106	P = 0.0899	5.5
Lingayen v Lamu	0.801***	0.954	P = 0.0000	0.1
The South China v Java Seas	-0.010[ns]	0.009	P = 0.4101	nd
The South China v Lamu	0.482***	0.408	P = 0.0000	0.5
The Java Sea v Lamu	0.462***	0.543	P = 0.0000	0.6

* = P < 0.05, ** = P < 0.01, *** = P < 0.001, ns = not significant, nd = not determined

[a]calculated from the equation: $FST \cong 1/(1 + 2N_{e(f)}\, m)$

0.254 in the banana shrimp, *P. merguiensis* (Daud 1995). Length heteroplasmy of the mitochondrial genome was not observed in this species.

Haplotype and nucleotide diversity in *P. monodon* were high compared with those previously reported in other marine invertebrates. For instance, they are 0.57 and 0.14%, respectively, for *C. virginica* collected from the Atlantic Ocean (Reeb & Avise 1990) and 0.66 and 0.16%, respectively, for the Japanese scallop (*Patinopecten yessoensis*) (Boulding et al. 1993). Nevertheless, the estimates within Lamu and Lingayen were lower than they were for the other conspecific samples. This result is in accord with that from allozyme analysis of *P. monodon* from the same sampling locations using 46 allozyme loci. The mean heterozygosity of Lamu (0.021) and Lingayen (0.020) was lower than the average heterozygosity of all the investigated samples from Southeast Asia (0.028) (Sodsuk 1996).

The reason for limited genetic diversity in these *P. monodon* samples is possibly due to a reduction in their effective population sizes as a result of a bottleneck effect. Additionally, historical biogeographic barriers that resulted from changes of sea level that occurred approximately 10,000 - 15,000 years ago separated Lamu and Lingayen from other conspecific samples for a period of time. Therefore they possibly suffered from reduced gene flow (Nei 1987, Dall *et al*. 1990, Sodsuk 1996). Nevertheless, the most likely explanation for lack of Lamu genetic variation is that the sample was established by a founder population having low mtDNA variability (Phillips 1994). Further investigation of genetic diversity levels of *P. monodon* from Lamu and Lingayen using nuclear DNA markers such as microsatellite loci will provide additional data for a clearer conclusion.

Theoretically, it is assumed that populations of nearly all species exhibit at least some degree of genetic differentiation among geographic locales (Ehrlich & Raven 1969). The establishment of stock composition of a given species can be revealed by the significance of an unbiased χ^2 analysis (such as a Monte Carlo simulation in this study) and by quantitative estimates of nucleotide diversity. If the samples show genetic differentiation, the amount of nucleotide diversity within samples would be less than that of nucleotide diversity between samples (Roff & Bentzen 1989, Ovenden 1990, Neish 1993). Generally, large genetic differences were observed between Lamu and all Southeast Asian samples. The average nucleotide diversity between pairs of samples (4.676 ± 0.000) was greater than that within samples (3.341 ± 0.003) implying population level genetic differentiation in *P. monodon*. The average nucleotide divergence between all pairwise comparisons of *P. monodon* was higher than that in other marine invertebrates. For instance, they are 0.011 in the Pacific oyster, *C. gigas* (Boom 1994) and 0.11% and 0.48% in S*tronglylocentrotus droebachensis* and *S. purpuratus*, respectively (Palumbi & Wilson 1990). This indicates that degree of population differentiation in *P. monodon* is greater than in those species.

Population subdivision in *P. monodon* may reflect histolical biogeographic barriers around the Malaysian archipelago and ocean current during the last glacial epoch. These effectively could have cut off *P. monodon* from Lamu (Kenya) and Lingayen (Philippines). At that period, the South China Sea was narrower than at present and the Java Sea still did not exist. As a result, Lamu and Lingayen may represent two isolated groups, with subsequent mixing in South East Asia giving rise to the recently established populations. However, the Malaysian archipelago still provides enough of a barrier to create a "genetic break" in haplotype frequencies between the east and west coast samples (Dall *et al* 1990, Benzie *et al*. 1992, Daud 1995, Sodsuk 1996).

Surprisingly, Kedah from the west coast phylogenetically clustered with North Java, situated approximately 900 km away and in a different coastal location rather than with proximal geographic samples such as those from Medan or Satun. This may be a natural phenomena related to a relic population that migrated into the area or it may have been

caused by man-made disturbances related to farming of *P. monodon* there (Klinbunga 1996a).

Gene flow or genetic exchange between samples was calculated at the haplotype (F_{ST}) level. Nevertheless, the estimated values were interpreted as a quantitative guideline, not a precise estimate, as to whether populations experience high, moderate or restricted gene flow. In the case of data inferred from nuclear genes, Slatkin (1987) and Avise (1994) proposed that an average exchange of one individual per generation (i.e., a value of $N_em = 1$ corresponding to an average value of Wright's $F_{ST} = 0.2$) between populations, irrespective of deme size, is marginally sufficient to prevent dramatic genetic differentiation by genetic drift alone.

Geographical differentiation and levels of gene flow of the tiger shrimp, *P. monodon*, based on allozyme analysis was first established from populations in Australia (Benzie *et al*. 1992). The *P. monodon* samples obtained from seven locations throughout the species's range in Australia were surveyed electrophoretically at eight polymorphic loci. Geographic differentiation between western compared with northern and eastern populations was evident ($P < 0.001$). The average number of migrants per generation among populations was estimated to be 7.8 ($F_{ST} = 0.0311 \pm 0.0036$) if the west coast sample (De Grey river) was included. Nevertheless, the values were approximately 8 times higher (52.9 individuals, $F_{ST} = 0.0047 \pm 0.003$) when that sample was excluded.

Ours is the first report of female gene flow in *P. monodon* estimated from mtDNA-RFLP data. Restricted female gene flow was observed between Lamu and samples from the east of the peninsula (Lingayen, South China Sea and Java Sea) while greater numbers of migrants among geographic samples within Southeast Asian *P. monodon* were observed. Nonetheless, these female gene flow levels were not high enough to homogenise the populations and differentiation of *P. monodon* was revealed by significant differences in geographic heterogeneity ($P = 0.0000$) and *F*-statistics ($P < 0.001$) among different geographical samples.

The planktonic larval stages of *P. monodon* last 10-20 days (Bailey-Brock & Moss 1992). The larvae of this species would, therefore, be assumed to be carried passively by wind-induced currents. The reason why high dispersal ability of *P. monodon* larvae has not resulted in genetic homogeneity may be explained by the surface currents of the South China Sea (Dall 1956). The prevailing current may inhibit gene flow between the east of the Malaysian peninsula and the Andaman samples, resulting in reproductively isolated populations observed in the Andaman *P. monodon*.

Based on all analyses above, the Southeast Asian *P. monodon* samples were allocated into 3 different stocks composed of A (Trat, Surat, North Java, South Java and Dungun), B (Kedah, Medan and Satun) and C (Lingayen). The Lamu sample was also different from the Southeast Asian *P. monodon* and should be regarded as a different population. The genetic diversity and population differentiation found from this study yield critical information for an effective management programme. Therefore, geographically and genetically different populations of *P. monodon* should be recognised as different units and must be managed separately (Carvalho & Hauser 1994).

Acknowledgments. This work would not have been possible without the assistance from several scientists who help us to collect *P. monodon* specimens around Southeast Asia and from Kenya. The authors would also like to thank Drs. J. Brown, S. Smith, M. Briggs and A. Roque for their kindness in providing *P. monodon* postlarvae during the early stages of the experiments. This research was subsidised by the UK Overseas Development Administration (project R4802) awarded to BJM and DJP and by a Thai Government Scholarship to SK.

LITERATURE CITED

Allendorf FW and Ryman N (1987) Genetic management of hatchery stocks, In: Ryman N, Utter N (eds.). Population Genetics & Fishery Management. Washington Sea Grant Program, University of Washington Press, Seattle, p.141-159

Avise JC (1994) Molecular markers, Natural history and Evolution. Chapman and Hall, London

Bailey-Brock JH, Moss SM (1992) Penaeid taxonomy, biology and zoogeography, In: Fast AW, Lester LJ (eds.). Marine Shrimp Culture: Principles and Practices, Elsevier Science Publishers, Amsterdam, p 9-23

Benzie JAH, Ballment E, Frusher S (1993) Genetic structure of *Penaeus monodon* in Australia: concordant results from mtDNA and allozymes. Aquaculture 111: 89-93

Benzie JAH, Frusher S, Ballment E (1992) Geographical variation in allozyme frequencies of populations of *Penaeus monodon* (Crustacea: Decapoda) in Australia. Aust J Mar Freshwater Res 43: 715-725

Birky CW Jr, Furest P, Maruyama (1989) Organelle gene diversity under migration, mutation and drift: equilibrium expectations, approach to equilibrium, effects of heteroplasmic cells, and comparison to nuclear genes. Genetics 121: 613-627

Boom JDG, Boulding EG, Beckenbach AT (1994) Mitochondrial DNA variation in introduced populations of Pacific oyster, *Crassostrea gigas*, in British Columbia. Can J Fish Aqua Sci 51: 1608-1614

Bouchon D, Souty-Grosset C, Raimond R (1994) Mitochondrial DNA variation and markers of species identity in two penaeid shrimp species: *Penaeus monodon* Fabricius and *P. japonicus* Bate. Aquaculture 127: 131-144

Boulding EG, Boom JDG, Bechenbach AT (1993) Genetic variation in one bottlenecked and two wild populations of the Japanese scallop (*Patinopecten yessoensis*): empirical parameter estimates from coding regions of mitochondrial DNA. Can J Fish Aquat Sci 50: 1147-1157

Carvalho GR, Hauser L (1994) Molecular genetics and the stock concept in fisheries Reviews in Fish Biology and Fisheries 4: 326-350

Chapman RW, Powers DA (1984) A method for the rapid isolation of mitochondrial DNA from fishes. Maryland Sea Grant College, University of Maryland, Publication number UM-SG-TS-84-05

Dall W, Hill BJ, Rothlisberg PC, Sharples DJ (1990) Marine Biology vol. 27, The Biology of the Penaeidae. Academic Press, London

Dall WL (1956) Wind and drift currents in the South China Sea. The Malaysian J Tropical Geography 8: 1-31

Daud SK (1995) Population Genetics of Penaeus monodon Fabricius and Penaeus merguiensis De Man in Malaysia, PhD. Thesis, University of Stirling, UK. 327 pp

Ehrlich PR, Raven PH (1969) Differentiation of populations. Science 165: 1228-1232

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease to high specific activity. Anal Biochem 132: 6-13

Felsenstein J (1993) Phylip (Phylogenetic Inference Package) version 3.5c. Distributed by the author, Department of Genetics, University of Washington, Seattle

Klinbunga S (1996a) Genetic variation and population structure of the giant tiger shrimp (*Penaeus monodon*) PhD thesis, University of Stirling, UK, 356 pp

Klinbunga S, Penman DJ, McAndrew BJ (1996b) An improved protocol for total DNA isolation and visualisation of mtDNA RFLP(s) in tiger prawn, *Penaeus monodon*. Thai J Aquat Sci 3:36-41

Lynch M, Crease TJ (1990) The analysis of population survey data on DNA sequence variation. Mol Biol Evol 7: 377-394

McElroy D, Moran P, Birmingham E, Kornfield I (1991) REAP (Restriction Enzyme Analysis Package) version 4.0, University of Orono, Maine

McMillan WO, Raff RA, Palumbi SR (1992) Population genetic consequences of developmental evolution in sea urchins (genus *Heliocidaris*). Evolution 46: 1299-1312

Mulley JC, Latter BDH (1981a) Geographic differentiation of Eastern Australian penaeid prawn populations Aust J Mar Freshwater Res 32: 889-895

Mulley JC, Latter BDH (1981b) Geographic differentiation of tropical Australian penaeid prawn populations. Aust J Mar Freshwater Res 32: 897-906

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonuclease. Proc Natl Acad Sci USA 76: 5269-5273

Nei M, Tajima F (1981) DNA polymorphism detectable by restriction endonucleases. Genetics 97: 145-163

Nei M, Tajima F (1983) Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. Genetics 105: 207-217.

Naish KA, Carvalho GR, Pitcher TJ (1993) The genetic structure and microdistribution of shoals of *Phoxinus phoxinus*, the European minow. J Fish Biol 43, 75-89.

Oohara I, Mori K (1989) Cloning and characterization of mitochondrial DNA from the Japanese oyster, *Crassostrea gigas*. Bull Natl Res Inst Aquaculture 16:89-99

Ovenden JR (1990) Mitochondrial DNA and marine stock assessment: a review. Aust J Mar Freshwater Res 41: 835-853

Ovenden JR, Brasher DJ, White RWG (1992) Mitochondrial DNA analyses of the red rock lobster *Jasus edwardsii* supports an apparent absence of population subdivision throughout Australia. Marine Biol 112: 319-326

Palumbi SR, Wilson AC (1990) Mitochondrial DNA diversity in the sea urchins *Strongylogentrotus purpulatus* and *S. droebachiensis*. Evolution 44: 403-415

Phillips CA (1994) Geographic distribution of mitochondrial DNA variants and the historical biogeography of the spotted salamander, *Ambystoma maculatum*. Evolution 48: 597-607

Reeb CA, Avise JC (1990) A genetic discontinuity in a continuously distributed species: mitochondrial DNA in the American oyster, *Crassostrea virginica*. Genetics 124: 397-406

Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membrane. Nucleic Acids Res 13: 7207-7221

Roff DA, Bentzen P (1989) The statistical analysis of mitochondrial DNA polymorphisms: X^2 and the problems of small samples. Mol Biol Evol 6: 539-545

Ryman N (1991) Conservation genetics consideration in fishery management. J Fish Biol 39 (suppl. A): 211-224

Slatkin M (1987) Gene flow and geographic structure of natural populations. Science 236: 787-792

Sodsuk S (1996) Genetic Population Structure of Penaeus monodon Fabricius using Allozyme and Mitochondrial DNA Analysis. PhD thesis. University of Stirling, UK, 303 pp

Suraswadi P (1995) Use of biological filtration and closed systems to reduce environmental impacts from coastal shrimp (Penaeus monodon) culture. MSc. Thesis. University of Stirling, UK, 150 pp

Ward RD, Skibinski, DOF and Woodwark, M (1992) Protein heterozygosity, protein structure, and taxonomic differentiation. Evol Biol 26: 73-159

Ward RD, Grewe PM (1994) Appraisal of molecular genetic techniques in fisheries. Reviews in Fish Biology and Fisheries 4: 300-325

Weir BS, Cockerham CC (1984) Estimating *F*-statistics for the analysis of population structure. Evolution 38: 1358-1370

Quantitative Genetics and Genetic Transformation for the Selection of Pathogen-Resistant Shrimp

[1]Virna Cedeño, [2]Evelyne Bachère, [1]Jenny Rodriguez, [3]Edouard Bédier, [3]Emmanuel Goyard, [2]Viviane Boulo, [2]Jean-Paul Cadoret, [1]Eric Mialhe

[1]French-ecuadorian cooperation, Embajada de Francia, Quito, Ecuador
[2]IFREMER, Laboratoire DRIM (Défense et Résistance chez les Invertébrés Marins), Université Montpellier II, Place Eugène Bataillon, 34000, France
[3]IFREMER, COP, Centre Océanologique du Pacifique, Papeete, Tahiti

ABSTRACT: From its beginning, shrimp aquaculture has been based on animals obtained from the sea, either as post-larvae for supplying farms, or as adults for producing post-larvae in hatcheries. Such animals are non-adapted to the artificial conditions of shrimp culture that leads to physiological and immunological stress with a subsequent increased sensitivity to pathogens. Moreover, these wild animals constitute a permanent introduction of pathogens in hatcheries and farms, resulting in epidemics or endemics because of the high density of animals. In addition to economic losses resulting from infectious diseases, traditional shrimp aquaculture is threatened by ecological damage related to the overexploitation of wild shrimp populations, the use of antibiotics leading to residues with the subsequent occurrence of resistant bacteria pathogenic for shrimp or humans, and the destruction of mangroves for moving farms to non-infected areas. All around the world, domestication of shrimp and selection of pathogen-resistant strains appear as the chief priorities for economic and ecological sustainability of shrimp aquaculture. Considering the complex molecular basis of animal growth and pathogen resistance and the nature and variety of shrimp diseases, a double strategy has been developed for shrimp genetic selection that is based on quantitative genetics and genetic transformation. For domestication and selection through quantitative genetics, several traits related to key immune effectors and pathogen resistance were considered as essential criteria in addition to growth and fitness characters. Genetic transformation was considered as a complementary way to obtain multi-resistant domesticated shrimp through the expression of homologous or heterologous immune genes and the expression of specific viral proteins or ribozyme sequences. Domestication and breeding programs through quantitative genetics can now be undertaken by shrimp producers, first because shrimp maturation and larval culture are well established procedures, second because several assays have been designed for individual evaluation of several essential quantitative immune characters and third because challenge tests have been optimized for family evaluation of resistance against highly pathogenic vibrios and viruses. Moreover, some microsatellites have been identified in shrimp genomes and AP-PCR technology has been shown to be suitable to characterize family or individual RAPD genetic patterns with DNA extracted from small hemolymph samples. These genetic markers allow several families to be mixed in a common pond for reliable comparisons of performance. Such breeding programs are urgently needed, but to be successful, they need to be organised by teams of specialists in shrimp genetics, immunology and pathology, thus taking into account the diversity of technologies and concepts that have to be managed. For genetic transformation of shrimp, much important progress has been made in the development of gene transfer technology. Transfection can be achieved through biolistic, lipofection and/or viral vectors. Heterologous promoters have been shown to be efficient to control gene expression. Research is currently focused both on the identification of specific transposable elements to construct integration vectors and on the evaluation of heterologous viral integration vectors.

KEY WORDS: shrimp, genetics, quantitative genetics, genetic transformation, breeding program, growth, pathogen resistance, immune characters, genetic markers

INTRODUCTION

Shrimp aquaculture is a crucial component in the economy of several developing countries, where there is a continuous impetus for production increases in order to decrease the fishing impact on overexploited wild populations. The productivity and the sustainability of shrimp aquaculture are, however, permanently menaced all around the world by epizootics of endemic and exotic orgin. The annual losses caused by infectious diseases on world shrimp production have been estimated at 3,000 million dollars per year, whereas the value of annual production is approximately 3,700 million dollars (Lundin, pers. comm.).

Infectious shrimp diseases are caused mostly by bacteria (particularly vibrio species that produce toxins) and viruses

Cedeño V, Bachère E, Jenny Rodriguez J, Bédier E, Goyard E, Boulo V, Cadoret J-P, Mialhe E (1998) Quantitative genetics and genetic transformation for the selection of pathogen-resistant shrimp. *In* Flegel TW (ed) Advances in shrimp biotechnology, National Center for Genetic Engineering and Biotechnology, Bangkok.

(the majority of which are related to highly pathogenic viruses of insects). Diseases may occur as outbreaks after the introduction of infected animals into hatcheries or farms. Many such animals come from local wild populations or from distant areas through international commercialization without sanitary regulations for pathogen screening. More frequently, diseases affect shrimp production as endemics, since the pathogens are present as reservoirs in continuously populated shrimp hatcheries and farms. Pathogens persist through the continuous supply of shrimp and also increase in pathogenicity through genetic changes such as mutation, transduction, transposition or transformation. These changes may include the generation of antibiotic resistant strains of bacteria that results from the routine use of antibiotics at low concentrations. This, in turn, can lead to high levels of antibiotic residues in shrimp farming areas. The occurrence and increase in antibiotic resistance affects not only shrimp but also human pathogens, creating high-risk situations for human health in countries with underdeveloped medical care.

Infectious diseases are also facilitated because shrimp aquaculture is presently based on wild animals that are adapted to natural conditions and not to the artificial conditions of shrimp hatcheries and farms, where water quality, microbiological flora and nutrition are vastly different from those in the sea. Intensive rearing conditions are stressful for shrimp and lead to physiological disturbances or immunodeficiencies that increase sensitivity to pathogens.

The impact of infectious diseases could be partially reduced by use of epidemiological surveys with efficient diagnostic methods (Mialhe et al. 1995), in parallel with use of probiotic bacteria that compete with pathogenic bacteria for colonization of the digestive tract in larvae and adult shrimp. However, as shrimp cannot be efficiently protected by vaccination because they lack an antibody-mediated immune response, genetic selection of pathogen resistant shrimps appears the only way to control shrimp mortalities simultaneously with an improvement in growth based on better adaptation of domesticated shrimp to rearing conditions.

Domestication may be easily achieved whatever the shrimp species. For example, some research centers have developed reliable methodologies for broodstock maturation and larval culture that have been successfully applied up to thirty generations (Ifremer COP). On the basis of these methodologies, a double strategy has been investigated for genetic selection based on growth rate and pathogen resistance. This double selection involves quantitative genetics and genetic transformation: shrimp first selected through quantitative genetics will subsequently be transformed with homologous or heterologous immune genes, viral protein genes or viral ribozyme sequences.

QUANTITATIVE GENETICS AND BREEDING PROGRAMME

Quantitative traits for individual and family selection related to pathogen resistance

Whereas growth rate and fitness can be directly and easily estimated, specific assays had to be developed for measuring quantitative traits related to the key immune effectors in live shrimp. Such immune traits correspond to original criteria for individual selection in shrimp breeding programs. Such programs must be conceived by considering simultaneously pathogen-resistance traits with growth rate and fitness traits in order to avoid the selection of large individuals with genetic immunodeficiency. The mistake of selecting for growth only has frequently resulted in the production of domestic animals and plants that is dependent on treatment with vaccines and chemicals. Moreover, selecting only for growth rate leads generally to an intensification of culture, making rearing conditions more stressful and generating a higher risk of disease.

During the last few years, the most relevant immune effectors have been studied by Ifremer and evaluated as individual selection traits. Specific reagents and assays were developed and optimized as tools for applied breeding programs (Bachère et al. 1995a, 1995b).

Individuals with superior or specific pathogen resistance survive an experimental infection and can be identified and subsequently used as broodstock in a breeding program based, partly or totally, on individual selection. This concept is not relevant for viruses, since there is the risk of selecting apparently healthy animals that in fact carry the virus and can subsequently propagate it inside the facilities of the breeding program. In the case of bacteria, the selection of broodstock animals that survive strong experimental infections represents an acceptable strategy for individual selection, since the animals can later be cleaned from pathogenic bacteria by antibiotic treatment. Consequently, the different challenge tests that have been developed and optimized for pathogenic bacteria or viruses will be preferentially considered as quantitative traits for family selection.

Quantitative immune traits for individual selection

Hemograms

The shrimp immune response to pathogens is based on cellular and humoral effectors. Hemocytes are the effectors of the cellular immune response and they are also involved in the synthesis of the majority of humoral effectors. Three major types of hemocytes have been identified (hyalinocytes, semi-granulocytes and granulocytes) that have specific functions (Bachère et al. 1995). Consequently, a hemogram (i.e., the number and the proportion of each hemocyte type), was considered as a potentially interesting trait for selection related to resistance. It has been previously shown in oysters that animals with higher numbers of hemocytes were significantly more resistant to an intracellular parasite (Hervio et al. 1995).

The individual and temporal variability of *Penaeus vannamei* hemograms were investigated on the basis of a large scale analyses of individual shrimp hemolymph samples. The shrimps were sampled at the same time during several molt cycles in order to avoid problems of intra-cycle fluctuations. An important variability of hemograms was found and some individuals with a stable and significantly higher number of hemocytes were identified. Consequently,

the hemogram constitutes an interesting quantitative trait for a breeding program aimed at pathogen resistance.

Hemocyte respiratory burst

Hemocytes, as cellular immune effectors, are directly involved in the elimination of invading pathogens through phagocytosis, associated with a process known as "respiratory burst". This consists of pathogen killing mediated by oxygen radicals produced by a set of hemocytic enzymes. Consequently, the phenomenon of respiratory burst can be considered as an immune oligogenic trait and that makes it an interesting candidate for selection of pathogen resistant shrimp. An *in vitro* miniaturized assay has been developed and optimized for individual quantification of shrimp hemocyte respiratory burst (Bachère et al. 1995b). Then, as for hemograms, the individual and temporal variability of *P. vannamei* respiratory burst were investigated based on a large scale analyses of shrimp considered at the same time during several molt cycles in order to avoid intra-cycle fluctuations. An important variability of the phagocytic oxidative activity (respiratory burst) was observed, and some individuals were found with a stable and significantly higher activity. They therefore had a higher potential capacity to eliminate and kill pathogens.

Plasma antibacterial activity

Pathogenic bacteria, in particular vibrios, are endemically associated with shrimp at all life stages. Bacterial pathogenicity is essentially based on capacity to colonize the shrimp digestive tract, kill host cells through toxin production and cause diarrhea. After the massive destruction of host cells, the disease frequently evolves as septicemia. Whereas the diarrhea can directly lead to death in larvae, evolution of the disease in juveniles and adults depends on their capacity to eliminate and kill the bacteria invading the haemolymph. Besides phagocytosis and the oxidative defense mechanism mediated by hemocytes, shrimp can kill invading bacteria through bactericidal activity of peptides present in the plasma. Recently, some related peptides named penaeidins, have been purified and characterized biochemically, antigenically and genetically (Destoumieux et al. 1997). A miniaturized assay, based on spectrophotometric quantification of bacterial growth reduction in the presence of shrimp plasma, has been developed, leading to the identification of individuals with a stable and extremely high capability to kill bacteria.

ELISA for plasma agglutinin

Agglutinins are key circulating molecules of shrimp plasma since their function is to recognize and to bind invading microorganisms. This binding process, known as opsonization, facilitates the subsequent phagocytosis by hemocytes (Bachère et al. 1995b). A monoclonal antibody specifically reacting with agglutinins of several shrimp species has been produced (Rodriguez et al. 1995) and an ELISA has been developed for an accurate quantification of plasma agglutinin. This humoral immune effector corresponds to a monogenic trait that makes it very attractive in terms of genetic selection.

The individual and temporal variability of *P. vannamei* plasmatic agglutinin concentration were investigated on the same type of large scale shrimp analyses used for hemograms and respiratory burst. Plasmatic concentration of agglutinin was shown to be highly variable. Among animals kept in the same conditions, some were found with a stable and significantly higher concentration of agglutinin, thus making them more efficient in the recognition and opsonization of invading microorganisms.

ELISA for plasma coagulogen

Coagulogen is another key circulating molecule of shrimp plasma, since its function is to form a coagulum at the site of wounds from which microorganisms can invade the shrimp body. This process, known as coagulation, needs to be very fast in order to reduce the volume of lost haemolymph and the risk of pathogen invasion (Bachère et al. 1995b). A monoclonal antibody specifically reacting with the coagulogen of several shrimp species has been produced (Rodriguez et al. 1995) and an ELISA has been developed for its accurate quantification. This humoral immune effector corresponds to a monogenic trait, that makes it very attractive in terms of genetic selection.

The individual and temporal variability of *P. vannamei* plasmatic coagulogen concentration were investigated as before and individuals with a stable and significantly higher potential capability for coagulation were identified.

Immune index

The preceding quantitative immune traits can be considered together in order to determine an individual immune index. A preliminary step consists of expressing each one as a percentage by referring to the range of observed values. Thus, the minimal and the maximal observed values were considered as 0% and 100%. For example, with hemograms ranging between 1 and 50 million hemocytes per mL, an animal with 20 million hemocytes per mL would have an index value of 40%. The simple addition of each percentage index corresponds to an overall index where all the traits were considered of equal importance in a breeding program. However, according to the breeding goal, some traits might be favored in particular lines. An immune index corresponding to the five quantitative traits previously described has been applied to the identification of adult shrimp with superior immune potential. These animals are candidates for broodstock in a breeding program orientated towards the selection of pathogen resistant lines.

Quantitative pathogen-resistant traits for family selection

It is important to state that all the quantitative traits previously examined for individual selection can also be considered for a breeding program based partly or totally on family selection.

Challenge tests for resistance to pathogenic bacteria

The larval production of shrimp is endemically affected by bacteriosis worldwide. In the case of *P. vannamei*, endemics are known as "bolitas syndrome" or "zoea II syndrome". These syndromes were shown to be vibriosis by comparison of the etiological microorganisms through biochemical and genetic analyses. Two vibrio isolates, *Vibrio harveyi* (S2) and *V. vulnificus* (E22), constitute useful reference strains. Recently, other pathogenic strains have been isolated, but were difficult to identify because of their inca-

pacity to grow in most identification media. This suggests a process of specialization by the pathogenic bacteria for parasitic life in the form of genetic mutations for some metabolic processes, a phenomenon well known in human medicine where non-cultivable, pathogenic bacteria have been identified (Carrera Int., pers. comm.).

Among the bacteria that have been isolated from the normal bacterial flora of *P. vannamei* larvae, one identified as *V. alginolyticus "strain Ili"* (Morales, pers. comm.) has been intensively investigated as a competitor to pathogenic bacteria for colonization of the shrimp digestive tract (San Miguel, pers. comm.). This species can prevent infection by pathogenic bacteria and has been successfully used as a probiotic in shrimp hatcheries and farms (Carrera Int., pers. comm.).

In the case of challenge tests performed to determine bacterial resistance of shrimp larvae, the pathogenic strain *V. vulnificus* (E22) and the probiotic strain *V. alginolyticus* (Ili) are being routinely used according to an optimized protocol of experimental infection (San Miguel, pers. comm.). The reliability of this protocol results from the use of disinfected Nauplius V larvae that allows the monocolonization of the digestive tract, either with Ili or E22, without any problem of mixed infections by unidentified bacteria previously present in the digestive tract. Thus, by mono-infection with probiotic bacteria, it is possible to estimate the control survival rate whereas the mono-infection with pathogenic bacteria permits the determination larval survival rate in comparison to the control. According to this protocol, it is possible to compare survival values amongst different families at any time and, for any family from one generation to another. This is essential for the efficiency and the reliability of a breeding program.

This protocol is not only useful for family selection but could also be applied to individuals by selecting animals surviving an infection arising from an extremely high number of pathogenic bacteria. In order to avoid the risk of pathogenic bacterial propagation, the selected animals would have to be treated by antibiotics and probiotics.

Because of the strong impact of septicemic vibriosis during the first weeks of shrimp culture, it is also very interesting to consider survival rates from experimental infections as a quantitative trait for family selection oriented to pathogen-resistance. The protocol of infection to reproduce septicemia in juveniles is much easier than the one just described for larvae, since it consists solely of the injection of pathogenic bacteria.

Challenge tests for resistance to viruses

Resistance to viruses could essentially result from immune effectors or from mutations (e.g., in the gene of a receptor on the host cell membrane). If the resistance corresponds to infection without mortalities, the selection may be hazardous since selected animals could permanently produce viruses that can mutate and become highly pathogenic. If the resistance corresponds to a refractory state (e.g., resulting from a receptor mutation) there would be a risk for the selected animals to be later infected by mutant viruses. Thus, selection for viral resistance in shrimp appears to be relatively uncertain through quantitative genetics. However, because of the terrible impact of viral epizootics, it is extremely important to consider virus resistance as a quantitative trait for family selection. Individual selection from animals surviving experimental infections could be considered, provided that the selected animals were checked for the absence of virus by using highly sensitive diagnostic assays.

In comparison to bacteria, reliable challenge tests for viral resistance are very difficult to perform because of the lack of shrimp cell lines for production and titration of viruses. The best alternative would be to use viral suspensions purified from infected animals and subsequently titered through LD50 assays. Moreover, it would be adequate to prepare large amounts of purified viral suspensions and to keep them frozen in order to analyze, with the same virus stock, the evolution of shrimp sensitivity to virus during the breeding program. The use of non purified suspensions that can be contaminated with other pathogens may lead to erroneous interpretations, in particular if comparisons between generations are made by infecting shrimp with different viral suspensions. Finally, challenge tests would have to be performed by injection in preference to ingestion for better reproducibility, making it necessary to obtain bacteria-free suspensions to avoid septicemia.

Due to the present impossibility of producing and titrating shrimp viruses, however, challenge tests for viruses such as BPV, IHHNV, or TSV would have to be relatively rudimentary since they would have to be performed with many samples during several years of a breeding program. The results of these challenges would have to be considered with extreme caution.

Genetic markers as useful tools for efficient breeding

Shrimp farmers know very well the extreme variability between ponds, tanks, seasons and years, in terms of water quality, primary productivity, zooplankton, bacteria, etc. An objective comparison between lines during the course of a breeding program depends on the possibility of mixing lines inside ponds with the possibility of later identifying animals belonging to each line. Different tag systems have been developed, in particular based on the injection of colored elastomer (Godin et al. 1996). Such systems are suitable for tagging juveniles or adults but are inappropriate for postlarvae or for mass tagging operations.

Genetic markers have been considered as the necessary alternative. Investigations have been made during the last few years in order to identify microsatellites and to evaluate the technique of AP-PCR to determine RAPDs in different shrimp species (Garcia and Benzie, 1995, Garcia et al. 1994, Tassanakajon et al. 1997). Thus, a few teams are presently able to characterize shrimp by RAPD, the unlimited availability of primers allowing specific markers to be obtained for any family in the course of a breeding program. This technology has been optimized for the evaluation of both immune traits and RAPD on the same hemolymph sample.

ORGANIZATION OF A BREEDING PROGRAMME FOR IMPROVEMENT OF GROWTH RATE, FITNESS AND PATHOGEN RESISTANCE

As previously mentioned, a breeding program for improvement of growth rate, fitness and pathogen resistance, depends on the organization of a multidisciplinary team that includes specialists working closely with producers. The specialists should include those concerned with shrimp maturation, larval culture, pathology, immunology and genetics. The success of the selection program will depend on the quality of the shrimp culture and on the reliability of the analyses for quantifying selection traits and determining the genetic markers.

Base population

The base population will include animals from several geographic origins and individuals from "domesticated" stocks, corresponding generally to animals produced in captivity for two or three generations .

Genotype-environment interactions

Whatever the species, shrimp aquaculture is carried out in a wide variety of environmental conditions, which makes it necessary to consider the possible interactions between genotypes and environment. However, it is necessary to create a hierarchy of the various environmental parameters, since it is impossible to repeat each selection experiment in all the different environments encountered for shrimp aquaculture. In a country like Ecuador, the most relevant environmental criterion corresponds to salinity, since farms can be located in area ranging from the sea to fresh water. Thus, a breeding program would have to consider at least these two environmental extremes.

Selection mode

The availability of assays to quantify traits for individual and family selection will enable the breeding program to develop on the basis of combined selection, which is considered unanimously to be the most efficient selection method. Thus, the best individuals (individual selection) from the best families (family selection) can be used as broodstock for the next generation. Under combined selection, the different families must be kept in environmental conditions as similar as possible, making it necessary to produce all the larvae in the same hatchery in as short a period as possible. The animals would also have to be tagged and pooled as early as possible. In order to estimate the genetic gain related to selection, it is important to include a control group.

Individual selection, which is relatively efficient for traits with high heritabilities, is currently the most frequently applied selection method for increased growth rate in aquaculture species. This could also be very useful for immune traits, some of which are possibly correlated and some monogenic. Individual selection, however, can cause inbreeding problems that must be permanently controlled and managed in the course of a breeding program. An interesting approach would be to establish homozygous lines through inbreeding and a very strong process of multi-trait selection, in parallel with analysis of heterozygous progeny performances of crossed-lines. It is interesting to note that a high level of consanguinity is acceptable for shrimp, since the strains kept at Ifremer-COP for many generations were established from a very small number of animals without any problems of growth or fitness.

GENETIC TRANSFORMATION

By referring to pathogen-resistant transgenic plants and vertebrates, genetic transformation has been considered for shrimp as the way to obtain multi-resistant strains against bacteria, through the expression of homologous or heterologous genes coding for antibacterial peptides. Strains resistant to viruses could be obtained through the expression of ribozymes corresponding to viral sequences. Moreover, in common with other organisms, for instance the fruit fly, *Drosophila melanogaster*, or the nematode, *Caenorhabditis elegans*, genetic transformation will be extremely useful for basic research on all the fields of shrimp biology.

Compared with quantitative genetics, shrimp genetic transformation is still in the research phase with investigations related to the identification of candidate genes and other studies focused on the development of reliable gene transfer technology. These topics have been recently reviewed (Mialhe et al. 1995, Mialhe et al. 1997, Bachère et al. 1997). In terms of selection of pathogen-resistant shrimps, quantitative genetics and genetic transformation must be considered as two complementary approaches.

Much important progress has been made for the development of gene transfer technology. Transfection can be achieved through biolistic (Gendreau et al. 1995), lipofection and/or viral vectors. Heterologous promoters have been shown to be efficient in the control of gene expression. Research is currently focused both on the identification of specific transposable elements to construct integration vectors and on the evaluation of heterologous viral integration vectors.

LITERATURE CITED

Bachère E, Mialhe E, Rodriguez J (1995a) Identification of defense effectors in the haemolymph of crustaceans with particular reference to the shrimp *Penaeus japonicus* (Bate): prospects and applications. Fish Shellfish Immunol 5:597-612

Bachère E, Mialhe E, Noël D, Boulo V, Morvan A, Rodriguez J. (1995b) Knowledge and research prospects in marine mollusc and crustacean immunology. Aquaculture 132:17-32

Bachère E, Cedeño V, Rousseau C, Destoumieux D, Boulo V, Cadoret JP, Mialhe E (1997) Transgenic crustaceans. World Aquacult 28:51-55

Destoumieux D, Bulet P, Loew D, Van Dorselaer A, Rodriguez J, Bachère E (1997) Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). J Biol Chem 272:28398-28406

García DK, Benzie JAH (1995) RAPD markers of potential use in penaeid prawn (*Penaeus monodon*) breeding programs. Aquaculture 130:137-144

García DK, Faggart MA, Rhoades L, Alcívar-Warren AA (1994) Genetic diversity of cultured *Penaeus vannamei* shrimps using three molecular genetic techniques. Mol mar Biol Biotechnol 3:270-280

Gendreau S, Lardans V, Cadoret JP, Mialhe E (1995) Transient expression of a luciferase reporter gene after ballistic intro-

duction into *Artemia franciscana* (Crustacea) embryos. Aquaculture 133:199-205

Godin DM, Carr WH, Hagino G, Segura F, Sweeney JN, Blankenship L (1996) Evaluation of a fluorescent elastomer internal tag in juvenile and adult shrimp *Penaeus vannamei*. Aquaculture 139:243-248

Hervio D, Bachère E, Boulo V, Cochennec N, Vuillemin V, Le Coguic Y, Cailletaux G, Mazurié J, Mialhe E (1995) Establishment of an experimental infection protocol for the flat oyster, *Ostrea edulis*, with the intrahaemocytic protozoan parasite, *Bonamia ostreae*: application in the selection of parasite-resistant oysters. Aquaculture 132:183-194

Mialhe E, Bachère E, Boulo V, Cadoret JP, Rousseau C, Cedeño V, Saraiva E, Carrera L, Calderon J, Colwell RR (1995) Future of biotechnology-based control of disease in marine invertebrates. Mol mar Biol Biotechnol 4:275-283

Mialhe E, Boulo V, Cadoret JP, Cedeño V, Rousseau C, Motte E, Gendreau S, Bachère E (1997) Gene transfer technology in marine invertebrates. In: Houdebine LM (ed) Transgenic animals: generation and use, Amsterdam, Harwood Academic Publishers, p151-155

Rodriguez J, Boulo V, Mialhe E, Bachère E (1995) Characterization of shrimp haemocytes and plasma components by monoclonal antibodies. J Cell Sci 108:1043-1050

Tassanakajon A, Pongsomboon S, Rimphanitchayakit V, Jarayabhand P, Boonsaeng V (1997) Random amplified polymorphic DNA (RAPD) markers for determination of genetic variation in wild populations of the black tiger prawn (*Penaeus monodon*) in Thailand. Mol mar Biol Biotechnol 6:110-115

Estimated Heritabilities for Early Growth Rate of the Black Tiger Prawn, *Penaeus monodon*, Fabricius

[1,3]P. Jarayabhand, [2]S. Uraiwan, [4]S. Klinbunga, [5]A. Tassanakajon [6]P. Srimukda, [1]P. Pattanachan, [7]R. Panakulchaiwit, [3,4,7]P. Menasveta

[1]Angsila Marine Biological Research Station, Department of Marine Science, Faculty of Science Chulalongkorn University, Chon Buri, 20210, Thaiand
[2]National Aquaculture Genetics Research Institute, Department of Fisheries Ministry of Agriculture and Cooperative, Patum Thani, 12120, Thailand
[3]Aquatic Resources Research Institute, Chulalongkorn University, Bangkok, 10330, Thailand
[4]Marine Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology Ministry of Science Technology and Environment, Phyathai Rd., Bangkok 10330, Thailand
[5]Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, 10330, Thailand
[6]Chanta Buri Coastal Aquaculture Center, Department of Fisheries, Chanta Buri, Thailand
[7]Department of Fisheries Science, Faculty of Agricultural Technology, KMITL Chalongkrung Rd., Bangkok, 10520, THAILAND

ABSTRACT: Heritabilities for growth of the black tiger prawn, *Penaeus monodon*, were estimated at the ages of 25 (PL_{15}) and 65 (PL_{55}) days by a full-sibs mating design. An artificial insemination technique (male:female = 1:1) was used to create twenty-one full-sib families in three batches (i.e., batch no. 1 included 9 full-sibs, batch no. 2 included 5 full-sibs, and batch no. 3 included 7 full-sibs) over a one month period. Growth rates measured as total length at 25 days were significantly affected by batch, rearing conditions and full-sibs family. Effects from batch numbers and replicates were not significant with respect to growth rate at the age of 65 days, but the effect from full-sibs families was. At the age of 25 days, heritability for growth rate measured as total length was 0.153±0.060, whereas the heritabilities at the age of 65 days were 0.073±0.037 for total length and 0.053±0.029 for wet weight. Decline in the estimated heritabilites from 25 days to 65 days was partly due to a confounding of common environmental factors in full-sibs variance at the early stage of life which decreased as the animals got older. In any case, this experiment demonstrated a significant genetic component controlling growth rate in the species. Advantages and disadvantages of the method used in this experiment for heritabiliity estimation are discussed. It was concluded that further investigations with a better experimenal design based on a full-sibs mating design were required to obtain better heritability estimates for growth rates of natural stocks of *P. monodon* in Thailand. Finally, a high phenotypic correlation between total length and wet weight in *P. monodon* meant that either total length or wet weight could be used to represent growth rate for a selective breeding program.

KEY WORDS**:** Genetics, *Penaeus monodon*, growth rate, heritability, full-sibs mating design

INTRODUCTION

Aquaculture of the black tiger prawn, *Penaeus monodon*, has been practiced in Thailand for more than 2 decades with very satisfactory results. Development of this type of aquaculture has consistently evoled from a very extensive system to an intensive one. Up to the present, *P. monodon* culture is widely known and accepted as a very important argicultural practice in Thailand. Despite the advanced production technology being practiced, the production cycle of *P. monodon* is still open. In other words, all broodstock used in nauplii production are captured from the wild. This practice arose historically from the ready availability of inexpensive captured broodscock and therefore, this peneaid species has not been domesticated. This practice makes it impossible to apply any selective breeding program in order to improve production efficiency by genetic manipulation.

Recently, due to many production problems related to such issues as disease, slow growth rate etc., the issues of domestication, genetic improvement and selective breeding for *P. monodon* have been consistently raised by most concerned parties (Jarayabhand et al. 1997). In general, increased growth rate is widely accepted as an important economic trait. With this aim, a three year reseach project was initiated in Thailand in 1996 to develop an appropriate selective breeding program for *P. monodon*. Estimation of heritability for growth rate was part of this project.

To calculate heritability, many methods are available. These include sib analysis (both half-sibs and full-sibs), offspring-parent regression and response to selection (Falcomer 1989). Lester (1988) reported on heritabilities with large errors for growth rate at larval stages of *P. stylirostris* and *P.*

Jarayabhand P, Uraiwan S, Klinbunga S, Tassanakajon A, Srimukda P, Pattanachan P, Panakulchaiwit R, Menasveta P (1998) Estimated heritabilities for early growth rate of the black tiger prawn, *Penaeus monodon*, Fabricius. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

vannamei. Strong influences of environmental factors on larval growth of these penaeid species were mentioned. In 1997, Benzie *et al*. reported on estimation of heritability using eighteen half-sib families of *P.monodon*. From this report, the strong influence of environmental factors controlling phenotype of this aquatic species was also emphasised. These factors resulted in highly variable data which lowered estimated heritabilities in his experiment. In 1997, Fjalestad *et al*. reported that estimated heritability for harvest weight in *P. vannamei* based on maternal half-sibs and full-sibs were 0.50±0.13 and 0.45±0.1, respectively. In the same year, Hetzel *et al*. (1997) also reported on realized heritability of harvested weight (PL_{185}) in *P. japonicus* with a value of 0.298. An asymmetry of realized heritabilities between high and low selection lines was also recognized. Estimated heritabilities using various calculation methods are shown in Table 1. Such information for local stocks of *P. monodon* in Thailand is still lacking. This paper describes our early attempt to estimate heritability for growth rate in a natural stock of *P. monodon* in Thailand.

MATERIALS AND METHODS

Broodstock

From Trad province situated on the eastern coast of the Gulf of Thailand, broodstock of *P. monodon* were caught by trawler and transported to a private hatchery. Males and females were kept separately in two 50 m^3 cement tanks provided with chlorinated sea water at salinity 30 ppt and 28-30°C. Artificial insemination (Pratoomchat et al. 1993) was accomplished by transfer of spermatophores from males to newly molted females (less than 24 hrs after molt) on a 1:1 basis. Following, this the females were subjected to unilateral eye-abalation to induce spawning of full-sib families. The broodstock were fed with a combination of fresh food including squid, polychaetes and clams until satiation. Female maturation stage was monitored daily. Fully matured females (stage IV) were transferred separately to 500-l spawning tanks filled with chlorinated sea water at salinity 30 ppt and 28-30°C. Spawning usually took place at night and newly hatched nauplii were obtained by the next evening.

Rearing of full-sib family larvae

Newly hatched nauplii of various full-sib families were transported to the shrimp hatchery at Angsila Marine Biological Research Station, Chon Buri. The nauplii from each full-sibs family were stocked into separate 500-l tanks. Initial stocking densities were controlled at 15,000 nauplii/tank. Filtered, dechlorinated sea water at salinity 28-30 ppt was used. Larvae of each full-sib family were reared separately in two replicate tanks according to our standard PL production protocol. Mixtures of *Chaetoceros sp*., cooked and fresh brine shrimp were given to the larvae at appropriate times and in appropriate amounts. At the age of 25 days (PL_{15}), the total length of 30 individuals from each tank was measured. Then, 150 individuals from each full-sib family were randomly sampled and put into 70x40x55 cm^3 cages of 1 mm mesh size at density of 50 individuals/cage (three replicates for each full-sib family). These cages were hung in 0.8x9x1 m^3 cement troughs with a maximum of 18 cages/tank. The tanks were continuously supplied with recirculated sand-filtered sea water in a closed system. The larvae were fed in excess four times a day with a commercial formulated feed (No. 1). At the age of 65 days (PL_{55}), total length and wet weight of all individuals in each cage were measured.

Statistical calculations

SYSTAT 6.0 for Windows (SPSS Inc. 1996) was used for statistical analyses and calculation of intraclass correlation or heritability (Becker 1992). For unequal family sizes, adjustments were made (Sokal & Rohlf 1981).

Computational models

25 DAYS $Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{k(j)} + \varepsilon_{ijk}$ (1)

where Y_{ijk} is the dependent variable
μ is a constant
α_i is the fixed effect from rearing condition i^k
β_j is the fixed effect from batch number j^k
$\gamma_{k(j)}$ is the random effect from full-sibs family k
ε_{ijk} is the error term.

65 DAYS $Y_{ijk} = \mu + \alpha_i + \gamma_{i(j)} + \varepsilon_{ijk}$ (2)

where α_i is the random effect from batch number i^k
$\gamma_{i(j)}$ is the fixed effect from replication j^k nested in full sibs family i^k
ε_{ijk} is the error term

Table 1. Estimated heritabilities using various methods in penaeid shrimp species.

Species	Heritability	Methods	Authors	Remark
P. stylirostris	0-1 with large errors	full-sib design	Lester 1988	larvae and PL
P. vannamei	0-1 with large errors	full-sib design	Lester and Lawson, 1990	larvae and PL
P. monodon	0.1 (sire;lt and wt) 0.5-0.6 (dam; lt and wt)	half-sib design	Benzie et al. 1997	6 weeks
	0.1 (sire; lt and wt) 0.3-0.4 (dam; lt and wt)	half-sib design	Benzie et al. 1997	10 weeks
P. vannamei	0.50±0.13	maternal half-sibs	Fjalestad *et al.* 1997	harvest weight
	0.45±0.10	full-sibs	Fjalestad *et al.* 1997	harvest weight
P. japonicus	0.277±0.083 (wt)	regression (offspring on mid parents)	Hertzel et al. 1997	PL185
	0.298 (wt)	response to selection	Hertzel et al. 1997	PL185

Table 2. Analysis of variance for partition of variance components used to calculate heritabilities at 25 and 65 days age in *P. monodon*.

Source	DF	Mean square	F-Ratio	Variance component	% variance component
25 days: (total length)					
Rearing conditions	1	15.313	13.476*	0.033	1.57
Batch number	2	247.229	217.571*	0.767	36.57
Between matings (Full-sibs number)	18	8.669	7.629*	0.161	7.68
Within progenies	968	1.136		1.136	54.0
65 days: (total length)					
Between matings (Full-sibs number)	13	269.860	3.983*	2.570	3.65
Within progenies	1100	67.751		67.751	96
65 days: (wet weight)					
Between matings (Full-sibs number)	13	0.148	3.166*	0.001	2.1
Within progenies	1100	0.047		0.047	97.90

DF = degrees of freedom; * significant at p-value<0.001; At 65 days for both total length and wet weight, effects from batch no. and replication were not significant (p-value>0.05) and they were dropped from the computational model.

RESULTS

Twenty-one full-sib families of *P. monodon* were obtained. Due to the limited number of spawners each day, nauplii available each day were brought into the station in three batches over 1 month (i.e., 9 full-sibs in Batch 1, 5 full-sibs in Batch 2, and 7 full-sibs in Batch 3). At the age of 65 days, all the PL from Batch 3 died. Partition of variance components used in the heritability estimation are shown in Table 2. At the age of 25 days, effects from batch number and rearing conditions were significant. Therefore, these factors were included in the calculation model. However, at the age of 65 days, the parameters batch number and replication were not significant and they were dropped from the calculation model (equation 2). Partitions of variance used for calculation of heritabilities at 25 and 65 days are shown in Table 2. At this age, an allometric equation between phenotypes, total length (TL; cm) and wet weight (WW; g) was also calculated and shown below.

$WW = 0.00485TL^{3.099}$ (P-value<0.001; n=1114; r^2=0.851)

DISCUSSION

At the beginning of this experiment, a half-sibs mating design was prepared as described by Benzie *et al.* (1997). However, this trial was not successful. The problems were due to low success in artificial insemination. In this experiment, only about 55% of the inseminated females spawned. The percentage was even lower when considered as complete half-sibs family number produced. Thus, it was difficult to calculate heritability by this design. Spawning synchronization was also another obstruction. Therefore, a full-sibs mating design was used instead. By this design, many full-sib families were produced. Intraclass correlations or heritabilities based on full-sibs were calculated. Later at mature age, these families will be reproduced and calculation of response to selection (realized heritability) will also be estimated.

It is known that heritability calculated by this method is confounded by non-genetic variance components such as common environments and maternal effects. These may result in overestimation of the obtained calculations. In this experiment, effects of some non-genetic variance components were significant. These variance components were to a certain extent removed from calculations, and this improved the accuracy of the obtained estimated heritabilities. In addition, these types of variance components were expected to decrease as the animals grew older. Such depletion was demonstrated again in this experiment and might partly be an explanation for reduction of the estimated heritabilities from age 25 days to 65 days. Calculated heritabilities in this experiment were low (Table 3), especially when compared with those calculated for *P. vannamei* (Fjalestad *et al.* 1997) and *P. japonicus* (Hertzel et al. 1997). However, they were in the same range (Table 1) as those reported by Benzie et al. (1997) for the same species. In both cases, the % variance component of error terms was high. Adjustment for non-genetic effects such as rearing conditions and batch at 25 days resulted in a reduction of the % variance component to 54% (Tabel 2), whereas error terms reported by Benzie et al. (1977) were 83.5-89%. Benzie et al. (1997) used a half-sibs

Table 3. Heratabilities for total length (cm) and wet weight (g) of *P. monodon.*

	Mean	CV	h^2	s.e.	n	# full-sib families
25 days						
total length	0.96	0.119	0.153	0.060	480	21
65 days						
total length	3.148	0.266	0.073	0.037	1114	14
wet weight	0.221	0.997	0.053	0.029	1114	14

mating design, whereas a full-sibs mating design was used in our experiment. The results obtained showed that the full-sibs design, if carefullly conducted, can also be used to calculate heritability for *P. monodon*. In addition, this experiment demonstrated a significant genetic component controlling growth rate in the species at both 25 and 65 days age. It should be pointed out that the best use of estimated heritability would be when animals were at market size.

Based on our situation, the full-sibs mating design was preferable to the half-sibs design. To minimize the error terms, further investigations need to be carried out with a better design (i.e., more full-sibs, more replicates, and less time difference between batches, etc.).

Finally, a very high phenotypic correlation between total length and wet weight in *P. monodon* meant that either total length or wet weight could be used to represent growth rate for a selective breeding program.

Acknowledgements. We would like to thank Decha Jantawong, Wiroj Hirunchaiyapuck, and Muay Prayoonsak for their technical assistance and Santi Songpaiboon for production of nauplii for this experiment. This research was funded by the National Science and Technology Development Agency (NSTDA), National Center for Genetic Engineering and Biotechnology (NCGEB) grant number BT-39-06-ATI-06-05.

LITERATURE CITED

Becker WA (1992) Manual of Quantitative Genetics. Academic Enterprises, Pullman,WA, USA

Benzie JAH, Kenway M, Trott L (1996) Estimates for the heritability of size in juvenile *Penaeus monodon* prawns from half-sib matings. Aquaculture 152:49-53

Falconer DS (1981) Introduction of Quantitative Genetics. Second edition. Longman, London and New York, 340 pp

Fjalestad KT, Carr WH, Lotz J, Sweeney JN, Gjedrem T (1997) Genetic variation and selection response in body weight and disease resistance in Pacific White Shrimp (*Penaeus vannamei*). An abstract of the sixth international Symposium on genetics in aquaculture, 24-28 June 1997, Institute of Aquaculture, University of Stirling, Scotland

Hertzel DJS, Crocos PJ, Davis GP, Moore SS, Preston N (1997) Response to selection for growth in the Kuruma prawn, *Penaeus japonicus*. An abstract of the sixth international symposium on genetics in aquaculture, 24-28 June 1997, Institute of Aquaculture, University of Stirling, Scotland

Jarayabhand P, Tassanakajon A, Klinbunga S, Menasveta P (1997) Domestication and Genetic Improvement:What are these and how are these important to Black Tiger Shrimp, *Penaeus monodon* Culture in Thailand? A paper presented at Technical Seminar on Shrimp Biotechnology. December 13, Room 101, NSTDA building, RAMA VI Road, Bangkok, Thailand (in Thai with English abstract) p.11-15

Lester LJ (1988) Difference in larval growth among families of *Penaeus stylirostris* Stimpson and *P. vannamei* Boone, Aquacult. Fish. Manage 19:243-251

Lester LJ, Lawson KS (1990) Inheritance of size as estimated by principal component analysis at two temperatures in *Penaeus vannamei*. Aquaculture 85:323 (abstract)

Pratoomchat B, Piyatiratitivorakul S, Menasveta P (1993) Sperm quality of pond reared and wild-caught *Penaeus monodon* in Thailand. Journal of the World Aquaculture Society 24(4):530-540

Sokal RR and Rohlf FJ (1981) Biometry. Second edition. W.H. Freeman and Company, San Francisco, 859 pp

SPSS Inc. (1996) SYSTAT 6.0 for Windows. SPSS Inc., Chicago

Triploidy induction in chinese shrimp *(Penaeus chinensis)* with special reference to a new chemical inducer 6-DMAP

Jianhai Xiang, Linghua Zhou, Fuhua Li, Changgong Wu

Institute of Oceanology, chinesc Academy of Scienccs Qingdao , 266071, China

ABSTRACT: The Chinese shrimp *Penaeus chinensis* is a very important commercial species for fisheries and aquaculture in China. Triploidy induction is one possible way of using genetic manipulation to make stock improvements. Usually, physical treatments including cold shock, heat shock and high pressure, or chemical treatment with Cytochlasin B (CB) have been used for this purpose. CB is a carcinogen and also a relatively expensive chemical. By contrast, 6-dimethylaminopurine (6-DMAP) is a puromycin analog that is cheaper and less toxic. It was initially reported to block the extrusion of first or second polar bodies in oocytes of starfish. After washing out the water soluble drug, the eggs were able to resume meiosis. This paper describes the first utilisation of 6-DMAP as an efficient inducer for triploidy in shrimp. Ploidy rates were detected by count methods and flow cytometry. The experimental results showed that the induced triploidy rate depended on three treatment factors: the timing of exposure post fertilization, the duration of exposure and the concentration of 6-DMAP. The highest triploidy rates achieved were over 75%.

Key words *: Penaeus chinensis*, 6-dimethylaminopurine, 6-DMAP, triploidy induction, genetics

Xiang J, Zhou L, Li F, Wu C (1998) Triploidy induction in chinese shrimp *(Penaeus chinensis)* with special reference to a new chemical inducer 6-DMAP. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Domestication and Selective Breeding of *Penaeus monodon* in Thailand

[1]Boonsirm Withyachumnarnkul, [2]Vichai Boonsaeng, [3]T.W. Flegel
[2]Sakol Panyim, [4]Chainarong Wongteerasupaya

Departments of [1]Anatomy, [2]Biochemistry and [3]Biotechnology, Faculty of Science
Mahidol University, Rama 6 Rd.. Bangkok 10400, Thailand
[4]Department of Biochemistry, Sri Nakarintaraviroj University, Sukumvit Rd., Bangkok, Thailand

ABSTRACT: This report describes progress in an ongoing project, "Domestication and Selective Breeding of *Penaeus monodon* in Thailand". Fast-growing and white-spot syndrome virus (WSSV)-free shrimp were selected from commercial grow-out ponds. They were reared for one year to become F_0 pond-reared broodstock which were then bred to produce F_1 offspring. These F_1 offspring were screened to be free of WSSV before being reared onward for another year to become F_1 broodstock. From these broodstock the F_2 offspring presently comprise nine full-sibling families. Fecundity of the F_1 broodstock was lower than, but approaching that of wild, captured broodstock. The improvement was sufficient to make commercial scale production of postlarvae from these broodstock foreseeable.

KEY WORDS: *Penaeus monodon*, breeding, domestication

INTRODUCTION

Farmed production of *P. monodon* in Thailand steadily increased for eight years prior to the years 1995-1997 during which time there was a leveling-off and even a slight drop in production. Crop loss has been due to several factors, but the most prominent cause has been outbreaks of yellow-head disease (YHD) and white spot syndrome (WSS) disease (Flegel et al. 1997). To a lesser degree, luminescent bacterial infections have contributed to the declining production. White-spot syndrome virus (WSSV), commonly called systemic ectodermal and mesodermal baculovirus (SEMBV) (Wongteerasapaya, et al. 1995) in Thailand, is the infectious agent of WSS, and it can be detected using polymerase chain reaction (PCR) assays (Wongwisansri 1996; Lo et al. 1996). Checking for sub-clinical WSSV infections of by PCR has been a common practice in Thailand to help farmers in screening out WSSV +ve PL before stocking. PLs that are positive by the PCR assay generally appear healthy until one or two months after stocking in earthen ponds, at which time 80% of the ponds develop clinical white-spot disease (Withyachumnarnkul, unpublished data). This finding, and that by Lo et al. (1997) who found WSSV in the ovaries of captured broodstock, leaves little doubt that the disease can be vertically transmitted. A survey currently underway in Thailand by the Shrimp Culture Research Center, Charoen Pokphand Group of Companies and the Thai Department of Fisheries (DOF), suggests that WSSV infections are widespread among wild broodstock. In addition to the disease problem, a decline in the growth rate of shrimp produced from currently available wild broodstock has also been observed.

Obviously, the use of wild broodstock as the only source of PL is not wise. As in farming of other agricultural species, domestication is a better alternative; better because it allows for stricter disease prevention and control, and better because genetic selection may also be achieved. In most cases as time passes, domestication also appears to enhance animal fecundity automatically. In shrimp farming, genetic selection for fast-growing and disease-resistant traits would be most desirable, as both would decrease the risk of loss due to infections. A good example is that of *P. vannamei* domestication. Domesticated stocks of this economic species of South, Central and North America have been available for more than five years. They are free of the major viral diseases that affect the species and especially infectious hypodermal and haematopoietic necrotic virus (IHHNV) which was formerly a serious threat to the industry. A selective breeding program for improved growth and disease resistance is underway. The advent of specific pathogen-free (SPF) or high-health stocks of *P. vannamei* could be considered a breakthrough, since farmers using them enjoyed more than doubled production during the interval 1992-1994 (Wyban et al. 1992). This SPF stock, however, turned out to be more sensitive than wild shrimp stocks to Taura syndrome virus (TSV)when it struck several shrimp farms during 1995-1996 (Wyban 1997). Thus, development of TSV-resistant, as well as fast-growing stocks of *P. vannamei* is being attempted (Carr et al. 1997).

This report describes progress to date in a 6 year-project aimed at the domestication of *P. monodon* for the economical production of SPF broodstock and PL. The project will

Withyachumnarnkul B, Boonsaeng V, Flegel TW Panyim S, Wongteerasupaya C (1998) Domestication and selective breeding of *Penaeus monodon* in Thailand. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

lead into the development of SPR stocks and improved growth performance through selective breeding.

MATERIALS AND METHODS

Selection of F_0 stocks

In successful *P. monodon* culture in normal Thai earthen ponds, shrimp production is in the order of 6 tons per hectare. The survival rate is usually 50% or more, and the standard target body weight (BW) at harvest after 4 months of cultivation is approximately 30 g average per piece. However, in many harvests, it is quite usual to find shrimp of ~65 g BW or higher. It is not yet known whether these fast-growing individuals result from genetic variation or other factors (an area of some interest in itself). Whatever the reason for their existence, these shrimp were considered to be good candidates for fast-growing stock in a selective breeding program. Thus, they were selected from a number of different ponds to constitute the F_0 generation.

These fast-growing shrimp were screened for the presence of pathogens by histological examination and PCR. One percent (~20 pcs) of the shrimp was sacrificed for routine histological examination by light microscopy and WSSV-PCR. Any evidence for the presence of WSSV infection by either histology or PCR in any of the tested shrimp from a pond was deemed sufficient grounds to reject all individuals from that pond for selection.

The fast-growing shrimp that passed these screening tests were stocked in 1600 m^2 ponds (i.e., one "rai" in Thai land measure) in a quarantine area. Stocking density was 4-8 pc/m^2 with males and females at the ratio of 1:3. Study in *P. japonicus* suggests that male shrimp produce pheromones that induce sexual maturation in females (Yano 1993). The quarantine system was designed and constructed to prevent the introduction of diseases, especially of viral origin.

Stocking of F_1 and F_2

When the female F_0 shrimp reached 150 g in BW and male 100 g in BW, they were transferred to maturation tanks in the hatchery. Breeding was performed by artificial insemination. The offspring (F_1) were reared for one year and then bred to produce F_2 offspring in the same manner. Spawning rate, fertilization rate, number of eggs per spawner and hatching rate were determined. The larvae from individual females were reared separately from nauplius to PL15. Each crop of PL15 was again screened for the presence of WSSV, by PCR and routine histology. If negative by this screen, they were stocked at a density of 18-25 pcs/m^2 in another separate quarantine area.

RESULTS

The 1% top-sized shrimp taken from commercial ponds weighed around 50-70 g. These were fast-growing individuals, a typical size distribution curve is shown in Figure 1. After being reared for 8 months in quarantine, they grew to the range of 150-180 g BW for females and 80-110 g BW for males. Survival rate during this 8 month rearing period was only 30%. After eyestalk ablation, 20% achieved ovarian maturation, reached Stage IV, and spawned. The remaining females either did not show any ovarian development or developed to Stage II or Stage III, but then regressed. From the spawners, the average number of eggs was approximately 300,000 per individual and the hatching rate was approximately 30%. The nauplii were subsequently reared in the hatchery to PL15 with an average survival rate of 30%. Therefore, PL15 were produced at the rate of approximately 30,000 pcs per spawner.

The fry that passed the WSSV screen were stocked in four 1600 m^2 earthen ponds (one family per pond) where they were reared for four months before being harvested. The ADG was 0.24-0.26, which was better than that of the

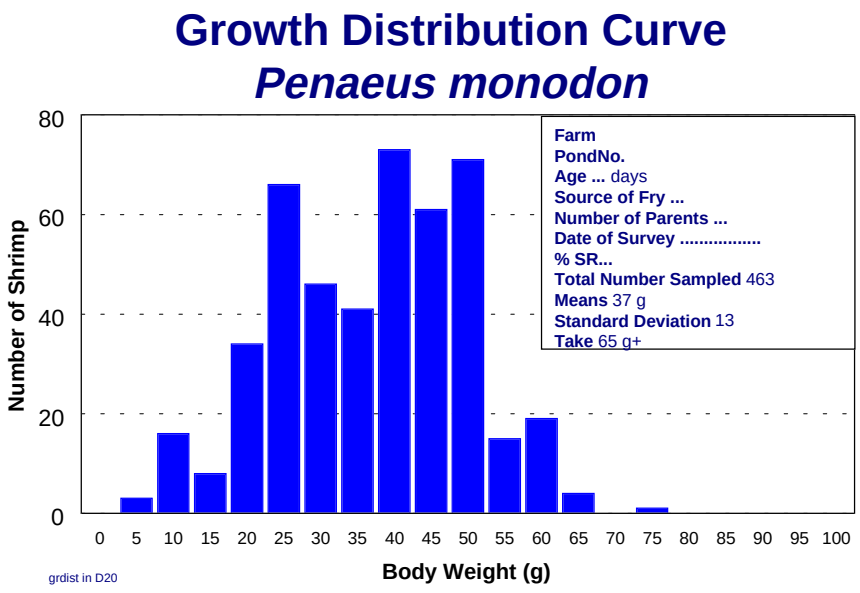


Figure 1. A typical histogram showing distribution of body weights of *P. monodon* at harvest.

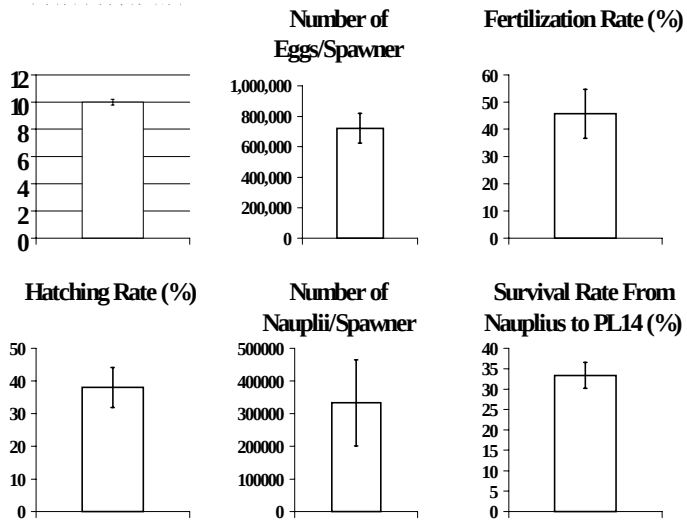


Figure 2. Data on the size, fecundity and offspring of the pond-reared F_1 spawners. The values are means ± S.E.

parent stock (0.18-0.22). The 1% top-sized shrimp from these ponds were selected and reared on for a further 8 months. These shrimp were designated the F_1 generation and they were used for breeding as described previously. This time, the spawning rate was 50% (i.e., half of the eyestalk-ablated females became spawners). Figure 2 shows the average size of the F_1 broodstock, and other parameters related to fecundity and survival rate of the F_2 PLs. The length of the F_1 spawners, measured from the base of the eyestalk to the end of the telson, was 10 inches and the average BW was 140 g. This size was comparable to that of wild broodstock. The number of eggs per spawner was higher than for the parental stock (approximately 700,000 eggs per spawner), and only slightly lower than the average 800,000 obtained from wild broodstock. Some of the F_2 broodstock produced more than one million eggs, and most spawned at least twice. The fertilization rate was variable, ranging from 0% to 95% and averaging approximately 45%. The hatching rate was also variable and averaged at 38%. The average number of nauplii produced was 300,000 pcs per spawner. As the survival rate from nauplius to PL14 was slightly over 30%, the production of PL14 was approximately 100,000 pcs per spawner.

The F_2 generation of pond-reared broodstock, consisting of nine full-sibling families, is now contained in a strict quarantine area. At the time of writing, the days of culture ranged from 60 to 105 days. They have been screened and found to be SEMBV free for two generations. Further screening for MBV, HPV and other viruses is under way.

DISCUSSION

Table 1 compares the performance of pond-reared *P. monodon* broodstock for the different generations studied. There is a trend of increasing fecundity, most notably for the spawning rate and the number of eggs per spawner. Millamena et al. (1986) reported approximately 30% spawning rate. The average number of eggs per spawner ranged from 120,000 to 333,000 and the hatching rate ranged from 4% to 36%. Menasveta et al. (1991) tried to improve the reproductive performance of pond-reared broodstock in a re-circulating water system and obtained slightly improved fecundity. San Miguel Foods, Inc. in the Philippines recently reported successful breeding of four generations of *P. monodon* pond-reared broodstock from 1991 to 1995 (Castro et al. 1997). The average number of eggs per spawner was improved up to 587,000 with a 42.7% hatching rate.

The improvement in fecundity seen here was probably due to improvements in nutrition and in the environment in which the broodstock were kept. Fresh, natural feeds containing high amounts of poly-unsaturated fatty acids (PUFAs), asthaxantin and cholesterol, were used. PUFAs like 20:4n6, 20:5n3 and 226n3 are known to be essential for ovarian development (Shimma 1977; Millamena et al. 1985) and cod-liver oil, blood worms (*Pereneresis nunta vallata*), squid and other marine species contain high amounts of these fatty acids (Millamena et al. 1986). In addition to these long-chained fatty acids, certain shorter chain fatty acids like linolenic acid (C18) have also been shown to enhance ovarian maturation in *P. japonicus* (Kanazawa et al. 1979).

Table 1. Comparison of the performance of pond-reared and wild broodstock.

	Wild Broodstock	Pond-Reared Broodstock		
		Millamena et al. Menasveta et al.	San Miguel	SCRD (F1 data)
Size of the Female (g)	90-200	50-100	80-160	150-200
Spawning Rate (%)	>80%	~30%	?	~50%
No. of Eggs/Spawner	>800,000	~200,000	~600,000	~700,000
Hatching Rate (%)	~80%	~20%	~40%	~40%
Breeding Generation		-	F4	F2

Water depth, salinity, temperature, photoperiod and light intensity may also play roles in shirmp maturation (Quinitio et al. 1993; Yano 1993). Light-dark cycle is an important factor in controlling reproductive function of several species. In mammals, for example, the environmental light message is conveyed to internal organs via the pineal gland (for review, see Reiter 1991). Crustaceans lack a pineal gland, but such messages might be carried through the optic lobe in the eyestalk, which contains a pineal-equivalent structure. The effects of photoperiod on the optic lobe biochemistry of the freshwater crustacean, *Macrobrachium rosenbergii* de Man, and the black tiger prawn, *P. monodon*, have been reported (Withyachumnarnkul et al.1990, 1992, 1995). Yano (1993) reported that a light:dark ratio of 14:10 h, with an intensity of 3-50 lux was optimum for the promotion of ovarian growth of *P. japonicus* in maturation tanks.

The finding that the F1 generation grew 10% faster than the parental stock suggests the possibility of inheritance for growth performance of *P. monodon*. However, more data are needed to draw definite conclusions, as the environment also plays an important role in growth.

This work showed an improvement in the performance of the pond-reared broodstock, with an averaged production of 100,000 PL15 per broodstock individual. This figure is about one-third of that for wild broodstock. Although lower, production at this rate is commercially viable. Moreover, the fry produced from pond-reared broodstock are healthier (i.e., WSSV-free). In the future, it is hoped that genetic improvement and better culture performance of the fry will compensate for lower production numbers.

Acknowledgments. This work was supported by the Shrimp Culture Research and Development, Inc. (SCRD), Thailand and the National Fisheries Institute, USA.

LITERATURE CITED

Castro MSC, Empig LT, Pacatang AE, Cruz AA (1997) Commercial production of *Penaeus monodon* nauplii and postlarvae from pond-reared broodstocks. In The Book of Abstracts of The Annual International conference & Exposition of the world Aquaculture Society. February 19-23, 1997, Seattle, Washington, USA. pp 68-69

Carr WH, Fjalestad KT, Lotz JM, Oyama RN, Godin DM, Chang CPL, Segura FJ, Roddy SC, Sweeney JN (1997) Advances in selective (SPF) pacific white shrimp, breeding for growth performance and disease resistance in specific pathogen free *Penaeus vannamei.* In The Book of Abstracts of The Annual International Conference & Exposition of the World Aquaculture Society, p68. February 19-23, 1997, Seattle, Washington, USA.

Flegel, T.W., Sitdhi Boonyaratpalin and Boonsirm Withyachumnarnkul. 1997. Current status of research on yellow-head virus and white-spot virus in Thailand. In T.W. Flegel and I. MacRae (eds.) Diseases in Asian Aquaculture III. Fish Health Section, Asian Fisheries Soc, Manila, 285-296.

Kanazawa A, Teshima S, Ono K (1979) Relationship between essential fatty acid requirements of aquatic animals of linolenic acid to highly unsaturated fatty acids. Comp Biochem Physiol 63B: 295-298

Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, Chen YT, Chou CM, Yeh PY, Huang CJ, Chou HY, Wang CH, Kou GH (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25: 133-141.

Lo CF, Ho CH, Chen CH, Liu KF, Chiu YL, Yeh PY, Peng SE, Hsu HC, Liu HC, Chang CF, Su MS, Wang CH, Kou GH (1997) Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis Aquat Org 30: 53-72.

Menasveta P, Fast AW, Piyatirativorakul S, Rungsupa S (1991) An improved, closed seawater recirculation maturation system for giant tiger prawn (*Penaeus monodon* Fabricius). Aquaculture Engineering 10: 173-181

Millamena OM, Pudadera RA, Catacutan MR (1985) Effect of diet on reproductive performance of wild ablated *Penaeus monodon* broodstock. In Proceedings of the First International Conference on the culture of Penaeid Prawns/Shrimps. Aquaculture Department, Southeast Asian Fisheries Forum. Asian Fisheries Society. Taki Y, Primavera JH, Llobrera JA (eds) Manila, the Philippines. pp 178-179

Millamena OM, Primavera JH, Pudadera RA, Caballero RV (1986) The effect of diet on the reproductive performance of pond-reared *Penaeus monodon* Fabricius broodstock. In The First Asian Fisheries Forum. Asian Fisheries Society,. Maclean JL, Dizon LB, Hosillos LV (eds) Manila, the Philippines. pp 593-596

Quinitio ET, Caballers RM, Gustilo L (1993) Ovarian development in relation to changes in the external genitalia in captive *Penaeus monodon*. Aquaculture 114: 71-81

Reiter RJ (1991) Pineal Gland: Interphase between the photoperiodic environment and the endocrine system, Trends in Endocrinol Metab 2: 13-19

Shimma Y (1977) On the lipids of adult carps raised on fresh meal and SCP feeds and hatchabilities of their eggs. Bull Freshwater Fish Res Lab 27: 35-48

Withyachumnarnkul B, Poolsanguan B, Poolsanguan W (1990) continuous darkness stimulates body growth of the juvenile giant freshwater prawn, *Macrobrachium rosenbergii* de Man. Chronobiology International 7(2): 92-97

Withyachumnarnkul B, Pongsa-Aswapaiboon A, Ajpru S, Siamwalla P, Trakulrungsi W, Samritthong C (1992) Continuous light increases N-acetyltransferase activity in the optic lobe of *Macrobrachium rosenbergii* de Man (Crustacea: Decapoda). Life Sci 51(19): 1470-1484

Withyachumnarnkul B, Pongtippatee P, Ajpru S (1995) N-Acetyltransferase, hydroxyindole-O-methyltransferase and melatonin in the optic lobes of the giant tiger shrimp *Penaeus monodon*. J Pin Res 18(4): 217-221

Wongteerasapaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon.* Dis Aquatic Org 21: 69-77

Wongwisarnsri S (1996) PCR-based method and *in situ* hybridization method for detection of white-spot virus in penaeid shrimp. Thesis for the Master Degree of Science, Faculty of Science, Mahidol University.

Wyban JA, Swingle JS, Sweeney JN, Pruder GD (1992) Development and commercial performance of high health shrimp using specific pathogen-free (SPF) broodstock *Penaeus vannamei.* In Proceeding of the Special Session of Shrimp farming. world Aquaculture society, Baton rouge, LA, USA. pp 254-260

Wyban JA (1997) Recent progress in domestication of marine shrimp. In Abstracts of the 2nd Asia-Pacific Marine Biotech-

nology Conference and 3rd Asia-Pacific Conference on Algal Biotechnology, 7-10 May, 1997, Phuket, Thailand. p 24

Yano I (1993) Ultraintensive culture and maturation in captivity of Penaeid shrimp. In CRC Handbook of Mariculture, 2nd Edition, Volume 1: Crustacean Aquaculture. McVey JP (ed) CRC Press, Boca Raton, pp 289-313

Shrimp Rearing

Study on the Impact of Intensive Marine Shrimp Farm Effluent on Sediment Quality in Kung Krabaen Bay, Eastern Thailand

[1]Siri Tookwinas, [1]Chanin Sangrungruang, [2]Osamu Matsuda

[1]Department of Fisheries, Chatuchak, Bangkok
[2]Department of Applied Biological Science, Hiroshima University, Japan

ABSTRACT: Investigations on the impact of shrimp farm effluent on bottom sediment quality in Kung Krabaen Bay indicated that the most affected areas were wastewater canals. The impact decreased with increasing distance form the bay shoreline. The organic matter in wastewater canals, water supply canals, the shoreline, and 500 m and 1,000 m from shoreline were 7.15, 6.58, 5.10, 3.37 and 1.69%, respectively. From the analysis of Kendall correlation coefficients, every parameter (except available phosphorus) showed positive correlation. The decrease in organic matter content from wastewater canals to the center of the bay was significantly different ($P<0.01$). Soil texture analysis showed that the sand content in the bay was affected by the coastal sea outside the bay during monsoon season while the silt content was affected by shrimp farm effluents during shrimp harvesting periods, which were significantly different among the survey stations ($P<0.01$). The quantity of shrimp harvesting affected C:N ratios and percentages of clay content in sediments which were also significantly different among the survey stations ($P<0.01$).

KEY WORDS: intensive shrimp farm, effluent, sediment quality, environmental impact, Thailand

INTRODUCTION

Kung Karbaen Bay is located at latitude 12.33 – 12.36 ^{o}N and longitude 101 53 – 101 55 ^{o}E in Chathaburi province in Eastern Thailand. The bay is fringed in the inner parts by mangrove forest which is about 500 – 800 meters wide from landward to seaward edge (Raine 1992). Intensive shrimp farm ponds are located around the bay behind the mangrove area with a total pond area of 348 ponds and approximately 142.76 ha. To understand the impact of shrimp farm effluent around Kung Krabaen Bay on the surrounding environment, it is important to assess the effect of these nutrients on sediment quality in the discharge canal and water receiving the shrimp pond effluent (i.e., Kung Karbaen Bay). This study analysed sediment quality and sediment settling rate in water supply canals, water discharge canals and Kung Krabaen Bay. The impacts of shrimp farm effluent on surrounding environment were then explored using Kendall correlation coefficients.

MATERIALS AND METHODS

Sediment from shrimp farms around Kung Krabaen bay

Sediment was collected in water supply canals, water drainage canals, Kung Krabaen's shoreline, 500 m from shoreline and 1,000 m from shoreline (i.e., the bay's center). The total number of sampling or survey stations was 23 (Fig.1). The sediment was collected in three PVC bottles, 4 inches in diameter and 25 cm long. Each bottle set was closed and weighted by a metal frame and placed at the survey station for 15 days per month. The sampling period was once per month for 12 months from June 1994-May 1995. The settling matter or sediment (gm/m2/day) in the bottle bottoms was calculated as sediment according to the following equation:

$$S = A \times Q \times 365/1{,}000 \times 1{,}000 \text{ ton}$$

where S = sediment (ton), A = area (m^2), Q = settling matter (sediment), Average per day ($gm/m^2/day$). Texture and organic matter were analyzed by the Walkely and Black method (Jackson 1958). Total nitrogen and available phosphorus were analyzed by the Kjeldahl method and the method of Murphy and Rilly, respectively (Anthanan et al. 1989). A mixture of soil and water (Soil : water = 1:1) was used to measure pH. The statistical analysis for quantity and quality of sediment as done by One-Way ANOVA and Dumcam's new Multiple Range Test (DMRT). The correlation coefficients were analyzed by the Kendall correlation coefficient method (SPSS for Window Version 6.0) (Watanachayakul 1989).

RESULTS AND DISCUSSION

The sediment was analyzed over a 12 month period (June 1994–May 1995). It was found that the sediment in the period of the Southwest monsoon (Jun.– Sept. 1994), the period of Oct. 1994 – Jan 1995 and the period of Feb.– May 1995 were significantly different ($P<0.05$). During the

Tookwinas S, Sangrungruang C, Matsuda O (1998) Study on the impact of intensive marine shrimp farm effluent on sediment quality in Kung Krabaen Bay, eastern Thailand. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

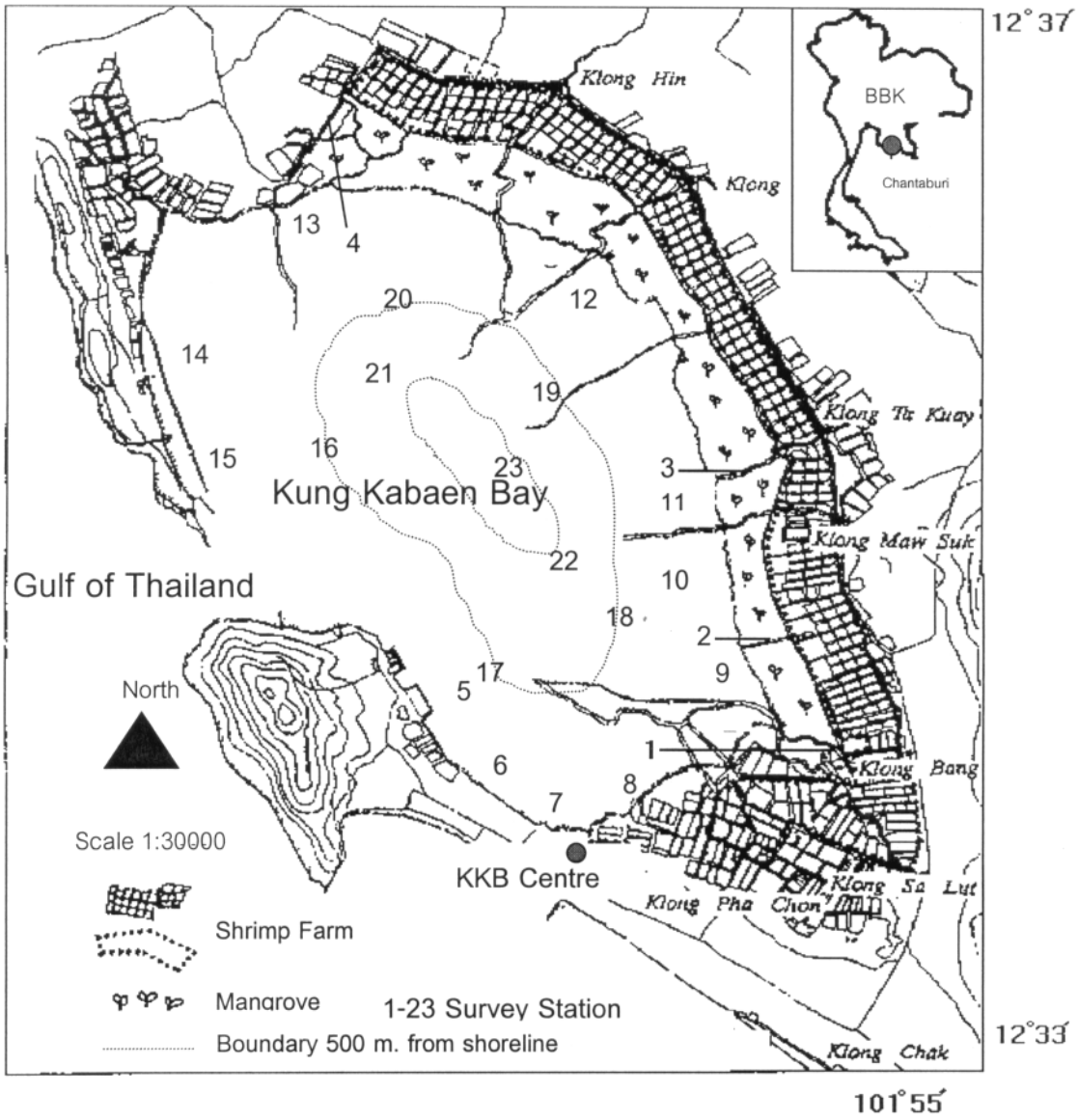


Figure 1. Survey stations in Kung Kabaen Bay.

monsoon period, the average value was 2296.73 ± 348.82 gm/m^2/day. During the period of Oct. 1994 to Jan. 1995 and during Feb – May 1995, the average values were 414.94 ± 60.88 and 843.43 ± 170.71 gm/m^2/day, respectively. In the monsoon season, the sediment was highest at the center of the bay (survey stations 21,22 and 23). The value decreased significantly ($P<0.05$) with increasing distance from the center of the bay. The sediment values were 8638.65 ± 2076.16, 3236.66 ± 708.51, 1334.90 ± 213.53, 739.99 ± 180.13 and 583.51 ± 106.68 gm/m^2/day, respectively, at the center, at 500 m from bay shoreline, at the bay shoreline, at water supply canals and at water drainage canals. (Table 1).

The sediment quality (pH, organic matter, total nitrogen, C:N ratio, available phosphorus, BOD_5 and texture are shown in detail in Tables 2 to 10. Organic matter in sediment was highest during Oct. 1994 – Jan. 1995 (winter season or shrimp harvesting period) and was significantly different ($P<0.05$) among the survey stations. The average values were 4.59 ± 0.33, 3.91 ± 0.31 and 3.45 ± 0.24% during the period of Oct. 1994 – Jan. 1995, the period of the monsoon and the summer period, respectively (Table 3). The organic matter was very high at areas near shrimp farms and decreased with increasing distance from the shrimp farm areas. The total nitrogen was highest during shrimp harvesting (Oct. 1994 – Jan. 1995) (Table 4) when it was 0.165 ± 0.008%. The C : N ratio in the sediment varied over a wide range during the shrimp harvesting period, and was significantly different ($P < 0.05$) from other periods. The BOD_5 value for the sediments fluctuated similar to the values for organic matter. It was highest during the shrimp harvesting period which was significantly different ($P< 0.05$) to other seasons. The clay was highest in the period of shrimp harvesting and this was significant different ($P<0.05$) to the monsoon season. The soil texture in water supply canals, water drainage canals and the bay shoreline consisted of silt, sandy loam, loam with silt 60 – 65%, clay 17 – 24% and sand loam 9 – 22%. The soil texture in the bay during the monsoon season was sandy loam.

Tables 1-10. Sediment characteristics. The values in the same row that have a different superscript are significantly different at P<0.05 Remark: WSC = Water Supply Canal, SL = Bay's shoreline, WDC = Water Drainage Canal, 500 m., 1000 m. = Distance from the bay's shoreline.

Table 1. Quantity of sediment (gm/m^2/day).

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	739.99±180.13^{c}	361.70±130.51^{a}	481.22±131.04^{b}	545.65±92.36^{b}
WDC	583.51±106.68^{c}	428.58±67.49^{a}	631.66±185.08^{b}	547.92±73.61^{b}
SL	1334.90±213.53^{c}	504.49±113.16^{a}	584.77±76.45^{b}	822.49±93.10^{b}
500 m.	3230.66±708.51^{b}	257.93±250.14^{a}	613.68±161.47^{b}	1437.74±316.49^{b}
1,000 m	8638.65±2076.16^{a}	342.89±121.84^{a}	2523.56±1142.50^{a}	3246.46±886.50^{a}
Mean ± SD	2296.73±348.82^{A}	414.94±60.88^{B}	843.43±170.71^{B}	1197.87±143.19

Table 2. pH in sediment.

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	7.22±0.19^{a}	6.84±0.46^{d}	7.25±0.20^{a}	7.13±0.12^{a}
WDC	7.14±0.17^{a}	7.09±0.14^{d}	7.22±0.24^{a}	7.15±0.10^{a}
SL	7.55±0.06^{a}	7.43±0.07^{c}	7.60±0.08^{a}	7.53±0.04^{a}
500 m.	7.86±0.08^{a}	7.77±0.06^{b}	7.33±0.49^{a}	7.65±0.18^{a}
1,000 m	8.24±0.52^{a}	8.09±0.05^{a}	7.42±0.74^{a}	7.60±0.39^{a}
Mean ± SD	7.62±0.10^{A}	7.49±0.06^{A}	7.45±0.15^{A}	7.49±0.06

Table 3. Organic matter in sediment (%).

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	6.09±0.88^{ab}	6.58±1.40^{a}	6.39±0.89^{a}	6.33±0.57^{a}
WDC	6.72±1.33^{a}	7.15±1.19^{a}	4.17±0.37^{b}	6.02±0.64^{a}
SL	4.28±0.39^{b}	5.10±0.49^{a}	3.79±0.35^{b}	4.39±0.24^{b}
500 m.	2.10±0.42^{c}	3.37±0.31^{b}	2.74±0.29^{b}	2.71±0.21^{c}
1,000 m	1.24±0.40^{c}	1.69±0.22^{b}	0.98±0.17^{c}	1.27±0.16^{d}
Mean ± SD	3.91±0.31^{AB}	4.59±0.33^{A}	3.45±0.24^{B}	3.97±0.17

Table 4. Total nitrogen in sediment (%).

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	0.219±0.017^{ab}	0.185±0.036^{a}	0.226±0.021^{a}	0.211±0.014^{a}
WDC	0.280±0.044^{a}	0.196±0.023^{a}	0.146±0.011^{b}	0.208±0.020^{a}
SL	0.183±0.013^{b}	0.186±0.012^{a}	0.166±0.011^{b}	0.178±0.007^{a}
500 m.	0.094±0.015^{c}	0.145±0.011^{a}	0.165±0.017^{b}	0.133±0.009^{b}
1,000 m	0.060±0.015^{c}	0.085±0.009^{b}	0.064±0.011^{c}	0.068±0.007^{c}
Mean ± SD	0.162±0.011^{A}	0.165±0.008^{A}	0.155±0.008^{A}	0.161±0.005

Table 5. C:N ratio in sediment.

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	15.83±1.08:1^{a}	19.80±2.09:1^{a}	16.25±1.32:1^{a}	17.10±0.89:1^{a}
WDC	14.98±2.51:1^{a}	21.10±2.77:1^{a}	16.99±1.42:1^{a}	17.69±1.38:1^{a}
SL	14.10±1.19:1^{a}	15.30±0.72:1^{b}	12.81±0.65:1^{b}	14.08±0.53:1^{b}
500 m.	11.69±0.89:1^{a}	14.02±1.34:1^{b}	9.84±1.11:1^{c}	11.84±0.67:1^{c}
1,000 m	10.80±1.62:1^{a}	10.73±1.23:1^{c}	8.37±1.13:1^{c}	9.56±0.75:1^{c}
Mean ± SD	13.51±0.71:1^{B}	15.32±0.63:1^{A}	12.31±0.53:1^{B}	13.65±0.37:1

Table 6. Available phosphorus in sediment (mg/kg).

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	64.28±6.40^{a}	62.57±4.63^{b}	69.84±4.25^{a}	65.65±3.05^{ab}
WDC	67.99±6.20^{a}	69.88±3.67^{ab}	64.82±7.45^{a}	67.57±3.33^{ab}
SL	66.41±2.65^{a}	69.58±1.53^{ab}	69.72±2.07^{a}	68.51±1.25^{ab}
500 m.	73.49±2.81^{a}	74.52±2.65^{a}	67.62±5.89^{a}	71.94±2.30^{a}
1,000 m	64.74±9.17^{a}	72.89±2.42^{ab}	60.12±6.82^{a}	63.90±3.77^{b}
Mean ± SD	67.75±1.92^{A}	70.55±1.12^{A}	67.56±1.95^{A}	68.34±0.99

Table 7. BOD_5 in sediment (mg/g soil).

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	4.61±0.22^{b}	4.08±1.02^{ab}	3.63±0.71^{ab}	4.13±0.37^{b}
WDC	6.91±1. 16^{a}	5.49±1.35^{a}	5.25±1.57^{a}	5.88±0.77^{a}
SL	3.84±0.32^{b}	3.84±0.33^{ab}	2.79±0.30^{b}	3.51±0.19^{b}
500 m.	1.97±0.29^{c}	2.99±0.39^{b}	2.69±0.33^{bc}	2.53±0.20^{c}
1,000 m	1.08±0.25^{c}	2.31±0.29^{b}	1.22±0.21^{c}	1.53±0.18^{d}
Mean ± SD	3.53±0.26^{AB}	3.64±0.25^{A}	2.87±0.25^{B}	3.34±0.15

Table 8. Sand in sediment (%)

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	7.64+1.96^{c}	17.30+4.60^{b}	9.71+2.00^{b}	10.78+1.70^{c}
WDC	14.34+4.11^{c}	22.05+3.70^{b}	11. 61+1.88^{b}	16.00+2.08^{c}
SL	14.99±2.46^{c}	15.77±2.01^{b}	20.30±2.40^{b}	17.14±1.37^{c}
500 m.	37.38±6.57^{b}	14.20±1.91^{b}	22.29±3.53^{b}	27.67±3.53^{b}
1,000 m	71.82±6.89^{a}	51.86±4.34^{a}	64.16±4.60^{a}	62.37±3.29^{a}
Mean ± SD	24.31±2.85^{A}	21.55±2.11^{A}	24.42±2.31^{A}	23.61±1.45

Table 9. Silt in sediment (%).

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	65.71±4.74^{a}	58.07±7.88^{a}	71.10±2.99^{a}	65.69±2.98^{a}
WDC	63.08±5.68^{a}	56.41±5.10^{a}	71.33±2.63^{a}	63.61±2.87^{a}
SL	67.13±2.98^{a}	61.68±2.15^{a}	59.14±2.46^{b}	62.83±1.53^{a}
500 m.	45.61±6.50^{b}	56.17±4.67^{a}	54.24±2.29^{b}	50.70±3.18^{b}
1,000 m	16.62±6.32^{c}	28.13±4.07^{b}	16.93±3.74^{c}	21.63±2.71^{c}
Mean ± SD	57.20±2.81^{A}	55.35±2.18^{A}	55.73±2.17^{A}	56.16±1.42

Table 10. Clay in sediment (%).

Location	Jun-Sept 94	Oct 94-Jan 95	Feb-May 95	Mean ± SD
WSC	26.65±5.28^{a}	24.04±3.69^{ab}	19.19±1.75^{ab}	23.39±2.39^{a}
WDC	22.58±4.93^{ab}	21.50±2.30^{b}	17.06±1.85^{ab}	20.38±1.90^{ab}
SL	17.88±2.04^{ab}	22.45±1.53^{ab}	20.18±1.45^{ab}	19.94±1.00^{ab}
500 m.	17.00±2.47^{ab}	29.63±3.58^{a}	23.47±1.82^{a}	21.64±1.60^{a}
1,000 m	11.56±1.13^{b}	20.02±2.49^{b}	15.90±1.19^{b}	16.00±1.15^{b}
Mean ± SD	18.49±1.38^{B}	22.88±1.09^{A}	19.86±0.87^{AB}	20.17±0.67

The relationships were analyzed by Kendall correlation coefficients. pH and sand were significantly different ($P<0.01$) with increasing distance from shrimp farming sites, with Kendall correlation coefficients of 0.4674 and 0.3618, respectively (Table 11). Organic matter, total nitrogen, C:N ratio, BOD_5 and silt decreased with increasing distance from shrimp farm sites, with Kendall correlation coefficients of –0.4467, - 0.3685, - 0.3367, - 0.3352 and - 0.3537, respectively. This indicated that the bay was impacted by organic matter from shrimp farms. The impact decreased with increasing distance from a shrimp farm site (point source).The correlation of pH in sediment and sand quantity were significantly different ($P < 0.01$). Organic matter, total nitrogen, BOD_5, silt and clay had correlations significantly different to the quantity of sediment ($P<0.01$) . The high quantity of sediment in the monsoon season contained very low organic matter and contained a very high portion of sand. The sand would have come from the coastal sea outside Kung Krabaen Bay, rather than from shrimp farms which contain very high portions of silt and clay. The sediment from coastal areas in the sea outside the bay would be transported into the bay by wave action during the monsoon season. However, the shrimp harvesting period (Oct. 1994 – Jan 1995) had an impact on quantity of clay in the bay. Shrimp harvesting also affected the C : N ratio which varied over a very wide range. It could make very low rate for organic matter decomposition. Sediment quantity at the bay center was highest during the monsoon season and lowest during the shrimp harvesting period and summer season. Sediment in water supply canals was higher than in water drainage canals. During the shrimp harvesting period, the quantity of sediment was highest at the bay shoreline. Praphruttham (1985) reported that decomposition in sediment would liberate hydrogen ions and decrease the pH. That contention was supported by our results. In the monsoon season, the pH in sediments was highest and it decreased during the period of shrimp harvesting. This was probably due to the high amount of organic matter in shrimp farm sediments and effluents.

The organic matter was very high in water drainage canals and decreased with increasing distance from the shrimp farm site. Tookwinas and Neumhom (1995) reported that

Table 11. Kendall correlation coefficients of sediment, chemical and physical properties of sediment the year round.

Season	.0049						
Sediment	.0631	-.1906**					
Soil-pH	.4674**	.0262	.2084**				
Org-Mat	-.4467**	-.0267	-.1679**	-.5131**			
Total-N	-.3685**	-.0047	-.2508**	-.4155**	.7121**		
C:N	-.3367**	-.0327	.0220	-.3800**	.5182**	.2232**	
Phos	.0632	-.0052	-.0235	.0534	.0505	.0743	-.2128
BOD5	-.3352**	-.0922	-.0963*	-.4152**	.5302**	.5336**	.2764**
Sand	.3618**	.0630	.2259**	.2545**	-.3565**	-.3598**	-.1531**
Silt	-.3537**	-.0646	-.1167*	-.1937**	.2956**	.2757**	.1658**
Clay	-.0816	.1269*	-.2797**	-.1776**	.2094**	.2171**	.0932*
Cul-Pond	.0021	-.4975**	-.0533	-.0548	.0463	.0058	.0675
Harvestp	.0114	.1089*	-.2690**	-.1770**	.0843	.0247	.1324**
	Station	Season	Sediment	Soil pH	Org-Mat	Total-N	C:N

Season						
Sediment						
Soil-pH						
Org-Mat						
Total-N						
C:N						
Phos						
BOD5	.1094**					
Sand	-.0467	-.2293**				
Silt	.0167	.1820**	-.6564**			
Clay	.0480	.1404**	-.1520**	-.1910**		
Cul-Pond	.1353**	.0828	-.0840	.0715	.0012	
Harvestp	.0194	.0440	.0043	-.0807	.2103**	.2900**
	Phos	BOD5	Sand	Silt	Clay	Cul-Pond

* - Signif. LE .05 ** - Signif. LE .01 (2-tailed)

the sediment from shrimp farms in Kung Krabaen Bay settled in the first hour after discharge from the pond and that most settled in water drainage canals. However, organic matter in the form of dissolved solids would be transported to the bay. The water volume in the bay is exchanged with seawater from coastal sea by up to approximately 86% (Sasaki and Inoue 1985). Therefore, organic matter can be transported to the the outer sea. Available phosphorus was not different at the survey stations during the period studied. The Soil Science Department (1992) indicated that phosphorus fixation is depended upon soil texture and soil quality, while seawater is the main reservoir of phosphorus (Long and Mason 1983). Boyd (1995) also indicated that many factors would be concern the phosphorus utilization in seawater. The Hydrology Division (1993) reported that the bottom sediment in coastal areas of Eastern Thailand consists of sandy clay which would be transported into the bay during the Southwest monsoon. The sand particles would settle faster than silt and clay (Eiumnoh 1983). Stapornvanit (1993) studied the effluent loading at shrimp farms around Kung Krabaen Bay and found that sand particles settled in the mud disposal areas near shrimp ponds while silt and clay were transported to water drainage canals and Kung Krabaen Bay. The results from our study were similar.

It can be concluded that the main impact of shrimp farm effluent would be on water drainage canals and the Kung Krabaen Bay shoreline. The sediment in Kung Krabaen Bay would be transported from coastal seas during the Southwest monsoon, as was similarly repoted in the study by Rimchala et al. (1983). Thus, the Southwest monsoon has the greatest influence on Kung Krabaen Bay sediment which can be transported from coastal seas into the bay over a period of years.

LITERATURE CITED

APHA et al. 1989. Standard methods for the examination of water and wastewater. 17 et al., American Public Health Association, Washington, D.C., 1430 p.

Anthanan, T. et al. 1989. Manual for soil and plant analysis, Soil Science Department, Faculty of Agriculture, Kasetsart University, Bangkok.

Boyd, C.E. 1995 Bottom soils, sediment and pond aquaculture, Auburn University, Alabama. 347 p.

Day, P.R. 1965. Particle fraction and particle size analysis, pp. 545 – 567. In C.A. Black (ed.); Method of soil analysis Part I, physical and mineral propertics including
statistics of measurement and sampling., Agronomy No 9, Amer. Soc. of Agron. Inc., Madison, Wisconsin.

Eiunnoh, A. 1983. Geomorphology, Soil Science Department, Faculty of Agriculture, Kasetsart University, Bangkok, 560 p.

Hydrology Division. 1993. Report on oceanography data in the Gulf of Thailand, Hydrology Department, Naval Acdemy, Bangkok, 89 p.

Jackson, M.L. 1958. Soil chemical analysis, Prentice – Hall, Inc., NewYork, 498 p.

Kilmer, V.J. and L.T. Alexander. 1949. Method of making mechanical analysis of soil, Soil Sci. 1949 : 8 –24.

Long, S.P. and C.F. Mason. 1983. Saltmarsh ecology, Chapman and Hall Ltd., New York. 160 p.

Musig, Y. and P. Yuthrutyanukul. 1991. Settling, sediment and soil quality in intensive marine shrimp farm at inner Gulf of Thai-

land, Fisheries Science Journal 1 (1) : 47 – 55.

Praphruttham, P. 1985 Soil chemistry, Soil Science Department, Faculty of Agriculture, Kasetsart University, Bangkok, 502 p.

Raine, R.M. 1992. Land use patterns, environmentally sensitive areas and changes in land use over time in the Coastal Zone of Chanthaburi Province, Thailand, 44 p.

Rimchala, P. et al. 1993. Report on soil survery in Kung Krabaen Bay Royal Development and Study Center, Soil Survery Division, Land Development Department, Ministry of Agriculture and Cooperative, Bangkok, 143 p.

Sasaki, T. and H. Inoue. 1985. Studies on fundamental environments in the Kung Krabaen Bay, pp. 77 – 98. In Mangrove estuarine ecology in Thailand, Thai – Japanese Cooperative Research Project on Mangrove Productivity and Development.

Soil Science Department. 1992. Introduction to soil science, Faculty of Agriculture Kasetsart University, Bangkok, 730 p.

Stapornvanit, K. 1993. The environmental impact of shrimp farm effluent, M.Sc. Thesis, AIT, Bangkok.

Tookwinas, S. and P. Neumhom 1995. Experiment on coagulation of an intensive marine shrimp farm effluent, International Seminar on Marine Fisheries Environment, DOF/JICA, 9 –10 March 1995. Rayong, Thailand.

Wattanachayakul, S. 1989 Fundamental of statistical analysis, Prakaiyphrit Printing Co. Ltd., Bangkok, 297 p.

Some Recent Innovations in Marine Shrimp Pond Recycling Systems

[1]Arlo W. Fast, [2]Piamsak Menasveta

[1]Hawaii Institute of Marine Biology, University of Hawaii at Manoa
P.O. Box 1346, Kaneohe, Hawaii 96744, U.S.A.
[2]Aquatic Resources Research Institute and Department of Marine Science
Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: Abundant shrimp seed availability beginning during the 1970's has resulted in numerous innovations and intensification with marine shrimp growout. Pond water recycling and recirculation systems are one such innovation which holds much promise for profitable and sustainable shrimp culture. These systems are based on earlier work with smaller, conventional water treatment systems. Pond water recycling systems can result in better disease control, less water use, higher water quality, crop diversification, and improved effluent quality. We review several types of existing and proposed pond water recycling designs for both large and small shrimp farm applications.

KEY WORDS: shrimp, grow-out ponds, water recycling

INTRODUCTION

Before abundant marine shrimp seed availability from hatcheries and from wild captured seed, shrimp pond growout techniques changed very little over the centuries. Shrimp were mostly cultured coincidentally with fish and other crustaceans in large, extensive pond systems characterized by low productivity (Fast 1991, 1992a). These primitive systems were basically catch, hold and harvest systems with little or no energy, feed, or material inputs. Shrimp yields were perhaps a few hundred kg/ha/yr. Growout was low-tech as judged by any standard.

After breakthroughs in large-scale, shrimp larviculture techniques during the 1970's, all aspects of shrimp culture underwent rapid and innovative changes. These changes were motivated by abundant availability of shrimp seed for pond culture, limited amounts of captured oceanic shrimp, and high profits from pond cultured shrimp. These conditions resulted in numerous growout innovations. Pond yields with the more intensive shrimp culture systems increased to >10,000 kg/ha/yr.

Fast (1992b,c,d) described many innovations in pond growout techniques that occurred during the 1980's and earlier. In our present paper, we describe some innovations which have occurred since then. Specifically, we review shrimp pond growout systems which use pond water recycling and recirculation.

POND WATER RECYCLING AND RECIRCULALTION SYSTEMS

Intensive monoculture of marine shrimp is potentially unstable and risky. It requires large applications of organic feed and mechanical energy per unit water volume. These applications focus productivity from much larger land and oceanic areas within a smaller area of shrimp growout pond (Folke & Kautsky 1992). The pond becomes the "tip of the funnel." As a result, large amounts of uneaten feed, feces and metabolic wastes accumulate in pond waters and pond soils. These wastes are degraded through microbial and other decomposition processes to produce among other things; ammonia, nitrite, nitrate and phosphate. These mineralized nutrients stimulate algal growth and lead to dense blooms in the pond. In addition to toxicity from some of these degradation products, collapse of algal populations can also cause shrimp stress and mortality through disease, oxygen depletion and increased metabolite toxicity. A conventional solution to these problems has been increased water exchange. Excess metabolites and algae are thus removed from the pond and replaced with water of lower metabolite and algal concentration and greater oxygen content. This water exchange or flushing solution is, however, not without potential perils of its own. In many cases, source waters for flushing contain disease organisms which infect the shrimp crop and cause massive mortalities. Industrial, domestic and agricultural pollution of source waters can likewise cause massive shrimp mortalities. In addition, source waters often contain high concentrations of suspended sediments which settle in the pond and cause shoaling of water depths that requires time consuming removal and disposal between crops. An alternative to high rates of water exchange and flushing is water recycling and recirculation. This allows water to be reused many times and reconditioned between each use. This has many advantages which will be reviewed herein.

Aquatic animal culture systems which use water recirculation and treatment have been used for many years

Fast AW, Menasveta P (1998) Some recent innovations in marine shrimp pond recycling systems. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

e-mail: arlo@hawaii.edu; mpiamsak@chula.ac.th

(Allen & Kinney 1981; Timmons & Losordo 1994). These water treatment methods have been used even longer for domestic and industrial wastewater treatment. Until recently, aquaculture recirculation systems were used mostly for experimental culture applications involving relatively small water volumes, or for relatively small commercial ventures involving high value crops such as ornamental fish (Anonymous 1987; Adey & Loveland 1991; Moe 1992), fish and crustacean seed for conventional growout or stock enhancement (Menasveta et al. 1989, 1991, Colt & White 1991, Wang 1993, Tseng et al. 1998), or for research (Yang et al. 1989). These conventional aquaculture recirculation applications typically range from small aquarium (~200 l), to perhaps 100 m^3 culture tanks, silos or raceways (Table 1).

Table 1. Comparisons between typical characteristics of conventional recirculation aquaculture systems, and commercial scale, marine shrimp recirculation pond culture systems.

Characteristic	Conventional Recirculation Systems	Shrimp Pond Recirculation Systems
Size	small (aquaria, tanks, silos, raceways)	large (ponds >1 ha)
System components		
Culture containment	tanks	ponds
Solids removal	settling; pressurized mechanical filtration	settling
Aeration	mechanical	photosynthesis and mechanical
Nitrification ($NH_3 \rightarrow NO_3$)	"biofilters"	photosynthesis and pond surfaces
Denitrification ($NO_3 \rightarrow N_2$)	requires anaerobic sub-component	pond mud
Ozonation/UV	possible	not likely
Energy I/P	intensive	less intensive
Solar I/P	none to small	large and important
Water Replacement (% daily flow-through)	0 to 10%	0 to <5%

System components of conventional water recirculation systems generally include provisions for: containment of the primary crop; solids removal; nitrification; and means of water recirculation and aeration (Table 1). Additional, optional components include: temperature control; denitrification; foam fractionation; and biocidal treatments such as ozonation and/or UV irradiation (Losordo 1998a,b). Daily water exchange rates, or flow-through rates typically range from near zero with denitrification, to 10% or more without denitrification. Application of these conventional recirculation systems to marine shrimp culture include broodstock maturation (Menasveta et al. 1991), larviculture (Huguenin & Colt 1989) and nursery operations (Sturmer et al. 1992).

During the past 15 years, recirculation systems have been applied commercially to marine shrimp growout on much larger scales than previously envisioned. Reasons for these applications relate mostly to: control of diseases from source waters; control of organisms in source waters; control of water quality problems with source waters; scarcity of high quality source waters; improved growth performance due to greater control over water quality; and concerns about environmental degradation caused by shrimp pond effluents.

Serious shrimp diseases can come from source waters and/or organisms in source waters. Therefore, by limiting use of source waters, by pre-treating these waters before use and by reconditioning effluent for recycle, chances of disease are greatly reduced. These precautions combined with use of specific pathogen free (SPF) or specific pathogen resistant (SPR) shrimp can greatly reduce disease incidence during growout. Recirculating pond systems can also reduce sedimentation within ponds, and improve discharge water quality to receiving waters.

A wide variety of shrimp pond recirculation schemes have been proposed and/or used. These share many characteristics with conventional recirculation culture systems, but also differ in some respects (Table 1). In addition to size differences, conventional and pond recirculation systems perhaps differ most markedly with respect to photosynthesis. With conventional systems, photosynthesis is typically absent. Aeration and ammonia conversion with conventional systems are achieved by mechanical means and by bacteria. With pond recirculation systems, photosynthesis plays a dominant role in oxygenation and ammonia conversion. In addition, energy and water exchange requirements are typically much lower with pond recirculation systems.

Although no two pond recirculation systems described in the literature to date are identical in all respects, they share the same objectives of reducing water exchange, reducing settlable solids, and reducing metabolite toxicity (especially nitrogen compounds). Satisfactory achievement of these objectives should result in more successful shrimp culture with less environmental degradation.

Shigueno (1985) described one of the earliest water recycle systems for ultra-intensive culture of the karuma prawn (*P. japonicus*). This system was developed during the 1970's and used relatively small (~0.2 ha), outdoor tanks. These circular concrete tanks had "false" bottoms with a sand layer through which water percolated and was collected for recirculation or discharge. The sand bottom functioned as a nitrification site (biofilter) and a place for the shrimp to burrow. Water flow-through was very high at 300% to 400% per day. Because of high capital and operating costs, these systems were unprofitable despite very high yields (>35,000 kg/ha/yr) and high prices for live shrimp (U.S.$80/kg) for the sushi market (Shigueno 1985. Fast 1992d).

Another ultra-intensive culture system using partial recirculation was developed in the U.S. and Mexico about the same time as the Shigueno round culture tanks in Japan. This was the covered raceway system developed by the University of Arizona and others (Colvin 1985, Liao 1986, Fast 1992d). This system used plastic lined raceways with aeration and partial water recirculation. An inflated plastic cover helped maintain optimal water temperatures. Yields of 70,000 kg/ha/crop with Pacific blue shrimp (*P. stylirostris*) were occasionally realized, but the system was plagued by disease problems of unknown origin and ultimately proved unprofitable.

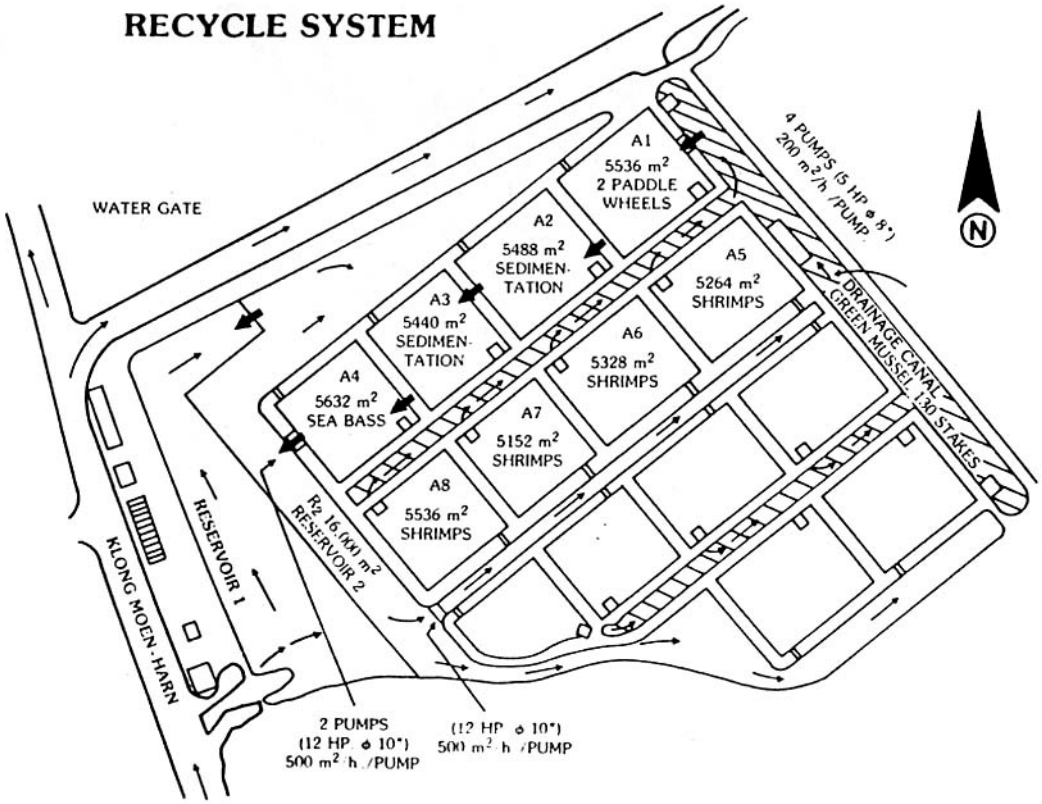


Figure 1. Recirculation shrimp pond system operated by Charoen Pokphand Group (CP) in Thailand (Anonymous 1996b). Source waters enter Reservoir Pond 1 for sedimentation and conditioning before flowing into Reservoir Pond 2, and then into shrimp growout ponds (A5-A8). Effluent from shrimp growout ponds goes into the Drainage Canal from whence some or all of it is circulated through four water treatment ponds (A1-A4) for aeration, solids removal and reconditioning. Green mussels are placed on stakes in the Drainage Canal. Sea bass are reared in pond A4 to help increase dissolved phosphorus and stabilize phytoplankton populations. (Note that pump water volumes should be in m^3 instead of m^2 as shown in the diagram).

Photosynthetic activities were minimal or virtually nonexistent with both of the above partial recirculation systems, and they were operated primarily as flow-through systems with only some recirculation.

More recently, Choroen Pakphand Group (CP) operated a recirculation pond system at their R&D center, Maeklong Area, Thailand (Anonymous 1996a). The system consisted of ten 0.5 ha shrimp culture ponds, two reservoir ponds, and four water treatment ponds (Fig. 1). Source water from a public canal was pumped into Reservoir Pond 1 where solids settled. Water then flowed into Reservoir Pond 2 which supplied water to shrimp growout ponds, which in turn discharged into a drainage system. Water then either went to drain or was pumped into water treatment ponds. The first version of this system included an aeration pond (A1) followed by two solids removal ponds (A2, A3) with green mussels (*Mytilus smaragdinus*) or oysters (*Crassostrea sp.*), and lastly a pond (A4) with sea weeds (*Graciliaria sp.*, *Polycavernosa sp.*) for nutrient stripping. Reclaimed water then flowed into Reservoir Pond 2 and returned to the shrimp growout ponds. This water treatment process reduced suspended organic solids by 30%, ammonia by 90% and nitrites by 60%. CP later modified this recirculation system as shown in Figure 1 (Anonymous 1996b). The revised configuration included green mussels on stakes in the drainage canal, two sedimentation ponds (A2, A3) following the aeration pond (A1), and culture of sea bass (*Lates calcarifer*) stocked at 2.6 pcs/m^2 in the final treatment pond (A4). This new treatment system resulted in greater dissolved phosphorus concentrations in pond waters and more stable algal blooms. Stable algal blooms are important since healthy, rapidly growing algae absorb NH_4 and CO_2, and create more desirable water quality. Without adequate phosphorus and with senescent algae, algal populations often crash resulting in NH_4 spikes, poor water quality and shrimp mortalities. Using this recirculation system and *P. monodon* stocked at densities of 30 PL/m^2, yields averaged 8,267 kg/ha/crop after 125 to 130 days culture. At the same time, sea bass yields were 6,288 kg/ha after 100 days culture.

In Indonesia, shrimp pond recirculation systems were developed as a result of poor source water quality and resultant negative impacts on shrimp yields. A typical Indonesian recirculation system consists of 50% shrimp culture and 50% water reclamation (Fig. 2; Anonymous 1996c). Shrimp pond effluent first flows through a sedimentation pond, followed by a fish/bivalve pond, and lastly through an aeration pond before return to the shrimp growout ponds. Milkfish (*Chanos chanos*), mullet (*Mugil spp.)* and/or green mussels or oys-

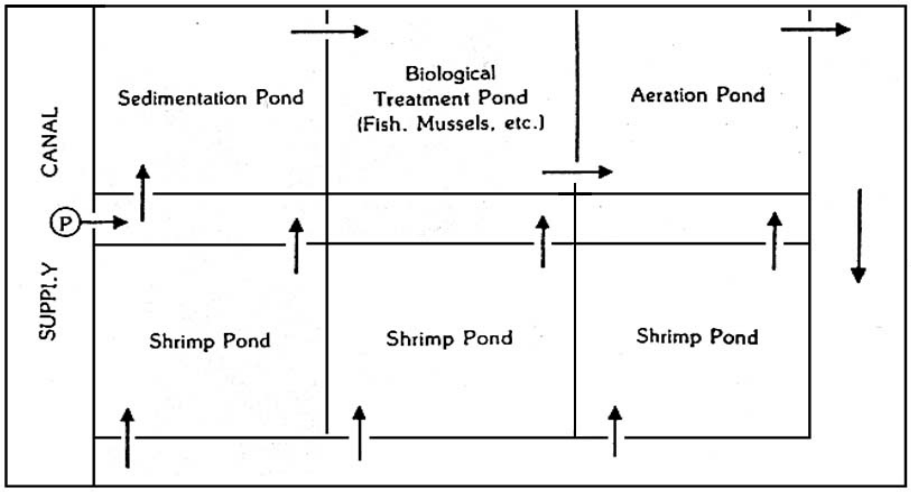


Figure 2. Typical recirculating shrimp farm system in Indonesia (Anonymous 1996c). Shrimp pond effluent flows to a sedimentation pond, followed by treatment with fish and/or bivalves. Milkfish, mullet, green mussles and/or oysters are commonly used. Waters are then aerated before returning to the shrimp growout ponds.

ters are commonly used. The system can be operated closed, or partly-closed. Using this system, average shrimp yields of 8,600 kg/ha/crop were reported after about 145 days culture when stocked at 50 PL/m2. Milkfish stocking densities were 1,000 pcs/ha.

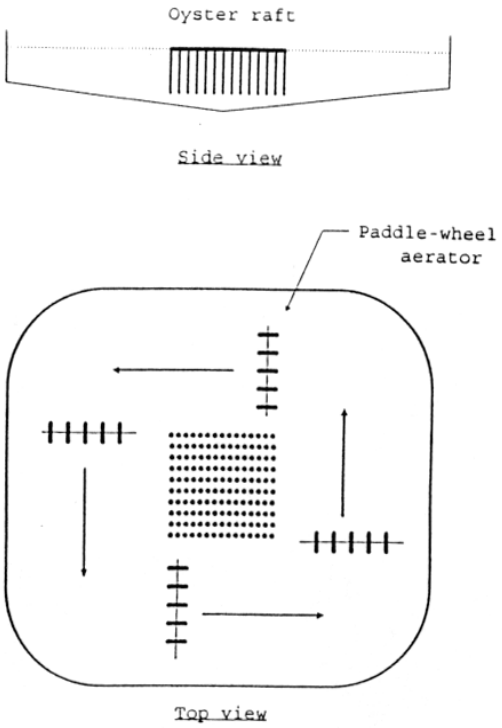


Figure 3. In-pond treatment system using an oyster raft proposed by Menasveta and Jarayabhand (1995). Oysters are preferred to green mussles because of their greater salinity tolerances and higher market value. Paddle wheel aerators would create circular water motions and concentrate wastes in the pond center.

Menasveta and Jarayabhand (1995) have proposed an in-pond treatment system (Fig. 3). This system consists of a floating raft from which oysters are suspended on strings or trays. Oysters would remove excess algae and other suspended solids, and provide an additional nitrification substrate. Oysters are preferred to green mussels because of their greater range of salinity tolerance and higher market value. Four paddlewheel aerators would move water in a circular motion, thus concentrating wastes in the center of the pond.

Another proposed system would consist of a small intensive shrimp culture pond nestled within a much larger extensive culture pond (Fig. 4; A.W. Fast, unpublished). Water would enter the intensive culture pond using low-energy water movers (Rogers & Fast 1988), or more commonly used push-

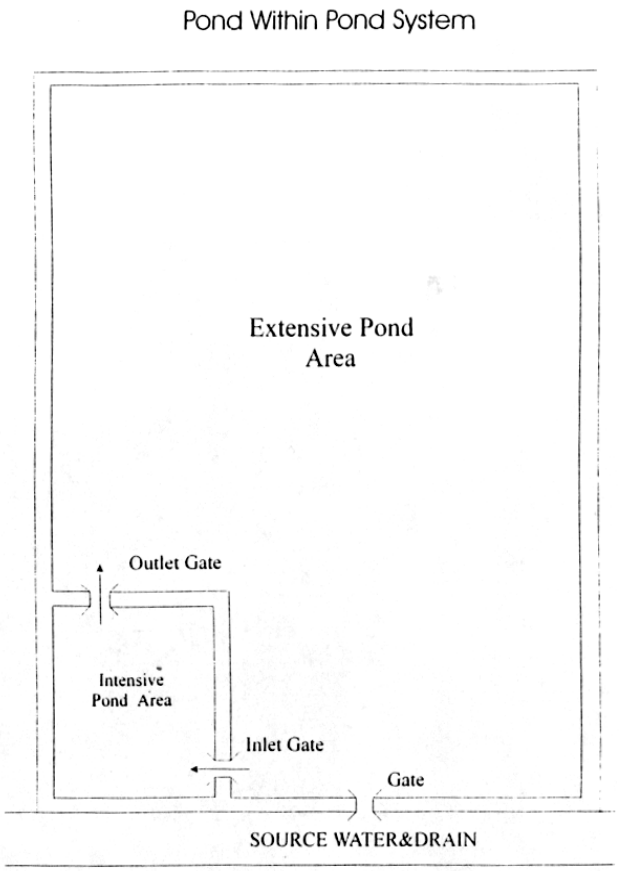


Figure 4. Proposed pond-in-pond culture system where shrimp are cultured intensively in a small pond, while source water and effluents recycle with a much larger extensive pond. The extensive pond serves as a waste treatment component and provides secondary cash crops. This system could easily be retrofited to existing extensive ponds withour creating new pond area.

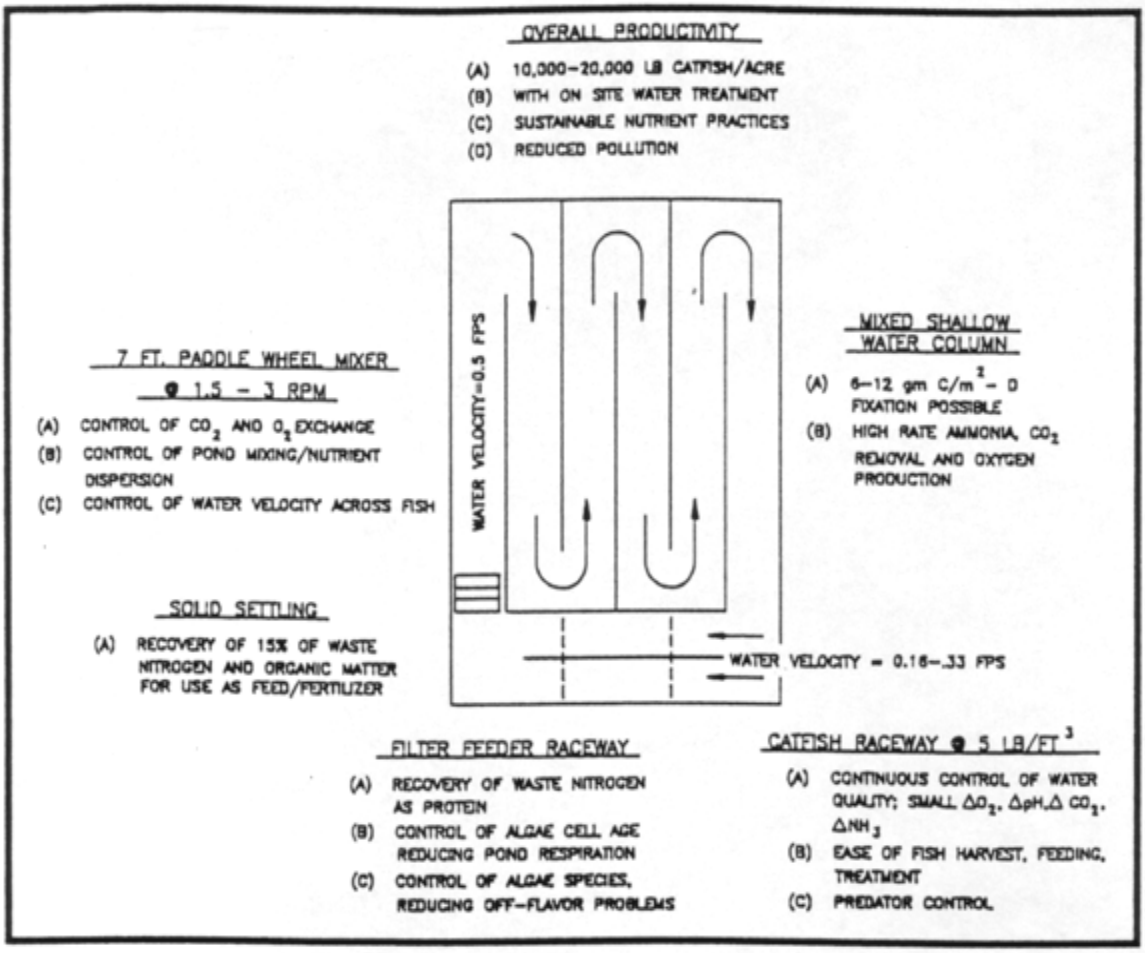


Figure 5. Schematic or Partitioned Aquaculture System (PAS) for pond culture under development at Clemson University, South Carolina, U.S.A. The primary crop is cultured in the raceway section, while phytoplankton and other solids are removed in the settling area where water velocities are less. Phytoplankton remove metabolic wastes and oxygenate the water. The system is computer controlled to maintain adequate oxygen concentrations and optimal algal growth. Figure courtesy of D.E. Brune (1998).

pumps. The intensive pond would be deeper and have mechanical aeration such as paddlewheels. Effluent from the intensive pond would return to the extensive pond where suspended solids would settle and nutrients would stimulate primary and secondary productivity for the secondary crop. Shrimp would be cultured at high densities in the intensive pond, while milkfish, mullet, tilapia, bivalves and/or shrimp could be cultured in the extensive pond at lower densities. A major advantage of this pond-in-pond system is that it could be retrofitted to existing extensive ponds without need to create more new pond areas from land now in other use.

Recently, Brune (1998) and Drapcho (1993) described their work at Clemson University with an algal based, partitioned aquaculture system (PAS) for freshwater channel catfish (*Ictalurus punctatus*). The PAS has been under development since 1988 and has produced catfish yields of 15,000 to 22,000 kg/ha/yr, compared with about 5,500 kg/ha/yr with conventional, intensive culture of catfish. The PAS consists of a fish culture raceway component which is designed to have uniform, well mixed flow with high phytoplankton growth (Fig. 5). This provides high dissolved oxygen concentrations while at the same time reduced ammonia. Following the raceway is a settling area where wastes settle for removal from the system. Tilapia (*Oreochromis niloticus*) and/or other filter feeding fish and plants can also be cultured in this settling areas to further utilize algal production and wastes, and to provide additional cash crops. Highest yields were realized when catfish were co-cultured with tilapia. Oxygen transfer and other control functions are accomplished by computer control of the paddlewheel circulator. Advantages of the PAS include: low water use and no water discharge; good disease control; good predator control; high crop yields; simplified grading of crop by size; easy crop harvest; and multi-cropping of more than one species. The PAS system has potential application to marine shrimp culture, but has not yet been used for this purpose.

Chien & Liao (1995) describe a somewhat different conceptual farm. Their proposed system includes both water treatment components (bivalves, seaweed, marsh, mangroves and settling), as well as waste water recycling. The reuse system includes first using reservoir or treated waters for intensive shrimp culture, followed by use in less intensive shrimp culture ponds before treatment and recycling. They also allow for a wide range of options, which are probably beyond the scope of our present ability to operate optimally.

CONCLUSIONS

Clearly, shrimp pond water recycling and recirculation are being used, and will continue to be used on a large scale for all the reasons identified above. However, it is also clear from the review of existing literature that while operating principals are well understood, there is little documentation of operating performance. More importantly perhaps, design criteria needed to size a given pond recycling system are largely lacking. This contrasts with conventional recirculation systems where these parameters are much better understood and where design criteria are described (Timmons & Losordo 1995). The reasons for this lack of detail for pond recirculation systems relates partly to uncertainties with performance predictability and partly to their relative newness. We anticipate much greater understanding of these pond systems over the next 10 years or so. While shrimp pond water recirculation and recycling will increase, it is not practical for all shrimp farms. A 1994 farm survey by the CP Shrimp Feed Marketing Department revealed that 70% of all intensive farms in Thailand were <1.6 ha (Anonymous 1996d). Most of these farms do not have adequate land area or perhaps the farmers do not have adequate skills to achieve meaningful water recycling. With these farms, some in-pond treatment methods seem more appropriate.

Acknowledgements. This study is financially supported by Thailand Research Fund (TRF).

LITERATURE CITED

Adey WH, Loveland K (1991) Dynamic aquaria. Academic Press, San Diego, CA, 643 p.

Allen LJ, Kinney EC (1981) Proceedings of the bio-engineering symposium for fish culture. American Fish. Soc., Bethesda, MD, 307 p.

Anonymous (1987) Practical koi keeping. Koi USA, Midway City, CA, 191 p.

Anonymous (1996a) The recirculation system, new technology for shrimp culture. In: Lin, CK, Nash, GL (compilers), Asian Shrimp News Collected Volume 1989-1995. Asian Shrimp Culture Council, C.P. Tower, 313 Silom Rd., Bangkok 10500, p 44-46

Anonymous (1996b) The recycle system for shrimp culture. In: Ibid. p 52-54

Anonymous (1996c) Successful Indonesian experience in shrimp-fish integration in recycle system. In: Ibid. p 56-58

Anonymous (1996d) Update on shrimp farm size, distribution and feed performance in Thailand. In: Ibid. p 91-92

Brune DE (1998) Recirculation in photosynthetic aquaculture systems. Aquaculture Magazine 24(3):63-71

Chien YH, Liao IC (1995) Integrated approach to prawn growout system design. In: Browdy CL, Hopkins JS (eds.), Swimming through troubled waters. Proceed. Special Session on Shrimp Farming, Aquaculture '95. World Aquacult. Soc., Baton Rouge, LA, p 167-182

Colt J, White RJ (1991) Fisheries bioengineering symposium. American Fish. Soc., Bethesda, MA

Colvin LB (1985) Intensive growout systems for shrimp. In: Chamberlain GW, Halby MG, Midget RJ (eds.), Texas Shrimp Farming Manual. Texas Agricult. Ext. Service, Texas A&M Univ., College Station, TX, IV-1-IV-14 p

Drapcho CM (1993) Modeling of algal productivity and diel oxygen profiles in the partitioned aquaculture system. Ph.D. thesis, Dept. of Agri. & Biol. Engr. Dept., Clemson Univ., Clemson, SC.

Fast AW (1991) Marine shrimp pond growout conditions and strategies: a review and prognosis. Reviews in Aquatic Sciences 3:357-399

Fast AW (1992a) Penaeid growout systems: an overview. In: Fast AW, Lester LJ (eds). Marine Shrimp Culture: Principles and Practices. Elsevier Science Publishers, Amsterdam. 347-356 p

Fast AW (1992b) Penaeid semi-intensive growout systems. In: Ibid. 371-382 p

Fast AW (1992c) Penaeid intensive growout systems. In: Ibid. 383-392 p

Fast AW (1992d) Penaeid ultra-intensive growout systems. In: Ibid. 393-402 p

Folke C, Kautsky N (1992) Ecological economic principles for aquaculture development. In: Cowey CB, Cho CY (eds), Feeding fish in our water: nutritional strategies in management of aquaculture waste. Univ. of Guelph, Ontario, Canada

Huguenin J, Colt J (1989) Design and operation guide for aquaculture seawater systems. Elsevier, Amsterdam.

Liao IC (1986) General introduction to the prawn system in Taiwan. Aquacultural Engineering 5:219-233.

Losordo TM (1998a) Recirculating aquaculture production systems: the status and future. Aquaculture Magazine (Jan./Feb.):38-45.

Losordo TM (1998b) Recirculating production systems: the status and future, part II. Aquaculture Magazine (March/April):45-53.

Menasveta P, Aranyakanonda P, Rungsupa S, Moree N (1991) Maturation and larviculture of penaeid shrimp in closed recirculating seawater system. Aquacultural Engineering 8:357-368

Menasveta P, Fast AW, Piyatiratitivorakul S, Rungsupa S (1991) An improved, closed seawater recirculation maturation system for giant tiger prawn (*Penaeus monodon* Fabricius). Aquacultural Engineering 10:173-181.

Menasveta P, Jarayabhand P (1995) Environmental planning in the coastal area for sustainable shrimp culture operation. Thai Jour. Aquatic Sci. 2(1):48-58.

Moe MA, Jr. (1992) The marine aquarium reference, systems and invertebrates. Green Turtle Publications, Plantation, FL, 512 p

Rogers GL, Fast AW (1988) Potential benefits of low energy water circulation in Hawaiian prawn ponds. J. Aquacultural Engineering 7:155-165.

Shigueno K (1985) Intensive culture and feed development in *Penaeus japonicus*. In: Taki Y, Primavera JH, Llobrera JA (eds), Proceed. First Internat. Confer. on the Culture of Penaeid Prawns/Shrimp. Southeast Asian Fish. Devel. Center, Iloilo City, Philippines, p 115-122

Sturmer LN, Samocha TM, Lawrence AL (1992) Intensification of penaeid nursery systems. In: Fast AW, Lester LJ (eds). Marine Shrimp Culture: Principles and Practices. Elsevier Science Publishers, Amsterdam, p 321-344

Timmons MB, Losordo TM (1994) Aquaculture water reuse systems: engineering design and management. Elsevier Science Publishers, Amsterdam. 346 p

Tseng KF, Su HM, Su MS (1998) Culture of Penaeus monodon in a recirculating system. Aquacultural Engineering 17:138-147.

Wang JK (1993) Techniques for modern aquaculture. American Soc. Agricultural Engineers. Niles, MI, 604 p

Yang WT, Halon RT, Lee PG, Turk PE (1989) Design and function of closed seawater systems for culturing loliginid squid. Aquacultural Engineering 8:47-65.

Preservation of Water Quality in Shrimp Ponds by Ozone

[1]M. Matsumura, [2]V.P. Migo, [3]D. Balobalo, [3]H.K. Young, [4]J.D. Albaladejo

[1]Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Japan
[2]National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Banos Laguna, Philippines
[3]Aqua-Ecotechnology Research Inc., Caloocan, Philippines
[4]Bureau of Fisheries and Aquatic Resources, Quezon City, Philippines

ABSTRACT: A novel application system of ozone for disinfection and water quality improvement in shrimp grow-out ponds was studied in this work. In the system a water-jet circulator was employed as an ozone distributor. This water-jet circulator enhanced the liquid mixing in shrimp ponds and diminished the vertical distribution of DO, which was also effective for sediment improvement. An appropriate ozone dosing rate for disinfection was determined through actual shrimp cultivation in polluted ponds with pathogenic *Vibrio*.

KEY WORDS : prawn culture, disinfection ozone, water-jet circulator

INTRODUCTION

In 1991, China attained the world record of 210,000 tons prawn production. However, the following year production dropped to 140,000 and finally to 30,000 tons in 1993. This virtual collapse of the prawn farming industry was caused by the prevalence of white spot syndrome virus (WSSV). Almost all prawn farms in the Asia-Pacific region have similar disease problems caused by pathogenic bacteria and viruses. These diseases are the result of the interaction of host (shrimp), pathogens and environment parameters like water and sediment quality. Therefore, to arrest the problems completely, we should approach prawn diseases in an integrated manner and develop the technologies for rapid detection of pathogens, disinfection, management of sediment and water, immunostimulation and also selective breeding for disease resistance. However, disinfection is now the most urgent requirement. Formalin and chlorine have been employed as disinfectants for pathogenic bacteria and viruses. The emergence of these diseases is associated with aquatic environment degradation which is pronounced in intensive culture ponds. Therefore, we have to focus our attention not only on disinfection but also on the improvement of water and sediment quality.

Ozone is one of the most powerful oxidizers known, and it has been utilized for disinfection, controlling taste and odor of water and removing color. Unlike chlorine and chlorine compounds, it produces no harmful byproducts. Once it has completed its task as a purifier and sanitizer, residual ozone reverts to natural oxygen which helps to aerate the water and improve the aquatic environment. Ozone is also very effective in breaking down ammonia and ammonia compounds, as well as organic matter refractory to biodegradation. Therefore, ozone appears to have prospects for use in water quality improvement of aquaculture ponds such as those used for prawn cultivation, where significant damage occurs due to bacterial and viral diseases. The application of ozone in intensive prawn farms is still limited in spite of its multiple functions as a disinfectant and oxidizer of toxic ammonia and nitrite. This is probably due to the lack of suitable *in situ* systems for ozone application in large water bodies.

The present work aimed to study the characteristics of ozone reaction with bromide and chloride ion which are involved in seawater, and to evaluate the performance of a novel ozone distribution system using an energy-efficient water-jet circulator.

BACKGROUND INFORMATION

Oxidants formed by ozone in sea water

When ozone is supplied into freshwater, decomposition of ozone is initiated by the formation of hydroxyl radical. Then it causes several chain reactions to form different radicals and oxidants like hydroperoxyl radicals and hydrogen peroxide, respectively. The hydroxyl radical is, however, mainly responsible for the high oxidative potential of ozone in freshwater. In the case of seawater containing bromide ion, the reaction is completely different from that in freshwater. This is shown by the following equations together with their reaction constants:

$$O_3 + Br^- \rightarrow O_2 + OBr^- \ (k_1 = 160\ M^{-1}s^{-1}) \quad (1)$$

$$O_3 + OBr^- \rightarrow 2O_2 + Br^- \ (k_2 = 330\ M^{-1}s^{-1}) \quad (2)$$

$$2O_3 + OBr^- \rightarrow 2O_2 + BrO_3^- \ (k_3 = 100\ M^{-1}s^{-1}) \quad (3)$$

$$H^+ + OBr^- \leftrightarrow HBrO \ (pKa = 8.68) \quad (4)$$

As we can see from the reaction constant k_1, ozone very quickly produces hypobromite ion, and the hypobromite ion can either be reduced back to bromide ion or oxidized to bromate ion as expressed by Eqs. (2) and (3), respectively. In the presence of bromide ion, ozone is catalytically destroyed, and dissolved free ozone is negligibly small. Ozone

Matsumura M, Migo VP, Balobalo D, Young HK, Albaladejo JD (1998) Preservation of water quality in shrimp ponds by ozone. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

also reacts with chloride. Analogous reactions to Eqs. (1) - (3), involving chlorine in place of bromine, have been proposed with the following rate constants; $k_1 = 0.003\ M^{-1}s^{-1}$, $k_2 = 110\ M^{-1}s^{-1}$ and $k_3 = 30\ M^{-1}s^{-1}$. Because the ratio of k_1 values for bromide ion to chloride ion is at least $5.3x10^4$, bromide ion reacts with ozone preferentially to chloride ion. Oxidative products of bromide ion are expected to dominate those of chloride ion even in seawater, where the molar ratio of Br^-/Cl^- is $1.5x10^{-3}$. Even if hypochlorite ion forms, it reacts quickly with bromide ion to form hypobromite ion as shown by Eq. (5). Therefore, the oxidants produced from bromide ion are mainly responsible for the high oxidative potential of ozone in seawater.

$$OCl^- + Br^- \rightarrow OBr^- + Cl^- \ (k_4 = 6.77x10^3\ M^{-1}s^{-1}) \quad (5)$$

Decomposition of harmful inorganic compounds

Shrimp are very sensitive to ammonia, nitrite and hydrogen sulfide, and their concentrations should be held lower than 0.1 ppm, 0.1 ppm and 0.033 ppm for NH_3-N, NO_2-N and H_2S, respectively. Ozone can easily convert these toxic inorganic compounds to harmless matter. The hypobromous acid formed by Eq. (4) reacts with ammonia to convert it to nitrogen gas and nitrate through the following reactions:

$$HBrO + NH_3 \rightarrow NH_2Br + H_2O \ (k_5 = 8x10^7 M^{-1}s^{-1}) \quad (6)$$

$$HBrO + NH_2Br \rightarrow NHBr_2 + H_2O \quad (7)$$

$$HBrO + NHBr_2 \rightarrow NBr_3 + H_2O \quad (8)$$

$$NHBr_2 + NBr_3 + 2H_2O \rightarrow N_2 + 3Br^- + 3H^+ + 2HBrO \quad (9)$$

$$NH_2Br + 3O_3 \rightarrow NO_3^- + Br^- + 2H^+ + 3O_2 \ (k_6 = 40\ M^{-1}s^{-1}) \quad (10)$$

The ratio of nitrate formation to nitrogen gas formation highly depends on the ratio of Br^- to NH_4^+-N. Under normal conditions in a shrimp pond (Br^- = 67 ppm, NH_4^+-N = 2 ppm, pH = 8, temp. = 28 °C), the Br^-/NH_4^+-N ratio is about 34 and almost all ammonia would be converted to nitrogen gas as shown in Fig.1. The conversion rate of ammonia to nitrogen gas is very fast, as can be seen from the rate constant of Eq. 6. Nitrite and hydrogen sulfide are also easily converted to nitrate and sulfate, respectively.

Inactivation of bacterial and viral pathogens

While the precise mechanisms of oxidant disinfection are unclear, a variety of mechanisms have been proposed. Cell walls and membranes are the primary sites attacked by oxidants. These primary sites in bacteria are composed of lipopolysaccharide, lipoprotein, peptidoglycan, phospholipid and protein. Generally, these components contain double bonds of unsaturated fatty acids, aromatic compounds and amino acids with thiol and amine groups which provide potential targets for oxidant action. Any alternation in lipids and membrane proteins would result in structural and functional changes in the membrane. In the case of viruses, the coat protein would be damaged first. This could denature it and destroy it ability to interact with host target molecules required for attachment and cell entry. If oxidants diffused into the virus through the damaged coat protein, DNA and RNA could be damaged.

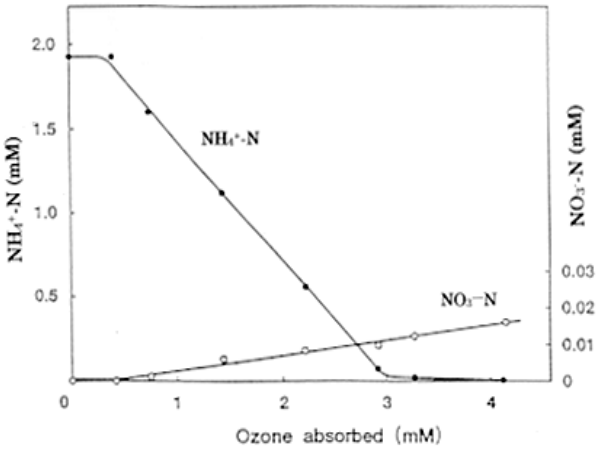


Figure 1. Ozonation of ammonium in sea water.

The inactivation of pathogens is affected by both total residual oxidant (TRO) concentration C and contact time t. This relationship has been expressed by the Chick-Watson equation ($K = C^n \bullet Et$). Figure 2 shows the effect of TRO concentration on inactivation of *Vibrio harveyi* pathogenic for shrimp. Almost 1.5 log units and 4 log units of inactivation occurred within a short contact time of 30 sec at an initial oxidation concentration of 1 and 2.3 mg/l, respectively. Based on this experimental result, effective disinfection of pathogenic bacteria in seawater coud be expected.

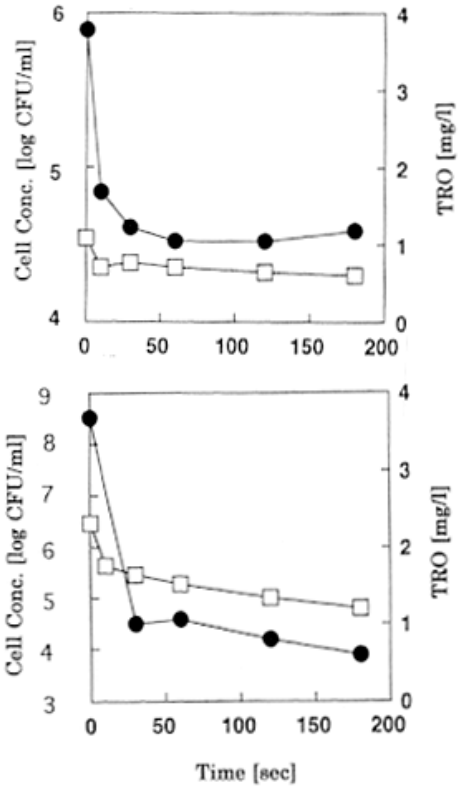


Figure 2. Inactivation of *Vibrio harveyi* in sea water (●=cell concentration; □=Total residual oxidant).

IN SITU APPLICATION OF OZONE IN SHRIMP PONDS

Water-jet circulator

There is a consensus among aquaculturists that water circulation in ponds is beneficial. Water circulation prevents thermal and chemical stratification. This makes the entire pond volume habitable and it eliminates oxygen depletion at the sediment-water interface. During daylight hours, surface water in ponds is often supersaturated with DO produced by phytoplankton, and water at greater depths may have low DO concentration. The paddle wheel aerator is commonly employed in intensive culture ponds, because it blends surface water with subsurface water. By mixing pond water, a uniform DO profile can be established, and the total DO content of a pond can be increased.

Figure 3 shows the 24-hour monitoring of DO at two different sampling points (SP-1 and SP-2) in an intensive culture pond with a surface area of 4500 m^2 and 1.5 m depth. Four paddle wheel aerators were installed in the pond. One had 6 blades and the others had 3 blades. They were driven by 1 horsepower motors. The monitoring was conducted on the 150th day after stocking. The locations of sampling points SP-1 and SP-2 were the mid point between two paddle wheel aerators and the center of pond, respectively. At the sampling point SP-1, there was a liquid mixing enough to diminish the vertical distribution of DO between the pond surface and the bottom. However, an obvious difference in DO between the surface and the bottom could be seen at sampling point SP-2. This was caused by poor liquid mixing. It was clear that uniform water quality in the pond could not be attained by the paddle wheel aerators. Therefore, we introduced a water-jet circulator to enhance liquid mixing.

The construction of the water-jet circulator was based on a double stage ejector as shown in Fig. 4. It was installed at the bottom of the pond as shown in the system construction. We could also introduce ozone mixed with air into the nacelle of the jet pump and make very fine bubbles which were easily dispersed in the sea water. When the driving liquid was supplied into the jet pump through a narrow slit at a volumetric flow rate of Q_1, the liquid was sucked at a flow rate of Q_2. The driving force for the liquid suction is provided by the pressure drop of the driving liquid. Then, the liquid is discharged from the nacelle at a flow rate of Q_1+Q_2 . This discharged liquid acts as the driving liquid for the second ejector. The final discharged flow rate becomes $Q_1+Q_2+Q_3$. The design of the ejector has been well established, and the energy efficiency of the jet pump fÅ is expressed by the following equation:

$$\eta = [Q_1/Q_2][(h_d - h_2)/(h_1 - h_d)] \equiv M \cdot H \quad (11)$$

where, h_1, h_2, h_d are the hydrostatic head at the pump connection points for drive line, suction line and discharge line,

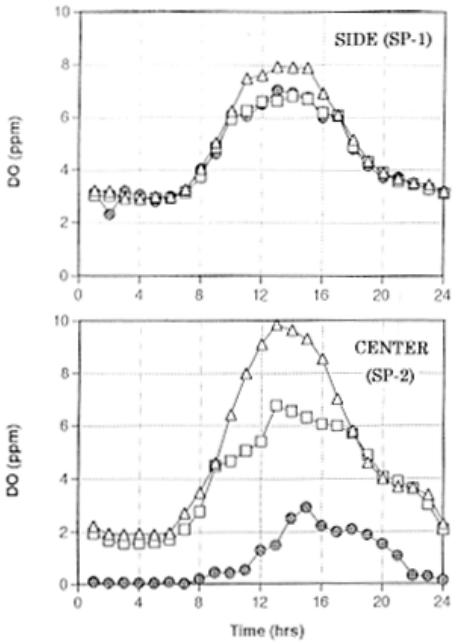


Figure 3. Dissolved oxygen (DO) in a shrimp pond aerated with paddle wheels (Δ=Top; □=Middle; ●=Bottom).

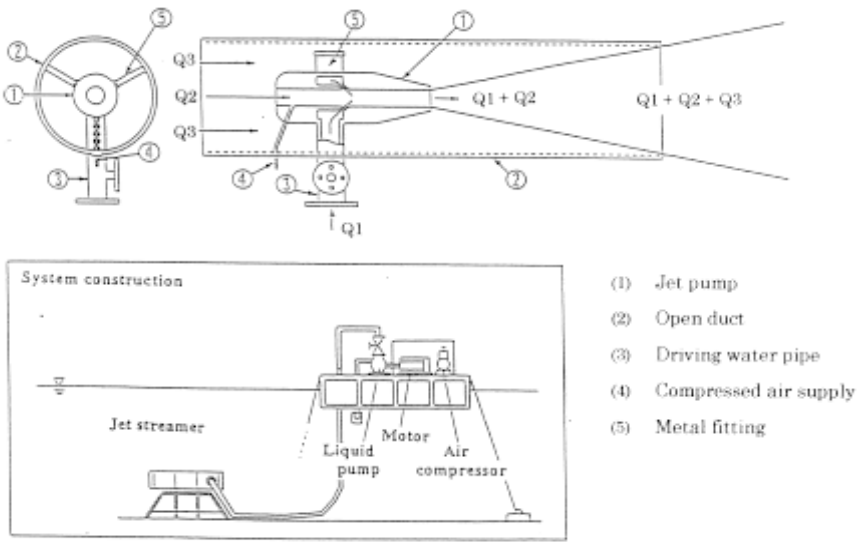


Figure 4. Schematic for construction of the water jet circulator.

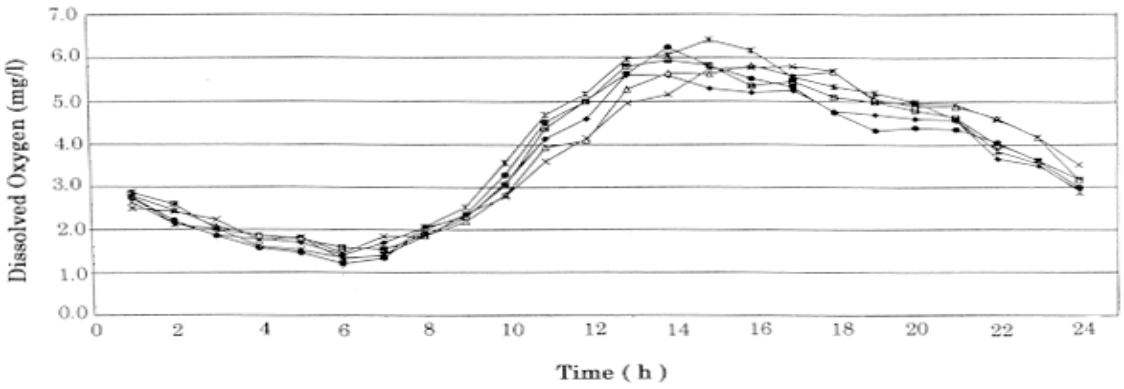


Figure 5. Vertical dissolved oxygen (DO) distribution in a pond using a water-jet circulator (♦=SP-1 top;□=SP-1 bottom, Δ=SP-2 top; X=SP-2 bottom; ✱= SP-3 top; ●=SP-3 bottom).

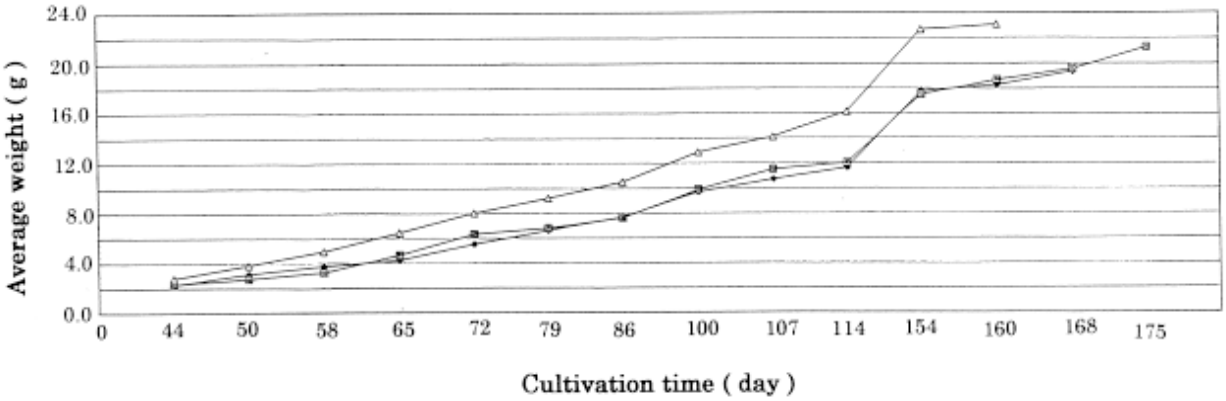


Figure 6. Average body weight of shrimp in ponds #3, #9 and #15 (♦=pond #3; □=pond #9, Δ=pond #15).

respectively. For a jet pump with a low discharge pressure but a high suction flow rate, a low area ratio of about 0.2 is recommended for the construction of the nozzle. In this case, M becomes 2.2, so the discharged liquid volume becomes 3.2 times as much as that of the drive liquid. For the double jet pump, this becomes 10.2 times.

Once the jet flow is established, the liquid surrounding the jet stream is entrained into the stream. The volumetric flow rate of entrained liquid Q_E is given by Eq.(12):

$$Q_E = [0.203(x/d_o - 1)]Q_d \quad (12)$$

where Q_d is the volumetric flow rate discharged from the water-jet circulator ($Q_1+Q_2+Q_3$), and d_o is the diameter of open duct shown in Fig. 4. X is the maximum distance from the exit edge of the open duct where the jet flow exits. If x/d_o is 40, Q_E in Eq.(12) becomes $8.2Q_d$, and then Q_E/Q_1 is 83.5. When a water-jet circulator with a driving liquid volume of 0.75 m^3/min is installed in a shrimp pond with a liquid volume of 6,000 m^3, the pond liquid passes through the circulator at a volumetric flow rate of 11,000 m^3/d. Therefore, the circulation time for the pond water becomes 0.54 d. A rather huge amount of liquid is entrained into the jet stream at a volumetric flow rate of 90,000 m^3/d, and this liquid flow will enhance the mixing of pond liquid. Figure 5 shows the 24- hour monitoring of DO in the same pond shown in Fig. 3. In this case, one water-jet circulator (driving liquid volume: 0.75 m^3/min, energy consumption: 2.2 kW) was installed together with two paddle wheel aerators. As you can see, the liquid mixing was greatly improved, and the vertical distribution of DO was diminished even at the center of the pond.

Effects of ozone on shrimp growth

The first experiment of shrimp cultivation using ozone was conducted in a shrimp farm located at Calatagan in the Luzon Island (Philippines) where no problems caused by pathogenic bacteria and viruses occurred. The main purpose of this experiment was to investigate whether ozone had any adverse effects on shrimp growth. An ultraviolet ozone generator containing two 110 w lamps was used under an air flow rate of 100 l/min. The exit ozone concentration was 100 ppm. The ozone dosing rate into the culture pond was 0.22 mg-O^3/h/m^3.

Figure 6 shows the average body weight over time for shrimp cultivated in three different ponds, pond #3 (surface area; 4000 m^2), #9 (4500 m^2) and #15 (4000 m^2). One water-jet circulator and two paddle wheels were installed in ponds #3 and #9 using sea water with an average salinity of 32 ppt, but ozone was employed only in pond #3. Pond #15 was operated with four conventional paddle wheels, and shrimp

culture was conducted using brackish water with an average salinity of 19 ppt. The stocking in these three ponds was started at the same time and their stocking ratio was about 45 psc/cm^2.

By comparing the results of ponds #3 and #9, it was clear that an ozone dosing rate of 0.22 mg-O_3/h/m^3 did not have any adverse effects on shrimp growth. However, the salinity had an obvious effect on shrimp growth. The survivals at harvest were about 60% in ponds #3 and #9, and 70% in pond #15. The concentration of chlorophyll a in ponds #3 and #9 was around 40 mg/m^3 (transparency: 20 - 30 cm), and in pond #15, 80 mg/m^3 (transparency: 15 - 20 cm), and the dominant algae were diatoms. One of the reasons which for the big difference in chlorophyll a concentration was considered to be enhanced liquid mixing in the vertical direction. It reduced the chance for phytoplankton to be exposed to the sun light. The liquid mixing was also effective in reducing the settling velocity of small organic particles like detritus of plankton and residual feed. This enhanced their oxidation in the water column. The sediment conditions in ponds #3 and #9 were completely different from that of pond #15. Ponds #3 and #5 showed no mud accumulation, even at the center.

Shrimp culture in polluted water with pathogenic *Vibrio*

In the Philippines, approximately 65% of the country's prawn farms are situated in the Visayas island group, and particularly on Negros Occidental. With recent disease outbreaks (luminous vibriosis caused by pathogenic *Vibrio* species like *V. harveyi*) only 30% of the prawn farms are operational, and of these, only 17% experience optimum harvests. Our second cultivation experiment was conducted using two ponds on a farm located in Bacolod City (Negros Occidental).

In this experiment in pond # 21 (pond surface area; 5000 m^2), two water-jet circulators coupled with an ozone generator with three 110 w UV lamps were installed together with two paddle wheel aerators (2 blades) driven by 1 hp motors. The control pond #15 (pond surface area 5400 m^2) was prepared with two paddle wheel units, each with 2 blades driven by a 1 hp motor and two paddle wheel units with 4 blades driven by 2 hp motors.

A pre-stocking experiment was done to determine how many ozone lamps were necessary to reduce or eliminate bacterial pathogens. The total plate count (TPC), total *Vibrio* count (TVC), luminous bacterial count (LBC), luminous *Vibrio* count (LVC) and yellow and green colonies on TCBS agar were monitored. Figure 7 shows the results of bacterial analysis (TOC and LVC) during the pre-stocking experiment. The jump in TPC was observed just after the introduction of water from a reservoir pond. Two UV lamps were turned on from the 15th day to the 34th day, four lamps from the 35th to 40th days, and 6 lamps from the 41st to 55th days. The rate of decrease in TPC increased with increasing number of UV lamps, but a negative effect on the plankton density was observed after 6 UV lamps were turned on. After this preliminary experiment, one water-jet circulator with a 2 UV lamp ozone generator was installed in the reservoir pond for pre-sterilization of water, and over-dosing of ozone into the grow-out pond was limited.

The stocking in control pond #15 and experimental pond #21 was done at the same time on August 3, 1997. The stocking ratio was 25 psc/m^2. Figure 8 shows the percentage survival of shrimp in these ponds. The percentage survival in the control pond dropped quickly, and it became less than 30% only one month after stocking. In pond #21, a fairly good survival was maintained for two months, but after that

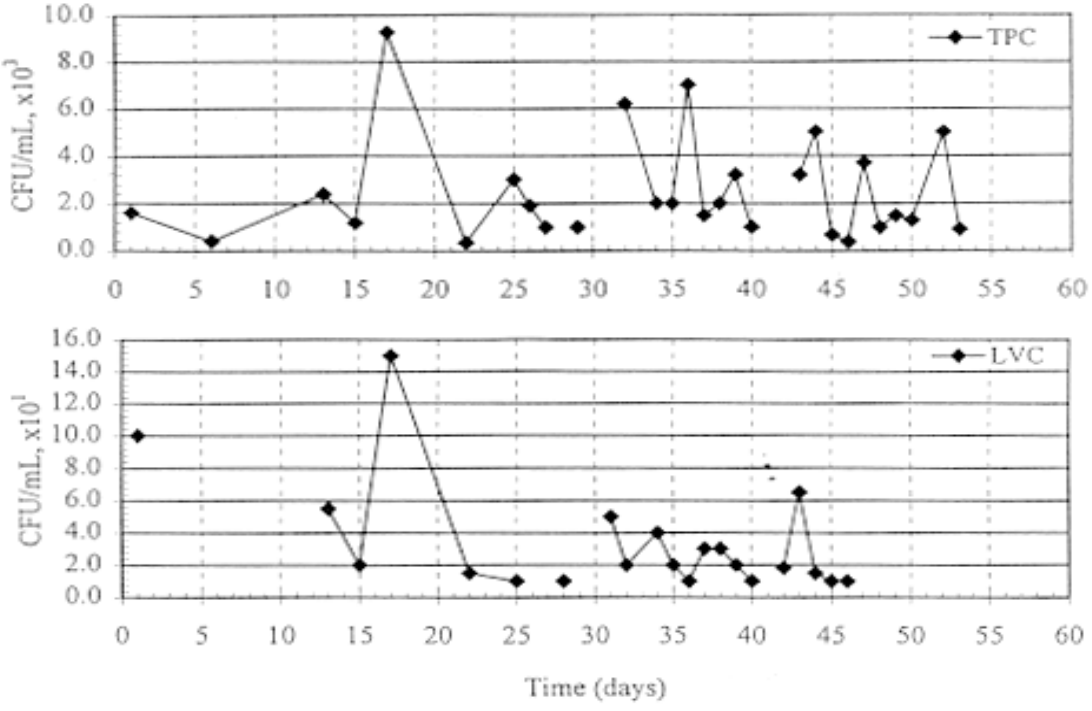


Figure 7. Effects of ozone dosing rate (i.e., number of generating lamps) on inactivation of bacteria (TPC = total plate count; LVC = luminous *Vibrio* count).

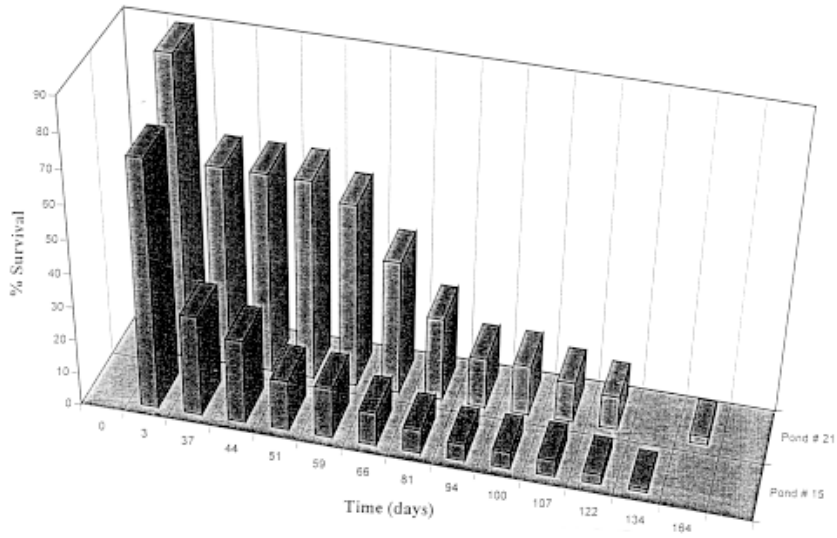


Figure 8. Percent survivl of shrimp in ponds #15 and #21.

it started to drop. In addition, its luminous *Vibrio* count (log CFU/ml) was held at a low level of around 1.3, and its DO was highly improved as shown in Fig. 9. Unfortunately, rainfall was very limited that year and an optimum salinity of about 20 ppt was not maintained. It started to increase two months after stocking and finally reached 39 ppt. The alkalinity of the pond water was around 30 ppm $CaCO_3$, which is much lower than the ideal level (•„70 ppm). This low alkalinity induced rapid fluctuations in pH as shown in Fig. 10. Dolomite and agri-lime was added to both ponds to correct the problem, but this did not increase the level of alkalinity to the optimum level. Water change also had a minimal effect, since the source also had low alkalinity. The level of chlorophyll a in these ponds was 20 - 25 mg/m^3, and the transparency was 50 -55 cm.. These values are far from ideal levels. The dominant alga was C*hlorella*, and diatoms were present at less than 10%. Especially, *Skeletonema* which secretes some anti-microbial compound was very rare. The low alkalinity and low plankton bloom may have resulted from the nature of the sandy pond bottom.

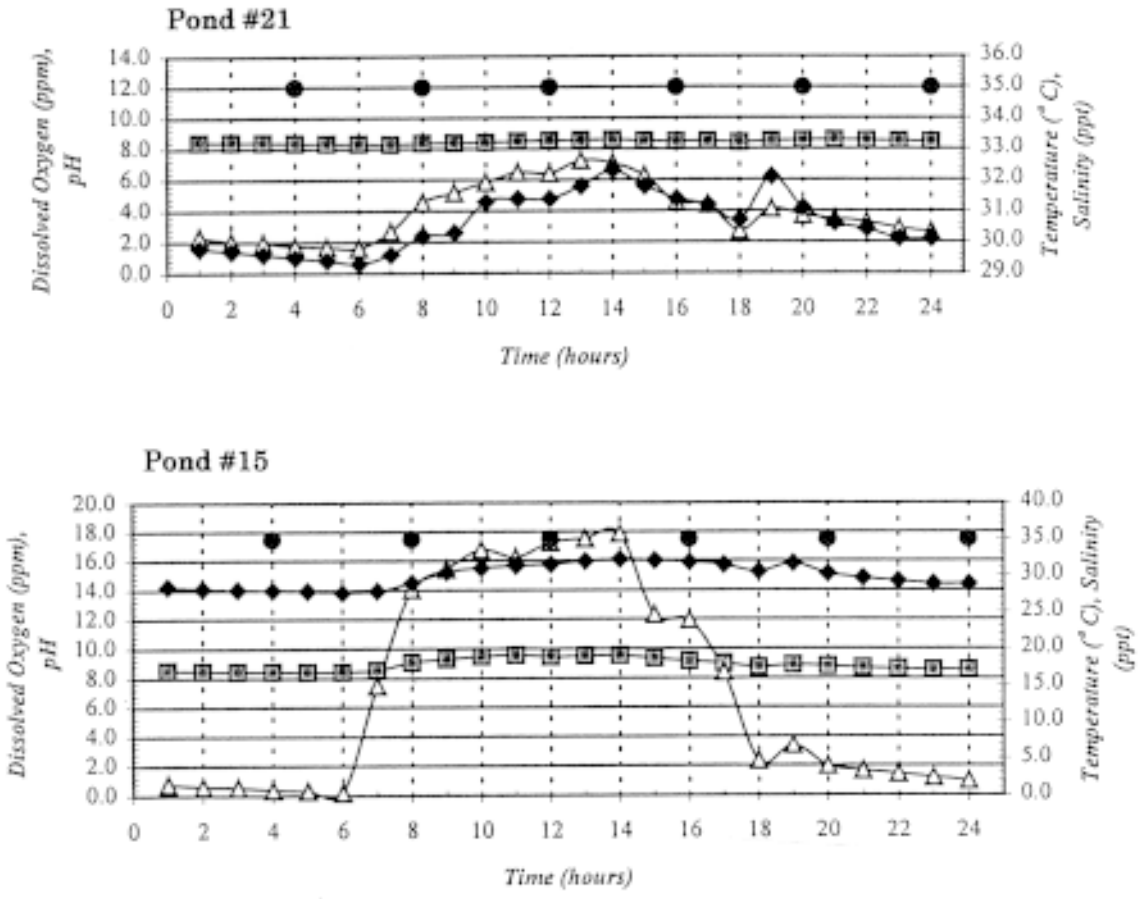


Figure 9. Dissolved oxygen (DO) at the centers of ponds #15 and #21over 24 hours.

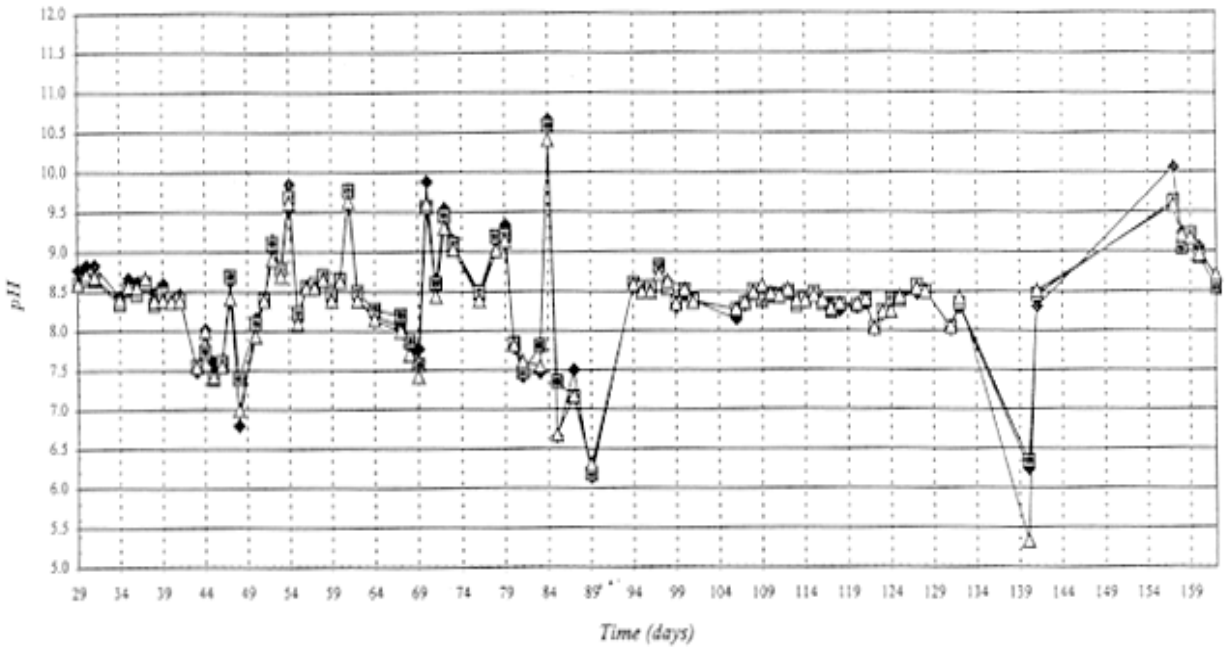


Figure 6. Record of daily pH in pond #15 (♦=SP-1; □=SP-2; Δ=SP-3).

CONCLUSIONS

The goal of increasing pond productivity was not achieved in the present experiments. However, we confirmed that the water-jet circulator was effective for enhancing liquid mixing in grow-out ponds and that appropriate application of ozone is very useful for inactivation of pathogenic bacteria. We also learned many things through these experiments. For example, it is clear that the original nature of pond soil and the algal bloom are very important in maintianing stable environmental conditions. The ecosystem in the shrimp pond is very complicated. We need to obtain much more information, especially about the interactions between the algae and bacteria, and we need to develop the technology to make beneficial algae dominant. We should also develop an energy efficient method to improve DO at night, and work harder to develop effective feed additives to enhance shrimp defense activity against pathogens.

Use of Probiotics for Improving Soil and Water Quality in Aquaculture Ponds

*Claude E. Boyd, Amit Gross

Department of Fisheries and Allied Aquacultures
Auburn University, Alabama USA 36849

ABSTRACT: A number of biological products including live bacterial inocula, enzyme preparations, and extracts of plant products are being promoted for use as water and soil quality conditioners in aquaculture ponds and particularly in shrimp ponds. Although there is much anecdotal information about these products and considerable promotional material from vendors, little independent research has been conducted. Several studies have shown no benefits of bacterial inocula, but one recent study at Auburn University demonstrated higher survival of fish and thus greater net production in ponds treated frequently with live *Bacillus* of three species than in control ponds. No improvements in water quality were noted, and the mechanism by which the bacteria improved survival is unknown. Laboratory studies of a bacterial inoculum demonstrated a greater rate of nitrogen loss. Liming of laboratory systems tended to improve bacterial activity. Pond studies also showed that applications of an enzyme preparation tended to enhance microbial mineralization of organic matter, but no effect on net fish production was observed. An extract of grapefruit seed caused greater survival of shrimp and higher production resulted. Again, the mode of action is unknown because water quality was not measurably improved. These few studies suggest that probiotics possibly can be beneficial in aquaculture ponds. Too little is known about their modes of action, the conditions under which they may be effective, and application rates and methods for general recommendation of their use. Nevertheless, the products are safe to humans and the environment, and their use poses no hazards. Thus, commercial producers are encouraged to conduct trials with these products, and researchers should conduct experiments with them.

KEYWORDS: probiotics, bacterial inocula, water conditioners

INTRODUCTION

There is considerable interest in use of probiotics to improve conditions for production in pond aquaculture. The most common probiotics are live bacterial inocula that sometimes are supplemented by yeast extracts with extracellular enzymes. Some companies sell extracellular enzyme preparations without live bacteria. Extracts of plant products also are used as probiotics. The most common are extracts of citrus seed and Yucca plants (*Yucca_schidigera*). Claims about the potential benefits of probiotics in aquaculture ponds include: enhanced decomposition of organic matter; reduction in nitrogen and phosphorus concentrations; better algal growth; greater availability of dissolved oxygen; less cyanobacteria (blue-green algae); control of ammonia, nitrite, and hydrogen sulfide; lower incidence of disease and greater survival; greater shrimp and fish production. Vendors usually sell probiotics for prophylactic treatments to protect against disease and to improve environmental conditions for culture. Few independent studies have been conducted on probiotics in aquaculture ponds. However, vendors have conducted trials on aquaculture farms and obtained testimonials from farmers. Results of trials and testimonials are used as the basis for exalted claims of the benefits of probiotics. Farmers usually do not have sufficient knowledge of microbiology and water quality to evaluate the claims of vendors. However, they are quite receptive to any new product that might enhance water and soil quality, reduce the incidence of disease, or increase production, and there is a brisk trade in probiotics for shrimp and fish farming.

BACTERIAL INOCULA

The argument for using bacterial inocula is that populations of beneficial bacteria in ponds can be increased by applying live bacteria or their propagules. Two types of inocula have been used. One type consists of the spores or other resting bodies of one or more species in a medium designed to prevent germination and retard growth. The media may be a liquid or granular material coated with bacterial propagules. Relatively small amounts of these inocula are placed in ponds with the assumption that propagules will quickly multiply and increase the abundance of the inoculated species. The second type of inoculum is a source of propagules that is inoculated into a nutrient solution and a culture of bacteria is produced for inoculating the pond. Initially, bacterial inocula were applied to ponds at 2- to 4-week intervals. Today, most vendors recommend that inocula be applied at more frequent intervals or even daily. Species of *Bacillus* are most commonly used, but species of

Boyd CE, Gross A (1998) Use of probiotics for improving soil and water quality in aquaculture ponds. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: ceboyd@acesag.auburn.edu

Nitrobacter, *Pseudomonas*, *Enterobacter*, *Cellulomonas*, *Rhodopseudo-monas*, and photosynthetic sulfur bacteria have been used as bacterial inocula (Boyd 1990). The number of bacteria introduced per application varies greatly, but the initial increase in the pond water normally ranges from 10^2 to 10^4 colony forming units (CFU) ml^{-1}.

The mode of action of bacterial inocula is claimed to be the enhancement of natural processes such as organic matter degradation, nitrification, ammonia removal, denitrification, sulfide oxidation, and degradation of toxic pollutants. Some vendors state that by increasing the abundance of useful bacteria, competitive exclusion of undesirable species occurs. They even declare that the bacterial inocula can reduce the incidence of fish and shrimp disease in ponds.

Ponds have a natural microbial flora capable of conducting the entire range of organic matter and nutrient transformation that occur in natural ecosystems. Bacterial populations respond to the availability of substrate. For example, if a pond has ammonia in the water, natural nitrifying bacteria will oxidize the ammonia to nitrate. If ammonia concentration increases, the population of nitrifying bacteria will respond to the increase in substrate by rapidly increasing in abundance. When ammonia concentrations decline, the abundance of nitrifying bacteria will decline. Nitrification rates are sensitive to ammonia concentration, but they also are regulated by environmental variables such as temperature, dissolved oxygen concentration, and pH. If nitrification does not increase in response to increasing ammonia concentration, it is because some environmental variable is not satisfactory, and not because there is a shortage of nitrifying bacteria. The same argument may be made for organic matter decomposition and other microbial processes. It does not seem likely that applications of bacterial inocula to most ponds would influence the rate of bacterially-mediated processes. Although some vague explanations of how application of beneficial bacteria can reduce the incidence of undesirable bacteria have been advanced, no convincing explanation of how bacterial inocula can reduce the frequency of disease is available.

Boyd et al. (1984) treated four channel catfish ponds with a bacterial inoculum containing seven species of bacteria. Water quality and fish production were compared between treated and control ponds. The inoculum was claimed by the vendor to contain more than 10^9 CFU ml^{-1}, and 0.25 mg l^{-1} of inoculum was applied at monthly intervals between June and September. Concentrations of inorganic nitrogen, total phosphorus, chemical oxygen demand, 5-day biochemical oxygen demand, and chlorophyll a; numbers of bacteria and phytoplankters per milliliter; and percentages of blue-green algae did not differ significantly ($p > 0.05$) between treatments. On three sampling dates between mid July and mid August, there were significantly higher ($p < 0.05$) dissolved oxygen concentrations in ponds treated with the bacterial suspension. Fish production did not differ between treatments ($p > 0.1$). Tucker and Lloyd (1985) conducted trials with the same bacterial inoculum used by Boyd et al. (1984) with almost identical results.

Chiayvareesajja and Boyd (1993) treated laboratory microcosms with up to 40 mg l^{-1} of a granular microbial inoculum, and no changes in total ammonia nitrogen concentrations related to concentrations in controls were observed ($p > 0.05$). Pond waters were treated with the inoculum at 5 mg l^{-1} initially, 2.5 mg l^{-1} after week 1, and 0.5 mg l^{-1} for the next two weeks. No reduction in ammonia concentration relative to the control ponds was noted ($p > 0.05$).

A commercial bacterial inoculum consisting of *Bacillus* spp. cultured on site was applied to three channel catfish *Ictalurus punctatus* ponds at Auburn, Alabama, USA, three times per week from May until October 1996 (Queiroz & Boyd 1998). There were few significant differences ($p < 0.1$) in concentrations of water quality variables between ponds treated with bacteria and control ponds. In addition, bottom soil carbon and nitrogen did not differ between treated and control ponds. However, survival and net production of fish was significantly greater ($p < 0.1$) in ponds that received the bacterial inoculum than in controls (Table 1). The mechanism by which the bacterial treatment influenced fish survival cannot be explained from data collected in this study.

Because of the high variability encountered in water quality among aquaculture ponds treated alike and the difficulty of establishing a given level of water quality impairment in production ponds, a laboratory study of a bacteria inoculum was conducted at Auburn University. Pond soil-water mesocosms were established by placing 2 liters of pond soil enriched with 5 g of fish feed (32% crude protein) and 20 liters of pond water enriched with nutrients for BOD dilution water (Eaton et al. 1995). The following treatments were replicated four times in mesocosms: (1) control (soil and water with nutrients); (2) soil, water with nutrients, and bacterial inoculum; (3) soil, water and nutrients, calcium oxide, and bacterial inoculum; (4) autoclaved soil, water and nutrients, and bacterial inoculum. These mesocosms provided conditions similar to those found in intensive aquaculture ponds, i.e., high nutrient concentrations and or-

Table 1. Average fish production data (± SD) for channel catfish ponds treated and untreated (control) with bacterial inoculum.

Variable	Treated	Control
Stocking rate (per hectare)	15,000	15,000
Fish surviving until harvest (per hectare)	13,150 ± 2,007[a]	8,425 ±2,850[b]
Average harvest weight per fish (g)	311 ±63[a]	400 ±41[a]
Net production (kg ha^{-1})	4,020 ±438[a]	3,301 ±838[b]
Feed conversion ratio	1.5 ±0.07	1.82 ±0.36

[a]Means for the same variable were different at a probability level of 0.1

Table 2. Grand means for soil-water mesocosms in which a bacterial inoculum was tested. Means with the same letter did not differ at p = 0.05 (horizontal comparisons only).

		Bacterial inoculum added		
	Control	Initially-sterile soil	Un-sterile soil	Un-sterile soil and calcium oxide
Total phosphorus (mg l^{-1})	2.68[a]	3.50[a]	3.43[a]	1.36[b]
Soluble reactive phosphorus (mg l^{-1})	1.73[a]	1.83[a]	1.87[a]	0.72[b]
Total ammonia nitrogen (mg l^{-1})	4.50[a]	5.45[a]	3.32[b]	2.72[b]
Organic nitrogen (mg l^{-1})	2.70[a]	3.50[a]	2.04[a]	2.31[a]
Nitrate-nitrogen (mg l^{-1})	0.27[a]	0.01[a]	0.19[a]	0.11[a]
Nitrite-nitrogen (mg l^{-1})	0.37[a]	0.03[a]	0.60[a]	0.17[a]
Chemical oxygen demand (mg l^{-1})	31.7 b	42.2[a]	42.7[a]	34.9[b]
Biochemical oxygen demand (mg l^{-1})	9.0[a]	8.2[a]	7.5[a]	10.8[a]
Dissolved oxygen (mg l^{-1})	8.2[a]	7.8[a]	7.1[a]	7.9[a]
pH (standard units)	7.7[a]	7.6[a]	7.4[a]	8.3[b]
Bacteria (cfu ml^{-1})				
Water	3.2×10^{5}[b]	2.9×10^{6}[a]	2.5×10^{5}[b]	2.3×10^{6}[a]
Soil	3.9×10^{6}[a]	4.0×10^{6}[a]	1.3×10^{6}[a]	7.0×10^{6}[a]

ganically-enriched bottom soil. The treatment with autoclaved soil provided mesocosms with few bacteria at the time of inoculation with bacterial product. The soil in microcosms had a pH of about 6, so a calcium oxide treatment was included to simulate liming.

The averages of water and soil analyses conducted over a 20-d period (Table 2) revealed bacteria abundance was greater in mesocosms treated with calcium oxide and in mesocosms with autoclaved soil than in the other treatments. Calcium oxide raised the pH to 8.34 and a pH of 8.0 to 8.5 is optimum for bacterial activity (Boyd & Pipoppinyo 1994). Sterilization of the soil eliminated the natural bacterial populations. This may have reduced competition of natural bacteria to provide a greater opportunity for growth for the inoculated bacteria.

Some differences ($p < 0.05$) were noted among treatments (Table 2). Total phosphorus and soluble reactive phosphorus concentrations in water were less in mesocosms treated with calcium oxide than in the others. This resulted from the precipitation of phosphorus by calcium oxide and was not related to bacterial activity. Total ammonia nitrogen concentrations were higher in waters of mesocosms containing autoclaved soil and control mesocosms than the other two treatments. Lower total ammonia nitrogen in the calcium oxide-treatment microcosm was related to the higher pH causing a greater loss of ammonia to the air by diffusion. The reason that application of bacteria to non-sterile soil resulted in lower total ammonia concentrations than found in control mesocosms or in mesocosms with initially-sterilized soils is not apparent. The chemical oxygen demand increased in the unlimed, microbial-treated mesocosms relative to the control. No differences in organic nitrogen, nitrate-N, nitrite-N, biochemical oxygen demand, or dissolved oxygen concentrations were noted among treatments.

This study failed to reveal a positive influence of the bacteria inoculum on water quality in the mesocosms. The only difference among treatments was the lower concentrations of total phosphorus, soluble reactive phosphorus, and total ammonia nitrogen in mesocosms treated with calcium oxide. Thus, liming to neutralize acidity in ponds with acidic soil and low alkalinity water may have a much greater effect on water quality than can be expected from bacterial augmentation. In ponds with higher pH and alkalinity, neither treatment is likely to enhance water quality.

ENZYMES

Reversible chemical reactions attain a state of equilibrium when the velocities of forward and reverse reactions become equal. The equilibrium state is influenced by temperature, pressure, concentrations of reactants, and catalysts. A catalyst can speed up both forward and reverse reactions to allow equilibrium to be reached quickly. For molecules to react, they must pass through a configuration known as the activated state in which they have the activation energy necessary to react. Catalysts reduce the activation energy to facilitate more rapid reaction of molecules. Catalysts are not used up in reactions, and they can be used over and over. In biochemical reactions, catalysts are specialized protein molecules called enzymes that are very specific in the reactions that they catalyze. They occur in living cells, and extracellular enzymes are produced and excreted by microorganisms. Enzymes are named for the reaction that they catalyze. For example, cellulase catalyzes the breakdown of cellulose into smaller molecules, and oxidases catalyze oxidations.

Bacteria excrete extracellular enzymes that degrade large molecules into smaller particles that can be absorbed for further degradation by enzyme-catalyzed reactions within their cells. It should be obvious that enzyme additions cannot speed up degradation of organic matter or toxic substances unless bacteria are present. Extracellular enzymes are only the first step in the degradation process. In cases where there is a high abundance of a substance, enzyme blocking may occur because of excess substrate.

A fermentation product that is rich in enzymes and contains stabilizers, nutrients, and minerals was applied to soil in laboratory respiration chambers (Boyd & Pippopinyo 1994). There were no significant differences ($p > 0.05$) in soil respiration between controls and soils treated at 50 and 200 mg kg^{-1} with the enzyme preparation. Queiroz et al. (1998) tested an enzyme preparation in channel catfish ponds. Although there were no significant differences ($p > 0.1$), ponds treated with the enzyme preparation tended to have higher concentrations of dissolved oxygen during summer months than control ponds. No differences in water quality or soil condition were noted between enzyme-treated and control ponds. However, there was a trend towards greater organic matter decomposition in soils treated with the enzyme product, but because of the high variation, the difference was not significant ($p > 0.1$). Fish survival was higher in the treated ponds ($p > 0.1$), but net fish production did not differ between treated and control ponds. Ponds used in this study were stocked at a moderate rate (15,000 fish ha^{-1}) and maximum daily feeding rate was only 75 kg ha^{-1}. Thus, water and soil quality were not severely impaired in the ponds. The enzyme product might produce greater benefits in ponds with higher stocking and feeding rates where the water and soil quality deteriorate greatly during the production period.

PLANT EXTRACTS

There has been preliminary research on the use of natural compounds extracted from plants for improving pond water quality or for controlling blue-green algae. Some of these products contain substances that are toxic to bacteria, while others contain compounds that are toxic to plants and especially to blue-green algae.

Yucca

Extracts of the Yucca plant contain glycocomponents which bind ammonia (Wacharonke 1994). Under laboratory conditions, 1 mg l^{-1} of a commercial Yucca extract reduced total ammonia nitrogen concentrations by 0.1-0.2 mg l^{-1}. Because pH data were not provided, the removal of unionized ammonia nitrogen cannot be computed. Pond treatments were made at 15-d intervals with 0.3 mg l^{-1} of the Yucca preparation per application. It was reported that ammonia concentrations were lower and shrimp survival better in ponds treated with Yucca extract than in control ponds (Wacharonke 1994). In a trial conducted at Auburn University, the commercial Yucca extract was applied at 0.3 mg l^{-1} at 2-week intervals to channel catfish ponds. Concentrations of total ammonia nitrogen in treated ponds averaged 0.1 mg l^{-1} lower than those of control ponds ($p > 0.1$). Further research on the use of Yucca extracts to reduce ammonia concentrations are needed to verify the benefits of this treatment.

Citrus seed extracts

There has been considerable use of citrus extracts for treating shrimp ponds in Ecuador to enhance soil and water quality. The most popular one, KILOL, is an extract of grapefruit seed. It is either applied directly to ponds or mixed with lime and applied to ponds. It also can be mixed into shrimp feed. KILOL has been approved by the United States Food and Drug Administration for use on foods and it does not cause environmental harm. One of the authors (CEB) designed and supervised an on-farm trial in Ecuador of KILOL and KILOMAR (KILOL powder mixed with agricultural limestone).

Five ponds were treated with KILOL and KILOMAR and KILOL was incorporated in feed at rates suggested by the manufacturer, and four ponds served as untreated control ponds. At the time of treatment, bottom soil conditions and water quality were similar among ponds. Ponds were stocked with an average of 15 postlarval *Penaeus vannamei*/m^2. Slightly more feed and fertilizer were applied to control ponds than to treated ponds. No large differences in soil and water quality were observed between treated ponds and control ponds, but on several dates, the color of water in treated ponds was yellowish brown, indicating large populations of diatoms, while waters of control ponds often were green in color. At harvest, shrimp in treated ponds had an average survival of 44.92%, a live weight production of 732 kg ha^{-1}, and a feed conversion ration (FCR) of 1.26 (Table 3). The control ponds had a survival of 35.86%, a production of 596 kg ha^{-1}, and a FCR of 1.83. Thus, treated ponds performed better than control ponds. Shrimp size at harvest was not different between control ponds and treated ponds. The greater production of shrimp in treated ponds was a result of higher shrimp survival in treated ponds than in control ponds. The farm manager reported fewer disease problems in treated ponds than in control ponds. However, it is not clear how KILOL and KILOMAR acted to reduce the incidence of disease and enhance shrimp survival.

Table 3. Summary from a shrimp farm in Ecuador of shrimp production data for control ponds (untreated) and ponds treated with KILOL plus KILOMAR.

Variable	Control	KILOL and KILOMAR
Stocking density (Postlarvae/m^2)	15.0	14.7
Feed applied (kg ha^{-1})	1,060	884
Average culture period (days)	141	143
Survival (%)	35.86	44.92
Average size of shrimp at harvest (g)	11.08	11.19
Production (kg ha^{-1})	596	732
Feed conversion ratio (kg feed/kg shrimp)	1.83	1.26

Potassium ricinoleate

Potassium ricinoleate, a natural compound derived from the saponification of castor oil and potassium hydroxide, was reported to be selectively toxic toward blue-green algae (van Aller & Pessoney 1982). Ricinoleate is structurally similar to other allelopathic compounds isolated from the aquatic angiosperm *Eleocharis microcarpa*. Additional field testing revealed that the percentage of blue-green algae in phytoplankton communities in ponds receiving potassium ricinoleate was not reduced (Tucker & Lloyd 1987, Scott et al. 1989).

Tannic acid

Tannic acid has also been reported to inhibit the growth of certain species of blue-green algae (Chung et al. 1995). However, Schrader et al. (1998) found that tannic acid was neither extremely toxic nor selectively toxic towards *Oscillatoria chalybea*, but they identified several other natural compounds that were selectively toxic towards *O. chalybea* in the laboratory. Also, decomposing barley straw has been identified as a method for controlling blue-green algae growth in reservoirs (Everall & Lees 1996), and Newman and Barrett (1993) found decomposing barley straw to be inhibitory towards the blue-green algae *Microcystis aeruginosa*. Phenolic compounds released during the decomposition of barley straw may undergo oxidation to form quinones that are toxic towards blue-green algae (Everall & Lees 1997).

DISCUSSION

The material reviewed above reveals that relatively little research has been done on probiotics in pond aquaculture. This is unfortunate because many companies are marketing these products, and they advertise that probiotics can enhance water and soil quality, improve production, and increase profits. The usual approach is to use low concentrations of probiotics for prevention and higher concentrations when a specific problem has been identified.

Results have shown very few positive benefits of probiotics to water or bottom soil quality. It may be that probiotics only produce improvements when specific problems occur, and possibly, these conditions never occurred in the ponds and laboratory trials reported above. Also, the advertising literature often is based on laboratory studies where high concentrations of the probiotics were used. In practice, it is too expensive to use such high concentrations, so the vendor recommends much lower doses for ponds. This is a common problem with the adoption of amendments in pond management. If there is an effect that is known to be produced by a certain agent, and if this effect would be good in ponds, the agent may be adopted for use with no idea of the appropriate concentration or of the conditions under which the effect is produced. There have been a few cases of improved survival and production of shrimp and fish through application of probiotics. However, the mechanism of action of probiotics are not known and the conditions under which improvements may be expected cannot be identified.

Obviously, considerable research should be conducted on probiotics in pond aquaculture by independent parties not interested in sales of the products. This research needs to elucidate the effects of probiotics on aquatic ecosystems, identify conditions under which probiotics can be beneficial, and develop appropriate doses and methods of application. In addition, the economic benefits that can accrue from probiotic use need to be determined. Until these findings are available, one should be cautious about probiotics. Although no damage to the fish or shrimp crop or to the environment should result from probiotics, one might spend considerable money on these products and receive little in return. Nevertheless, if using probiotics in your pond will make you feel better and you are willing to risk the investment, there is no reason not to use them.

LITERATURE CITED

Boyd CE (1990) Water quality in ponds for aquaculture. Alabama Agricultural Experiment Station, Auburn University, Alabama

Boyd CE, Pippopinyo S (1994) Factors affecting respiration in dry pond bottom soils. Aquaculture 120:283-294

Boyd CE, Hollerman WD, Plumb JA, Saeed M (1984) Effect of treatment with a commercial bacterial suspension on water quality in channel catfish ponds. Prog Fish-Cult 46:36-40

Chiayvareesajja S, Boyd CE (1993) Effects of zeolite, formalin, bacterial augmentation, and aeration on total ammonia nitrogen concentrations. Aquaculture 116:33-45

Chung K-T, Zhao G, Stevens, Jr. SE (1995) Inhibitory effects of tannic acid and its derivatives on growth of the cyanobacteria Nostoc sp. strain MAC and *Agmenellum quadruplicatum* PR-6. J Aquat Animal Health 7:341-344

Eaton AD, Clesceri LS, and Greenburg AE (1995) Standard methods for the examination of water and wastewater, 19th edition. American Public Health Association, Washington, DC

Everall NC, Lees DR (1996) The use of barley-straw to control general and blue-green algal growth in a Derbyshire reservoir. Water Res 30:269-276

Everall NC, Lees DR (1997) The identification and significance of chemicals released from decomposing barley straw during reservoir algal control. Water Res 31:614-620

Newman JR, Barrett PRF (1993) Control of *Microcystis aeruginosa* by decomposing barley straw. J Aquat Plant Man 31:203-206

Queiroz JF, Boyd CE (1998) Effects of a bacterial inoculum in channel catfish ponds. J World Aquacult Soc 29:67-73

Queiroz JF, Boyd CE, Gross, A (1998) Evaluation of a bio-organic catalyst in channel catfish, *Ictalurus punctatus*, ponds. J. Appl Aquacult: in press

Schrader KK, DeRegt MO, Tidwell PD, Tucker CS, Duke SO (1998) Selective growth inhibition of the musty-odor producing cyanobacterium *Oscillatoria chalybe*a by natural compounds. Bull Environ Contam Toxicol 60:651-658

Scott JH, Bayne DR, Boyd CE (1989) Effects of potassium ricinoleate on water quality, phytoplankton, and off-flavor in channel catfish ponds. J. Aquat Plant Man 27:26-31

Tucker CS, Lloyd SW (1985) Evaluation of a commercial bacterial amendment for improving water quality in channel catfish ponds. Mississippi Agriculture and Forestry Experiment Station, Mississippi State University, Res Rpt 10:1-4

Tucker CS, Lloyd SW (1987) Evaluation of potassium ricinoleate as a selective blue-green algicide in channel catfish ponds. Aquaculture 65:141-148

van Aller RT, Pessoney GF (1982) U.S.M. algal research team makes major off-flavor/water quality discovery. Aquaculture Magazine 8:18-22

Wacharonke C (1994) Shrimp farming: a breakthrough in controlling nitrogen metabolism and minimizing water pollution. In: Lyons TP, Jacques KS (eds) Biotechnology in the feed industry. Nottingham University Press, Loughborough, United Kingdom, p 247-252

Application of Phytoplankters as Live Diet and Biocontrol in Improving the Hatchery Production of Commercially Important Penaeid Shrimp in Asia

[1]Jesse Dapon Ronquillo, [2]Toshio Saisho, [2]Shigehisa Yamasaki

[1]College of Fisheries, University of the Philippines in the Visayas, Miagao, Iloilo, Philippines 5023
[2]Faculty of Fisheries, Kagoshima University, Kagoshima City, Japan 890

ABSTRACT: Four genera and eight species of penaeid shrimp (*Penaeus monodon, P. japonicus, P. semisulcatus, P. latisulcatus, P. chinensis, Metapenaeus ensis, Trachypenaeus curvirostris,* and *Metapenaeopsis barbata*) found in the Indo-West Pacific region were spawned. The general objectives of the study were: (1) to develop innovative, more efficient and less expensive methods of producing healthy "seedlings" of penaeid shrimp; and (2) to develop rearing protocols for the larval production of other species of penaeid shrimp which are not traditionally cultured. Live shrimp were bought from fishermen and spawned in the laboratory. The hatched nauplii were transferred to larval rearing tanks. The first protozoeae (PZ-1) were provided with phytoplankters (*Chaetoceros calcitrans, C. gracilis, Tetraselmis tetrathele*). The myses were likewise fed with phytoplankters and/or zooplankters (*Brachionus plicatilis* and *Artemia salina*) until the first postlarval stage (PL-1). To analyze and correlate the development of the feeding structures in every stage of larval development, the appendages and feeding structures (maxillae, maxillules, maxillipeds, and mandibles) of the larvae were dissected by fine needles, drawn using a camera lucida, and correlated with survival rate and developmental stage. Nutritional values and particle sizes of the different phytoplankters and zooplankters were evaluated. Mortality due to the presence of harmful bacteria was also noted. Based on the inferred morphological function of the feeding structures, observation of larval feeding habits, faster rates of metamorphoses, and high survival rates on mixed phytoplankter diets until the first postlarval strge (PL-1), the early larval stages of penaeid shrimp are filter feeders. However, when the postlarvae of *Penaeus monodon, P. joponicus, P. chinensis,* and *P. semisulcatus* metamorphosed into the first postlarval stage, morphological changes were observed: the plumose setae on maxillae and maxillipeds were lost; endopods and exopods became vestigial; and inner margins of the first to third pereiopod chelae became serrated and functional. These morphological changes corresponded with a change in feeding habits from filter feeding to raptorial omnivore feeding as the planktonic larvae adapted to benthic life. The postlarvae of *Penaeus latisulcatus* and *Metapenaeus ensis* remained as filter feeders until about the fifth postlarval stage (PL-5), but those of *Trachypenaeus curvirostris* and *Metapenaeopsis barbato* were able to subsist on phytoplankters even at PL-15. These results question the practice of feeding the protozoeae and mysis substages of penaeid shrimp with expensive *Artemia* nauplii when the early larval stages are still filter feeders and benefit much from cheaper but nutrilionally efficient nalural diets of phytoplankton. Diatoms and prasinophytes exhibit antagonistic effects on or inhibit the growth of virulent bacteria and are rich in nutrients, which are limiting in artificial diets and zooplankters (*Artemia* nauplii and *Brachionus plicatilis*). Thus, they are ideal live feeds for penaeid shrimp larvae. The presently developed hatchery technique was also applicable to other species of penaeid shrimp which are not traditionally cultured. Adoption of this new technique in shrimp hatchery management could boost the production of healthy "seedlings" and greatly reduce operating costs.

KEYWORDS : Prawn/shrimp hatchery; penaeid shrimps; larval rearing; biocontrol; live diet

Ronquillo JS, Saisho T, Yamasaki S (1998) Application of phytoplankters as live diet and biocontrol in improving the hatchery production of commercially important penaeid shrimp in Asia. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Manipulation of Bacterial Populations in Shrimp Larval Cultures Fed Artificial Diets

N. Misciattelli, D.A. Jones, N. Simoes, J.W. Latchford, P. Bridson

School of Ocean Sciences,University Wales Bangor, Menai Bridge
Anglesey, N.Wales, UK.LL59 5EY, UK

ABSTRACT: While microencapsulated diets (MED) have been used to totally replace microalgae in penaeid hatcheries where 5 µm filtered ocean quality sea water is used, in coastal water subject to bacterial blooms total algal replacement results in variable larval survival levels. *Penaeus indicus* larval rearing trials conducted over 2 years in the UK using 5 µm filtered sea water subject to seasonal bacterial blooms confirms the link between high bacterial populations and poor survival on MED. These trials also demonstrate that if a single dose of live algae (SDLA) is given at the first protozoea (PZ1) stage, survival levels on MED are not significantly different from controls fed live diet. Experiments reveal that the factor responsible in SDLA for improving survival in MED fed cultures is likely to be an algal metabolite or exudate which modifies the bacteria in the culture water. The action of this algal metabolite does not appear to be bacteriacidal, but reduces or prevents swarming behaviour in *V. harveyi* and other species. Further experiments demonstrate that some micro and macro algae may produce N-acyl-homoserine lactone (AHL) mimics which prevent swarming and virulence in *V. harveyi*. It is suggested that these compounds may be used to control bacterial populations in MED fed penaeid larval cultures.

KEY WORDS: shrimp larval culture, *Vibrio harveyi*, AHL mimics, artificial diets, algae

INTRODUCTION

The use of artificial feeds, either microencapsulated (MED) or microparticulate (MPD), as partial replacement for conventional microalgae is now universal through out the penaeid hatchery industry (Wilkenfeld 1992). However complete replacement of microalgae by artificial feeds has only been achieved in hatcheries using 5 µm filtered ocean quality seawater retaining the natural bacterial community (Avalhe and Rothius 1990, Ottogali 1991, 1992). Alabi *et al*. (1997) also showed that successful total microalgal replacement is dependent upon the establishment of a balanced bacterial community originating either from 5 µm filtered seawater or as in fish culture, the conditioning effect of microalgae (Skjermo *et al.* 1997). As a result Jones *et al.* (1997) suggested the inoculation of a single dose of live algae (SDLA) before use of artificial feeds to condition hatchery water when it is taken from coastal water of variable bacterial quality.

The first part of this paper describes the variable survival of *Penaeus indicus* Edwards larvae fed MED in a series of trials at the School of Ocean Sciences (SOS) throughout 1996 using 5 µm filtered coastal sea water subject to both algal and bacterial blooms (Blight *et al.* 1995). In 1997 a similar series of trials demonstrated that the addition of SDLA to MED fed larvae is sufficient to remove this variability in survival. By contrast, presterilisation of culture water proved ineffective in reducing the variability (Alabi *et al*. 1997). To identify the factors in SDLA responsible for improving larval survival, a further series of experiments were run in the summer of 1997. Results showed that survival was related to the presence of a metabolite or exudate from the live microalgae.

Recent outbreaks of viral disease affecting *P.monodon* and *P. indicus* in India and *P. monodon* in Indonesia have been associated with vibriosis. These outbreaks were initiated by sudden changes in coastal water due to cyclonic or monsoon rains (Prayatno & Latchford 1995, Krishna *et al*. 1997). Species such as *Vibrio harveyi* are universally present in coastal water and appearance of pathogenic or luminous forms is often associated with salinity, temperature or nutrient changes, some of which can be induced experimentally (Prayatno & Latchford 1995).

Robson *et al*. 1997 reported that this bacterium utilises an N-acyl-homoserine lactone (AHL) signalling system to activate the transformation to swarming phase which produces luminescence, exoprotease and /or virulence. These authors suggested that, as only a small number of genes are involved, it may be possible to block the AHL pathway by using AHL mimics which will bind to the bacterial AHL receptors, prevent inter-bacterial signaling and prevent the expression of virulence.

One aim of the work reported here was to substantiate existing evidence for antibacterial activity of algae (Hornsey & Hide 1974, Reichelt & Borowitzka 1984, Kellam & Walker 1989). A second aim was to further investigate the potential of algae for bacterial control in larval cultures fed MED.

Misciattelli N, Jones DA, Simoes N, Latchford JW, Bridson P (1998) Manipulation of bacterial populations in shrimp larval cultures fed artificial diets. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

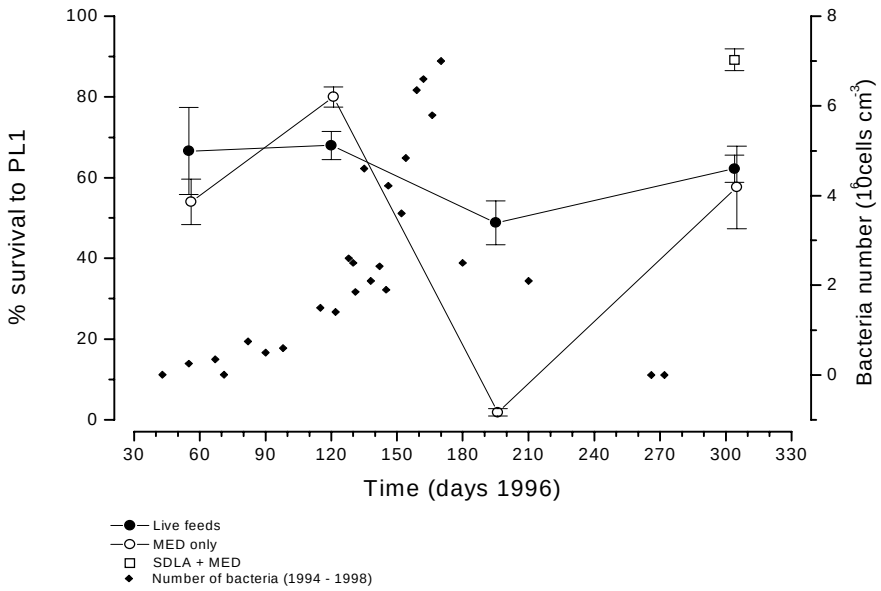


Figure 1. Percentage survival of *Penaeus indicus* larvae up to PL in trials fed on MED and live feeds in 5µm filtered seawater from the Menai Strait in 1996. Live feeds consisted in 25 cells/ml of *Tetraselmis chuii* and 35 cells/ml of *Skeletonema costatum* to PZ3, followed by *Artemia* nauplii at 2-5/ml. MED was fed at 4mg/l day to PZ3 followed by *Artemia* at 2-5/ml. MED + SDLA, was fed as before, but with single dose of mixed live algae (10-15 cells/ml) given at PZ1 stage. Bacterial counts taken from Menai Strait close to intake water pipe (Blight *et al*. 1995, Dr. Rubina pers. com., 1996 and authors own data 1997/98).

MATERIALS AND METHODS

Larval cultures

P.indicus larvae were derived from broodstock originating from Saudi Arabia held in a recirculation system with 2 round 4 metric ton black tanks. The larvae were stocked at protozoea 1 (PZ1) at a density of 100 l^{-1} in 2 l round bottom glass flasks to assess survival and growth performance on different live and artificial diets (From 3-8 replicates per treatment). Seawater from the Menai Strait (SOS) was filtered through 5 µm standard cartridge filters and temperature maintained at 28± 1 °C. Salinity varied between 30-35 ppt. A mixture of *Skeletonema costatum* and *Tetraselmis chuii* was used as the live diet control (50 cells ml^{-1}) and the same mixture at 10-15 cells ml^{-1}as a single dose of algae (SDLA) at PZ1. An experimental MED formulation (INVE Technologies Belgium) was fed in all trials at 4 mg l^{-1}, up to mysis 3 (M3) stage. Newly hatched *Artemia* (EG INVE aquaculture) were also given to all trials at 2-5 *Artemia* ml^{-1} from PZ3 onwards. Flasks were thoroughly washed and sterilised after each trial. Light followed a controlled 14 hr day and 10 hr night cycle. Regular checks on pH and concentration of ammonia and nitrite were also performed following standard methods (Parsons *et al.* 1984, Boyd & Tucker 1992), and levels remained within acceptable limits for penaeid larval culture (Chin & Chen 1987, Chen & Chin 1988).

Every other day the flasks were emptied and the larvae counted and staged according to the method of Silas *et al.* (1978). All artificial diets were hydrated daily and introduced into the larval culture at 08:00,12:00,20:00,24:00 hr. Algae were grown in semi continuous culture and algal cell density was estimated using a haemocytometer.

Algal extracts

The red alga *Polysiphonia lanosa* (Linnaeus) was collected from Rosneigr (Anglesey. N. Wales) at low tide. *Tetraselmis chuii* was maintained in artificial culture and was harvested by centrifuging at 5000 g for 15 min. All algae were freeze dried. The algae were extracted using dichloromethane. The dichloromethane was rotary evaporated and the extract resuspended in sterile seawater (autoclaved).

Effect of algal extracts on bacterial growth

Bacteriacidal tests were conducted using specific media (as advised by the National Collection of Industrial Marine Bacteria or NCIMB, for 7 bacterial species. These were Bacterium I, Bacterium II, *Serratia liquefaciens*, *Pseudomonas fluorescens*, *Vibrio harveyi* and *Vibrio ordalii*. *Tetraselmis chuii* and *Polysiphonia lanosa* extracts were tested. The latter species was chosen, as Hornsey and Hide (1974) reported that it had a strong anti-microbial activity. Growth inhibition was estimated using 5 mm diameter wells in agar as described by Kellam & Walker (1989) and using 5 mm and 25 mm GFC milipore filterpaper discs following Duff *et al.* (1966).

Swarming motility inhibition

Swarming was examined using 2 bacteria isolated from Menai Strait sediment and water column. These bacteria exhibited pronounced swarming ability. Swarming was examined using sea water complex (SWC) agar plates supplemented with various concentrations of algal extract. The plates were dried in a laminar flow cabinet for 1h to ensure complete dryness of the surface of the plate. The bacteria were grown for 24 hr in a shaking incubator (150 rpm) in SWC liquid medium at 28°C. Each plate was then stab inoculated with 5 ml of bacterial culture into the middle of the plate. The swarming distance was measured using vernier callipers at set intervals.

Protease activity

Bacterial protease activity was assessed by use of skimmed milk agar plates. Each plate was stab inoculated with 5 ml of bacteria into the middle of the plate and the diameter of the 'halo' measured.

Bacterial counts

Bacterial counts in the Menai Strait samples were done using the DAPI fluorescent stain method (Porter & Feig, 1980). The stain concentration was kept standard at 50 mg-

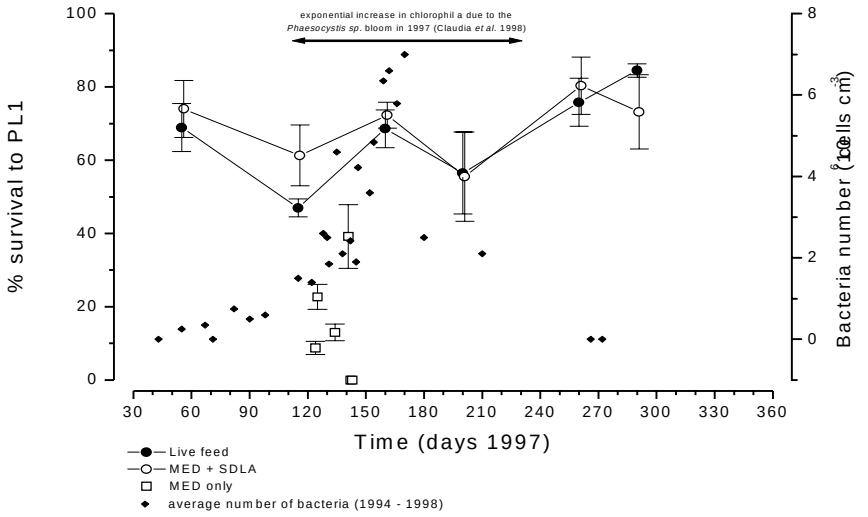


Figure 2. Percentage survival of *Penaeus indicus* larvae to PL1 in trials fed on MED and live feeds in 5µm filtered seawater from the Menai Strait in 1997. Live feeds as Figure 1. MED as Figure 1 plus single dose of mixed algae (10-15 cells/?l) given at the PZ1 stage. Bacteria as Figure 1. *Phaeocystis* bloomed from day 110-210 in 1997.

ml^{-1}. In the algal bactericidal experiment bacteria counts were done using total culturable counts (TCC) on SWC.

RESULTS AND DISCUSSION

Larval survival with and without SDLA

Fig. 1 plots the percentage survival to post larva 1 (PL_1) in a series of trials with *P. indicus* using larvae fed on standard live feeds (microalgae to PZ3 followed by *Artemia* to PL_1). These trials ran throughout 1996, using Menai Strait sea water filtered to 5 µm. There were strong seasonal changes in the primary production in this water with an annual early summer *Phaeocyctis* bloom followed by a peak in bacterial numbers. Winter sea bacterial numbers averaged 1 x 10^3 ml $^{-1}$ but rose sharply following the collapse of the algal bloom. They ranged from 5 to 7 x 10^6 ml $^{-1}$ over the period 1994 – 1998. Data plotted (Fig. 1) is from Blight *et al* (1995) together with spot values for later years.

In 1996 larval survival on live feeds was high in winter months when coastal water bacterial numbers were low, but declined as bacterial numbers rose following the *Phaeocyctis* bloom. Overall fluctuations in survival ranged from 49 - 66.6% for larvae fed algae. By contrast, survival ranged from 0 – 80% when MED was substituted for microalgae, and culture collapse coincided with the period when bacterial levels in the coastal water were elevated (Fig. 1). At times when coastal bacterial levels were reduced, survival of larvae on MED was similar to that of larvae fed microalgae, but when SDLA was given in addition to MED at the first feeding PZ1 stage in the experiment (Fig. 1), survival improved to 89% as opposed to 62% on live feeds and 57% on MED.

Fig. 2 shows the results of a similar series of larval trials run throughout 1997. Again, these indicated higher survival on live feeds during winter months. However, when MED fed larvae were given SDLA in 5 µm filtered sea water in trials run at the same time as the live feed controls, there was no significant difference in survival (W= 314.0, P= 0.56) between MED and live feed controls. Although lowest survival on MED (56%) coincided with the period of high bacterial numbers in the Menai Strait, the larval cultures did not collapse. This contrasted with the controls fed on MED alone which gave only 0 - 40% survival (Fig. 2).

In an attempt to isolate the factor in SDLA responsible for promoting survival, even in the presence of high bacterial numbers, a further series of experiments were conducted during the summer period (Table 1). In Experiments 1 to 3, SDLA consisting of 8-10 cells ml $^{-1}$ *Skeletonema* and 8-10 cells ml $^{-1}$of *Tetraselmis* were harvested as normal. However, the algal cells were removed by filtration and only the algal culture water (AW) was added to MED. Due to shortage of larvae, it was not always possible to run a full set of controls. However, the results still clearly showed that addition of algal water to MED gave significantly higher survival than seen with MED alone (Table 1, Experiments 2 and 3). In Experiment 1 (Table 1) addition of algal water gave similar survivals to those for MED plus SDLA and for live feed controls, indicating that the algae in the SDLA did not contribute to larval nutrition. Similarly, the high survival rate on MED with algal water filtered to 0.2mm excluded any contribution by bacteria from the algal culture.

In Experiments 2 to 5 (Table 1), survival rates for MED without SDLA were variable, ranging from 0 - 57%, as previously seen when using 5 µm filtered sea water during this period of the year (Fig. 1). Sterilisation of this sea water before use (Table 1 Experiment 4) caused a total collapse of the larval culture. In contrast, Experiments 5 to 7 showed the consistently higher survival for larval cultures fed on MED with SDLA. Results (Table 1) indicate that enhanced larval survival, even in the presence of high bacterial numbers, was probably due to a microalgal exudate or metabolite. Thus, a further series of experiments was designed to test the effects of algal extracts on bacteria.

These results confirmed earlier suggestions (Jones *et al*. 1997) that the addition of a single dose of live microalgae (10–15 cell ml^{-1}) to MED fed penaeid larval cultures prevents culture collapse. Kumlu and Jones (1995) demonstrated that the nutrition supplied by 10–15 algal cells ml^{-1} is insufficient to support observed postlarval yields. This was confirmed in the present work, where survival rates on MED

Table 1. Percentage survival of *P. indicus* at PL_1 fed on different regimens and with different water treatments. The feeds were a live feed control (mixed microalgae to PZ3, *Artemia* nauplii to PL1), total algal replacement by MED (4mg $l^{-1}day^{-1}$ to PZ3, *Artemia* nauplii to PL1) or MED in combination with a single dose of live algae at first feed (SDLA). The water treatments were 5µm filtered water or autoclaved water and no change of water throughout the culture period. There were 7 Trials, each done with 3-5 replicates. AW = water from algal culture. The 0.2µm filtered AW was filtered to exclude bacteria.

Feed	Sea water	*P. indicus* % survival to PL_1 in experiments 1-7							Mean %
	treatment	1	2	3	4	5	6	7	survival
live feed control	5µm filtered	63±5.9	68±2.6	88±10.1	61±6.5	62±6.7	84±6.2	76±6.5	72
MED + SDLA	5µm filtered	89±4.9				89±5.4	73±3.5	80±7.8	83
MED + AW	5µm filtered	89±12.2	49±2.2	76±4.9					71
MED + AW filtered 0.2µm	autoclaved	83±3.2							83
MED	5µm filtered		0	3±1.3	43±10.7	57±6.6			34
MED	autoclaved				0				0

supplemented by algal water alone were similar to those for live feed larval controls (Table 1). Similarly, a probiotic effect caused by transferring the bacterial community associated with the algal growth culture can be excluded since 0.2 mm filtered algal culture water was equally successful in promoting larval survival when added to cultures of MED fed larvae (Table 1). As the positive effect of SDLA did not appear to be nutritional or due to probiotic bacteria, the presence of a chemical in algal water was further explored.

Effect of algal extracts on bacteria

In initial tests, compounds from the microalga *Tetraselmis chuii* and the macroalga *Polysiphonia lanosa* were extracted with dichloromethane and tested for antibacterial activity against a series of bacterial species (See materials and methods). Based on the lack of inhibition zones using either agar well or filter-paper disc methods, neither algal extract inhibited growth of any of the bacterial species tested. Bacteria I and II were isolated from Menai Strait sea water/ sediment and the other tested species were obtained from NCIMB. The absence of an inhibitory effect on bacterial growth was confirmed by inoculating Menai Strait sea water with an extract of *Polysiphonia*. The extract actually enhanced bacterial growth.

As the extracts did not appear to be bacteriacidal, they where subsequently tested for effect on the swarming behaviour of Bacteria I and II. Figs. 3a and 3b show the effect of *Tetraselmis chuii* extract at concentrations of 0.017 g (1 ml) and 0.033 g (2 ml) dry wt algae $plate^{-1}$ on the swarming of Bacteria I and II. The average swarming distance of untreated Bacterium I at 36 hr was 30 mm while it was reduced to 22 and 17 mm, respectively, by 1 ml and 2 ml of extracts. For untreated Bacterium II at 36 hr it was 35 mm while it was reduced to 28 and 24 mm, respectively, by 1 ml and 2 ml of extract. Over 50 % of the data was shown to be normally distributed (A^2 = 0.189-0.488, p=0.387-0.057) and

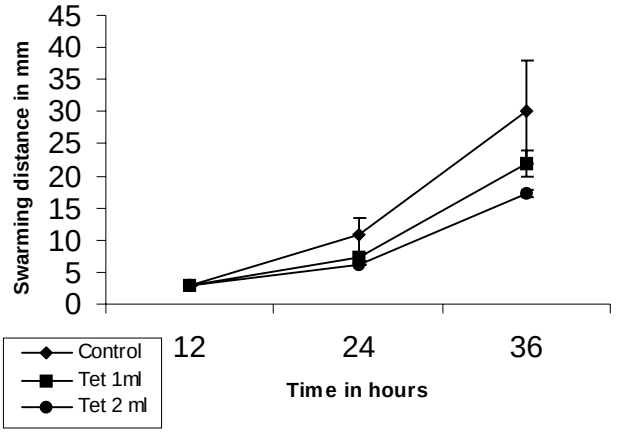


Figure 3a. Shows the effect of *Tetraselmis chuii* extract inoculated at two different concentrations (1ml; 2ml) on Bacterium I (Bact I) swarming activity. The experiment was conducted in triplicate taking triplicate measurements from each vessel. The results shown is the mean of the 9 samples and the standard deviation.

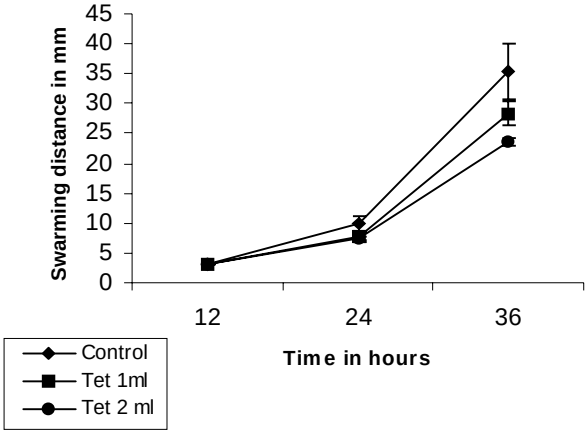


Figure 3b. Shows the effect of *Tetraselmis chuii* extract inoculated at two different concentrations (1ml; 2ml) on Bacterium II (Bact II) swarming activity. The experiment was conducted in triplicate taking triplicate measurements from each vessel. The results shown are the mean of the 9 samples and the standard deviation.

there was no significant heterogeneity of variance (Levens test =2.032, p =0.128). A two way ANOVA using a general linear model indicated that the mean swarming distances of the two bacteria (Bacteria I and II) were significantly different (F=8.25, p=0.011) and that the inclusion of *Tetraselmis* extract significantly reduced the swarming distance (F=13.06, p<0.001). The interaction term was not significant (F=0.03, p=0.974). Schaffe's comparison indicated that the mean differences in the presence of 1or 2 ml of *Tetraselmis* extract were not significantly different (mean difference = 4.67, 95 % CI of diff = - 2.55 – 11.89).

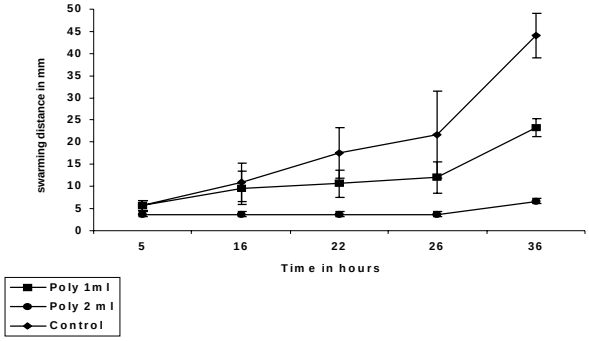


Figure 4a. The effect of *Polysiphonia lanosa* (Poly) extract inoculated at two different concentrations (1ml; 2ml) on Bacterium I (Bact I) swarming activity. The experiment was conducted in triplicate taking triplicate measurements from each vessel. The results show the mean of the 9 samples and the standard deviation.

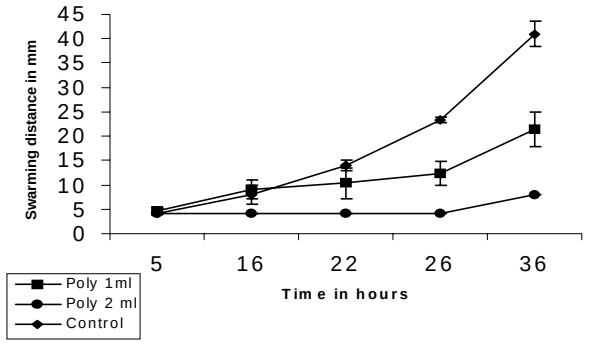


Figure 4b. Shows the effect of *Polysiphonia lanosa* (Poly) extract inoculated at two different concentrations (1ml; 2ml) on Bacteria II (Bact II) swarming activity. The experiment was conducted in triplicate taking triplicate measurements from each vessel. The results shown is the mean of the 9 samples and the standard deviation.

Figs. 4a and 4b show the effect of *Polysiphonia lanosa* extracts at concentrations of 0.017 g (1 ml) and 0.033 g (2 ml) dry wt algae plate^{-1} on the swarming of Bacteria I and II. The average swarming distance of untreated Bacterium I was 44 mm while it was 23 and 7 mm, respectively, with 1 ml and 2 ml of extract. For untreated Bacterium II at 36 hr it was 42 mm while it was 21 and 8 mm, respectively, with 1 ml and 2 ml of extract. The data was found to be normally distributed for Bacterium I (A^2 =0.189-0.488, p =0.057-0.631). Heterogeneity of variance was established (Bartlett test = 5.38, p=0.068) and an ANOVA was therefore conducted (F = 106.11, p<0.001).The ANOVA showed that there were a significant differences amongst treatments, and Tukey's pair-wise comparison indicated significant differences between *Polysiphonia* 1 ml and 2 ml (mean difference = 15.67±7.89). *Polysiphonia* 1 ml and 2 ml were also significantly different from the control (mean difference =37.34±7.89, 20.67±7.89, respectively). Bacterium II was also normally distributed (A^2 = 0.197-0.204, p= 0.596-0.565) and heterogeneity of variance was also shown (Bartlett test = 0.174, p= 0.676). An ANOVA was conducted showing significant differences amongst the treatments (F = 66.45, p< 0.001). Tukey's pair-wise comparison showed significant differences between *Polysiphonia* 1 ml and 2 ml (mean difference =15.67±8.89). *Polysiphonia* 1 ml and 2 ml were also significantly different from the control (mean difference = 37.34±8.89, 20.67±8.89, respectively)

As the initiation of swarming behaviour in bacteria is regulated by the AHL pathway (Robson *et al*. 1997), the extract from *Polysiphonia* was tested for effects on other bacterial characteristics such as exoprotease and light production known to be controlled by this pathway. *Polysiphonia* was used for these and other experiments, because it was easier to collect in larger volumes than the microalga *Tetraselmis* and because it produced similar inhibitory behaviour with the bacteria.

Fig. 5a and 5b show the effects of *Polysiphonia lanosa* extract at concentrations of 0.017 g (1 ml) and 0.033 g (2 ml) dry wt algae plate^{-1} on the production of exoprotease by both *Vibrio harveyi* and *Listonella angullarum.* The average exoprotease hydrolysis zone for untreated *Vibrio harveyi* was 4.67 mm at 48 hr while it was 3.33 and 2.0 mm, respectively, with 1 ml and 2 ml of *Polysiphonia.* The average

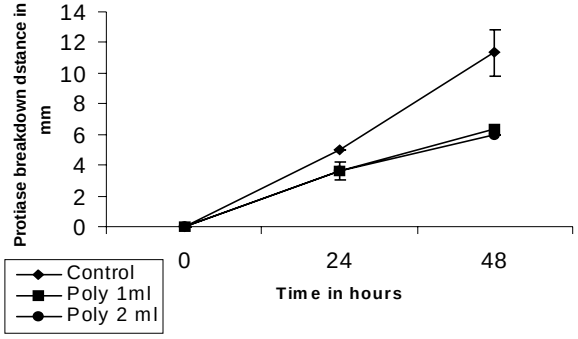


Figure 5a. Shows the effect of *Polysiphonia lanosa* (Poly) extract inoculated at two different concentrations (1ml; 2ml) on *Vibrio harveyi* protease production. The experiment was conducted in triplicate taking triplicate measurements from each vessel. The results shown are the mean of the 9 samples and the standard deviation.

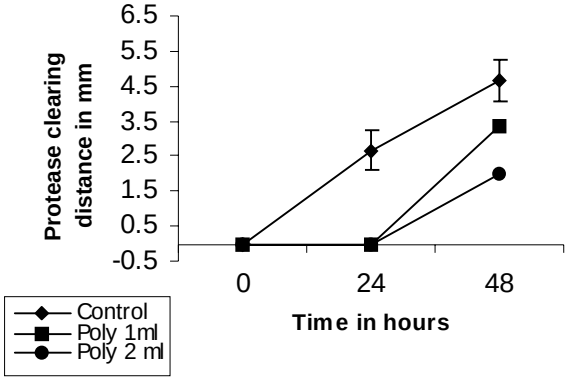


Figure 5b. Shows the effect of *Polysiphonia lanosa* (Poly) extract inoculated at 2 different concentrations (1ml;2ml) on *Listonella angullarum* protease production. The experiment was conducted in triplicate taking triplicate measurements from each vessel. The results shown are the mean of the 9 samples and the standard deviation .

exoprotease hydrolysis zone for untreated *Listonella angullarum* at 48 hr was 11.33 mm while it was 6.33 and 6.0, respectively, with 1 ml and 2 ml of extract. Normality was established for the *Listonella angullarum* data (A^2=0.230-0.488, p= 0.0057-0.487) and heterogenicity of variance was also established (Bartlett test = 1.323, p=0.250). The ANOVA showed a significant difference amongst treatments (F=28.16, p=0.006). Tukey's pair-wise comparison established that *Polysiphonia* 1 ml was not significantly different from *Polysiphonia* 2 ml (mean difference = 0.33, 95 % CI of diff = -3.02-3.69) but that *Polysiphonia* 1 ml and 2 ml were significantly different from the control (mean difference -5.0±3.35, -5.33±3.35). Normality was also established for *Vibrio harveyi* data (A^2=0.488, p= 0.0057), as was heterogeneity of variance (Bartlett test =0.500, p=0.630). The ANOVA showed a significant difference amongst treatments (F=8, p= 0.047) and Tukey's pair-wise comparison established that *Polysiphonia* 1 ml was not significantly different from *Polysiphonia* 2 ml (mean difference = 1.33?1.67,). However, both *Polysiphonia* 1 ml and 2 ml were significantly different from the control (mean difference =1.33±1.67, 2.66±1.67). Furthermore the algal extracts also significantly interfered with bioluminescence in *V. harveyi* (data not shown).

Since bacterial swarming is often associated with bacterial virulence, the *Polysiphonia* extract was tested in a culture experiment in which *P. indicus* larvae challenged with BP04, a *V. harveyi* strain isolated from Indonesian hatcheries (Prayatno & Latchford 1995). In this experiment *P. indicus* larvae were cultured in 0.2 mm filtered seawater and fed MED at 4 mg $l^{-1}D^{-1}$. Control flasks (8) were challenged with BPO4 at 10^4 bacteria ml^{-1} and experimental flasks were challenged with the same concentration of BPO4 together with *Polysiphonia* extract at a concentration of 0.01 g dry wt algae l^{-1}. Fig. 6 shows that while average survival after 24 hr in control flasks was less than 2 %, survival in experimental flasks was 83%. Normality of the data was established (A^2=0.535, p=0.121, A^2=0.629, p=0.069), as was heterogeneity (Bartlett test = 7.533, p=0.006). A two sample T test indicated that the survival of larvae in the presence of the extract was significantly higher than that of the control (t=23.05, p<0.001, mean diff= 80.33±7.93).

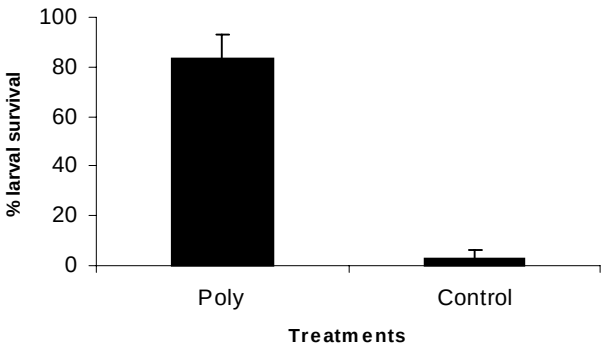


Figure 6. *Penaeus indicus* larval experiment. Stocked at Nauplius 6 inoculated with BPO4 *(Vibrio harveyi)* at a concentration of 10^4.bacteria per ml plus 1 ml of bacterial nutrient media. Larvae fed immediately after metamorphosis (zoea 1).

The high survival of larvae in the BP04 challenge treated with algal extract indicated that bacterial swarming was reduced together with virulence. Thus, we suggest that control of swarming by the active factor in SDLA may contribute to the success of larval cultures fed MED during periods of bacterial blooms.

CONCLUSIONS

Season fluctuations in bacterial numbers occur on a regular annual basis following collapse of phytoplankton blooms (Blight *et al*. 1995). These fluctuations are typical of coastal temperate regions, but are also similar to those which occur in tropical regions as a result of monsoons, cyclones or storms (Krishna *et al.* 1997). In all situations, these fluctuations may stimulate bacterial blooms which often contain virulent, swarming *Vibrio* strains, together with high numbers of viral particles (Berg *et al* . 1989). Presterilisation of larval culture water is not always effective in these circumstances, as opportunistic pathogens rapidly recolonise cultures (Baticados & Pitogo 1990, Alabi et al 1997). While addition of probiotic strains of bacteria may alleviate the situation (Garriques & Arevalo 1995), both probiotic and pathogenic bacterial populations constantly change and thus probionts may provide only a temporary solution.

Although the algal compound responsible for reduction in swarming has not yet been identified, it is likely that it will be an AHL mimic exhibiting alloinhibition of the natural AHL regulated pathway (Robson *et al*. 1997). It has been shown that the marine alga *Delisea pulchra* inhibits fouling by the production of furanones which interfere with AHL mediated expression of bioluminescence, swarming motility and exoenzyme synthesis in different bacterial species (Kjelleberg *et al*. 1997). However, the present work is the first to demonstrate both that the production of AHL analogues or mimics may be widespread amongst the algae and that virulence in *V. harveyi* may be controlled using this mechanism.

The potential biotechnological application of this approach to the control of virulent bacteria in shrimp and other aquaculture systems is far reaching. Unlike antibiotics and bactericides which kill bacteria leading to the selection for resistance in any survivors, the suppression of a behavioural trait such as swarming and virulence may not induce resistance selection as bacterial numbers are not affected. In addition, no new compounds would be introduced into marine ecosystems since the present AHL mimics are derived from marine algae.

Before bacterial swarming behaviour can be initiated, the AHL concentration in the medium must rise sufficiently for communication to occur between bacteria (Robson *et al* . 1997). An alternative approach to the control of virulence would be to ensure dilution of either the medium or bacterial numbers so that this critical AHL concentration is never attained. This may explain why presterilisation of culture water is seldom successful, since rapid bacterial repopulation usually follows (Alabi *et al*. 1997). The potential for recirculation of hatchery water with a continual removal of bacteria and nutrients is currently under study.

Acknowledgements. This work was funded by INVE Technologies NV. One of us (N.M.) is grateful for a grand from the Danis Research Academy.

LITERATURE CITED

Alabi AO, Yudiati E, Jones DA, Latchford JW (1997) Bacterial levels in penaeid larval cultures. In: Flegel TW, MacRae IH (eds) Diseases in Asian aquaculture, fish health section. Asian Fisheries Society, Manila

Avalhe O, Rothius AJ (1990) F.A.O. launches shrimp project in Madagascar Fish Farm Inter 17: 6-28

Baticados MCL, Pitogo CL (1990) Chlorination of seawater used for shrimp culture. Israeli Journal of Aquaculture-Bamidgen 42 (4):128-130

Berg O, Borsheink KY, Bratbak G, Heldal M (1989) High abundance of viruses found in aquatic environments. Nature 340 (6233): 467-468

Blight PS, Bantley TL Lefévre D, Robinson C, Rodrigues R, Rowlands J, Williams PB (1995) Phasing of autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem. Mar Ecol Prog Ser 128: 61-75

Boyd CE, Tucker CS (1992) Water quality and pond soil analyses for aquaculture. Auburn University, Alabama. 183 pp

Chen JC, Chin TS (1988) Acute toxicity of nitrite to tiger prawn, *Penaeus monodon*, larvae. Aquaculture 69: 253-262

Chin TS, Chen JC (1987) Acute toxicity of ammonia to larvae of tiger prawn *Penaeus monodon*. Aquaculture 66: 247-253

Duff DCB, Bruce DL, Antia NJ (1966) The antibacterial activity of marine planktonic algae. Can J Microbiol 12: 877-884

Garriques D, Arevalo G (1995) An evaluation of the production and use of a live bacteria isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador. In Browdy CL, Hopkins JS (eds) Swimming through troubled water. Proceedings of the special session on shrimp farming.Aquaculture'95. World Aquaculture Society, Baton Rouge, Louisiana, pp 53-59

Hornsey IS, Hide D (1974) The production of antimicrobial compounds by British marine algae. I. Antibiotic- producing marine algae. B Phycol J 9: 353-361

Jones DA, Freeman M, Latchford J, Misciattelli N, Ribeiro FALT, Rowlands SC (1997) Progress in the use of artificial feeds in penaeid larval culture.2nd Asia-Pacific Marine Biotecnology Conf. And 3rd Asia-Pacific Conf. On Algal Biotchnology. Phuket Thailand.p 107 Abstract

Kellam SJ, Walker JM (1989) Antibacterial activity from marine microalgae in laboratory culture. B Phycol J 24: 191-194

Kjelleberg S, Steinberg P, Givskov MM, Gram L, Manefield M, deNys R (1997) Do marine natural products interfere with prokaryotic AHL regulatory systems. Aquatic Microbiol Ecol 13 (1): 85-93

Krishna RR, Rao KG, Babu PH (1997) White spot disease. World Aquacul 28: 1-19

Kumlu M, Jones DA (1995) The effect of live and artificial diets on growth, survival and trypsin activity of larvae of *Penaeus indicus*. J World Aquaculture Soc 26: 406-415

Ottogali L (1991) Total substitution of microparticles for algae for *Penaeus stylirostris* larval rearing in New Caledonia. J World Aquaculture Soc 22: 46A

Ottogali L (1992) Nueva gestion del agua en las crias de peneidos en Nueva Caledonia Mem. In Calderon J, Sandoval V (eds) 1er Congreso Ecuatoriano de Acuicultura. CENAIM, Guayaquil, Ecuador, pp. 87-91

Parsons TR, Maita Y, Lalli CM (1984) A manual of chemical and biological methods for seawater analysis. Pergamon Press, 173pp

Porter KG, Feig JS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Ocean 25 (5): 943-948

Prayatno SB, Latchford JW (1995) Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio* .Effect of salinity and pH on infectiosity. Aquaculture 132: 105-112

Reichelt JL, Borowitzka MA (1984) Antimicrobial activity from marine algae: Results of a large-scale screening programme. Hydrobiologia 116: 158-168.

Robson NDA, Cox RJ, McGowan SJ, Bycroft BW, Salmond GPC (1997) Bacterial N-Acyl- homoserine-lactone- dependent signalling and its potential biotechnological application. TIBTECH 15: 458-464

Silas EG, Muthu S, Pillai NN, George KV (1978) Larval development *Penaeus indicus*. CMFRI Bull 28: 2-12

Skjermo J, Salvesen I, Oie G, Olsen Y, Vadstein O (1997) Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquaculture International, 5: 13-28

Wilkenfield JS (1992) Commercial hatchery status report: an industry panel viewpoint. In: Wyban J (ed) Proceedings Special Session on Shrimp Farming. World AquacultureSociety. Florida, USA, pp.71-86

Effects of Astaxanthin on Larval Growth and Survival of the Giant Tiger Prawn, *Penaeus monodon*

Jintana Darachai, *Somkiat Piyatiratitivorakul, Prasat Kittakoop Charoen Nitithamyong, Piamsak Menasveta

Aquatic Resources Research Institute, Department of Marine Science and Marine Biotechnology Research Unit, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: The aim of this study was to evaluate effects of natural and synthetic astaxanthins on growth, survival and low salinity resistance of *Penaeus monodon* larvae. Four diets: algal astaxanthin-added diet (AAD), synthetic astaxanthin-added diet (SAD), non astaxanthin supplemented diet (NAD) and natural food (NF) were prepared to feed three *P. monodon* larval stages (zoea, mysis and postlarvae). The results indicated that zoea fed AAD, NF and NAD survived significantly better than zoea fed SAD. For the mysis stage, larvae fed NF and AAD had better survival rates than those fed NAD and SAD. After 15 days of rearing the postlarval stage to PL-15, AAD showed the best survival rate and it was significantly higher than that of PL-15 that had been fed NF. PL-15 fed AAD and NF were significantly longer than those fed SAD and NAD ($P<0.05$). In low salinity challenge tests, the postlarvae fed AAD showed higher tolerance than postlarvae fed other diets.

KEY WORDS: astaxanthin, *Penaeus monodon*, larvae, growth, survival, *Haematococcus pluvialis*

INTRODUCTION

Larviculture of marine shrimp has become increasingly important due to an expansion of shrimp culture in coastal zones. Each year in Thailand, at least 40 billion postlarvae of *Penaeus monodon* are needed to support shrimp aquaculture. In order to ensure good quality shrimp postlarvae, good quality food, either natural or artificial needs to be used. Since it is difficult or impossible to control natural live feeds which vary in quality seasonally, larval feed developments have focused on artificial feeds. These artificial feeds are still in need of more intensive study. One of the components of artificial feeds is astaxanthin, the main carotenoid found in the integument of shrimp. It is an orange to red carotenoid pigment that contributes to consumer appeal for foods in the marketplace. However, it is also important for health, since it is involved in intra-cellular protection by stabilizing cell membranes and improving health and immunology through the removal of oxygen free radicals (Roche News 1993). Since animals lack the ability to synthesize carotenoids, the pigments must be included in thier feed. There is considerable interest in using natural sources of astaxanthin for feed, because synthetic astaxanthin is expensive and difficult to prepare. Algae in the genus *Haematococcus* are known to produce astaxanthin. *H. pluvialis* has been especially studied because of its rapid growth and proficient production of astaxanthin (Johnson & An 1991). It can be used to produce the carotenoid in large quantity, free from toxicity. In the present experiments, natural astaxanthin from *H. pluvialis* was produced and compared with synthetic astaxanthin for effects on shrimp larval health, growth and survival.

MATERIALS AND METHODS

Algal culture for astaxanthin

H. pluvialis NIES144 obtained from the National Institute for Environmental Studies (NIES), Japan was cultured under optimal conditions for growth (temperature 25°C, 10 h in darkness and 14 h at 1.5 klux illumination). When the culture reached 10^6 cells per ml, the cells were continuously stressed with 10 klux illumination to stimulate astaxanthin accumulation.

Effect of astaxanthin on *P. monodon* larvae

The present study was carried out at the Department of Marine Science and the Marine Biotechnology Research Unit, Chulalongkorn University. A completely randomized design was used for four diets with five replicates. The diets were; natural food (NF: *Chaetoceros* sp. for zoea and the first mysis stages, and newly hatched *Artemia salina* for mysis II to postlarva-15), control diet (NAD: a semi-purified diet that was astaxanthin-free), algal astaxanthin-added diet (AAD: a semi-purified diet with added astaxanthin from *H. pluvialis*) and a synthetic astaxanthin-added diet (SAD: a semi-purified diet with added synthetic astaxanthin). The amount of astaxanthin added in the diet was controlled at 200 mg/kg diet. The feeding experiments were carried out at three larval stages; zoea I-III, mysis I-III and postlarvae 1-15.

P. monodon nauplii obtained from a commercial hatchery in Chonburi Province were used in this experiment. Nauplii parents were from Andaman Sea stock. The feeding tests were started at the beginning of zoea I, mysis I and

Darachai J, Piyatiratitivorakul S, Kittakoop P, Nitithamyong C, Menasveta P (1998) Effects of astaxanthin on larval growth and survival of the giant tiger prawn, *Penaeus monodon*. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: psomkiat@chula.ac.th

postlarva-1, respectively. The initial larval density for each stage was 100 larvae/l for zoea, 80 larvae/l for mysis and 30 larvae/l for postlarvae.

The larval rearing units consisted of conical shaped aquaria containing 4 l sea water. The rearing system was static with partial water change (about 2/3 of total volume) every morning. Filtered and chlorinated seawater at 30 ppt salinity was used and water temperature was 27-30 °C. All rearing units were provided with gentle, continuous aeration.

All larval stages were fed *ad libitum* with five feed additions; 08:00, 11:00, 14:00, 17:00 and 20:00 h. The first feed for zoea I in all treatment groups was *Chaetoceros* sp. and then the designed diets were substituted as appropriate for the later developmental stages. Sizes of the semi-purified diet particles used in the experiments were ≤ 106 microns for zoea and mysis and 106-250 microns for postlarvae.

Experimental diets

The basal diet in these experiments was in a semi-purified, microparticulate form. The diet was modified from Teshima and Kanazawa (1984) and the composition is shown in Table 1. Astaxanthin sources were Carophyll Pink (synthetic carotenoid, 8% astaxanthin, coated with gelatin, carbohydrates and antioxidants and provided by Roche Ltd., Switzerland) and algal astaxanthin (astaxanthin produced by *H. pluvialis* NIES144, 1.2% astaxanthin with 38% protein). The control diet contained no supplemental carotenoid. The amount of wheat flour was altered to compensate for astaxanthin elimination and for added protein from the algae. The natural diet used consisted of *Chaetoceros* sp. and newly hatched *Artemia* sp.

Table 1. Percentage composition of the experimental diets.

Ingredients	% Use	NAD	AAD	SAD
Casein	56.0	56.0	54.7	56.0
Wheat flour	15.0	15.0	15.0	15.0
Fish oil (refined tuna)	6.0	6.0	6.0	6.0
Na- citrate	0.3	0.3	0.3	0.3
Na-succinate	0.3	0.3	0.3	0.3
Glucosamine-HCl	0.8	0.8	0.8	0.8
Mineral mix	9.4	9.4	9.4	9.4
Vitamin mix*	3.9	3.9	3.9	3.9
Cholesterol 90%*	1.0	1.0	1.0	1.0
Lecithin	2.0	2.0	2.0	2.0
Rice bran	6.1	6.1	6.1	6.1
Carrageenan	5.0	5.0	5.0	5.0
Synthetic astaxanthin*	0.52	-	-	0.52
Cysts of *H. pluvialis*	3.5	-	3.5	-
Wheat flour**	-	0.89	-	0.37

*Provided by Rovithai Ltd., Thailand.; **For protein compensation; Mineral mix 100 g contains; K_2HPO_4 2.00 g, $CaSO_4$ 3.58 g, $MgSO_4.7H_2O$ 3.04 g and $Na_2HPO_4.2H_20$ 0.79 g; NAC = no added astaxanthin control diet; AAD = algal astaxanthin diet; SAD = synthetic astaxanthin diet

Salinity stress tests

Postlarvae-15 fed different diets were challenged in stress tests. Ten larvae from each treatment replication were immediately transferred from rearing salinity (30 ppt) to 2 ppt salinity. Then, the number of dead shrimp was recorded at 10 min intervals for a period of two hours.

Data collection

Survival of the larvae for each dietary treatment was recorded when the larvae reached the early phase of the next developmental stage. Survival rate was calculated as the final number surviving as a proportion of the initial number of larvae in the test. For effect of diets on growth, ten postlarvae-15 from each replicate were randomly sampled for length measurement. The length was determined from the tip of telson to rostrum under a x10 magnification projection microscope. Water quality such as salinity and temperature was determined daily, while ammonium, nitrate and pH were monitored every 2 days. The astaxanthin content in *H. pluvialis* cysts, in synthetic astaxanthin, in diets and in shrimp tissues was determined using the spectrophotometric technique described by Boussiba and Vonshak (1991) and Davies (1976) and by HPLC (Weber 1990).

Statistical analysis

All experimental data were analyzed using the Statistic Analysis System (SAS) Program (SAS 1985) or the SPSS-PC program for probit analysis. Means were compared using Duncan's new multiple-range test.

RESULTS

Cultivation of *H. pluvialis* NIES144

During cultivation for growth, the *H. pluvialis* cells were green, biflagellates swimmers with a size of approximately 10µ. The specific growth rate (µ) and doubling time were 0.26 and 2.67 days, respectively. After culturing up to 25 days, the density of the cells reached 10^5-10^6 cells/ml. Encystment was then promoted by increasing the light intensity to 10 klux. The cells lost their flagellae and formed persistent thick walled cysts. Simultaneously, they accumulated massive amounts of astaxanthin. Astaxanthin deposition first occurred around the nucleus and then proceeded radially until the entire protoplast turned red. The size of the cysts was 50 µ (4-5 times larger than the vegetative cells). Fully matured cysts only were harvested 10-12 days after initiating cell stress. The yield of dried cysts was approximate 0.2 g/l. Freeze dried cyst composition was protein 39.14%, fat 1%, carbohydrate 17.01%, ash 3.25% and moisture 7.45%. The average astaxanthin quantity produced of *H. pluvialis* was 1.44±0.35% of algal dry weight.

Proximate analysis of diets

Proximate analysis of the artificial diets gave protein 47%, fat 8%, fiber 1%, ash 7% and moisture 5%. The astaxanthin content in the algal astaxanthin-added diet (AAD), the synthetic astaxanthin-added diet (SAD), and the control diet (NAD) was 188.90 ± 4.84, 208.60 ± 4.26 and 0 ppm, respectively.

Effect of astaxanthin on survival

Survival of shrimp zoea, mysis and postlarvae fed different diets are shown in Table 2. Survivals of zoea fed NF, NAD, and AAD were significantly higher than survival of zoea fed SAD ($P<0.05$). At the mysis stage, the survival of larvae fed AAD and NF was similar but significantly higher than that of mysis fed NAD and SAD. At the postlarval stage, the best survival was obtained with AAD and this was sig-

nificantly higher than that of postlarvae fed NF. However, there were no significant differences in survival amongst larval groups fed AAD, SAD and NAD.

Table 2. Percent survival of *Penaeus monodon* larval stages fed different diets.

Diets	Survival of larval stage (%)		
	Zoea	Mysis	Postlarvae
NF*	82.0 ± 3.30[a]	76.7 ± 8.61[a]	55.2 ± 6.14[b]
NAD	74.0 ± 5.19[a]	57.3 ± 5.01[b]	68.6 ± 5.73[ab]
AAD	82.5 ± 3.53[a]	69.7 ± 12.05[a]	76.0 ± 9.46[a]
SAD	27.8 ± 4.01[b]	58.1 ± 0.29[b]	64.4 ± 11.86[ab]

Means in the same column with different superscripts are significantly different (P<0.05); *NF (Natural food) from zoea I to mysis II was *Chaetoceros* sp. and for mysis II to postlarva-15 was newly hatched *Artemia salina*.

Effect of astaxanthin on growth

Growth as measured in terms of total length of postlarva 15 fed different sources of astaxanthin diet are shown in Table 3. The mean lengths for larval groups fed NAD and SAD were significantly lower than those for larvae fed AAD and NF (P<0.05) but there was no significant difference between the groups fed NF and AAD.

Table 3. Length of *Penaeus monodon* postlarva-15 fed different diets.

Diets	Length of PL 15 (cm) Mean ± SD
NF	0.9843 ± 0.073 [ab]
NAD	0.9674 ± 0.074 [b]
AAD	1.0019 ± 0.067 [a]
SAD	0.9652 ± 0.075 [b]

Means with different superscripts are significantly different at P<0.05

Low salinity tolerance

Larvae from all of the diet treatment groups started to die within 10 minutes of beginning the test. In the first hour, the highest cumulative mortality (CM) was for the larval group fed NF (76%), followed by those fed NAD (68%), SAD (66%) and AAD (62%). The mortality rate declined after the first hour. At 90 minutes, CM for larvae fed AAD and SAD was 76%, while it was 80% and 86%, respectively for those fed NF and NAD. After 2 h, remaining larvae from all the treatment groups had adapted to 2 ppt salinity and showed only slight mortality up to 140 minutes. Using probit analysis, the effect of diet on time to 50% CM for postlarva 15 was determined and the results are shown in Table 4. They indicated that time to 50% CM for shrimp fed AAD was longer than that for shrimp fed SAD, NAD and NF.

Table 4. Time to 50% cumulative mortality (CM) in low salinity resistance test of larvae fed different diets.

Diets	Time to 50% CM (min)
NF	27.99
NAD	32.23
AAD	44.86
SAD	36.63

Water quality

The water quality of larval rearing units for all treatment groups is shown in Table 5. Water quality parameters were similar for the four dietary treatments and were in the normal range for shrimp cultivation throughout the whole period of the experiment.

Carotenoids in postlarvae fed different diets

Because of their small size, carotenoid analysis of the postlarva 15 was determined on a wet weight basis using a spectrophotometric method. The results of the carotenoid assay are shown in Table 6. Carotenoids in all the diet groups were significantly different (P<0.05). The highest carotenoid accumulation occurred in the shrimp fed NF, followed by the shrimp fed AAD, SAD and NAD, in order of highest to lowest accumulation.

Table 6. Carotenoid concentration (µg/g body wet weight) in PL-15 of *Penaeus monodon* fed different diets.

Source feed	Shrimp carotenoid content (ppm)
NF	179.54 ± 0.65 [a]
NAD	97. 33 ± 3.42 [b]
AAD	122.57 ± 5.62 [c]
SAD	109.07 ± 0.47 [d]

Means with different superscripts are significantly different at P<0.05

Table 5. Water quality of the rearing units for different stages of *Penaeus monodon* larvae.

Diets	Larval stages	Ammonium (mg/l)	Nitrate (mg/l)	pH	Temp. (°C)	Salinity (ppt)
Natural diet	Z_1-Z_3	0.5-1	25	7.7	28-30	30
	M_1-M3	0.5-1	25	7.7	28-29	30
	PL_1-PL_{15}	0.5-1	10	7.7	27-28	30
Artificial diets	Z_1-Z_3	1-2	10	7.4-7.7	28-30	30
	M_1-M_3	2-3	25	7.4-7.7	28-29	30
	PL_1-PL_{15}	1-2	10	7.4-7.7	27-28	30

DISCUSSION

Haematococcus cultivation

H. pluvialis grew well under laboratory conditions at 25°C. With high light intensity stress, it accumulated astaxanthin and turned red. Astaxanthin esters are the primary pigment in *H. pluvialis* cysts, typically ranging from 60% to 80% by weight of the total pigment content (Spencer 1989). Kobayashi et al. (1992) showed that *H. pluvialis* NIES144 accumulated astaxanthin mainly in esterified form [monoester (69%) and diester (20%)]. By contrast, free astaxanthin is the major compound in synthetic astaxanthin. The predominant configuration isomer in *H. pluvialis* is 3S, 3'S (Johnson & An 1991). This 3S, 3'S configuration is also found in lobster (*Homarus gammarus*) eggs and yeast (*Phaffia rhodozyma*).

Effect of dietary astaxanthin on shrimp larvae

From our results, the highest survival rate of zoea and mysis was obtained with shrimp fed AAD followed by NF, NAD and SAD, in descending order. This indicated that shrimp larvae accept natural better than synthetic astaxanthin. Commonly, the early stages of shrimp larvae are fed with phytoplankton (e.g., *Chaetoceros* sp.) and zooplankton (e.g., *Artemia* sp.) from which they derive natural carotenoids (and convert them to astaxanthin). In the case of AAD, it contained *H. pluvialis* that had produced natural astaxanthin and accumulated various nutrients (from the rich cultivation medium) and these could be utilized directly by the larvae. By contrast, the free form of astaxanthin in SAD may have been difficult for early larval stages to utilize and this may have affected their survival. Indeed, the zoea fed SAD had the lowest survival.

In postlarval stages, there were no significant differences in survival rate for larval groups fed AAD, SAD and NAD. The postlarvae fed NF had the lowest survival (significantly lower than the postlarvae on the AAD diet). This indicated that *Artemia* nauplii as the sole feed to postlarvae could not provide sufficient nutrients for good survival. Chindamaikul and Phimonchinda (1990) reported that the survival of *P. monodon* from zoea to postlarva-15 was higher when they were fed natural feed (*Chaetoceros* sp. and *Artemia* sp.) plus artificial feed than it was when they were fed only natural feed or only artificial feed.

Efficacy of astaxanthin was higher for postlarvae fed AAD than for those fed NF (*Artrmia* sp.). Tanaka et al. (1975) explained that *Artemia* sp. accumulated mainly canthaxanthin and that two biochemical steps were required for shrimp to convert it to astaxanthin. By contrast, the astaxanthin in *H. pluvialis* cysts could be used directly. The source of astaxanthin may affect survival rate of shrimp larvae differently at different stages. For example, the survival of early larval stages fed the diet containing synthetic astaxanthin (SAD) was poor but survival was higher for larvae at the postlarval stage. Utilization of free astaxanthin may be poor for zoea and mysis but may be better for postlarvae. Both zoeal and mysis stages naturally consume algae and small living organisms (e.g., rotifers and *Artemia*) which are natural sources of astaxanthin.

The postlarva-15 fed natural diets containing natural astaxanthin were larger than those fed diets containing synthetic astaxanthin or no astaxanthin. The best postlarval growth was in the group fed AAD and this was significantly better than that for groups fed SAD. This indicated that astaxanthin from *H. pluvialis* (mostly in esterified form) performed significantly better than free, synthetic astaxanthin. Brown et al. (1991) reported that shrimp fed a dry diet plus algae grew faster and had better survival than shrimp fed a dry diet only. This was probably because the algae contained essential nutrients that were lacking in the dry diet. Similarly, *H. pluvialis* NIES144 was cultured in a rich medium containing thiamin as a growth factor and its dry cysts contained approximately 40% protein and 1% fat. These factors may explain why *H. pluvialis* cysts in the diet were more advantageous than synthetic pigments. Cohen (1986) reported that dietary xanthophylls from algae are incorporated into the exoskeleton of prawns and lobsters (as astaxanthin) where they play a major role in pigmentation. Other functions are poorly defined. Boonyaratpalin et al. (1994) found that addition of synthetic astaxanthin and canthaxanthin pigment to diets did not effect growth and feed efficiency of *P. monodon* juveniles.

The pigment content of shrimp fed pigment-free diets was less than that of groups fed pigmented diets. This confirmed that shrimp larvae can accumulate carotenoids (mainly astaxanthin) from their diets. The astaxanthin content of shrimp fed NF (*Artemia* sp.) was higher than that of other groups. It may because *Artemia* sp. contains various carotenoids, such as astaxanthin, violaxanthin, zeaxanthin, echinenone, b-carotene, lutein in addition to its main pigment canthaxanthin. Some of these carotenoids can be converted to astaxanthin. Moreover, all carotenoids have similar maximum absorption wavelengths that may overlap or interfere during analysis by spectrophotometer. The larvae fed AAD contained higher amounts of carotenoids. This may have resulted for several reasons. For example, shrimp larvae may gain esterified astaxanthin and accumulate it in body tissue better than free astaxanthin. Secondly, the larvae may be more accustomed to natural astaxanthin than synthetic astaxanthin. Third, they may have accept AAD better than SAD. However, the factors controlling pigment absorption, transportation and excretion among various shrimp tissues are not known. Herring (1969) speculated that pigment absorbed in excess an animal's requirement is later excreted.

Determination of 50% cumulative mortality upon low salinity challenge showed that larvae fed AAD endured better than larvae fed NF, SAD and NAD. In addition, tolerance of shrimp fed pigmented diets (AAD and SAD) was higher than those fed a pigment-free diet (NAD). Astaxanthin seemed to be helpful to the postlarvae and to prolong their life upon this acute environmental stress.

The esterified astaxanthin in AAD was a storage form of the pigment and it was probably accumulated in the lipid portion of the larval hepatopancreas before transfer to other sites in the body by haemolymph (Ghidalia 1985). This was probably also true for the free astaxanthin in SAD. However, *P. monodon* has been reported to accumulate esterified astaxanthin (85.7%) more than free astaxanthin (14.3%) (Latscha 1989).

Because astaxanthins contain a long conjugated double bond system, they are relatively unstable and usually scavange oxygen radicles in cells (Stanier et al. 1971). When the shrimp were transferred from high to low salinity, they were exposed to stress and needed to expend more energy to maintain osmotic stability. There would have been the potential for generation abnormally high levels of oxygen radicles. However, the dynamics of this interaction and how it relates to adequate or excess astaxanthin content in a cell were not examined in this study. Although the mechanism by which astaxanthin improved the response to stress cannot be explained, the information that natural astaxanthin (from *H. pluvialis*) is more efficacious than synthetic astaxanthin for growth, survival and stress resistance of shrimp larvae should be useful for further research on shrimp larval nutrition.

Acknowledgements. This research was supported by a grant from the Thai National Center for Genetic Engineering and Biotechnology. The authors would like to thank Mr. Sari Donnau for his technical support.

LITERATURE CITED

Boonyaratpalin M, Wannagowat J, Borisut C, Boonchuay S (1994) Effect of various dietary canthaxanthin and astaxanthin levels on pigmentation of giant tiger shrimp. Technical paper no.18/ 1994. National Institute of Coastal Aquaculture, Songkhla (in Thai).

Boussiba S, Vonshak A (1991) Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Plant Cell Physiol. 32(7) : 1077-1082

Brown MR, Jeffrey SW, Garland CD (1991) Nutritional aspects of microalgae used in mariculture; A literature review. Fishing Industry Research and Development, Trust Account 86/81. Department of Primary Industry, Canberra, ACT.

Chindamaikul T, Phimonchinda T (1990) The studies on comparison of nursing *Penaeus monodon* Fabricius with different feed. Technical paper No. 34/1990, Phuket Coastal Aquaculture, Department of Fisheries (in Thai).

Cohen Z (1986) Products from microalgae. Handbook of Microalgal Mass Culture. Richmond ed. Florida: CRC Press, pp. 421- 454

Davies BH (1976) Carotenoids. Chemistry and Biochemistry of Plants Pigments, 2 nd ed, vol 2. London: Academic Press

Ghidalia W (1985) Structural and Biological Aspects of Pigment. *In*: Bliss DE, Mentel LH (eds). The Biology of Crustacea. vol 9, Integument, Pigment and Hormonal Processes. London: Academic Press

Herring P (1969) Pigmentation and carotenoid metabolism of the marine isopod *Idotea metallica*. Mar. Bio. Ass. UK. 49: 767-779

Johnson EA, An GH (1991) Astaxanthin from microbial sources. Critical Reviews in Biotechnology 11(4): 297-326

Kobayashi M, Kakizono T, Yamagushi K, Nishiro N, Nagai N (1992) Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. Fermentation and Bioengineering 74(1): 17-20

Latscha T (1989) The role of astaxanthin in shrimp pigmentation. Advances in Tropical Aquaculture 319-325

Roche News (1993) Roche Aquaculture Center, Vol. 1/1993. Bangkok.

SAS, 1985. The Statistic Analysis System, SAS Institute Inc. USA, 95 pp

Spencer K (1989) Pigmentation supplements for animal feed composition. US Patent No. 4,871,551

Stanier RY, Kunizawa MM, Cohen-Bazire G (1971) Purification and property of unicellular blue-green algae (Order Chroococales). Bacterial Rev. 35: 171-201

Tanaka Y, Malsuguchi H, Katayama T, Simpson KL, Chichester CO (1975) The biosynthesis of astaxanthin-XVIII. The metabolism of the carotenoids in the prawn *Penaeus japonicus* Bate. Bull. Jpn. Soc. Sci. Fish. 42 (2): 197-202

Teshima S, Kanazawa A (1984) Effects of protein, lipid and carbohydrate levels in purified diets on growth and survival rates of the prawn larvae. Bull. Jpn Soc. Sci. Fish. 50 (10): 1709-1715

Weber S (1990) Determination of added stabilized astaxanthin in fish feeds and premix with HPLC. Roche-publication, Index No. 2101, 59-61

Low Salinity Culture of *Penaeus monodon* Fabricius and Its Effect on the Environment

***Yont Musig, Sathit Boonnom**

Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand

ABSTRACT: In 1997, a survey was made in the central part of Thailand of farming areas where low salinity culture of *Penaeus monodon* was being practised in fresh-water environments. The purpose of the study was to determine the effect of such culture on the surrounding environment. This farming technique was being practised over a total area of 11,504 ha in 12 central provinces. Culture areas could be categorised into 3 types: 1) freshwater areas with normal soil, 2) freshwater areas with saline soil, and 3) freshwater areas that become salty during the dry season due to the penetration of sea water upstream along waterways connected to the sea. Closed culture systems without water exchange were being used. In normal practice of this technique, water of 5 to 9 ppt salinity is prepared by mixing transported seawater brine with fresh water in earthen ponds that are filled to about 30 to 50% of pond volume. After stocking with post larvae, fresh water is gradually added as required such that the salinity of water during the last month of culture falls to between 1-5 ppt. For small size farms (SF), the effluent is released directly into receiving waters. In some medium size and large farms (MLF), effluents are treated and water is recirculated to rearing ponds. In MLF culture systems, no effluent is released into the surrounding environment and dikes and ditches are built surrounding the farms to prevent the spread of salt water. Analysis of pond effluents revealed that their average characteristics were 38.7 mg/l BOD_5, 0.63 mg/l of inorganic nitrogen, 0.037 mg/l of total phosphorus, 182.4 mg/l of total suspended solids, and 0.2-5.0 ppt salinity. Analysis of electrical conductivity of soil in and around the farms indicated that most of the salt accumulated in the top 0-15 cm layer of pond bottom soils. However, in some ponds, the salt penetrated down to depths of 70-100 cm. Analysis of soil samples surrounding the shrimp ponds indicated mostly that the salt spread for only short distances from the ponds.

KEY WORDS: *Penaeus monodon*, low salinity culture, environmental impact, fresh water

Musig Y, Boonnom S (1998) Low salinity culture of *Penaeus monodon* Fabricius and its effect on the environment. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

A Closed Recycle System for Sustainable Black Tiger Shrimp Culture in Freshwater Areas

[1]Chalor Limsuwan, [2]Pornlerd Chanratchakool

[1]Faculty of Fisheries, Kasetsart University, Bangkaen, Bangkok, Thailand
[2]Aquatic Animal Health Research Institute, Bangkaen, Bangkok, Thailand

ABSTRACT: The culture of marine shrimp in inland freshwater areas of central Thailand was made possible because farmers could purchase brine water from salt farms and transport it to their farms. Inland shrimp culture has been so successful that the number of farms in Thailand has increased steadily since 1994. However, this development has taken place without adequate research into the most appropriate and environmentally sustainable culture systems. This is important, as the environment and surrounding farmland must be protected form any negative impacts brought on by the new activity. It is not just the environment that could be affected. Problems created by inland shrimp farming could result in social tensions and real economic losses. Thus, researchers feel there is a necessity and an urgency to carry out impact assessment surveys on inland shrimp culture. A typical closed recycle system for shrimp culture consisting of growout ponds, a reservoir, disposal ponds and a canal surrounding the farm was used in this study. Shrimp were stocked in experimental ponds with either 5, 7 or 10 ppt salinity during the summer production cycle and in ponds containing 3 or 5 ppt salinity during the rainy season. Water from the summer production cycle was recycled and used again during the rainy season period. The survival and growth rates from shrimp stocked in all ponds was compared. A salinity analysis on soil samples from the bottom of each experimental pond was conducted every month after stocking the shrimp and once after harvest. The results revealed that closed recycle systems employing low salinity water are appropriate for inland shrimp culture. More details are discussed in the paper.

KEY WORDS: fresh water shrimp culture, sustainability, environmental impact

Chalor C, Chanratchakool P (1998) A closed recycle system for sustainable black tiger shrimp culture in freshwater areas. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

The Shrimp Defence System

Shrimp Immunity and Disease Control: An Integrated Approach*

Evelyne Bachère

UMR 219, Défense et Résistance chez les Invertébrés Marins, IFREMER/CNRS, CC80
Université Montpellier 2, 2 Place E. Bataillon, Montpellier 34095 FRANCE

ABSTRACT: The prevention and the control of diseases are now considered priorities for shrimp aquaculture in the vast majority of shrimp producer countries. The maintenance and development of this industry are at stake as shrimp aquaculture faces increasingly significant ecological and pathological problems on a global scale. The intensification of shrimp production based on progress in zootechnology occurred without significant progress in scientific knowledge on shrimp physiology. Within that field, research in immunology has been a subject of only minor interest, particularly when compared to similar research on other crustaceans and insects. In fact, shrimp immunology is a key element in establishing strategies for disease control in shrimp aquaculture. Thus, research on the development of assays for evaluating and monitoring the shrimp immune state must be emphasized. Performing regular immune "check-ups" would permit not only the detection of shrimp immunodeficiencies, but also the control and improvement of environmental quality. To do this, immune effectors must be identified and characterised. However, the future sustainability of shrimp aquaculture will also depend greatly on the selection of disease-resistant animals, making parallel research in immunology and genetics essential. A research project, Shrimp Immunity & Disease Control, has been established with the primary aim of increasing basic knowledge on penaeid immunity, by creating a collaborative network and by exchanging information and results. By opening this network to other research areas related to shrimp pathology, physiology, genetics or environment, the project will ultimately contribute to the development of strategies for prophylaxis and control of shrimp diseases.

KEY WORDS: penaeid shrimp, immunity, prophylaxis, selection, pathogen resistance, disease control

INTRODUCTION

Shrimp aquaculture started in the 1970s as an industrial activity with a rapid development that has been linked to zootechnic progress and a huge increase in the number of hatcheries and farms. Shrimp farming presently represents roughly 30% of the world shrimp market. This activity concerns about 50 inter-tropical countries in South East Asia and Central and South America. The Eastern hemisphere is the major producer of shrimp and Thailand was the world's leading producer with 160,000 metric tons of *Penaeus monodon* farmed in 1996. Ecuador is the second largest producer in the world, with a production estimated at 120,000 metric tons of *P. vannamei* in 1996 (Rosenberry 1996). In Ecuador, shrimp farming and processing generate about 200,000 jobs and together they constitute the second largest activity after the production of bananas.

The industry saw important growth during the 1980s, but now shrimp production is regularly and dramatically affected by a series of problems linked to environmental degradation and to infectious and non-infectious diseases. Together, these problems have caused a recent decrease of world production from 721,000 tons in 1992 to 609,000 tons in 1993 (World Shrimp Farming 1993). The causative agents of infectious diseases in shrimp are mainly viruses and bacteria belonging to *Vibrionacea*. All around the world, moreover, non-infectious diseases are often suspected to be the consequence of environmental degradation.

Consequently, the control of diseases has become a global priority to assist in making shrimp production ecologically and economically sustainable. To a large extent, the durability of production is dependent on the equilibrium amongst (i) environmental quality, (ii) disease prevention by pathogen diagnosis and epidemiological surveys and (iii) the health status of the shrimp. Shrimp aquaculture is also dependent on the selection of shrimp resistant to diseases. Therefore, the prevention and the control of shrimp diseases would appear to require an integrated approach. Knowledge in shrimp immunity must be improved while also considering other research areas related to pathology, shrimp physiology, and genetics.

Recently, a collaborative research project " Shrimp Immunity & Disease Control "(SI&DC) has been initiated and is now supported as a Concerted Action by the European Commission (DG XII) in the programme INCO-DC, International Cooperation with Developing Countries (project n°IC18CT970209). Herein, we present the objectives and the programme of this Concerted Action and we propose a collaborative approach aimed at the prevention and the control of diseases in shrimp aquaculture.

Bachère E (1998) Shrimp immunity and disease control: an integrated approach. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*Project supported by the European Commission, DG XII, in the programme International Cooperation with Developing Countries, INCO-DC ; Contract n° ERBIC18CT970209.

OBJECTIVES OF THE SI&DC CONCERTED ACTION

There is an urgent need to organize and develop research on penaeid shrimp if we are to ensure sustainable shrimp aquaculture by preventing and controlling disease. By doing this, new methodologies and new concepts for shrimp aquaculture could be established and then developed both with and for producers. With this goal in mind, the SI&DC Concerted Action supported by the DGXII (European Commission) (Sept.1997-Sept.2000) is meant to increase basic knowledge on penaeid immunity by creating an open collaborative network of research laboratories involved in the immunity of shrimp and other invertebrate groups. In this relatively recent area, communication and collaboration must also be improved between ongoing research projects in different parts of the world. The greatest benefits for collaborating countries, beyond the avoidance of redundancy, will lie in cooperation rather than competition and in exchange of information. This will improve efficacy, which is the definition of a concerted action (Loukopoulos 1995, in Newsletter on Biomedical & Health Res., vol 6). Through the SI&DC project, it is expected that results and reagents will be exchanged, that technologies and methodologies will be transferred and standardised, and that training and career development of young scientists in producing countries will also be improved.

Considering the importance of shrimp aquaculture and its limitations due to disease problems, the lack of specialists in the fields of pathology, immunology and genetics of penaeids, particularly in the producing countries is surprising. The crisis situation can be explained by the fact that the industry developed rapidly with very little scientific knowledge to support the new farming techniques. The training of local scientists specialised in shrimp pathology, immunology and genetics is thus of prime importance.

The original project approved by the European Union started with 10 scientists specialising in immunology. They are from European, Latin American and Asian countries and have membership status with the SI&DC Concerted Action. They are:

Arala-Chaves M., ICBAS, Porto, Portugal (imuno@icbas.up.pt)
Bachère E., IFREMER-CNRS, Montpellier, France (ebachere@ifremer.fr)
Barraco M., Univ. Santa Catarina, Florianopolis, Brazil (barracco@mbox1.ufsc.br)
Bulet P., CNRS, Strasbourg, France (bulet@ibmc.u-strasbg.fr)
Cuellar-Anjel J., CENIACUA, Bogota, Colombia (ceniacua@ns.axisgate.com)
Jiravanichpaisal P., MBRU, Bangkok, Thailand (pikul@biotec.or.th)
Le Moullac, G., IFREMER, French Polynesia (glemoull@ifremer.fr)
Rodriguez, J., CENAIM, Ecuador (cenaim@espol.edu.ec)
Saulnier, D., IFREMER, French Polynesia (dsaulnie@ifremer.fr)
Söderhäll, K., Univ. Uppsala, Sweden (kenneth.Soderhall@fysbot.uu.se)
Vargas-Albores F., CIAD, Hermosillo, Mexico (fvargas@cascabel.ciad.mx)

The SI&DC project may initiate both new ideas and novel supported projects. Moreover, in the framework of the SI&DC Concerted Action, one of the objectives is to transfer and to exchange information and results with other areas, not only on pathology and genetics, but also on nutrition, reproduction, environmental biology and ecotoxicology. Research domains such as these can be related with immunity in order to develop strategies for the prevention and control of shrimp diseases. It is expected therefore, that other scientists specialised in such domains will join the SI&DC integrated approach as associate members.

EXPECTED OUTCOMES OF THE SI&DC PROJECT

For the prevention of shrimp diseases, as for human health and that of vertebrates generally, it is crucial to develop quantitative assays for the evaluation and monitoring of immune status. The establishment of regular health controls will permit the detection of shrimp immunodeficiencies. In turn, this will help in the development of strategies to decrease disease susceptibility and lead to the control and improvement of environmental quality (Bachère et al. 1995).

By reference to plant and animal genetics (Strittmatter & Wegener 1993, Müller & Brem 1994), a long term strategy to assist shrimp production would be to select disease resistant shrimp by developing parallel research in immunology and genetics. The measurement of defence reactions as well as the characterisation of immune genes could be exploited in quantitative genetic selection for increased resistance to disease (Bachère et al. 1995, Cedeno et al. 1998, this issue). Genetic transformation may constitute another promising strategy for obtaining resistant strains. This might be accomplished by modifying the expression of immune encoding genes or by conferring new traits of resistance on shrimp through the expression of foreign genes characterised in other species (Mialhe et al. 1995, Bachère et al. 1997, Mialhe et al. 1997).

PROGRAMME OF THE SI&DC PROJECT

The programme concerns the main shrimp species used in aquaculture, namely *Penaeus monodon* (South East Asia), *P. vannamei* and *P. stylirostris* (Latin America and Pacific), *P. japonicus* (Europe, Japan), *P. paulensis* (Brazil) and the crayfish *Pacifastacus leniusculus*.

The scientific programme of the SI&DC project aims at the characterisation of cellular and humoral defence effectors in shrimp, taking into account knowledge and experience acquired in other arthropods. The innate defence mechanisms in arthropods are based on both cellular and humoral components of the circulatory system which interplay for detecting and eliminating foreign and potentially harmful microorganisms and parasites. The immune response in arthropods can be subdivided into different phases: (i) an immedi-

ate and inductive stage corresponding to the recognition of the non-self factors, and to the initiation of immune reactivity, (ii) a cellular and synthesis stage of effectors, and (iii) a final humoral and cellular stage of recovery (as reviewed by Ratcliffe 1993).

SPECIFIC ACTIONS

Within the overall framework of the SI&DC project, specific actions deal with functional, biochemical, antigenic and genetic characterisation of effectors involved and expressed in response to pathological injuries or to stressful conditions (Figure 1). These actions are based on background knowledge already acquired in shrimps, as well as information from other better studied crustacean species and other arthropods.

Action 1 – Non-self recognition

The first immune process is the recognition of invading microorganisms which is mediated both by the hemocytes and/or plasmatic proteins. There is little information about the molecular mechanisms that mediate recognition. However in crustaceans, several types of modulator proteins have been described that recognise cell wall components of microorganisms.

In the freshwater crayfish, *Pacifastacus leniusculus*, a ß-1,3-glucan-binding protein has been characterized and cloned, and a hemocyte receptor has been partially characterised that binds the plasmatic glucan-binding protein after the latter has reacted with ß-1,3-glucan (reviewed by Söderhäll et al. 1996). In the shrimp, *P. californiensis*, a protein which responds to b-1,3-glucan has been purified and characterised (Vargas-Albores et al. 1996). Recently, a peptidoglycan recognition protein capable of activating the proPO cascade has been purified from the hemolymph of *Bombyx mori* (Yoshida et al. 1996). Various lipopolysaccharide-binding proteins (LPS-BP) have been characterised in different arthropods. For example, a lectin entraps bacteria within the hemolymph and participates in tissue regeneration in the insect *Periplaneta americana* (Jomori & Natori 1992, Natori & Kubo 1996); lipophorin detoxifies LPS from *Bombyx mori* hemolymph (Kato et al. 1994); and hemolin, a circulating protein with immunoglobulin-like domains characterised in the blood of some lepidopterans, can bind to bacteria and possibly function as an opsonin (Lanz-Mendoza et al. 1996). In addition, various proteins with LPS-binding properties have been characterised in the Chelicerate, *Tachypleus tridentatus*. These include lectins (tachylectins) and a serine proteinase (factor C) which induces a coagulation cascade (for review, see Iwanaga et al. 1998). In shrimp and crayfish plasma, LPS-binding proteins that are agglutinins have also been purifed, but their exact role remains to be determined (Vargas-Albores et al. 1993b, Kopacek et al. 1993a).

Considering the importance of bacterial diseases in shrimp, special attention must be devoted to the study of the molecules or mechanisms involved in the recognition of bacteria belonging to the *Vibrionacea* group.

Action 2 – Circulating hemocytes and hematopoieitic cells

Recognition molecules may interact with and activate the hemocytes which play an important and central role in host defence. In crustaceans, according to numerous works dealing with the identification of hemocyte cell types, a classification scheme has been commonly adopted which recognises three types of circulating haemocytes, namely hyaline, semigranular and granular cells (Bauchau A 1981, Martin & Graves 1985). Based on morphological and cytochemical characterisation, some functions and involvement in different defence reactions have been attributed to the different cell types. These include the involvement of hyaline cells in coagulation (Omori et al. 1989), and of granular and semigranular cells in phagocytosis (Gargioni & Barracco 1998) and in the proPO system (Johansson & Söderhäll 1985). Flow cytometry analysis of the hemocyte populations of the shrimp, *P. japonicus*, also revealed three populations according to cell size, structure and granularity (Sequeira et al. 1995)., However, divergent results with hemogrammes and determination of cell populations are still obtained by different authors. Such contradictory results are particularly evident when analyses are based on the behaviour and involvement of hemocyte populations in response to immune stimulation. Recently, a characterisation of shrimp hemocytes based on specific monoclonal antibodies has led to the identification of proteins specifically expressed in different cell types (Rodriguez et al. 1995). New approaches relating flow cytometric analyses and molecular markers specific for hemocytic immune proteins will permit better determination of the role and function of different cell populations. These approaches should also help to establish cell lineage, a topic which is largely still debated in crustaceans.

Action 3 – Immediate defence systems

The non-self recognition factors activate several immediate defence systems mediated by the hemocytes. The prophenoloxidase (proPO)-activating system is indubitably one of the better studied system in crustaceans, particularly in crayfish. This is an immediate defence process which leads to the reaction of melanisation. Numerous components associated with it have been characterised and functions can now be proposed for most of them (Söderhäll et al. 1996, Söderhäll & Cerenius 1998). The activation (triggering) in crustacean hemolymph of a rapid and powerful coagulation reaction is closely related to activation of the proPO system and it has been studied in some detail in crayfish. It not only prevents blood loss upon wounding but also performs an important defence reaction by participating in the engulfment of foreign invading organisms (Kopacek et al. 1993b). In shrimp, some components of these defence reactions have been identified or have begun to be characterized. These include the proPO-activating system (Vargas-Albores et al. 1993a, Perazzolo & Barracco 1997), a plasmatic clotting factor and α2-Macroglobulin characterised by means of specific monoclonal antibodies (Rodriguez et al. 1995, Bachère et al. 1995). Defence reactions also include the hemocytic process of encapsulation, phagocytosis and production of microbicidal, cytotoxic, reactive oxygen intermediates such as those demonstrated in crabs (Bell & Smith 1993) and shrimp (Song & Hsieh 1994, Bachère et al. 1995). The role and real function of these immediate defence reactions have yet to be analysed in regard to pathological injuries, and it is necessary initially to fully characterise their components in shrimp.

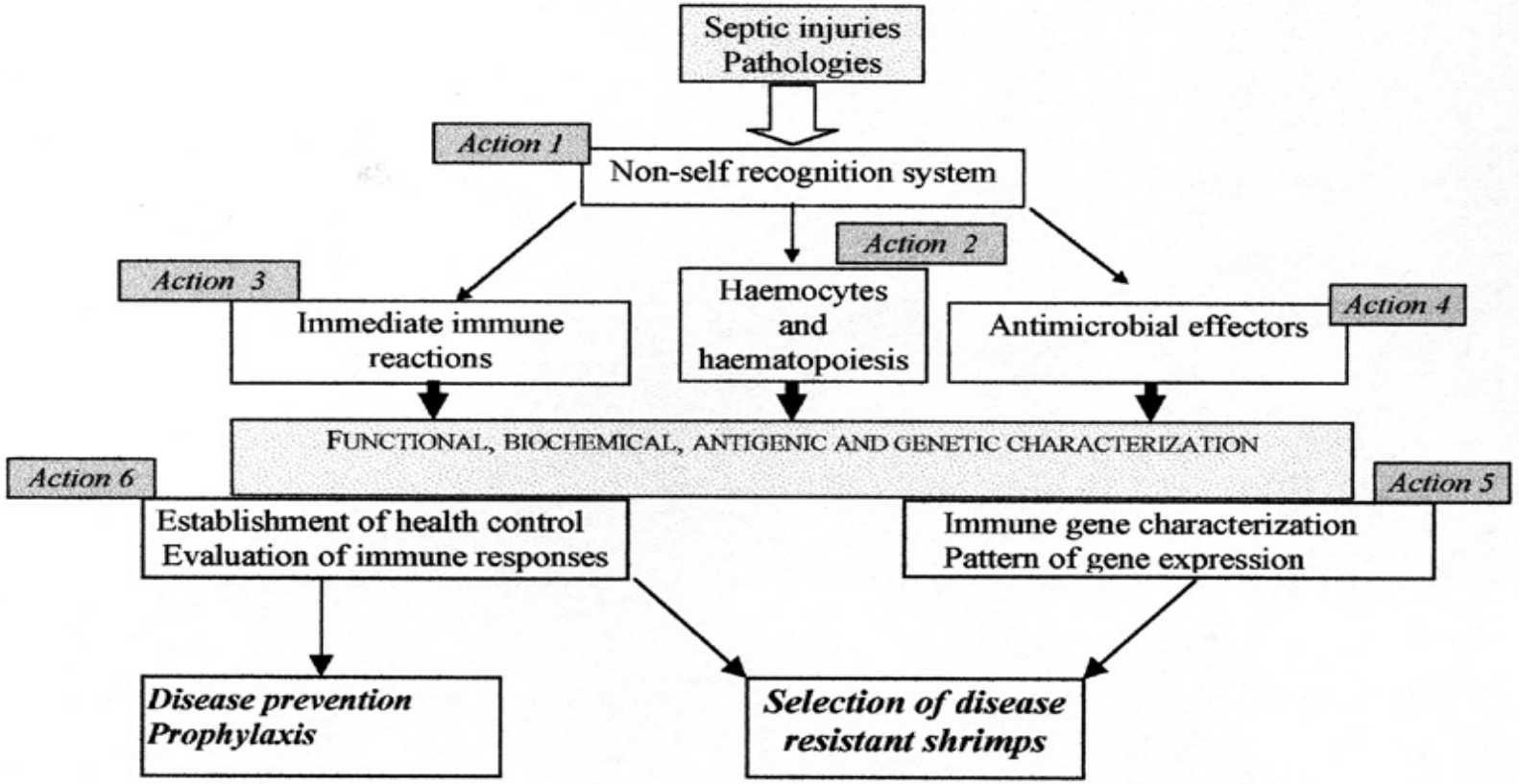


Figure 1. Specific actions of the Shrimp Immunity and Disease Control (SI&DC) project and the expected outcomes for the prevention and the control of disease in penaeid shrimp.

Action 4 – Antimicrobial proteins or peptides

The innate immune response of arthropods also relies on the production of antimicrobial peptides which possess activity against a large range of pathogens. In the horseshoe crab (Chelicerata), endotoxin activation of hemocytes results in the release by exocytosis of the contents of two types of granules - clotting factors essential for hemolymph coagulation (e.g., proteases and tachylectins) and antimicrobial peptides (e.g., tachyplesins, big defensin, tachycitin and tachystatins) (reviewed in Iwanaga et al. 1998). In insects, the synthesis of antimicrobial peptides induced by injury is the hallmark of the immune response of higher insect orders. A septic injury induces the rapid and transient transcription of several genes encoding potent antibacterial and antifungal peptides that are released into the blood where they act to destroy the invading microorganisms. The activation cascades that control the expression of the antimicrobial peptide genes in higher insects show striking structural and functional similarities with activation cascades involved in cytokine-induced expression of acute phase proteins in mammals (Hultmark 1993, Hoffmann & Reichhart 1997). The production of antimicrobial peptides is in fact widespread in the living kingdom, from bacteria to plants (Broekaert et al. 1995) and from vertebrates to invertebrates (for review, see (Hetru et al. 1994).

Surprisingly, in crustaceans, until now the innate defence reaction involving the synthesis of antimicrobial peptides has been poorly studied. To date, constitutive hemocytic proteins have been isolated in the crab *Carcinus maenas* and a 6.5 kDa antimicrobial peptide has been partially characterised (Schnapp et al. 1996). In the penaeid shrimp, *P. vannamei*, three antimicrobial peptides have been purified from the hemocytes and the plasma. They have been fully characterised and their cDNA cloned. According to their biochemical and structural features, the three peptides could not be associated to other peptide families hitherto described, and they were named penaeidins after the genus *Penaeus* (Destoumieux et al. 1997). Research on the role of the penaeidins in the immune response against pathogens is continuing, as is research on the characterisation of other peptides in shrimp.

Action 5 - Immune effector encoding genes and regulation of gene expression

Besides biochemical or antigenic characterisation and determination of the biological activities of immune effectors, one may focus on the cloning and characterisation of their encoding genes. Analyses of the expression of effector genes during the response to infections or to immune stimulation will lead to a better understanding of their involvement in defence reactions against specific harmful pathogens. In this objective, determination of the mechanisms that control and regulate effector gene expression is of prime importance.

Action 6 – Health management and evaluation of immune responses

Compared to mammals, the availability and knowledge of criteria for evaluating the health status of shrimp or other invertebrates are practically non-existent. The establishment of health controls for shrimp firstly requires the development and standardisation of quantitative assays for both immune reactions and the expression of effectors. These assays must be miniaturised for simultaneous individual analyses of different effectors. Furthermore, they have to be adapted for analysis of individually variable physiological parameters such as moult cycle, age, sex or breeding stage. The aim is to establish intervals of normality for immune factors. In addition, the effect of defined physico-chemical or environmental parameters needs to be considered. With these assays and standards, the effect of organic or chemical pollutants and antibiotics used in aquaculture could be studied. So also could the real effect of immunostimulants, which are until now empirically used. For validation of health controls at the population level, immune responses of cultured shrimp should also be analysed under stressful conditions and/or in the presence of pathological injuries.

CONCLUSIONS

The SI&DC integrated approach as proposed here would greatly contribute to the improvement of knowledge about shrimp physiology. These findings could be exploited in analysing the expression of immune effectors and in the evaluation of shrimp immune responses. Such knowledge could be used both for the establishment of prophylactic measures and for further application in the genetic selection of disease resistant shrimp.

Litterature cited

Bachère E, Cedeno V, Rousseau C, Gendreau S, Robalino J, Destoumieux D, Boulo V, Cadoret JP, Mialhe E. (1997) Transgenic crustaceans. World Aquaculture 28:51-55

Bachère E, Mialhe E, Rodriguez J (1995) Identification of defence effectors in the haemolymph of Crustaceans with particular reference to the shrimp *Penaeus japonicus (Bate)*: prospects and applications. Fish & Shellfish Immunol 5:597-612

Bauchau A G (1981) Crustaceans. In: Rowley R (ed) Invertebrate blood cells, New-York, Academic Press, p 386-420

Bell KL, Smith VJ (1993) In vitro superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.). Dev Comp Immunol 17:211-219

Broekaert WF, Terras FRG, Cammue BPA, Osborn RW (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol 108:1353-1358

Destoumieux D, Bulet P, Loew D, Van Dorsselaer A, Rodriguez J, Bachère E (1997) Penaeidins: A new family of antimicrobial peptides in the shrimp *Penaeus vannamei* (Decapoda). J Biol Chem 272:28398-28406

Gargioni R, Barracco MA (1998) Hemocytes of the palaemonids *Macrobrachium rosenbergii* and *M. acanthurus*, and of the Penaeid *Penaeus paulensis*. J Morphol 236:209-221

Hetru C, Bulet P, Cociancich S, Dimarcq JL, Hoffmann D, Hoffmann JA (1994) Antibacterial peptides/polypeptides in the insect host defense: a comparison with vertebrate antibacterial peptides/polypeptides. In: Hoffmann JA, Janeway CA, Natori JS (eds) Phylogenetic perspectives in immunity: the insect host defense, Boca Raton, CRC Press, p 43-66

Hoffmann JA, Reichhart J-M (1997) *Drosophila* immunity. Trends Cell Biol 7:309-316

Hultmark D (1993) Immune reactions in *Drosophila* and other insects: a model for innate immunity. Trends Genet 9:178-183

Iwanaga S, Kawabata S-I, Muta T (1998) New types of clotting factors and defense molecules found in Horseshoe Crab

hemolymph: Their structures and functions. J Biochem 123:1-15

Johansson MW, Söderhäll K (1985) Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. J Comp Physiol 156:175-181

Jomori T, Natori S (1992) Function of the lipopolysaccharide-binding protein of *Periplaneta americana* as an opsonin. FEBS 296:283-286

Kato Y, Motoi Y, Taniai K, Kadono-Okuda K, Yamamoto M, Higashino Y, Shimabukuro M, Chowdhury S, Xu J, Sugiyama M (1994) Lipopolysaccharide-lipophorin complex formation in insect hemolymph : a common pathway of lipopolysaccharide detoxification both in insects and in mammals. Insect Biochem Molec Biol 24:547-555

Kopacek P, Grubhoffer L, Söderhäll K (1993a) Isolation and characterization of a hemagglutinin with affinity for lipopolysaccharides from plasma of the crayfish *Pacifastacus leniusculus*. Dev Comp Immunol 17:407-418

Kopacek P, Hall M, Söderhäll K (1993b) Characterization of a clotting protein, isolated from plasma of the freshwater crayfish *Pacifastacus leniusculus*. Eur J Biochem 213:591-597

Lanz-Mendoza H, Bettencourtn R, Fabbri M, Faye I (1996) Regulation of the insect immune response : the effect of hemolin on cellular immune mechanisme. Cell Immunol 169:47-54

Martin GG, Graves L (1985) Fine structure and classification of shrimp hemocytes. J Morphol 185:339-348

Mialhe E, Bachère E, Boulo V, Cadoret JP, Saraiva E, Carrera L, Calderon J, Colwell R (1995) Biotechnology-based control of disease in marine invertebrates: development of molecular probe diagnostics and disease-resistant transgenic shrimps and molluscs. Mol Marine Biol Biotech 4:275-283

Mialhe E, Boulo V, Cadoret J-P, Cedeno V, Rousseau C, Motte E, Gendreau S, Bachère E (1997) Gene transfer technology in marine invertebrates. In: Houdebine JM (ed) Transgenic animals: generation and use, Amsterdam, Harwood Academic Publishers, p 151-155

Müller M, Brem G (1994) Transgenic strategies to increase disease resistance in livestock. Reprod Fertil Dev 6:605-613

Natori S, Kubo T (1996) Role of lectins in development and morphogenesis in insects. In: Söderhäll K, Iwanaga S, Vasta GR (eds) New Directions in Invertebrate Immunology, Fair Haven, SOS publications, p 175-187

Omori SA, Martin GG, Hose JE (1989) Morphology of hemocyte lysis and clotting in the ridgeback prawn, *Sicyonia ingentis*. Cell Tissue Res 255:117-123

Perazzolo LM, Barracco M (1997) The prophenoloxidase activating system of the shrimp *Penaeus paulensis* and associated factors. Dev Comp Immunol 21:385-395

Ratcliffe NA (1993) Cellular responses of Insects: unresolved problems. Parasites and Pathogens of Insects. Academic Press, Inc, p 267-304

Rodriguez J, Boulo V, Mialhe E, Bachère E (1995) Characterisation of shrimp haemocytes and plasma components by monoclonal antibodies. J Cell Sci 108:1043-1050

Rosenberry B (1996) World shrimp farming 1996. Rosenberry R, (ed) Shrimp News International. 167 p

Schnapp D, Kemp GD, Smith VJ (1996) Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. Eur J Biochem 240:532-539

Sequeira T, Vilanova M, Lobo-Da-Cunha A, Baldaia L, Arala-Chaves M (1995) Flow cytometric analysis of molt-related changes in hemocytes types in male and female *Penaeus japonicus*. Biol Bull 189:376-380

Söderhäll K, Cerenius L (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. Cur Opinion Immunol 10:23-28

Söderhäll K, Cerenius L, Johansson MW (1996) The prophenoloxidase activating system in invertebrates. In: Söderhäll K, Iwanaga S, Vasta GR (eds) New Directions in Invertebrate Immunology, Fair Haven, SOS publications, p 229-253

Song YL, Hsieh YT (1994) Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. Dev Comp Immunol 18:201-209

Strittmatter G, Wegener D (1993) Genetic engineering of disease and pest resistance in plants: present state of the art. Z Naturforsch 48C:673-688

Vargas-Albores F, Guzman MA, Ochoa JL (1993a) An anticoagulant solution for haemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*). Comp Biochem Physiol 106A:299-303

Vargas-Albores F, Guzman MA, Ochoa JL (1993b) A lipopolysaccharide-binding agglutinin isolated from Brown shrimp (*Penaeus californiensis* Holmes) haemolymph. Comp Biochem Physiol 104B:407-413

Vargas-Albores F, Jimenez-Vega F, Söderhäll K (1996) A plasma protein isolated from brown shrimp (*Penaeus califoriensis*) which enhances the activation of prophenoloxidase system by b-1,3-glucan. Dev Comp Immunol 20:299-306

Yoshida H, Kinoshita K, Ashida M (1996) Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. J Biol Chem 271:13854-13860.

Review of Crustacean Immunity

Kenneth Söderhäll

Department of Phsyiological Mycology, University of Uppsala, Villavägen 6, 752 36 Uppsala, Sweden

ABSTRACT: Crustaceans lack immune memory and therefore have to rely on innate immune reactions. One such reaction is the clotting process which is very efficient and rapid. It is based on a clotting protein present in plasma and a transglutaminase in blood cells. The clotting protein has been cloned and belongs to the vitellogenin superfamily of proteins. Clotting proteins have also been purified from shrimp and they are very similar in properties to those of crayfish. Another innate immune defence process is the socalled proPO-system which is a non-self recognition system. Upon activation by microbial products, it generates several factors which aid in the elimination of foreign particles or parasites. This system has been studied in greatest detail in freshwater crayfish and most of the proPO-components have been purified and cloned. Recently, we have also been able to clone some proPO-componenets from a shrimp, *Peneaus monodon*. Cellular immune reactions are important in defence and two communicating proteins have been isolated and cloned. They are the beta-1,3-glucan binding protein and peroxinectin. Both of these proteins are associated with the proPO-system. Of great surprise was the finding that peroxinectin had a functional peroxidase domain but that its peroxidase activity was not involved in its cell adhesion activity. Instead, a KGD motif was found to be of importance. Recently antibacterial peptides have been characterised in shrimp and they have been named penaeidins. In this talk I will attempt to review our recent findings of the proPO-system in crayfish and shrimp and how it functions in crustacean defence.

KEY WORDS: crustaceans, immunity, shrimp, proPO, peroxynectin, peroxidase, transglutaminase, vitellogenin, penaeidin,

Söderhäll K (1998) Review of crustacean immunity. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Evidence for the haemocytic origin of lymphoidal spheroids in *Penaeus monodon*

Margaretha Anggraeni, *Leigh Owens

Department of Microbiology and Immunology, James Cook University, Townsville, Queensland 4811, Australia

ABSTRACT: The lymphoid organ of penaeid prawns is believed to function within the immune system, but its actual role is still debatable. The production of spheroids within the lymphoid organ has been associated with disease states but again their role is unknown. We present evidence that the spheroids are made of accumulations of large granular haemocytes in the haemal sinuses of the lymphoid organ. These spheroids are possibly removed via moulting as the animal grows. Within the groups of prawn haemocytes, hyalinocytes have neither prophenoloxidase (proPO) nor peroxidase activity; small granular haemocytes have only proPO activity, whilst large granular haemocytes have both. Therefore, histological assays for proPO and peroxidase were conducted on lymphoid organs to discriminate the role of haemocytes in this organ. Only the spheroid cells were positive for proPO and peroxidase. Occasionally a cell in the stromal matrix stained positive. The relative lack of small granular haemocytes was surprising. Staining with a universal crustacean, 18S rRNA gene probe showed the spheroid cells to be metabolically highly active relative to stromal matrix cells. A gene probe for spawner-isolated mortality virus (SMV), a probable ssDNA virus, mostly localised in the spheroid cells. Apoptosis assays demonstrated that the spheroid cells were highly apoptotic. This result must be treated with some caution as the apoptosis assay would also bind to DNA strands of viruses such as SMV. Time-course experiments suggested that the spheroids were periodically removed and it is hypothesised they were removed upon moulting. In summary, spheroid cells are most likely accumulations of exocytosed large granular haemocytes that are probably at the end of their functional life. Coupled with the work of Martin and Hose, a function for the lymphoid organ is postulated. It seems likely that large granular haemocytes are the major viral removal mechanism for penaeid prawns and it may explain why so many viruses are visualised in the lymphoidal spheroids (e.g., lymphoidal parvovirus, lymphoid organ virus, lymphoid organ vacuolisation virus, gill-associated virus, rhabdovirus of penaeid shrimp, white spot syndrome virus, Taura virus, and spawner-isolated mortality virus).

KEY WORDS: *Penaeus monodon*, lymphoid organ, granular haemocytes, sphaeroids, apoptosis

Anggraeni M, Owens L (1998) Evidence for the haemocytic origin of lymphoidal spheroids in *Penaeus monodon*. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: leigh.owens@jcu.edu.au

Immuno-physiology of the Black Tiger Shrimp (*Penaeus monodon*)

Kidchakan Supamattaya

Aquatic Animal Health Research Center, Faculty of Natural Resources, Prince of Songkla University Had Yai, Songkhla 90110, Thailand

ABSTRACT: Basic immuno-physiological characteristics of the black tiger shrimp (*Penaeus monodon*) were studied. These included numbers and types of haemocytes, phagocytic activity of hemocytes, and microscopic and ultrastructural morphology of hemocytes. Phenoloxidase activity in the cytoplasm of hemocytes, serum electrolytes and osmolarity were also determined. These parameters were analyzed in healthy shrimp under normal laboratory and normal pond culture conditions. The effects of molting cycle, size, sex, water quality and aquaculture chemicals on immuno-physiological functions of shrimp were also studied.

KEY WORDS: *Penaeus monodon*, immunity, immuno-physiology, phenoloxidase, haemocytes

Supamattaya K (1998) Immuno-physiology in the black tiger shrimp (Penaeus monodon). *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Studies on IgY for Passive Immunization of Shrimp Against White Spot Syndrome Virus

***[1]Victoria Alday-Sanz, [1]Sarayut Thaikua, [2]A.N. Yousif**
[2]L.J. Albright, [1]T.W. Flegel

[1]Department of Biotechnology, Faculty of Science, Mahidol University, Rama 6 Rd, Bangkok 10400, Thailand
[2]Department of Biological Sciences, Simon Fraser University, Burnaby, B.C. V5A 1S6, Canada

ABSTRACT: In this study, we administered egg yolk immunoglobulins (IgY) against white spot syndrome virus (WSSV) to *Penaeus monodon* as a possible method of preventing or treating the disease. A semicontinous method for the production of WSSV was developed in the mud crab, *Scylla serrata*. The carapace was drilled over the heart and fitted with a rubber serum plug, providing direct access to the heart by hypodermic syringe. Crabs were infected with shrimp WSSV infected tissue extract (approximately $5x10^5$ viral particles/crab). After 4 days allowed for viral replication, haemolymph was collected from the heart daily or on alternate days if the crabs appeared somewhat weak. WSSV was purified by means of a sucrose gradient to a concentration of 10^9 viral particles/ml as determined by PCR tests of serial dilutions. The virus was inactivated with formaldehyde (0.6% final concentration) and used for the production of Ig in egg yolk (IgY). Hens were inoculated with inactivated WSSV and eggs were collected five weeks after the first inoculation. Egg yolks were washed with distilled water to remove traces of albumin and mixed 1:3 with precooled (-20°C) isopropyl alcohol. The precipitate was treated the same way another 3 times followed by the same treatment using acetone. The final residue was filtered, washed with a small amount of acetone and allowed to dry at room temperature. This powder was used as IgY. IgY were used for the development of an ELISA to detect WSSV and to detect anti-WSSV activity. Oral and injection routes for IgY administration were tested, and *in vitro* studies were carried out to determine anti-WSSV IgY activity after exposure to the digestive enzymes and haemolymph. For oral administration, pellets were coated with 2% IgY and fed to the shrimp twice daily for 7 days followed by feeding with non-coated pellets. Haemolymph samples were collected from 3 shrimp 8 hr after the first feeding and every 24 hr thereafter for 14 days. No IgY activity was detected in any of these haemolymph samples. After IgY injection, haemolymph samples were collected from 3 shrimp at 15 min and thereafter for up to 17 days on 21 occasions. IgY activity was detected in the samples collected after 15 and 30 min, but in no sample thereafter. *In vitro*, IgY were incubated with hepatopancreatic and stomach extracts diluted from 1:10 to 1:1000 from 15 min to 4 hr. The ELISA results showed a sharp decrease of IgY activity within the first 15 min and a subsequent slow but steady decrease over the next 4 hr. Even IgY incubated with extracts diluted at 1:1000 showed some loss of activity. For *in vitro* study on the effect of haemolymph, IgY were incubated with haemolymph and activity was checked on 10 occasions after 5 min and up to 5 hr thereafter. IgY activity was partially reduced within the first 5 min of incubation but remained stable thereafter for the remainder of the 4 hr incubation time. The difference from results obtained *in vivo* may have resulted because heamolymph for the *in vitro* tests was diluted 1:2 in lobster haemolymph buffer anticoagulant. It was concluded that orally administered IgY was quickly inactivated by digestive enzymes and that injected IgY was fully or partially inactivated by haemolymph. These results suggested that the use of IgY for passive immunization of shrimp would be unlikely to succeed.

KEY WORDS: IgY, egg yolk immunoglobulin, shrimp, passive immunization, white spot syndrome virus, WSSV

INTRODUCTION

The WSSV epizootic in Asia has been the cause of serious losses for shrimp farmers and is largely responsible for lost production in the range of 3 billion dollars per year (Lundin 1997). Due to the wide range of potential hosts of WSSV (Flegel 1997), control of the disease is extremely difficult. Disease control by passive immunization has been widely used in mammals (Guarino *et al*. 1995) including fish (Nakamura *et al*. 1990, Gutierrez *et al*. 1993) with variable results. The success of oral administration appears to be dependent on the gastric conditions of the animal, since a significant amount of IgY given orally is considered to be degraded and inactivated at this stage. Shimizu *et al*. (1988) and Ohtani *et al.* (1992), reported that the activity of IgY examined by ELISA was quite stable on incubation with trypsin or chymotrysin but it was sensitive to pepsin, especially at pH lower than 4.5. Shrimp rely mainly in trypsin and to a lesser degree, chymiotripsin for protein digestion (Chuang *et al*. 1985, Yen & Tsen 1985, Glass *et al.* 1989, Lan & Pan 1991). Therefore, we considered that it might be possible to succeed in using oral passive immunization to protect shrimp from WSSV infection. Parenteral administration of Ig to shrimp has been reported to successfully prevent mortality after bacterial challenge for a 17 days period

Alday-Sanz V, Thaikua S, Yousif AN, Albright LJ, Flegel TW (1998) Studies on IgY for passive immunization of shrimp against white spot syndrome virus. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: frvas@mahidol.ac.th

(Lee *et al.* 1997). In this study, we administered egg yolk immunoglobulins (IgY) against white spot syndrome virus (WSSV) to *Penaeus monodon* to assess their potential for use in preventing or treating WSSV disease.

MATERIALS AND METHODS

Production of the virus

The initial WSSV sample was provided by the Charoen Pokphand (CP) Shrimp Research Center from WSSV experimentally infected *Penaeus monodon* shrimp. This sample was amplified by injecting it in laboratory maintained *Penaeus monodon*. Haemolymph and subcuticular epidermis were subsequently collected from the moribund shrimp and homogenised (1:2) in lobster haemolymph buffer (LHB) (Boonyaratpalin, *et al*. 1993). This stock of WSSV was used for further production of the virus. Mud crabs (*Scylla serrata*) were used to produce WSSV, since they appear to tolerate high levels of WSSV viremia without showing any sign of disease (Supamattaya *et al.* 1998, Kanchanaphum *et al.* 1998). Their carapace was drilled over the heart and fitted with a rubber serum plug (Fig. 1), providing direct access to the heart by hypodermic syringe. Crabs were injected with shrimp WSSV tissue extract (containing approximately $5x10^5$ viral particles/crab). After allowing 4 days for viral replication, 0.5ml of haemolymph (1:2 in LHB) was collected from the heart daily or on alternate days if the crabs appeared somewhat weak. The haemolymph was stored at –70ºC until sufficient was collected for further processing.

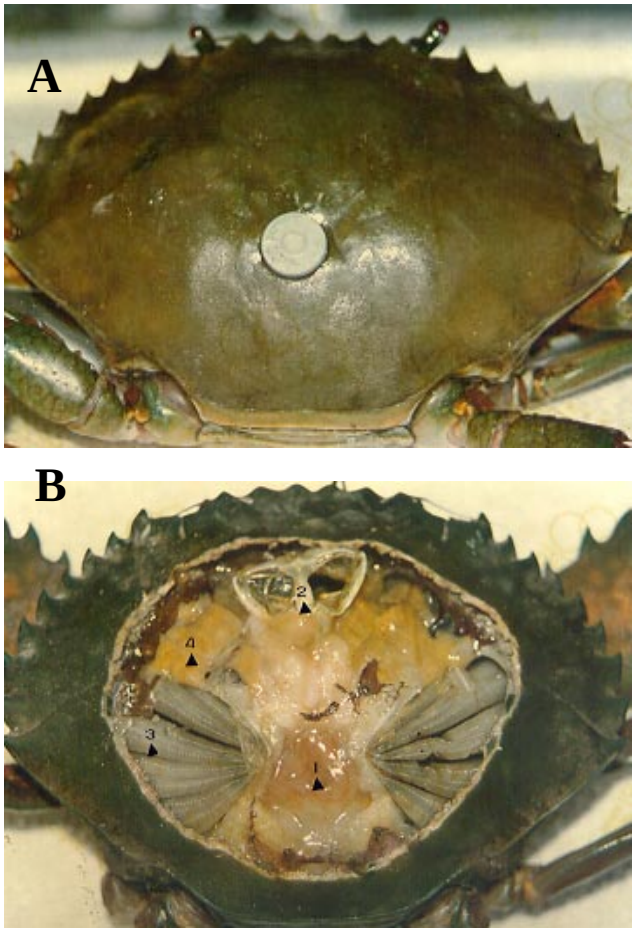


Figure 1. Mud crabs (*Scylla serrata*) prepared for semi-continuous production of WSSV. (A) External view of a crab fitted with a rubber serum plug providing direct access to the heart by hypodermic syringe. (B) View of internal anatomy after removal of the carapace (1 = heart , 2 = stomach, 3 = gills, 4 = hepatopancreas).

Purification of the virus

Purification was carried out following the modified method described by Takahashi *et al.* (1996). Stored haemolymph was pooled and thawed before centrifugation at 3000g for 30 minutes at 4ºC. The supernatant was then filtered through a 0.45um membrane and the filtrate ultracentrifuged at 100,000g for 1h. at 4ºC. The pellet was then soaked in a small volume of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) at 4ºC overnight. The pellet was then gently resuspended and layered onto the top of a discontinuous gradient of 10 and 50% (w/v) sucrose and ultracentrifuged at 100,000g for 1h. at 4ºC. The viral band, visible on top of the 50% sucrose solution, was collected with a Pasteur pipette and washed with TE buffer by centrifuging at 100,000g for 1h. at 4ºC. Finally the pellet was resuspended in 50µl of TE buffer and frozen at -70ºC.

Estimation of viral concentration

Serial dilutions of WSSV infected haemolymph were used as the template for specific WSSV PCR according to the protocol by Kanchanaphum *et al.* (1998), such that the lowest number of viral particles to give a clearly visible amplicon band was considered approximately equal to 500 viral genomes. This protocol was based on calculations derived from PCR assays of a known quantity of purified, serially diluted WSSV DNA and assuming a viral genome size of approximately 160 kbp.

Inactivation of the virus

WSSV was inactivated by adding formaldehyde to achieve final concentration of 0.6% in the suspension. This inactivated viral suspension (IVS) (approximately 10^9 estimated viral particles per µl).

Anti-WSSV IgY production

Hens were injected in the breast with 30 µl of IVS mixed with twice the volume of complete Freud's adjuvant. Injections were repeated every 7 days for two weeks using the same dose of antigen but mixed with incomplete Freud's adjuvant. Eggs began to be collected five weeks after the first inoculation. They were cracked and the albumin was removed. The intact yolk was washed gently with distilled water to remove as much albumin as possible. The yolk sac was then cut open and the yolk material was mixed 1:3 with precooled (-20ºC) isopropyl alcohol at 5ºC for 20-30 min. The precipitate was allowed to settle for 5 min before the supernatant was decanted. This procedure was repeated three times with isopropyl alcohol and twice with precooled (-20ºC) acetone, for complete removal of lipids. The final residue was filtered (Whatman filter paper no. 1, UK), washed with a small amount of acetone, and left to dry at room temperature. The resultant powder was a mixture of yolk proteins, including anti-WSSV IgY, but for the purpose of this paper it will be referred to simply as IgY. IgY were stored at

5°C until used. Eggs collected from the same hens prior to immunization were used to extract the control IgY.

Development of an ELISA

An ELISA was developed using anti-WSSV IgY and control IgY.

IgY oral administration trial

Commercial shrimp pellets were coated with 2% anti-WSSV IgY using fish oil and fed to 50 *P. monodon* juvenile shrimp twice daily for 7 days. Feeding then continued with uncoated pellets. Haemolymph samples were collected from the ventral sinus of the shrimp (1:2 LHB), 8 hr after the first feeding with coated pellets and every 24 hr thereafter for 14 days. Three shrimp were sampled each time. Haemolymph of shrimp fed with untreated pellets was used as the control. Samples were checked for anti-WSSV IgY activity in the haemolymph by ELISA. The experiment was repeated twice.

IgY injection trial

IgY powder was resuspended in LHB (1g:20ml) and 50ml of the suspension was injected intramuscularly at the 5th abdominal segment of 50 *P. monodon* juvenile shrimp. Haemolymph samples were collected from the ventral sinus of the shrimp (1:2 LHB) at 15 min, 30 min, 1 hr, 2 hr, 4 hr, 24 hr and every 24 hr thereafter for 17 days after IgY injection. Three shrimp were sampled at each time interval. Haemolymph of shrimp collected prior to the IgY injection was used as the control. Samples were checked for anti-WSSV IgY activity in the haemolymph by ELISA. The experiment was repeated twice.

In vitro digestibility of IgY

Hepatopancreases and stomachs were removed from 5 *P. monodon* juvenile shrimp and homogenized in four times their weight of 0.05M Tris buffer (pH 7.5). The homogenate was ultrasonicated for 10 minutes in an ice bath and centrifuged at 5000g at 4°C for 30 minutes. The supernatant was filtered (0.22µm) and regarded as crude enzyme extract (CE). IgY diluted 1:2000 in LHB was incubated at 28°C (1:1) with CE diluted 1:10, 1:20, 1:100. 1:500 and 1:1000. Samples were collected at 15 min, 30 min, 1 hr, 2 hr and 4 hr after the start of incubation and further digestion was avoided by freezing at –20°C. Control samples were not exposed to the enzyme extract. Samples were checked for anti-WSSV IgY activity by ELISA . The experiment was repeated twice.

In vitro incubation of IgY and haemolymph

Haemolymph collected from the ventral sinus (1:2 in LHB) of 3 *P. monodon* juvenile shrimp, was incubated at 28°C (1:1) with IgY diluted 1:2000 in LHB for 5 min, 10 min, 15 min, 30 min, 1 hr, 2 hr and 4 hr. Control samples were not exposed to haemolymph. Samples were checked for anti-WSSV IgY activity by ELISA. The experiment was repeated twice.

RESULTS

Crabs were bled for 15 to 20 days and gave an estimated haemolymph concentration of WSSV over this period of 10^5-10^6 viral particles/µl. The purified virus suspension had an estimated concentration of 10^9 viral particles/µl . The development of the ELISA showed that dilution of the IgY powder at 1:2000 gave a strong specific reaction. The suspension of WSSV in the antigen used for the ELISA had a concentration of approximately 10^5 viral particles/ml. As the titration of the virus could not be estimated more accurately, a stock of the virus suspension was prepared and aliquoted at the beginning of the study, so that every experiment could be carried out with the same viral suspension. This allowed for comparison of results amongst the various tests. The ELISA could also be used to detect WSSV in infected animals.

In the oral administration trial, no anti-WSSV IgY activity was detected by ELISA in any of the haemolymph samples. ELISA results from the injection trial showed anti-WSSV IgY activity in haemolymph samples collected at 15 and 30 min after injection, but in no sample thereafter. ELISA results on the *in vitro* digestibility of IgY showed a sharp decrease in IgY activity within the first 15 min after mixing with CE, followed by a slow but steady decrease over the next 4 hr. IgY activity of samples incubated with CE diluted 1:10 and 1:20 was almost nil after 4 h incubation and even CE diluted at 1:1000 showed some loss of activity. ELISA values for the controls remained constant during 4 hr incubation at 28°C (Table 1). ELISA results from *in vitro* incubation of IgY and haemolymph showed a partial reduction in IgY activity within the first 5 min of incubation but a stable activity thereafter for the remainder of the 4 hr incubation time. ELISA values for the controls remained constant during 4 hr incubation at 28°C (Table 2).

Table 1. ELISA results from the *in vitro* digestibility of IgY by crude enzyme extract (CE). OD_{450} data are shown for two replicates at each incubation time interval.

CE	Incubation time									
Dilution	15 min		30 min		1 hr		2 hr		4 hr	
None	0.009	0.006	0.003	0.029	0.076	0.054	0.017	0.033	0.047	0.045
1:10	0.221	0.261	0.153	0.199	0.111	0.070	0.098	0.124	0.096	0.073
1:20	0.486	03245	0.410	0.417	0.255	0.288	0.285	0.192	0.080	0.079
1:100	0.898	0.841	0.870	0.986	0.819	0.692	0.718	0.715	0.517	0.491
1:500	1.314	1.336	1.135	1.108	1.396	1.329	1.320	1.481	1.117	1.027
1:1000	1.990	1.834	1.840	1.892	1.784	1.812	1.796	1.765	1.466	1.426
Control	2.320	2.203	2.250	2.174	2.059	2.042	2.034	2.061	2.021	2.056
Blank	0	0	0	0	0	0	0	0	0	0

Table 2. ELISA results from the *in vitro* incubation of IgY with haemolymph. OD_{450} data are shown for two replicates at each incubation time interval.

Incubation time	Shrimp 1	Shrimp 2	Shrimp 3	Control	Blank
5min	1.513	1.689	1.728	2.128	0
	1.589	1.098	1.603	2.186	0
10min	1.528	1.598	1.635	2.028	0
	1.680	1.603	1.654	2.256	0
15min	1.728	1.402	1.709	2.062	0
	1.652	1.592	1.713	2.164	0
30min	1.629	1.098	1.667	2.222	0
	1.603	1.032	1.543	2.088	0
1hr	1.418	1.467	1.566	2.067	0
	1.523	1.574	1.666	2.058	0
2hr	1.527	1.488	1.532	2.066	0
	1.534	1.383	1.654	2.102	0
4hr	1.510	1.341	1.761	2.022	0
	1.530	1.282	1.698	2.091	0

DISCUSSION

The use of crabs allowed for the semi-continous production of WSSV. It proved to be a convinient method of viral preparation since the crabs survived much longer than WSSV infected shrimp. Chicken egg yolk was a good source of IgY. Lesli and Clem (1969) reported that more than 100-200 mg of antibody can be obtained from one egg. We obtained what we have referred as IgY (a mixture of IgY and other yolk proteins). Once diluted 1:2000, it could be used as specific anti-WSSV IgY that may be developed into an inexpensive diagnostic test for WSSV in shrimp without further purification.

Two major difficulties were identified with respect to possible use of IgY for passive immunization in shrimp. First, orally administered IgY would be inactivated by digestive enzymes, and second, any IgY absorbed into the haemolymph would also be at least partially inactivated. By contrast, a previous study carried out in gold fish (an agastric species not producing pepsin) showed that orally administered rabbit IgG was transported from the alimentary canal into blood circulation with preserved antigen-binding activity (Nakamura *et al*. 1990). Our result with the shrimp seems to contradict the facts that shrimp apparently rely mainly in trypsin and to a lesser degree chymotripsin for protein digestion (Chuang *et al*. 1985, Yen & Tsen 1985, Glass *et al*. 1989, Lan & Pan 1991) and that the activity of IgY examined by ELISA has been reported to be quite stable on incubation with trypsin or chymotrysin (Shimizu *et al*. 1988, Ohtani *et al*. 1992). This suggests that another enzyme(s) in the shrimp digestive extact degraded the IgY. It might also be argued that the IgY was pH sensitive. For example, Petschow and Talbott (1994) showed that some reduction of the neutralising activity of Ig occurred after gastric-phase digestion, and that this was due to acidic pH and not proteolytic degradation. In the case of the shrimp, the hepatopancreas is the major site for proteolysis (Glass *et al*. 1989) which is carried out in extracellular and intracellular steps. The endopeptidases, trypsin and chimiotrypsin, are excreted into the digestive tract and have highest activity at pH 7.5, the physiological pH of the hepatopancreas. The exopeptidases carbopeptidase A and B and aminopeptidase, are intracellular and have highest activity at pH 4.0 (Al-Mohanna and Nott 1986). However, our *in vitro* digestibility trials were carried out at pH 7.5, so there was no possibility of inactivation by low pH, and the results suggested that IgY inactivation was due to the effect of unidentified digestive enzymes. Whatever the reason, our oral administration trials showed that no anti-WSSV activity could be detected in the haemolymph of shrimp fed with IgY coated pellets. Perhaps any IgY that was absorbed was recognised as foreign material and inactivated by unknown components in the haemolymph.

Lee *et al*. (1997) reported that parenteral administration of Ig to shrimp successfully prevented mortality from bacterial challenge for 17 days, although they did not test for Ig activity in the haemolymph. The results from our IgY injection trial showed that anti-WSSV IgY activity could be detected for only 15 to 30 min after injection but not thereafter. Thus, the IgY appeared to be rapidly neutralised after injection. By contrast, *in vitro* incubation of IgY with haemolymph, showed rapid but only partial inactivation. This happened within the first 5 min of incubation, after which activity remained stable. The difference from the injection trial results may have resulted because the heamolymph for the *in vitro* tests was diluted 1:2 in LHB anticoagulant. This may have diluted its capacity to inactivate the IgY. If so, it would suggest that sufficient doses of IgY might be able saturate this neutralizing capability and achieve higher measurable levels.

Further work needs to be done before ruling out the possible use of passive immunization in shrimp. Ultimately, viral challenge tests would be required. However, the present results suggest that practical use of IgY for passive immu-

nization of shrimp would first require the development of adaptations to protect the reagent from digestion, promote absorption and to overcome haemolymph neutralization.

Acknowledgements. We would like to thank the National Science and Technology Development Agency (NSTDA) for support of a Post Doctoral Fellowship for Dr. Alday-Sanz and for funding to carry out this work. We would also like to thank Dr. Boonsirm Withyachumnarnkul and the CP Shrimp Research Center for assistance in obtaining WSSV viral extracts.

LITERATURE CITED

Al-Mohanna SY, Nott JA (1986) B-cells and digestion in the hepatopancreas of *Penaeus semisalcatus* (Crustacea: Decapoda). Biol Assoc UK 66: 403-414

Boonyaratpalin S, Supamataya K, Kasornchandra J, Direkbusarakom S, Ekpanithanpong U, Chantanachookin C (1993) Non-occluded baculo-like virus the causative agent of yellow-head disease in the black tiger shrimp *Penaeus monodon.* Gyobo Kenkyu (Fish Pathol) 28:103-109

Chuang JL, Lee MF, Jenn JS (1985) Comparison of digestive enzyme activities of five species of shrimp cultured in Taiwan. J Fish Soc Taiwan 12: 43-53

Flegel TW (1997) Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. Wld J Microbiol Biotechnol 13 (4): 433-442

Glass HJ, MacDonald NL, Moran RM, Stark JR (1989) Digestion of protein in different marine species. Comp Biochem Physiol 94B (3): 607-611

Guarino A, Carnani RB, Russo S (1995) Developments in the treatment of rativurs gastroenteritis. Clinical Immunother 3 (6): 476-484

Gutierrez MA, Miyazaki T, Hatta H, Kim M (1993) Protective properties of egg yolk IgY containing anti-*Edwarsiella tarda* antibody against paracolo disease in the Japanese eel, *Anguilla japonica*. J Fish Dis 16 113-122

Kanchanaphum P, Wongteerasupaya C, Sitidilokratana N, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1998) Experimental transmission of white spot syndrome virus (WSSV) from crabs to shrimp *Penaeus monodon*. Dis Aquat Org (in press)

Lan CC, Pan BS (1991) Effect of substrates on proteolytic enzyme activites of midgut gland polyhedrosis virus J Virol 27: 754-767

Lee KK, Liu PC, Kou GH, Chen SN (1997) Passive immunization of the tiger prawn *Penaeus monodon*, using rabbit antisera to *Vibrio harveyi*. Lett Appl Microbiol 25: 34-37

Lesli GA, Clem L W (1969) J Exper Medicine 130: 1337-1352

Lundin CG (1997) Global attempts to address shrimp disease. Proceedings to the Second Asian Pacific Marine Biotechnology Conference, Phuket, Thailand, National Center for Genetic Engineering and Biotechnology, Bangkok

Nakamura O, Kobayashi M, Suzuki Y, Aida K, Hanyu I (1990) Transport of orally administered rabbit IgG into blood circulation of goldfish. Nippon suisan Gakkaishi 56 (11) 1749-1753

Petchow, Talbott (1993) Reduction in virus neutralizing activity of bovine colostrum immunoglobulin concentrate by gastric and digestive enzymes. J Pediatric Gastro Nutr 19: 228-235

Shimizu M, Fitzsimmons RC, Nakai S (1988) J Food Sci 53: 1360-1366

Supamattaya K, Hoffmann RW, Boonyaratpalin S, Kanchanaphum P (1998) Experimental transmission of white spot syndrome virus (WSSV) from balck tiger shrimp *Penaeus monodon* to the sand crab *Portunis pelagicus*, mud crab *Scylla serrata* and krill *Acetes* sp. Dis Aquat Org 32: 79-85

Ohtani H, Matsumoto K, Saeki A, Hosno A, Lebens M (1992) Lebensm. Wiss. Technol 24: 152-258

Yen CM, Tsen HY (1985) Application of swine blood meal for shrimp feed prepartion. A study on the relatioship between the proteolytic enzymes in the digestive tracts of grass prawn and different feed protein. J Chinese Agricul Chem Soc 23 (3-4): 328-338

Takahashi Y Itami T, Maeda M, Suzuki N, Kasornchandra J, Supamattaya K, Khongpradit R, Boonyaratpalin S, Kondo M, Kawai K, Kusuda R, Hirono I, Aoki T (1996) Polymerase chain reaction (PCR) amplification of bacilliform virus (RV-PJ) DNA in *Penaeus japonicus* Bate and systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *Penaeus monodon* Fabricius. J Fish Dis 19: 399-403

Effect of Oxygen Depletion on Some Parameters of the Immune System in Black Tiger Shrimp (*Penaeus monodon*)

Sataporn Direkbusarakom, Yaowanit Danayadol

National Institute of Coastal Aquaculture Songkhla Thailand 90000

ABSTRACT: Environmental stress (e.g., oxygen depletion) is a common risk in intensive shrimp culture ponds that are stocked at a high density or where the shrimp are over fed. This stress can lead to the occurrence of disease. This study aimed to investigate the effect of oxygen depletion on the immune system in black tiger shrimp, *P. monodon*, in order to better understand how it causes disease. The experiments consisted of 2 treatments (i.e., control groups at DO about 6 ppm and oxygen depleted groups at DO about 1.8-2.0 ppm) in 40 liter aquaria using shrimp of 10-15 g. Oxygen depletion was achieved by stopping aeration for about 6 h. During the experiment water temperature and pH were 29-30°C and 7.9-8.1, respectively. Haemolymph from the experimental shrimp was sampled to determine phagocytic activity and efficiency in clearing luminescent bacteria. Based on 5 trials, the results showed that phagocytic activity was 34.7% for control groups and 28.3% in oxygen depleted groups. The average clearance efficiency of the oxygen depleted groups was approximately 50% less than that of the control groups. These results suggested that low dissolved oxygen environmental stress can have a significant negative affect on the shrimp immune system and may therefore lead to disease outbreaks and mortality.

KEYWORDS: shrimp, oxygen, phagocytosis, clearance efficiency Introduction

INTRODUCTION

Intensive cultivation of the black tiger shrimp, *Penaeus monodon*, began in Thailand in the mid 1980's (Flegel *et al*. 1995) and the production rose rapidly to reach a record export quantity of 190,650 metric tonnes in 1994. Since 1995, the production gradually decreased to 130,728 metric tones in 1997 (Department of Economics and Commerce 1997). Diseases are considered to be the most importanct causes for this decrease. Outbreaks of yellow head disease were the most serious problem in central and southern Thailand during 1993-1994, while white spot disease has been the most serious problem from 1994 to 1996. Also, from mid 1996 until now, luminescent bacterial disease is increasingly considered to be the cause for unsuccessful shrimp culture.

According to Snieszko's (1974) well-known diagram, appropriate states of the host, pathogen, and environment are necessary in order for disease outbreaks to occur. One important state of the environment is the presence of sufficient oxygen to maintain systems vital to the health of an aquatic ecosystem. Since all large aquatic organisms, plant or animal, depend on oxygen, its concentration in a pond is a good indicator of water quality. Low levels of oxygen are commonly found in intensive shrimp ponds that are stocked at high density or where shrimp are being over fed.

Thus, this study aimed to investigate the effect of low oxygen levels on the function of the immune system of the black tiger shrimp, *P monodon*, in order to better understand the cause of disease outbreaks in culture ponds.

MATERIALS AND METHODS

Shrimp and test protocol

The experiments were conducted in 2 treatments consisting of 10-15 g shrimp in a control group (DO about 6 ppm) and a low oxygen group (DO about 1.8-2 ppm) in 40 liter aquaria. The condition of low oxygen was generated by stopping the aeration for about 6 h. During these experiments water temperature and pH were 29-30°C and 7.9-8.1, respectively. The haemolymph of the experimental shrimp was sampled to determine phagocytic activity and efficiency of clearing luminescent bacteria.

Phagocytosis *in vivo*

Twelve shrimp in each treatment group were injected with 0.1 ml of yeast suspension (*Saccharomyces cerevisiae*) containing 10^7 cells/ml in 0.85% NaCl. After 30 min, 0.2 ml of haemolymph was collected from the base of the 3th walking leg and mixed with 0.4 ml of lobster haemolymph medium (LHM) containing 0.5% L-cystein. The haemocytes were washed with LHM 3 times by centrifugation at 5000 rpm for 2 min. Then the haemocyte suspension was adjusted to 10^6 cells/ml before it was spread on a glass slide and incubated in a moist chamber for 45 min at room temperature. Finally, they were fixed with 2% glutaraldehyde for 10 min, washed with LHM, stained with Giemsa for 30 min, and then observed under the light microscope. Two hundred haemocytes were counted and the phagocytosis rate (PR) was calculated according to the following formula:

$$PR = \frac{\text{Phagocytic hemocytes}}{\text{Total haemocytes}} \times 100$$

Direkbusarakom S, Danayadol Y (1998) Effect of oxygen depletion on some parameters of the immune system in black tiger shrimp (*Penaeus monodon*). *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Clearance efficiency

For these tests, each group contained 12 black tiger shrimp (average size about 12.6-16.9 g) and tests were carried out with five replications. Each shrimp was injected with 0.2 ml of *V. harveyi* (1.4×10^{10} CFU/ml) into the muscle of the sixth abdominal segment. After waiting 30 min, 0.2 ml of haemolymph was collected from the base of 3th walking leg and mixed with 0.8 ml LHM. The number of bacteria in the haemolymph was then counted by the spread plate method using BTB-teepol agar. Percentage inhibition (PI) was calculated according to Adams (1991) as follows:

$$PI = 100 - \frac{\text{Mean of CFU in tested group}}{\text{Mean of CFU in control group}} \times 100$$

Effect of oxygen depletion

For these tests, each group contained 6 black tiger shrimps of 12-15.6 g and tests were carried out with five replications. In test groups, aeraters were stopped 6 h per day for 5 consecutive days and mortality as observed over 7 days.

RESULTS

Phagocytosis in control shrimp reared under normal conditions ranged from 32 to 37%, while it was lower under low oxygen conditions (25-31%). An ANOVA comparison showed a significant difference ($P < 0.05$) between the control and test groups (Table 1).

Table 1. Percentage of phagocytosis in black tiger shrimp reared normally or under low oxygen.

Treatment	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Avg.
Control	37	32	34	35	35	35
Low oxygen	31	26	29	25	31	28
P-value	0.009	0.006	0.002	0.001	0.005	

The bacterial clearance efficiency of the oxygen depleted groups was also less than that for normal control groups and ranged from 12.45 to 143.29%. The clearance efficiency amongst the 5 trials was highly variable and possibly depended on shrimp health. The shrimp used in Trials 2 and 3 were weaker than those used in the other trials. Thus, clear and consistent results were not found between the test and control groups (Table 2).

Table 2. Bacterial clearance efficiency in black tiger shrimp reared under normal and low oxygen conditions.

	Trial Number					
Treatment	1	2	3	4	5	Avg.
Control	0	0	0	0	0	0
Low oxygen	-37	-12	-15	-41	-143	-50

Mortality was observed only in the groups reared under low oxygen conditions and cumulative mortality ranged from 17-67% (Table 3).

Table 3. Cumulative mortality of black tiger shrimp reared under normal and low oxygen conditions.

	Trial Number					
Treatment	1	2	3	4	5	Avg.
Control	0	0	0	0	0	0
Low oxygen	17	67	67	33	50	47

DISCUSSION

Dissolved oxygen is the one of the most important factors in aquaculture pond management. Oxygen depleted conditions cause high stress and mortality (Madenjian *et al.* 1987). The optimal oxygen concentration for shrimp culture ponds is 5 ppm to saturation (Musig 1980) and the critical concentration for shrimp ponds is 3.7 ppm (Chen 1985).

Phagocytosis is an important cellular defense mechanism in crustaceans (Ratcliffe *et al*. 1985). An increase in the percentage of active phagocytes in the freshwater crayfish, *Parachaeraps bicarinatus*, paralleled a rise in the protective immunity of vaccinated animals (McKay & Jenkin 1970). In this study, the percentage of phagocytic activity in the low oxygen test groups was lower than in the normal control groups. Moreover, the average of accumulative mortality was 47% in the low oxygen groups after stopping aeration for 6 h per day on 5 consecutive days. After stopping aeration, the dissolved oxygen gradually decreased from 6 ppm to 1.8-2.2 ppm. This low oxygen level depressed the immune system of the shrimp and it can lead to bacterial infections that are usually the final cause of shrimp death (Flegel *et al.* 1995). Indeed, in our experience, luminescent bacteria isolated from diseased shrimp do not cause mortality in healthy shrimp. In spite of this, luminescent bacteria have become the most serious problem for shrimp farmers in Thailand since late 1997.

Other stress factors that should be considered are temperature (higher than normal 1997-1998) and low quality of shrimp feed pellets. Boyd (1989) reported that high temperature and high organic matter at the pond bottom are very effective in reducing the concentration of dissolved oxygen in the pond. Accumulated anaerobic sediments are an invitation to disease (Clifford 1992), and they have been widely implicated in outbreaks of various diseases caused by opportunistic pathogens (Lightner 1993). This information suggests that oxygen depletion might often be the primary cause of shrimp mortality by opportunistic bacteria. If this is so, then one effective measure for control of bacterial diseases in shrimp culture ponds would be to maintain high oxygen levels in the water near the pond bottom. This is because it would not only provide oxygen for shrimp but also promote the removal of substances such as ammonia and prevent other substances such as iron, manganese and hydrogen sulfide from coming out of the mud (Boyd 1979).

Chamberlain (1988) reported that as much as 50% of the feed applied to a pond is not eaten by the shrimp, but becomes part of the detritus. This detritus accumulates on the pond bottom and can lead to anaerobic conditions. To

avoid overfeeding, the oxygen content at the pond bottom should be continuously recorded and feeding tables must consider not only temperature and shrimp weight but also saturation of dissolved oxygen.

LITERATURE CITED

Boyd CE (1979) Water quakity in warmwater fish ponds. Agriculture experimnt station, Auburn University, Auburn, Alabama.

Boyd CE (1989) Water quality management and aeration in shrimp farming. Fisheries and Allied Aquaculture Department Series No. 2 Alabama Agriculture Experiment Station, Aubure University, Alabama

Chamberlin GW (1988) Rethinking shrimp pond management, Coast. Aqua. 5(2):19.

Chen J (1985) Water quality criteria for farming the grass shrimp, *Penaeus monodon*. Proceeding of the First International Conference on the culture of Penacid prawns, SEAFDEC, Illoilo, Philippines, p 165

Clifford III HC (1992) Marine shrimp pond management; A review, In Wyban J (ed.) Proceedings of the special session on shrimp farming. World Aquaculture Society, Baton Rouge, Louisiania USA. p 110-137

Department of economic commerce (1997) Report of aquactic export , Ministry of commerce.

Flegel TW, Fegan DF, Sriuratana S (1995) Environmental control of infectious shrimp diseases in Thailand. In: Shariff M, Arthus JR, and Subasinghe RP (eds.) Diseases in Asian Aquaculture II, Fish Health Section, Asian Fisheries Society, Manila p 65-79

Lightner DV (1993) Diseases of cultured penaeid shrimp. In McVey JP (ed.) CRC handbook of mariculture, Crustacean aquaculture, Vol. 1 , CRC Press, Boca Ralton , Florida U.S.A. 394-486

Madenjian CP, Rogers GL and Fast AW (1987) Predicting might time dissolved oxygen loss in prawn pond of Hawii: Part I Evaluation of traditional method. Aqua. Eng. 6:191-208

McKay, D and Jenkin CR (1970) Immunity in the invertebrates : The role of serum factors in phagocytosis of erythrocytes by haemocytes of th freshwater crayfish (*Parachaeraps bicarinatus*). Aust. J. Exp. Biol. Med. Sci. 48:139-150

Musig Y (1980) Water quality and mangment in marine shrimp culture. Seminar of shrimp culture and nutrition, Diethelm pharmachem Ltd. and Rovithai Ltd. Bangkok, p 1-9

Ratcliffe NA (1985) Invertebrate immunity: A primer for the non-specialist. Immunology letters 10: 253-270

Snieszko SF (1974) The effects of environmental stress on outbreaks of infectious diseases of fishes. J. Fish Biol. 6:197-208

Molecular Cloning and Characterization of Prophenoloxidase in the Black Tiger Shrimp, *Penaeus monodon*

Kallaya Sritunyalucksana, Lage Cerenius, Kenneth Söderhäll

Department of Physiological Botany, University of Uppsala
Villavägen 6, S-75236, Uppsala, Sweden

ABSTRACT: A cDNA encoding shrimp, *Penaeus monodon*, prophenoloxidase (proPO) was obtained by screening a hemocyte library by plaque hybridization using a proPO cDNA fragment probe from the freshwater crayfish, *Pacifastacus leniusculus*. The 3,069 bp cDNA contained an open reading frame of 2,121 bp and a 948 bp 3'-untranslated region. The molecular mass of the deduced amino acid sequence (688 amino acids) was 78,669 Da with an estimated pI of 5.8. Two putative copper binding sites were present and were found to be highly conserved amongst arthropod proPOs. No signal peptide was detected in the shrimp proPO, as has been previously shown to be the case for the arthropod proPOs so far cloned. The cleavage site of zymogen activation is suggested to be between the amino acid positions No. 44 (Arg) and No. 45 (Val). A tentative complement-like motif (GCGWPQHM) was also observed. Northern blot analysis indicated a 3.2 kb-transcript and demonstrated that shrimp proPO mRNA was synthesized in haemocytes, and not in the hepatopancreas. Phylogenetic analysis showed that shrimp proPO is more closely related to another crustacean proPO (i.e., crayfish) than to insect proPOs.

KEY WORDS: *Penaeus monodon*, cDNA, proPO, gene, haemocytes

Sritunyalucksana K, Cerenius L, Söderhäll K (1998) Molecular cloning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Comparison of Shrimp High Density Lipoprotein and Beta Glucan Binding Protein

*Gloria Yepiz-Plascencia, Francisco Vargas-Albores, Flor Jimenez-Vega
Lydia M. Ruiz-Verdugo, Gabriela Romo-Figueroa

Centro de Investigación en Alimentación y Desarrollo (CIAD)
PO Box 1735, Hermosillo Son. 83000, Mexico

ABSTRACT: In the hemolymph of marine shrimp, a high density lipoprotein (HDL) and a ß-glucan binding protein (BGBP) have been found. These proteins are involved in the transport of lipids and in the recognition of foreign matter, respectively. Similarities between the color and molecular mass of these proteins were initially detected. To do a detailed comparison, HDL and BGBP were purified, and characterised from three shrimp species, white shrimp *Penaeus vannamei*, brown shrimp *P. californiensis* and blue shrimp *P. stylirostris*. HDL was purified by two sequential density gradient ultracentrifugation steps while BGBP was purified by carbohydrate (laminarin) affinity chromatography. Both proteins were monomeric with approximately the same molecular mass by SDS-PAGE (~100-112 kDa). Both were recognized by ConA and WGA, indicating that they are glycoproteins. Since shrimp HDLs contain lipids, they have a lower density than the majority of shrimp plasma proteins. This allowed their purification by density gradient ultracentrifugation. Purified HDL from *P. vannamei* had densities of 1.12-1.14 g/ml, while those from *P. californiensis* and *P. stylirostris* had average densities of 1.139 and 1.137 g/ml, respectively. These proteins also had similar amino acid composition and high sequence identity in the N-terminus. Polyclonal antibodies were prepared against *P. vannamei* HDL (anti-HDL) and *P. californiensis* BGBP (anti-BGBP). Both antibodies recognized the six proteins: HDL and BGBP from *P. vannamei*, HDL and BGBP from *P. californiensis* and HDL and BGBP from *P. stylirostris*. Therefore, BGBP and HDL appear to be the same protein. This suggests a very close relationship amongst molecules of the immune system in different shrimp species.

KEYWORDS: crustacea, BGBP, defense system, HDL, lipoprotein, plasma, shrimp

INTRODUCTION

Crustacean blood contains circulating cells and proteins, including lipoproteins (Lps). These proteins are involved in oxygen transport, coagulation and in the defense system. Plasma Lps transport lipids from different tissues, including polyunsaturated fatty acids and cholesterol that are provided mainly by the diet (Teshima & Kanazawa 1971, Van den Oord 1964, Zandee 1964). Similar to vertebrate Lps, crustacean Lps are classified according to their density as high, low and very low density Lps (Kanost et al. 1990, Stratakis et al. 1992), although to our knowledge, only HDL and VHDL have been detected (Hall et al. 1995, Komatsu & Ando 1992, Komatsu et al. 1993, Lee 1990, Spaziani et al. 1995, Yepiz-Plascencia et al. 1995). The HDLs appear to be more abundant than VHDLs and contain more lipid. Therefore, they may be more important as lipid transportation vehicles.

Two different types of HDLs have been reported in crustaceans. One of them seems to be non sex-specific, while the other is found in ovigerous females and related to vitellogenesis (Lee 1990, Lubzens et al. 1997). The non sex-specific HDL appears to be simpler in apolipoprotein composition than the female-specific HDL, since it contains only one high molecular mass subunit (100-112 kDa), whereas vitellogenin has several medium size apolipoproteins (Lubzens et al. 1997, Stratakis et al. 1992). The lipid moiety of crustacean Lps is composed mainly by phospholipids (Lee 1990, Lubzens et al. 1997, Stratakis et al. 1992), in contrast to insect Lps that contain mainly diacylglycerol (Kanost et al. 1990); therefore, crustacean Lps differ from their insect counterparts in their protein and lipid constituents.

Penaeid plasma non sex-specific HDL from *Penaeus japonicus* (Teshima & Kanazawa 1980), *P. semisulcatus* (Tom et al. 1993) and *P. vannamei* (Yepiz-Plascencia et al. 1995) have buoyant densities of 1.10 to 1.17 g/ml. White shrimp *P. vannamei* has one apo-HDL of ~ 98 kDa in SDS-PAGE that is glycosylated with mannose and/or glucose (Yepiz-Plascencia et al. 1995); it also contains approximately 50% lipids with phospholipid as the most abundant component (Ruiz-Verdugo et al. 1997).

Also present in crustacean hemolymph are two types of proteins that have been proposed to be involved in the non-self recognition process: agglutinins that react with bacterial

Yepiz-Plascencia G, Vargas-Albores F, Jimenez-Vega F, Ruiz-Verdugo LM, Romo-Figueroa G (1998) Comparison of Shrimp High Density Lipoprotein and Beta Glucan Binding Protein. *In* Flegel TW (ed) Advances in shrimp biotechnology, National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: gyepiz@cascabel.ciad.mx

lipopolysaccharides (LPS) (Kopacek et al. 1993, Vargas-Albores et al. 1993a) and the so-called ß-1,3-glucan binding proteins (BGBP) (Duvic & Söderhäll 1990, Duvic & Söderhäll 1993, Thörnqvist et al. 1994, Vargas-Albores et al. 1996, Vargas-Albores et al. 1997). These proteins *per se* are unable to destroy foreign matter. Cellular defense systems have to be involved (Barracco et al. 1991, Vargas-Albores 1995). Although ß-glucans can activate directly the prophenoloxidase (proPO) activating system of arthropods (Söderhäll 1982, Söderhäll 1992, Söderhäll et al. 1990, Söderhäll et al. 1988, Vargas-Albores 1995, Vargas-Albores et al. 1993b), BGBP amplifies the activation.

BGBPs have been purified from insects (Ochiai & Ashida 1988, Söderhäll et al. 1988) and crustaceans, including the freshwater crayfish *Pacifastacus leniusculus* (Duvic & Söderhäll 1990) and two marine shrimp species *P. californiensis* (Vargas-Albores et al. 1996) and *P. vannamei* (Vargas-Albores et al. 1997). In addition, BGBP has been identified in other crustaceans (Duvic & Söderhäll 1993, Thörnqvist et al. 1994) by using a monospecific antiserum against crayfish BGBP (Duvic & Söderhäll 1990) that detected a similar protein.

Both, HDL and BGBP from penaeid shrimp plasma were studied independently. Examination of their characteristics revealed striking similarities. They have similar pigmentation, molecular mass and plasma concentration; in addition, their amino acid compositions and N-terminal sequences were compared. Furthermore, cross-recognition by antibodies prepared independently against the purified proteins was tested to determine whether they are the same protein participating in two different functions: i.e., lipid transport and activation of the proPO system.

HDL PURIFICATION AND CHARACTERIZATION

Density gradient ultracentrifugation has been used to isolate lipoprotein from many organisms, including crustaceans. It relies on the density of the proteins due to their lipid components. Using this principle, two sequential KBr density gradient ultracentrifugation steps at 400,000*g* for 2 hr yielded a yellowish-orange non sex-specific lipoprotein from juvenile male and females *P. vannamei* plasma (Ruiz-Verdugo et al. 1997). Approximately the same amount was obtained from both females and males (0.93 and 0.73% of the total plasma protein) (Yepiz-Plascencia et al. 1995). The lipoprotein had an yellowish-orange color and a density of 1.1 to 1.17 g/ml with a peak at 1.14 g/ml; this value classified the Lps as high density Lps. The protein concentration, density and neutral lipid content of the fractions obtained from the first density gradient ultracentrifugation are shown in Fig. 1.

Reducing SDS-PAGE analysis revealed that *P. vannamei* HDL contained one apolipoprotein of approximately ~100 kDa, and furthermore, that the native HDL was strongly recognized by Concanavalin A. In addition, it gave a weak reaction with other lectins, indicating that it was a glycoprotein (Yepiz-Plascencia et al. 1995).

The same methodology was successfully applied for the purification of HDL from *P. californienesis* and *P. stylirostris*, revealing that these three penaeid HDLs had similar characteristics. This indicated that the same approach could probably be used to purify other crustacean plasma HDLs.

The most abundant amino acids in the shrimp HDL were aspartic acid/asparagine and glutamic acid/glutamine, with 13 and 12 mol %, respectively. The methionine comtent was very low (Table 1). This was also the case for *Potamon*

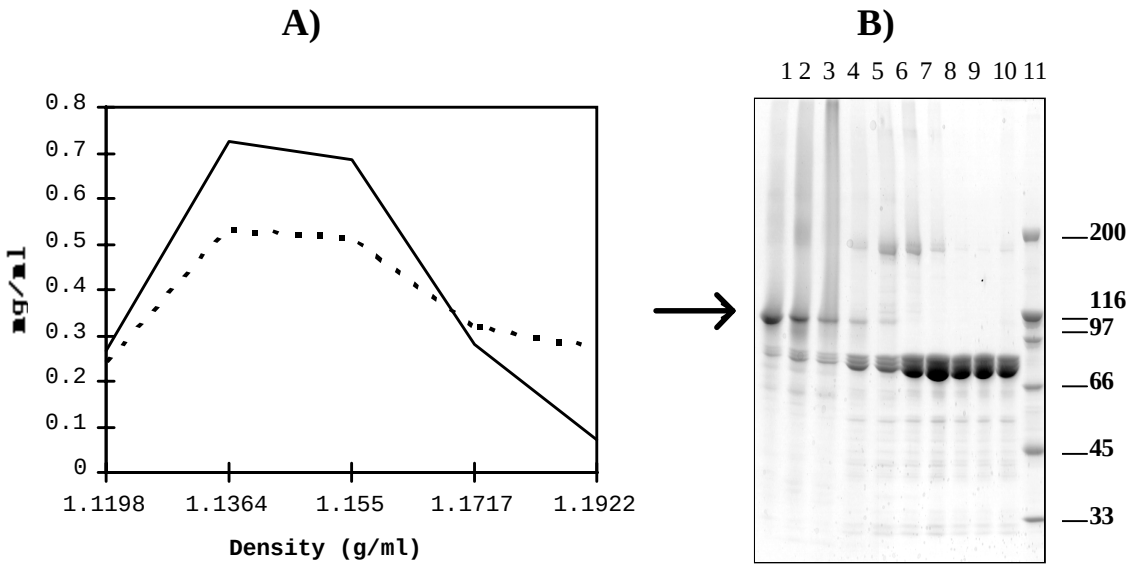


Figure 1.First density gradient ultracentrifugation step of *P. vannamei* plasma. Panel A), density of the fractions in g/ml, protein (—) and lipid (….) concentrations in mg/ml. Panel B, SDS-PAGE of the fractions from panel A. Arrow marks the position of HDL apolipoprotein.

Table 1. Amino acid composition (mol%) of *P. vannamei* HDL and BGBP, *P. californiensis* BGBP, *P. stylirostris* BGBP and *P. leniusculus* BGBP.

Amino acid	Penaeus vannamei HDL	Penaeus vannamei BGBP	Penaeus californiensis BGBP	Penaeus stylirostris BGBP	Pacifastacus leniusculus BGBP
Ala	5.43	6.53	7.14	6.98	5.74
Arg	4.87	4.83	5.91	5.70	5.00
Asx	13.19	12.36	12.87	15.34	12.22
Cys	1.07	n.d.	n.d.	n.d.	0.54
Glx	12.40	10.95	10.19	13.06	11.41
Gly	10.99	8.92	10.22	9.30	7.52
His	1.94	1.79	1.50	2.25	3.17
Ile	5.96	6.08	5.90	5.60	6.03
Leu	7.01	8.86	8.04	7.09	7.51
Lys	5.90	6.37	6.66	7.35	6.84
Met	1.54	0.95	0.79	0.58	1.18
Phe	5.87	5.34	5.21	4.28	5.55
Pro	2.92	3.56	3.14	1.92	3.20
Ser	7.25	8.44	9.01	7.47	7.70
Thr	6.14	6.31	5.91	5.02	6.23
Tyr	3.00	2.98	2.79	3.32	3.37
Val	4.53	5.62	4.55	4.74	6.78

n.d. = not determined; Asx = asparagine + aspartic acid; Glx = glutamine + glutamic acid

potamios (Stratakis et al. 1992), *Manduca sexta* ApoLp-1 (Shapiro et al. 1983) and human ApoA-1 (Ghiselli et al. 1985). Furthermore, in all these cases the sums of the neutral amino acids Gly, Val, Ala, Ile and Leu are approximately 34%, which may be related to hydrophobic regions that interact with lipids.

As previously mentioned, white shrimp, brown shrimp and blue shrimp HDLs have only one apolipoprotein with a molecular mass of approximately 100 kDa. When separated by SDS-PAGE and transferred to nitrocelulose, these apolipoproteins were recognized by the lectins ConA and WGA, indicating the presence of oligosaccharides.

ApoHDLs of approximately 100 kDa have also been reported from *Callinectes sapidus* (Lee & Puppione 1988) and *Limulus polyphemus* (Lee 1990). Furthermore, in *Cancer antennarius* the same apolipoprotein is present in three HDL subclasses, where changes in lipid concentrations give rise to different density Lps (Spaziani et al. 1995).

BGBP PURIFICATION AND CHARACTERIZATION

Similar to freshwater crayfish (*Pacifastacus leniusculus*) plasma (Duvic & Söderhäll 1990), dialysis of shrimp plasma against a low ionic strength solution, resulted in the formation of a yellowish-orange precipitate. This occurred with *P. vannamei*, *P. californiensis* and *P. stylirostris* plasma and the precipitated material was rich in BGBP. *P. californiensis* BGBP was recognized by an anti-*Pacifastacus leniusculus* BGBP antibody, which was originally used to purify shrimp BGBP by immunoaffinity chromatography. The purified *P. californiensis* BGBP amplified activation of the proPO system (Vargas-Albores et al. 1996).

In the case of the shrimp *P. vannamei*, BGBP was purified by laminarin-affinity chromatography, demonstrating the interaction with ß-glucans as previously described for *P. californiensis* BGBP (Vargas-Albores et al. 1997)(Fig. 2). In addition, an antibody against *P. vannamei* BGBP was prepared and it recognized BGBP from the three shrimp species: *P. vannamei*, *P. californiensis* and *P. stylirostris* (Fig. 2). In all cases, the shrimp BGBP was a monomeric protein with approximately 100 kDa molecular mass. The three BGBPs were also recognized in protein blots by the biotinylated lectins ConA and WGA, indicating the presence of glucose and/or mannose residues and N-acetylglucosamine.

Purified BGBPs from the three marine shrimp species and from the freshwater crayfish *P. leniusculus* had similar amino acid compositions and N-terminal amino acid sequences. By contrast, BGBP from the cockroach *Blaberus craniifer* (Söderhäll et al. 1988) and the silkworm *Bombyx mori* (Ochiai & Ashida 1988) have different molecular masses and amino acid compositions. There is a higher proportion of Gly, Glx and Ser in *B. craniifer* BGBP and a higher proportion of Pro in *B. mori*. Furthermore, they are smaller polypeptides (a 62 kDa monomeric protein in *B. mori* and a dimer of 63 and 52 kDa in *B. craniifer*).

In addition to freshwater crayfish (Duvic & Söderhäll 1990), BGBP has been identified in the plasma of other freshwater crustaceans (*Procambarus clarkii* and *Astacus astacus*) (Duvic & Söderhäll 1993) and one marine crustacean (*Carcinus maenas*) (Thörnqvist et al. 1994). BGBP appears to be present in crustaceans as a monomeric protein of 95-110 kDa.

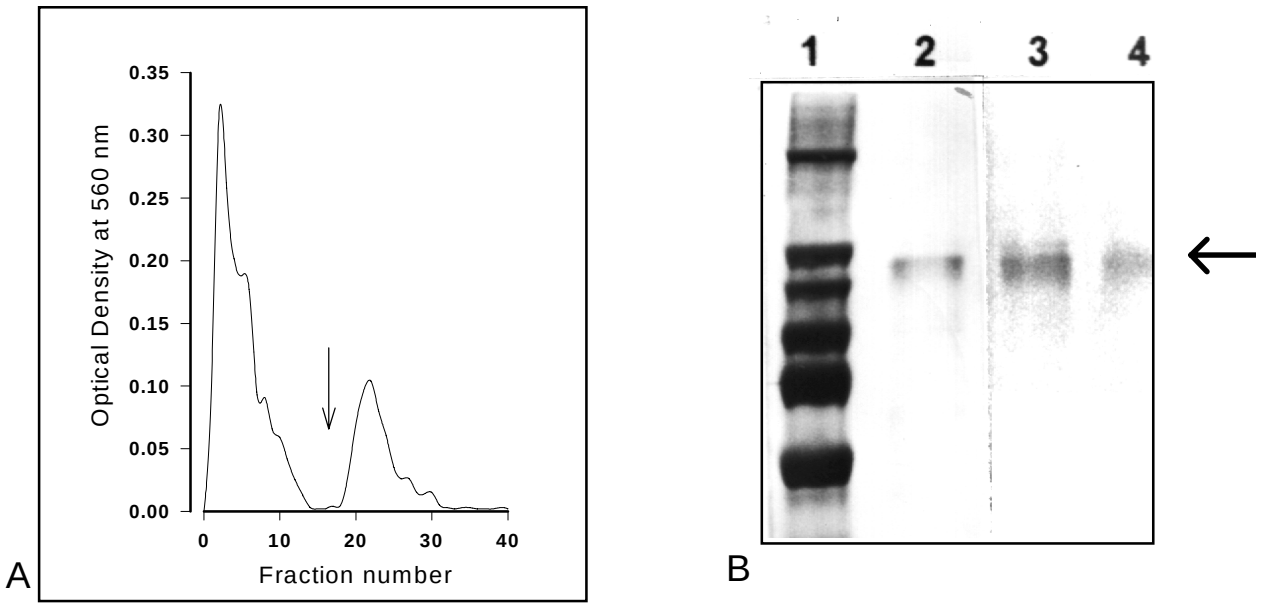


Figure 2. Purification of *P. vannamei* BGBP by laminarin affinity chromatography. Panel A) Chromatogram from laminarin-affinity column; arrow marks application point of Tris-HCl + 200 mM NaCL, pH 8.0, where BGBP begins to elute. Panel B) SDS-PAGE of affinity purified BGBP (lane 2); immunoblot of *P. vannamei* and *P. stylirostris* BGBP (lane 3 and 4, respectively), incubated with anti-*P. californiensis* BGBP antibodies.

COMPARISON OF SHRIMP HDL AND BGBP

Initial observations showed that HDL from *P. vannamei* and BGBP from *P. californiensis* were similar to Lps in the three shrimp species studied. They are composed of a single glycosylated polypeptide of ~100 kDa. To determine antigenic similarities, cross-reactions with independently prepared monospecific polyclonal antibodies were tested. The polyclonal antibodies against *P. californiensis* BGBP recognized the HDLs and BGBPs from *P. californiensis*, *P. vannamei* and *P. stylirostris.* Similarly, the anti-*P. vannamei*-HDL antibodies recognized the HDLs and BGBPs from the other three shrimp species. These results indicated that similar epitopes in all these proteins were recognized by the two antibodies (Figs. 2 and 3).

Likewise, the amino acid composition of the shrimp proteins were very similar and also similar to that of *P. leniusculus* BGBP (Table 1). The same was true for N-terminal amino acid sequences, although only the N-terminal part of the proteins has been obtained. Nevertheless, these proteins appear to be highly conserved among marine and freshwater crustaceans. There are only three amino acid differences between *P. californiensis* and *P. vannamei* HDL/BGBP and they correspond to conservative replacements. Moreover, there are 14 of 25 identical residues and 8 conservative substitutions among the N-terminal sequences of

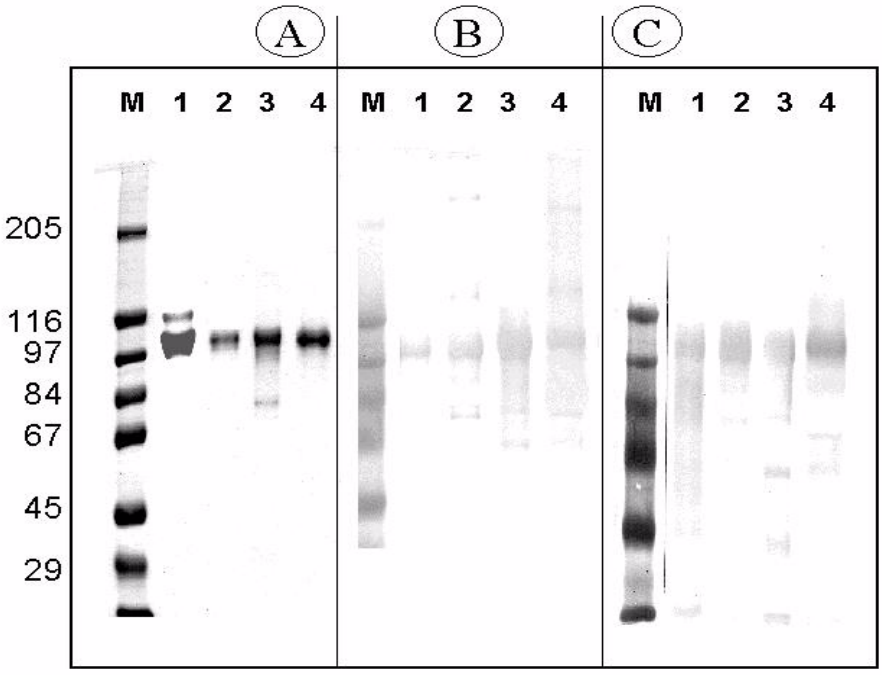


Figure 3. SDS-PAGE and western blots of shrimp BGBP and HDL. A) SDS-PAGE, B) anti-BGBP recognition C) anti-HDL recognition M, molecular markers; 1, *P. californiensis* HDL, 2, *P. californiensis* BGBP; 3, *P. vannamei* HDL; 4, *P. vannamei* BGBP.

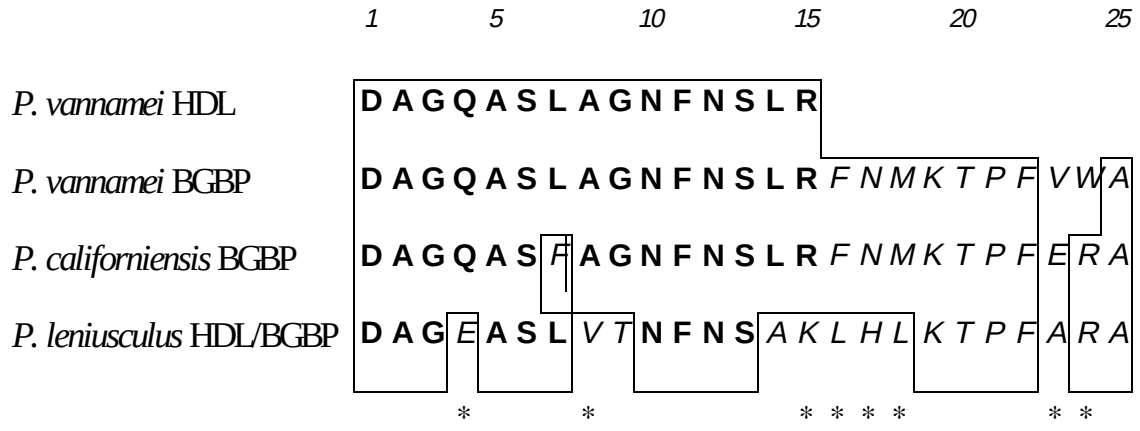


Figure 4. N-terminal amino acid sequence alignment of HDL and BGBP from marine shrimp and freshwater crayfish. Boxed residues are identical and (*) indicates conservative replacements.

these molecules among the marine shrimp and the freshwater crayfish *P. leniusculus*. Interestingly, residue number 7 is different in brown shrimp and residue 24 is different in white shrimp (Fig. 4).

These results suggest that shrimp HDL and BGBP are the same protein. At the least, they share the same molecular mass, contain linked oligosaccharides, have the same amino acid composition and N-terminal sequence. Furthermore, polyclonal antibodies prepared independently to proteins purified from two shrimp species recognized both, HDL and BGBP. Moreover, the polyclonal antibodies were raised against two different protein conformations, the native HDL and the SDS-denatured BGBP. Both antibodies were able to recognize the four SDS-denatured proteins, suggesting that at least some of the native protein epitopes remain available after SDS-denaturation of the proteins.

There must be some common epitopes also in other crustacean BGBPs, since the anti-crayfish BGBP antibody cross-reaction was initially used to purify brown shrimp BGBP (Vargas-Albores et al. 1996) and to detect BGBP in other crustaceans such as *Procambarus clarkii*, *Astacus astacus* (Duvic & Söderhäll 1993) and *Carcinus maenas* (Thörnqvist et al. 1994). Recently, Hall et al. (1995) found similar results in *P. leniusculus*. BGBP is a recognition protein involved in the shrimp defense system. It binds b-glucans and activates the proPO system (Vargas-Albores 1995, Vargas-Albores et al. 1996, Vargas-Albores et al. 1997), and HDL is the major plasma lipoprotein (Ruiz-Verdugo et al. 1997, Yepiz-Plascencia et al. 1995). These results indicate a direct relationship between the transport of essential lipids provided by the diet and a recognition protein component of the shrimp defense system. The effect of reduced dietary lipid in the shrimp defense system remains to be investigated.

Acknowledgemets. This research was partially supported by CONACyT (Mexico) grant O564P-N9506, and IFS grant A/2500-1. We are grateful to A. Sierra (CIBNOR, Mexico) for obtaining anti-brown shrimp BGBP, M. García Bañuelos for preparing the anti-white shrimp HDL and R. Sotelo Mundo (Univ. Arizona) for searching and comparing protein amino acid sequences.

LITERATURE CITED

Barracco MA, Duvic B, Söderhäll K (1991) The ß-1, 3-glucan-binding protein from the crayfish *Pacifastacus leniusculus*, when reacted with a ß-1, 3-glucan, induces spreading and degranulation of crayfish granular cells. Cell Tissue Res 266:491-497

Duvic B, Söderhäll K (1990) Purification and characterization of a ß-1, 3-glucan binding protein from the plasma of the crayfish *Pacifastacus leniusculu*s. J Biol Chem 265:9332-9337

Duvic B, Söderhäll K (1993) ß-1,3-glucan-binding proteins from plasma of the fresh-water crayfishes *Astacus astacus* and *Procambarus clarkii*. J Crust Biol 13:403-408

Ghiselli G, Rhode MF, Tannenbaum S, Krishnan S, Gotto AM (1985) Origin of apolipoprotein A-1 polymorphism in plasma. J Biol Chem 259:15662-15668

Hall M, Van Heusden MC, Söderhäll K (1995) Identification of the major lipoproteins in crayfish hemolymph as proteins involved in immune recognition and clotting. Biochem. Biophys. Res. Comm. 216:939-946

Kanost MR, Kawooya JK, Law HL, Ryan RO, Van Heusden MC, Ziegler R (1990) Insect hemolymph proteins. Adv Insect Physiol 22:315-320

Komatsu M, Ando S (1992) A novel low density lipoprotein with large amounts of phospholipids found in the egg yolk of crustacea sand crayfish *Ibascus ciliatus*: its function as a vitellogenin degrading proteinase. Biochem Biophys Res Comm 186:498-502

Komatsu M, Ando S, Teshima S (1993) Comparison of hemolymph lipoproteins from four species of crustacea. J Exp Zool 266:257-265

Kopacek P, Grubhoffer L, Söderhäll K (1993) Isolation and characterization of a hemagglutinin with affinity for lipopolysaccharides from plasma of the crayfish *Pacifastacus leniusculus*. Develop Comp Immunol 17:407-418

Lee R (1990) Lipoproteins from the hemolymph and ovaries of marine invertebrates. Adv Comp Environ Physiol 7:187-207

Lee R, Puppione DL (1988) Lipoproteins I and II from the hemolymph of the blue crab *Callinectes sapidus*: lipoprotein II associated with vitellogenesis. J Exp Zool 248:278-289

Lubzens E, Ravid T, Khayat M, Daube N, Tietz A (1997) Isolation and characterization of the High-density lipoproteins from the hemolymph and ovary of the penaeid shrimp *Penaeus semisulcatus* (de Hann): apoproteins and lipids. J Exp Zool 278:339-348

Ochiai M, Ashida M (1988) Purification of a ß-1, 3-glucan recognition protein in the prophenoloxidase activating system from

hemolymph of the silkworm, *Bombyx mori*. J Biol Chem 263:12056-12062

Ruiz-Verdugo LM, García-Bañuelos M, Vargas-Albores F, Higuera-Ciapara I, Yepiz-Plascencia GM (1997) Amino acids and lipids of the plasma HDL from the white shrimp *Penaeus vannamei* Boone. Comp Biochem Physiol 118B:91-96

Shapiro JP, Keim PS, Law JH (1983) Structural studies on lipophorin, an insect lipoprotein. J Biol Chem 259:3680-3685

Söderhäll, K (1982) Prophenoloxidase activating system and melanization - a recognition system of arthropods? A review. Develop Comp Immunol 6:601-611;

Söderhäll K (1992) Biochemical and molecular aspects of cellular communication in arthropods. Boll Zool 59:141-151

Söderhäll K, Aspán A, Duvic B (1990) The proPO system and associated proteins -Role in cellular communication in arthropods. Res Immunol 141:896-907

Söderhäll K, Rögener W, Söderhäll I, Newton RP, Ratcliffe NA (1988) The properties and purification of a *Blaberus craniifer* plasma protein which enhances the activation of haemocyte prophenoloxidase by a ß-1, 3-glucan. Insect Biochem. 18:323-330

Spaziani E, Wang WL, Novy LA (1995) Serum high-density lipoproteins in the crab *Cancer antennarius*- IV. Electrophoretic and immunological analyses of apolipoproteins and a question of female-specific lipoproteins. Comp Biochem Physiol 111B:265-276

Stratakis E, Fragkiadakis G, Carpeli-Moustazi E (1992) Isolation and characterization of a non sex-specific lipoprotein from the hemolymph of fresh water crab *Potamon potamios*. Biol Chem Hoppe-Seyler 373:665-677

Teshima S, Kanazawa A (1971) Biosynthesis of sterols in the lobster, *Panulirus japonica*, the prawn, *Penaeus japonicus*, and the crab, *Portunus trituberculatus*. Comp Biochem Physiol 38B:597-602

Teshima S, Kanazawa A (1980) Lipid constituents of serum lipoproteins in the prawn. Bull Jap Soc Sci Fish 46:57-62

Thörnqvist PO, Johansson MW, Söderhäll K (1994) Opsonic activity of cell adhesion proteins and ß-1, 3-glucan binding proteins from two crustaceans. Develop Comp Immunol 18:3-12

Tom M, Shenker O, Ovadia M (1993) Partial characterization of three hemolymph proteins of *Penaeus semisulcatus* de Haan (crustacea, decapoda, penaeidae) and their specific antibodies. Comp Biochem Physiol 104B:811-816

Van den Oord AHA (1964) The absence of cholesterol synthesis in the crab, *Cancer pagurus* L. Comp Biochem Physiol 104B:811-816

Vargas-Albores F (1995) The defense system of brown shrimp (*Penaeus californiensis*): Humoral recognition and cellular responses. J Mar Biotechnol 3:153-156

Vargas-Albores F, Guzmán-Murillo A, Ochoa JL (1993a) A Lipopolysaccharide-binding agglutinin isolated from brown shrimp (*Penaeus californiensis* HOLMES) haemolymph. Comp Biochem Physiol 104A:407-413

Vargas-Albores F, Guzmán-Murillo MA, Ochoa JL (1993b) An anticoagulant solution for haemolymph collection and prophenoloxidase studies of Penaeid shrimp (*Penaeus californiensis*). Comp Biochem Physiol 106A:299-303

Vargas-Albores F, Jiménez-Vega F, Söderhäll K (1996) A plasma protein isolated from brown shrimp (*Penaeus californiensis*) which enhances the activation of prophenoloxidase system by ß-1, 3-glucan. Develop Comp Immunol 20:299-306

Vargas-Albores F, Jiménez-Vega F, Yepiz-Plascencia G (1997) Purification and comparison of ß-1, 3-glucan binding protein from white shrimp (*Penaeus vannamei*). Comp Biochem Physiol 116B:453-458

Yepiz-Plascencia G, Sotelo-Mundo R, Vazquez-Moreno L, Ziegler R, Higuera-Ciapara I (1995) A non-sex-specific hemolymph lipoprotein from the white shrimp *Penaeus vannamei* Boone. Isolation and partial characterization. Comp Biochem Physiol 111B:181-187

Zandee DI (1964) Absence of sterol synthesis in some arthropods. Nature 202:1335-1336

Probiotics and Immunostimulants

Activation of shrimp cellular defence functions by microbial products

***Francisco Vargas-Albores, Jorge Hernández-López, Teresa Gollas-Galván, Karla Montaño-Pérez, Flor Jiménez-Vega, Gloria Yepiz-Plascencia**

CIAD, P.O. Box 1735, Hermosillo, Son., 83000, Mexico.

ABSTRACT: Despite their relatively short life and assumed lesser complexity, crustaceans have mechanisms to detect foreign matter. For example, they recognize characteristics such as lipopolysaccharides (LPS) and β-glucans present in bacterial and fungal surfaces. In shrimp, these microbial components can activate cellular defence functions such as phagocytosis, melanization, encapsulation and coagulation. In spite of a direct stimulation, two kinds of recognition proteins have been detected in shrimp plasma. The LPS-binding agglutinin acts like an opsonin to increase the phagocytic rate. After reaction with β-glucans, the beta glucan binding protein (BGBP) induces degranulation and the activation of pro-phenoloxidase (proPO). The activation of proPO triggers cellular functions that inactivate, immobilise or destroy invaders. These plasma proteins stimulate cellular function only after reaction with LPS or β-glucans, in a manner resembling the secondary activities of vertebrate antibodies.

KEY WORDS: shrimp, cellular defence, proPO, BGBP

INTRODUCTION

Similar to other crustaceans, penaeid shrimp have molecules, cells and systems involved in defensive mechanisms to prevent invasions by microorganisms. As with vertebrates, an efficient defence system is expected to include recognition, effector and regulator components. Depending on the responding immunological defence mechanisms have been divided into delayed and immediate responses. As with most other invertebrates, it has been very difficult to demonstrate that a delayed response can be established in shrimp. However, some of their immediate response components have been studied and most of them have similar characteristics to those found in vertebrates and in other invertebrates.

Another interesting question concerning invertebrates, including shrimp, is the specificity of the defence system. While vertebrates apparently recognise and establish an immune response against an unlimited number of chemical structures, the invertebrates have a poor, if any, spectrum. There are few examples of invertebrates responding to challenge with proteins and other substances.

Besides other functions, the defence system in invertebrates is strongly compromised in pathogen elimination. Thus, the most important foreign signals that they have to recognise are those localized on the pathogen surface. In shrimp, two microbial compounds have been shown to be involved in the stimulation of cellular functions, i.e., lipopolysaccharides (LPS) and β–glucans. Although the primary interaction in both cases could be similar (i.e., directly or through a plasma protein), the cell targets and the activated systems are different. Microbial compounds are able to activate cellular functions directly, but the participation of plasma proteins improves the efficiency of the immune system response. These proteins, defined as recognition proteins, react with microbial compounds and either prevent unnecessary cellular activation or, if necessary, increase the response of effector cells.

RECOGNITION PROTEINS

In shrimp, two kinds of proteins are involved in the recognition of microbial products, and their activation of cellular functions has been described (Vargas-Albores 1995). The first group is constitutes multivalent sugar-binding agglutinins, also named hemagglutinins or lectins. The second group constitutes molecules that are apparently monovalent and do not induce agglutination, even though they are able to bind sugar residues. Agglutinating activities have been detected in plasma of *Penaeus monodon* (Ratanapo & Chulavatnatol 1990), *Penaeus stylirostris* (Vargas-Albores et al. 1992), *Penaeus californiensis* (Vargas-Albores et al. 1993a), *P. japonicus* (Bacheré et al. 1995) and *Penaeus indicus* (Maheswari et al. 1997). However, only the agglutinins from *P. monodon* and *P. californiensis* have been purified and their main properties studied. From *P. monodon* plasma, a 420-kDa glycoprotein formed by identical 27-kDa subunits was isolated by affinity chromatography using a fetuin-agarose column (Ratanapo & Chulavatnatol 1990). This lectin, named monodin, can react with NANA (N-acetyl neuraminic acid) and other N-acetyl amino sugars (GalNAc, GlcNAc and ManNAc) as determined by inhibition studies. Monodin induces the agglutination of *Vibrio vulnificus*, a major infective bacterium for prawns, and this agglutination

Vargas-Albores F, Jorge Hernández-López J, Gollas-Galván T, Montaño-Pérez K, Jiménez-Vega F, Yepiz-Plascencia G (1998) Activation of shrimp cellular defence functions by microbial products. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: fvargas@cascabel.ciad.mx

can be specifically inhibited by NANA (Ratanapo & Chulavatnatol 1992).

Similarly, the agglutinin isolated from *P. californiensis* plasma by affinity chromatography can be inhibited by monosaccharides (GalNAc, GlcNAc, NANA) and glycoproteins (fetuin, submaxillary bovine mucin). Although specificity has not been completely defined, this agglutinin can react with bacterial LPS and is also capable of agglutinating several Vibrios, including *V. parahaemolyticus*. Furthermore, the reaction can be inhibited by LPS (Vargas-Albores et al. 1993a). This shrimp LPS-binding agglutinin (LPS-BA) has an apparent molecular weight of 180-170 kDa and is formed of four 41-kDa subunits.

The second recognizing protein detected in shrimp plasma has the capability to react with beta glucan, and therefore it is named beta glucan binding protein or BGBP. The first crustacean BGBP was reported in crayfish (*Pacifastacus leniusculus*) by Söderhäll and colleagues (Duvic & Söderhäll 1990) who purified and characterized it exhaustively (Barracco et al. 1991, Duvic & Söderhäll 1992, Duvic & Söderhäll 1993, Thörnqvist et al. 1994). Antibodies against crayfish BGBP have been used to detect this protein in other crustaceans, including shrimp (Duvic & Söderhäll 1993, Thörnqvist et al. 1994, Vargas-Albores et al. 1996).

BGBP has been purified from *P. californiensis*, *P. vannamei* and *P. stylirostris* plasma as a 100-kDa monomeric protein. As shown in Table 1, the amino acid content of these shrimp BGBPs is nearly identical to the homologues from the freshwater crayfish, *P. leniusculus*. Major differences could be noted when comparing BGBP from crustaceans and insects, including differences in molecular masses and amino acid composition. *Bombyx mori* BGBP has a molecular mass of 62 kDa (Ochiai & Ashida 1988), significantly smaller than crustacean BGBP. In *Blaberus craniifer*, BGBP was reported as a 91 kDa protein that under reducing conditions split into subunits of 63 and 52 kDa (Söderhäll & Hall 1988), but such dimeric structure has not been reported in crustaceans.

When the known N-terminal amino acid sequence of crustacean BGBPs (*P. californiensis*, *P. vannamei* and *P. leniusculus*) are compared, high homology is observed. Crayfish BGBP was recently cloned and sequenced (Cerenius et al. 1994), but until now no other crustacean or insect BGBP complete sequence is available. Even though amino acid sequence comparison among BGBP can only be done with the N-terminus of the protein, the sequence appears to be highly conserved between shrimp and crayfish (Fig. 1). There are only 3 different residues between white and yellowlegs shrimp BGBPs and notably, all are substitutions by conserved amino acids. Whether this region corresponds to a specific domain important for protein function or is representative of the entire protein conservation, remains to be analysed once the complete sequence of more BGBPs are obtained. Although these kinds of proteins have also been found in insects (Ochiai & Ashida 1988, Söderhäll & Hall 1988), molecular masses and amino acid compositions are different to shrimp BGBP (Vargas-Albores et al. 1996). Unfortunately, studies on other beta glucan binding proteins that stimulate the proPO system are not yet available.

Table 1. Amino acid composition (mol %) of β-1,3-glucan binding proteins from the white shrimp (Vargas-Albores et al. 1997), brown shrimp (Vargas-Albores et al. 1996) and crayfish (Duvic & Söderhäll 1990).

Amino acid	White Shrimp	Brown Shrimp	Blue Shrimp	Freshwater Crayfish
Ala	6.53	7.14	6.98	5.74
Arg	4.83	5.91	5.70	5.00
Asx	12.36	12.87	15.34	12.22
Cys	n.d.	n.d.	n.d.	0.54
Glx	10.95	10.19	13.06	11.41
Gly	8.92	10.22	9.30	7.52
His	1.79	1.50	2.25	3.17
Ile	6.08	5.90	5.60	6.03
Leu	8.86	8.04	7.09	7.51
Lys	6.37	6.66	7.35	6.84
Met	0.95	0.79	0.58	1.18
Phe	5.34	5.21	4.28	5.55
Pro	3.56	3.14	1.92	3.20
Ser	8.44	9.01	7.47	7.70
Thr	6.31	5.91	5.02	6.23
Tyr	2.98	2.79	3.32	3.37
Val	5.62	4.55	4.74	6.78

n.d. not determined

THE SHRIMP PROPHENOLOXIDASE (PROPO) SYSTEM

In shrimp, like in all crustaceans, melanization is due to the action of phenoloxidase (PO) which promotes hydroxylation of phenols and oxidation of o-phenols to quinones, in response to foreign matter invading the hemocoele and during wound healing (for reviews see (Ashida & Yamazaki 1990, Johansson & Söderhäll 1989, Söderhäll 1992, Söderhäll et al. 1994). These quinones are subsequently transformed to melanin by non-enzymatic reactions. Although a direct antimicrobial activity has been described for melanin, the production of reactive oxygen

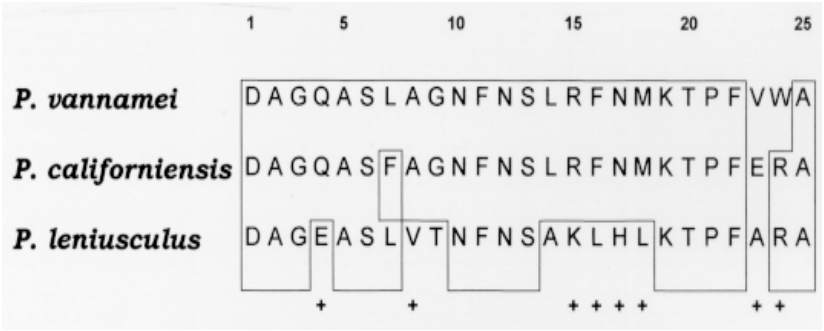


Figure 1. Alignment of *P. vannamei* (Vargas-Albores et al. 1997), *P. californiensis* (Vargas-Albores et al. 1996) and *P. leniusculus* (Duvic & Söderhäll 1990) BGBP N-terminal sequences. Identical sequences are boxed. Plus signs indicate conservative replacement between white shrimp and crayfish.

species such as superoxide anions and hydroxyl radicals during the generation of quinoids (Nappi et al. 1995, Song & Hsieh 1994) has also an important antimicrobial role. In addition, biological reactions like phagocytosis, encapsulation and nodulation are also activated.

Crustacean PO is located inside hemocytic granules as an inactive pro-enzyme called prophenoloxidase (proPO) and its transformation from proPO to PO involves several reactions known as the proPO activating system. As in other crustaceans (Ashida et al. 1983, Smith & Söderhäll 1983, Söderhäll & Hall 1984, Söderhäll & Unestam 1979), the shrimp proPO system is specifically activated by β-1,3-glucans (Vargas-Albores 1995, Vargas-Albores et al. 1996, Vargas-Albores et al. 1997) and LPS (Gollas-Galván et al. 1997, Hernández-López et al. 1996). Therefore, the crustacean proPO system has been considered as a recognition system (Ashida et al. 1983, Ashida & Yamazaki 1990, Johansson & Söderhäll 1989, Johansson & Söderhäll 1992, Lanz et al. 1993, Ratcliffe 1985, Ratcliffe et al. 1991, Ratcliffe et al. 1985, Söderhäll 1992, Söderhäll et al. 1990, Söderhäll et al. 1994). In addition, the proPO system has been proposed as the invertebrate counterpart of the vertebrate complement system since it can be activated by β-1,3-glucans (Ashida 1981, Leonard et al. 1985, Smith & Söderhäll 1983, Vargas-Albores et al. 1993b), has a cascade reaction, and involves proteinases (Aspán et al. 1990a, Söderhäll 1992, Söderhäll et al. 1994). However, other than these similarities, a direct lytic activity by the proPO system has not been detected.

Shrimp proPO can be obtained from hemocyte lysate supernatant (HLS) or by recovering the granular content by centrifuging the cells at 10000 g. However, neither HLS nor granular content could be directly activated by LPSs, including those from *Escherichia coli, Vibrio cholerae, Pseudomonas aeruginosa, Shigella flexneri, Klebsiella pneumoniae, Serratia marcencens* and *Salmonella thypi* (Hernández-López et al. 1996) if Ca^{2+} was absent. On the other hand, by adding Ca^{2+} (5 mM or more), activation of proPO is observed, but this activation occurs even in the absence of LPS. Moreover, during incubation of whole shrimp hemocytes with bacteria or laminarin, the activation of proPO can be detected only if Ca^{2+} (5 mM or more) is present. In absence of Ca^{2+}, proPO (but not PO) could be recovered from the supernatant, indicating that laminarin or LPS induced hemocyte degranulation only, but did not activate proPO.

The proposed proPO activation model for crustaceans (Johansson & Söderhäll 1989, Söderhäll 1992, Söderhäll et al. 1994) involves a proteolytic cleavage mediated by a serine-proteinase (Aspán et al. 1990a, Aspán et al. 1990b), namely proPO activating enzyme (PPAE). Although in shrimp, PPAE has been not purified, its presence and participation on proPO activation has been detected. Shrimp PPAE is contained as a zymogen inside the hemocyte granules, together with proPO and is also released during microbial stimulus, centrifugation or cellular lysis. The activation of PPAE is Ca^{2+}-dependent and the active enzyme can be inhibited by either melittin or soybean trypsin inhibitor (STI).

In shrimp, the activation of proPO involves in two steps. The first one is the degranulation that occurs when hemocytes are stimulated by bacteria, LPS or ß-glucans, and inactive forms of both proPO and PPAE are released. The second one requires the participation of Ca^{2+} for the conversion of inactive PPAE to an active proteinase that, in turn, transforms proPO to active PO (Gollas-Galván et al. 1997). Thus, under *in vivo* conditions, PPAE is activated by plasmatic Ca^{2+} after hemocyte degranulation, which is induced by external stimuli (Gollas-Galván et al. 1997), like LPS and ß-glucans.

THE COAGULATION SYSTEM

Another system activated by microbial products is coagulation. This is an essential defence response for crustaceans because it prevents both loss of hemolymph through breaks in the exoskeleton and the dissemination of bacteria throughout the body (Martin et al. 1991). According to Tait (1911), shrimp posses type C coagulation (i.e., there is a requirement for plasma proteins and cellular components). The key plasma protein which constitutes the clot has been named clotting protein or CP. It appears to be present in relatively high concentrations in hemolymph.

CP has been purified and characterized from other crustaceans, including the spiny lobster, *Panulirus interruptus* (Fuller & Doolittle 1971a), crayfish, *P. leniusculus* (Kopácek et al. 1993) and in the white shrimp, *P. vannamei* (Montaño-Pérez et al. 1998). In all cases, CP was reported as a lipoglycoprotein with a molecular mass about 420 kDa, and with two identical subunits linked by disulfide bridges. In addition, the amino acid composition of the white shrimp CP (Table 2) is similar to its homologues from crayfish (Kopácek et al. 1993), lobster (Fuller& Doolittle 1971a) and sand crab (Madaras et al. 1981), even though the latter species has a different type of coagulation (Ghidalia et al. 1981).

Table 2. Amino acid composition (mol %) of clotting protein from white shrimp (Montaño-Pérez et al. 1998), crayfish (Kop?cek et al. 1993), lobster (Fuller& Doolittle 1971a) and sand crab (Madaras et al. 1981).

Amino acid	*Penaeus vannamei*	*Pacifastacus leniusculus*	*Panulirus interruptus*	*Ovalipes bipustulatus*
Asx	10.39	9.05	9.90	11.94
Thr	7.68	8.72	7.12	6.66
Ser	7.92	7.65	8.19	6.75
Gxl	11.61	12.30	10.91	12.22
Pro	5.64	4.99	5.25	5.74
Gly	7.17	5.84	6.17	7.40
Ala	5.44	5.72	5.46	6.01
Cys	n.d.	1.53	1.31	1.27
Val	6.97	6.48	6.90	6.66
Met	1.38	1.49	1.69	1.75
Ile	5.69	6.31	5.12	5.09
Leu	8.79	8.32	9.60	8.14
Tyr	3.28	3.13	3.18	2.31
Phe	4.65	5.19	4.10	3.98
His	3.77	3.18	4.16	3.61
Lys	5.02	6.28	4.18	5.64
Arg	4.56	3.84	4.75	4.81

n.d. not determined

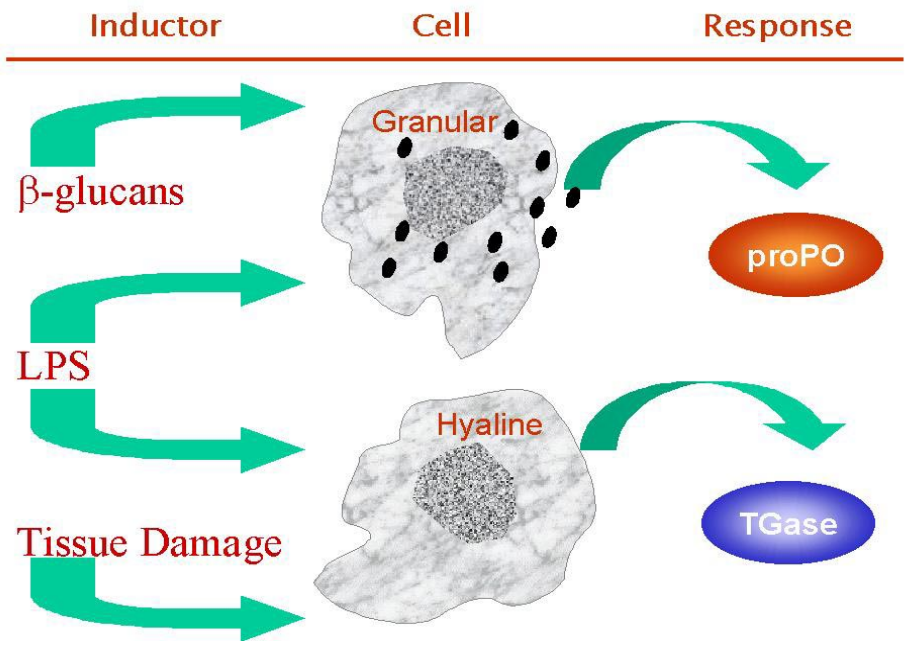


Figure 2. b-glucans and LPS stimulate shrimp hemocytes. Granular cells are stimulated by both microbial compounds producing degranulation, whereas hyaline cells are stimulated by LPS (and tissue damage) releasing TGase.

Apparently, cleavage of the N-terminus is not involved in the crustacean event, since the same N-terminal sequence is present before and after gelation (Fuller& Doolittle 1971b). However, the basis of coagulation is a cellular transglutaminase (TGase) supplied by hemocytes (probably hyaline cells) under external stimulus. The TGase-catalyzed reaction results in the formation of intermolecular ε–(γ-glutamyl)-lysine cross-links between the side chain of a glutamine residue on one polypeptide and the side chain of a lysine residue on another polypeptide (Lorand & Conrad 1984).

In shrimp, as well as in crayfish, polymerization of purified shrimp CP occurs by adding guinea pig liver TGase in the presence of Ca^{2+}. Polymerization of *P. vannamei* CP was also possible with either *P. californiensis* or *P. stylirostris* hemocyte lysate, in the presence of Ca^{2+}. Thus, considering that the reaction was also observed using guinea pig TGase, polymerization of CP is not species-specific. In addition, since polymerization of shrimp CP can be catalyzed by guinea pig liver TGase, it is possible that other cellular factors are not required for clot formation. Therefore, in spite of structural or probable differences in origin, vertebrate and invertebrate coagulation systems have the same mechanisms, although different strategies. In both cases, the clot is formed by the action of a Ca^{2+}-dependent TGase on a clottable plasma protein, fibrinogen or clotting protein, respectively. However, in vertebrates, a previous proteolytic processing reaction is required (fibrinogen to fibrin) to obtain a substrate for the TGase which is found in plasma. By contrast, shrimp TGase is compartmentalised in hemocytes (as in some other invertebrates) and acts immediately after release. Considering that prior proteolytic cleavage is not required, stimulation of hyaline hemocyte with bacterial LPS appears to be enough to induce the release of TGase and lead to subsequent plasma coagulation.

CELLULAR ACTIVATION

Both LPS and ß-glucans are capable of stimulating shrimp hemocytes to release cellular components. ß-glucans stimulate the proPO activating system, whereas LPS can stimulate both the proPO system and the coagulation system by activating different hemocyte populations. In addition to direct activation, the participation of recognition proteins (LPS-BA and BGBP) is very important in shrimp both for preventing undesirable activation and for stimulating cell activation. In despite of some differences, shrimp recognition proteins share a characteristic with vertebrates antibodies: the activation of cellular mechanisms after reaction with antigens (Fig. 2).

When mice erythrocytes (used as target cells) were incubated with shrimp hemocytes and LPS-BA, a stimulation in phagocytosis was observed. In addition, when the erythrocytes were previously incubated with sub-agglutinating doses of LPS-BA, washed and followed by incubation with shrimp hemocytes, similar stimulation of phagocytosis was recorded. Thus, LPS-BA acts as an opsonin rather than a direct activator of shrimp hemocytes. These results help to explain why *in vivo* spontaneous hemocyte agglutination does not occur, even though the agglutinin seems to bind to hemocyte membranes. Similarly, BGBP potentiates laminarin stimulation of the proPO system. However, the real stimulator is the complex BGBP-laminarin, as demonstrated in crayfish where a corresponding receptor on the hemocyte surface has been

reported. These results indicate that recognition proteins require a prior reaction with ß-glucans before they can stimulate cellular functions.

On the other hand, direct hyaline cell stimulation by LPS induces the release of a polymerizing TGase-type enzyme that in turn causes clot formation. Thus, in shrimp several defensive responses are activated by microbial products (LPS and b-glucans). Even though these products can directly activate proPO and the coagulation system, plasma recognition proteins that enhance the cellular defence mechanism also play key roles. Paradoxically the signal to activate the defence mechanisms is given by pathogens themselves through their surface components like LPS or glucans.

LITERATURE CITED

Ashida M (1981) A cane sugar factor suppresing activation of prophenoloxidase in haemolymph of the silkworm, Bombyx mori. Insect Biochem 11:57-65

Ashida M, Ishizaki Y, Iwahana H (1983) Activation of prophenoloxidase by bacterial cell walls or β–1,3-glucans in plasma of the silkworm, *Bombyx mori*. Biochem Biophys Res Commun 113:562-568

Ashida M, Söderhäll K (1984) The prophenoloxidase activating system in crayfish. Comp Biochem Physiol 77B:21-26

Ashida M, Yamazaki H (1990) Biochemistry of the phenoloxidase system in insects: with special reference to its activation. In Ohnishi E, Ishizaki H (eds) Molting and metamorphosis., Springer-Verlag: Berlin p 239-265

Aspán A, Hall M, Söderhäll K (1990a) The effect of endogeneous proteinase inhibitors on the prophenoloxidase activating enzyme, a serine proteinase from crayfish haemocytes. Insect Biochem 20:485-492

Aspán A, Sturtevant J, Smith VJ, Söderhäll K (1990b) Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells. Insect Biochem 20:709-718

Bacheré E, Mialhe E, Rodriguez J (1995) Identification of defence effectors in the haemolymph of crustaceans with particular reference to the shrimp *Penaeus japonicus* (bate) - prospects and applications. Fish Shellfish Immunol 5:597-612

Barracco MA, Duvic B, Söderhäll K (1991) The β–1,3-glucan-binding protein from the crayfish *Pacifastacus leniusculus*, when reacted with a β–1,3-glucan, induces spreading and degranulation of crayfish granular cells. Cell Tissue Res 266:491-497

Cerenius L, Liang Z, Duvic B, Keyser P, Hellman U, Tapio-Palva E, Iwanaga S, Söderhäll K (1994) Structure and biological activity of a 1,3-β–D-glucan-binding protein in crustacean blood. J Biol Chem 269:29462-29467

Duvic B, Söderhäll K (1990) Purification and characterization of a β–1,3-glucan binding protein from the plasma of the crayfish *Pacifastacus leniusculus*. J Biol Chem 265:9332-9337

Duvic B, Söderhäll K (1992) Purification and partial characterization of a β–1,3-glucan-binding-protein membrane receptor from blood cells of the crayfish *Pacifastacus leniusculus*. Eur J Biochem 207:223-228

Duvic B, Söderhäll K (1993) β–1,3-glucan-binding proteins from plasma of the fresh-water crayfishes *Astacus astacus* and *Procambarus clarkii*. J Crust Biol 13:403-408

Fuller GM, Doolittle RF (1971a) Studies of invertebrate fibrinogen. I. Purification and characterization of fibrinogen from spiny lobster. Biochemistry 10:1305-1310

Fuller GM, Doolittle RF (1971b) Studies of invertebrate fibrinogen. II. Transformation of lobster fibrinogen into fibrin. Biochemistry 10:1311-1315

Ghidalia W, Vendrely R, Montmory C, Coirault Y, Brouard MO (1981) Coagulation in decapod crustacea. Comparative studies of the clotting process in species from group A, B and C. J Comp Physiol 142:473-478

Gollas-Galván T, Hernández-López J, Vargas-Albores F (1997) Effect of calcium on the brown shrimp (*Penaeus californiensis*, Holmes) prophenol-oxidase system activation. Comp Biochem Physiol 117A:419-425

Hernández-López J, Gollas-Galván T, Vargas-Albores F (1996) Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes). Comp Biochem Physiol 113C:61-66

Johansson MW, Söderhäll K (1989) Cellular immunity in crustaceans and the proPO system. Parasitol Today 5:171-176

Johansson MW, Söderhäll K (1992) Cellular defence and cell adhesion in crustaceans Anim Biol 1:97-107

Kopácek P, Hall M, Söderhäll K (1993) Characterization of a clotting protein, isolated from plasma of the freshwater crayfish *Pacifastacus leniusculus*. Eur J Biochem 213:591-597

Lanz H, Hernández S, Garrido-Guerrero E, Tsutsumi V, Ar?chiga H (1993) Prophenoloxidase system activation in the crayfish *Procambarus clarkii*. Develop Comp Immunol 17:399-406

Leonard C, Ratcliffe NA, Rowley AF (1985) The role of prophenoloxidase activation in non-self recognition and phagocytosis by insect blood cells. J Insect Physiol 31:789-799

Lorand L, Conrad SM (1984) Transglutaminases. Mol Cell Biochem 58:9-35

Madaras F, Chew MY, Parkin JD (1981) Purification and characterization of the sand crab (*Ovalipes bipustulatuss*) coagulogen (fibrinogen). Thrombos Haemostas 45:77-81

Maheswari R, Mullainadhan P, Arumugam M (1997) Characterisation of a natural haemagglutinin with affinity for acetylated aminosugars in the serum of the marine prawn, *Penaeus indicus* (Edwards,H.Milne). Fish Shellfish Immunol 7:17-28

Martin GG, Hose JE, Omori S, Chong C, Hoodbhoy T, McKrell N (1991) Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod crustaceans. Comp Biochem Physiol 100B:517-522

Montaño-Pérez K, Yepiz-Plascencia G, Higuera-Ciapara I, Vargas-Albores F (1998) Purification and characterization of the clotting protein from the white shrimp (*Penaeus vannamei*). Comparative Biochemistry & Physiology In press

Nappi A J, Vass E, Frey F, Carton Y (1995) Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. Eur J Cell Biol 68:450-456

Ochiai M, Ashida M (1988) Purification of a β-1,3-glucan recognition protein in the prophenoloxidase activating system from hemolymph of the silkworm, *Bombyx mori*. J Biol Chem 263:12056-12062

Ratanapo S, Chulavatnatol M (1990) Monodin, a new sialic acid-specific lectin from black tiger prawn (*Penaeus monodon*). Comp Biochem Physiol 97 B:515-520

Ratanapo S, Chulavatnatol M (1992) Monodin-induced agglutination of *Vibrio vulnificus*, a major infective bacterium in black tiger prawn (*Penaeus monodon*). Comp Biochem Physiol 102B:855-859

Ratcliffe NA (1985) Invertebrate immunity - a primer for the non-specialist. Immunol Lett 10:253-270

Ratcliffe NA, Brookman JL, Rowley AF (1991) Activation of the prophenoloxidase cascade and initiation of nodule formation in locusts by bacterial lipopolysacharides. Develop Comp Immunol 15:33-39

Ratcliffe NA, Rowley AF, Fitzgerald SW, Rhodes CP (1985) Invertebrate immunity: Basic concepts and recent advances. Int Rev Cytol 97:183-350

Smith V J, Söderhäll K (1983) β–1,3 glucan activation of crustacean hemocytes *in vitro* and *in vivo*. Biol Bull 164:299-314

Söderhäll K (1992) Biochemical and molecular aspects of cellular communication in arthropods. Boll Zool 59:141-151

Söderhäll K, Aspán A, Duvic B (1990) The proPO system and associated proteins -Role in cellular communication in arthropods. Res Immunol 141:896-907

Söderhäll K, Cerenius L, Johansson MW (1994) The prophenoloxidase activating system and its role in invertebrate defense. Ann NY Acad Sci 712:155-161

Söderhäll K, Hall L (1984) Lipopolysaccharide-induced activation of prophenoloxidase activating system in crayfish haemocyte lysate. Biochem Biophys Acta 797:99-104

Söderhäll K, R?gener W, Söderhäll I, Newton RP, Ratcliffe NA (1988) The properties and purification of a *Blaberus craniifer* plasma protein which enhances the activation of haemocyte prophenoloxidase by a β–1,3-glucan. Insect Biochem 18:323-330

Söderhäll K, Unestam T (1979) Activation of serum prophenoloxidase in arthropod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of crayfish phenoloxidase. Can J Microbiol 25:406-414

Song YL, Hsieh YT (1994) Immunostimulation of tiger shrimp (Penaeus monodon) hemocytes for generation of microbicidal substances: Analysis of reactive oxygen species. Developmental and Comparative Immunology 18:201-209

Tait J (1911) Types of crustacean blood coagulation. J Mar Biol Assoc UK 9:191-198

Thörnqvist P-O, Johansson MW, Söderhäll K (1994) Opsonic activity of cell adhesion proteins and β–1,3-glucan binding proteins from two crustaceans. Develop Comp Immunol 18:3-12

Vargas-Albores F (1995) The defense system of brown shrimp (*Penaeus californiensis*): Humoral recognition and cellular responses. J Mar Biotechnol 3:153-156

Vargas-Albores F, Guzman-Murillo A, Ochoa J L (1993a) A Lipopolysaccharide-binding agglutinin isolated from brown shrimp (*Penaeus californiensis* HOLMES) haemolymph. Comp Biochem Physiol 104A:407-413

Vargas-Albores F, Guzman-Murillo MA, Ochoa J-L (1992) Size-dependent haemagglutinating activity in the haemolymph from sub-adult blue shrimp (*Penaeus stylirostris* Stimpson). Comp Biochem Physiol 103A:487-491

Vargas-Albores F, Guzman-Murillo MA, Ochoa J-L (1993b) An anticoagulant solution for haemolymph collection and prophenoloxidase studies of Penaeid shrimp (*Penaeus californiensis*). Comp Biochem Physiol 106A:299-303

Vargas-Albores F, Jiménez-Vega F, Söderhäll K (1996) A plasma protein isolated from brown shrimp (*Penaeus californiensis*) which enhances the activation of prophenoloxidase system by β–1,3-glucan. Develop Comp Immunol 20:299-306

Vargas-Albores F, Jiménez-Vega F, Yepiz-Plascencia G (1997) Purification and comparison of β–1,3-glucan binding protein from white shrimp (*Penaeus vannamei*). Comp Biochem Physiol 116B:453-458

Immunostimulation of Shrimp Through Oral Administration of *Vibrio* Bacterin and Yeast Glucan

T.N. Devaraja, S.K. Otta, G. Shubha, *Indrani Karunasagar P. Tauro, Iddya Karunasagar

Department of Fishery Microbiology, University of Agricultural Sciences, College of Fisheries Mangalore - 575 002, India.

ABSTRACT: The effect of oral administration of Vibrio bacterin and yeast glucan to black tiger shrimp, *Penaeus monodon* was studied. Vibriocidal activity was detectable in hemolymph and hemocyte lysate. The activity peaked at 48 h post treatment but persisted even at 72 h. Generation of reactive oxygen species in hemocytes could be detected by nitroblue tetrazolium (NBT) assay. Enhanced phenol oxidase activity was observed in hemocytes of treated shrimp. When a combination of bacterin and glucan was administered, the response was much higher compared to individual treatments. The results suggest that immunostimulation of shrimp can be achieved through oral administration of a combination of Vibrio bacterin and yeast glucan.

KEY WORDS: Immunostimulants, *Penaeus monodon*, *Vibrio* bacterin, glucan.

INTRODUCTION

Shrimp aquaculture has been facing serious disease problems and therefore, several investigators have considered the possibility for immunoprophylaxis. Itami et al. (1989) noted that immunised shrimps *(Penaeus japonicus)* were better protected against challenge. Adams (1991) observed that bactericidins and other humoral factors were induced in *P. monodon* following treatment with vibrio bacterin. Sung et al. (1991) reported that *P. monodon* larvae treated with Vibrio bacterin grew faster. Induction of resistance in *P. monodon* against challenge by *Vibrio vulnificus* after treatment with beta-glucan was reported by Sung et al. (1994). They further noted that beta-glucans stimulated phenoloxidase activity in hemocytes of *P. monodon.* Activation of hemocytes as indicated by increased respiratory burst activity following treatment with beta-glucans has been recorded by Song and Hsieh (1994). Enhancement of the pro-phenol oxidase system (proPO) in hemocytes by treatment with beta-glucans was observed by Sung et al. (1994). However, most of these reports examined the response after treatment by immersion or injection. The preferred route of delivery in aquaculture systems would be the oral route. Further, interactions between immunostimulants have not been examined. In this study the response of black tiger shrimp, *Penaeus monodon* to oral administration of immunostimulants was evaluated and the combined effect of bacterin and glucan was also investigated.

MATERIALS AND METHODS

Shrimp

Healthy *P. monodon* weighing 22-27 g were obtained from local farms. They were maintained in 300 l tanks containing 150 l sea water and fed with commercial diet at 4% body weight three times a day.

Treatment with immunostimulants and assessment of immune response

Vibrio bacterin contained heat killed cells of *V. harveyi* (local strain, isolated from moribund shrimp) at a density of 10^9/ml. Yeast B-1,3 glucan was a product obtained from Mangalore Biotech Laboratory. Immunostimulants were added to the feed at following levels before feeding: bacterin, 5, 10, 15 and 20%; glucan, 0.1, 0.2, 0.3 and 0.4%. The following combinations of bacterin + glucan were also tried : 5+0.1, 10+0.2, 15+0.3 and 20+0.4%. Diet containing immunostimulants was given at the rate of 4% body weight for three successive feedings in one day at the start of the tests. Thereafter, normal diet was given. At 24, 48 and 72 h after feeding with immunostimulant containing diet, the immune response was assayed. For immunological assays, hemolymph was collected from the sixth abdominal segment using a 26 gauge needle on a 2 ml syringe containing 0.2 ml anticoagulant (0.0l M Tris - HCl, 0.25 M sucrose, 0.1 M sodium citrate, pH 7.6). The hemolymph was centrifuged at 300xg for 10 min at 4°C and the supernatant was used as plasma supernatant (PS). The pellet was resuspended in Hanks Balanced salt solution (HBSS). Hemocyte lysate supernatant (HLS) was prepared by a modification of the protocol described by Sung et al. (1994). The hemocytes were washed in HBSS (instead of cacodylate buffer) and lysed by sonication.

Vibriocidal activity was measured by modification of procedure of Adams (1991). Briefly, *V. harveyi* was cultured overnight in tryptic soy broth (TSB) at 30°C. Bacteria were

Devaraja, TN, Otta SK, Shubha G, Karunasagar I, Tauro P, Karunasagar I (1998) Immunostimulation of shrimp through oral administration of *Vibrio* bacterin and yeast glucan. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail:mircen@giasbg01.vsnl.net.in

pelleted by centrifugation, washed in phosphate buffered saline (PBS) and resuspended in it to the original volume. To 100µl PS or HLS, 1µl bacterial suspension was added and incubated for 1 h. Serial tenfold dilutions were then prepared in PBS and plated on tryptic soy agar (TSA). After 24 h at 30°C, colonies were counted. Percentage inhibition was calculated as follows :

$$\% \text{ inhibition} = 100 - \frac{\text{cfu/ml in treated shrimp}}{\text{cfu/ml in untreated shrimp}} \times 100$$

Production of reactive oxygen species was measured by nitroblue tetrazolium (NBT) assay described by Song and Hsieh (1994). Briefly 100µl aliquots of hemocyte suspension in HBSS were transferred to flat bottom 96 well microtitre plates and incubated at 37°C for 30 min. NBT solution was removed and 100µl methanol was added to each well. Methanol was removed and wells washed thrice in 70% methanol, air dried and coated with a solution of 120µl KOH (2M) and 140µl DMSO to dissolve cytoplasmic formazan.The optical density was then measured at 630 nm using a Precision Microplate Reader (Emax, Molecular Device, USA).

Phenoloxidase activity was measured as detailed by Sung et al. (1994). HLS (50µl) was transferred to a flat bottom microtitre plate and 100µl L-DOPA (3-4 Dihydroxyphenyl L alanine, 1.6 mg/ml in HBSS) was added. After 10 min, OD was measured at 490 nm using a microplate reader. Protein content of HLS was measured by Folin reaction (Lowry et al. 1951). Units of enzyme activity were calculated based on the definition that increase in absorbance of 0.001/min/mg protein was one unit (Söderhäll & Unestam 1979). All experiments were repeated thrice and mean values are presented. Friedman's non-parametric test (Weber 1973) was used to analyse the statistical significance of the results.

RESULTS AND DISCUSSION

Results in Table 1 show that vibriocidal activity was induced in the plasma and hemocyte fractions of *P. monodon* treated orally with vibrio bacterin. Peak response was recorded at 48 h after treatment and the activity persisted even at 72 h. A maximum of 85.8% inhibition was recorded in the plasma of shrimp groups fed at 20% level. The maximum inhibition in hemocyte fractions was 79.2% in groups fed at 10% bacterin. Even at 72 h after treatment, there was more than 75% inhibition in all batches. Induction of bactericidal activity in *P. monodon* following bacterin treatment has been reported by Adams (1991) who administered bacterin by immersion and injection and by Sung et al. (1996) who administered bacterin by immersion. The results presented here show that administration of *Vibrio* bacterin through feed is also effective in inducing vibriocidal activity in *P. monodon* and that the activity persists for over 72h after treatment.

Table 1. Vibriocidal activity in plasma and hemocytes of *P. monodon* fed with *Vibrio* bacterin at different levels.

Treatment (% bacterin)	Percentage inhibition of *Vibrio harveyi*					
	Plasma			Hemocyte lysate supernatant		
	24h	48h	72h	24h	48h	72h
5.0	70.1	80.9	79.6	65.5	76.5	75.5
10.0	69.5	82.9	81.8	67.2	79.2	79.0
15.0	72.0	84.9	82.8	70.1	78.7	76.3
20.0	71.9	85.8	83.9	69.5	76.9	76.0

Treatment of *P. monodon* with yeast glucan at different levels also induced vibriocidal activity in plasma and hemocyte fractions (Table 2). Peak activity was noted at 48h and maximum activity was recorded in the groups treated with 0.4% glucan. Only marginally higher activity (75% inhibition) was noted in the hemocyte lysate fraction when compared to plasma (72.2 % inhibition). Results of Sung et al. (1996) indicated that the treatment of *P. monodon* with glucan by immersion brought about induction of anti-bacterial activity in the plasma which could be recorded up to 24 h. Results presented here show that vibriocidal activity was detectable in both the plasma and the hemocyte fractions of *P. monodon* treated with the glucan by the oral route and that the activity persisted for more than 72 h.

Table 2. Vibriocidal activity in plasma and hemocytes of *P. monodon* fed with glucan at different levels.

Treatment (% bacterin)	Percentage inhibition of *Vibrio harveyi*					
	Plasma			Hemocyte lysate supernatant		
	24h	48h	72h	24h	48h	72h
0.1	55.2	67.8	66.1	60.8	69.1	67.8
0.2	60.2	66.2	65.9	63.3	70.9	70.1
0.3	58.8	71.1	68.2	64.2	73.0	69.5
0.4	61.9	72.2	67.9	63.2	75.0	69.3

Itami et al. (1994) suggested that oral administration of beta glucan enhanced disease resistance of Kuruma prawn, *P .japonicus*. They noted high phagocytic activity in the hemocytes of *P. japonicus* treated with 0.01% glucan for 3 days or 0.005% glucan for 10 days. Results of the present study indicate that oral administration of 0.1% glucan for one day can induce vibriocidal activity in both hemocytes as well as plasma.

Results in Table 3 indicate the combined effect of bacterin and glucan. It can be seen that in combination, the activity is significantly ($p < 0.05$) enhanced. Over 90% inhibition was noticed in all batches at 48h and even at 72h, over 83% inhibition was still recorded. This suggested that the bacterin and glucan combination was more effective than either of the components used individually.

Generation of reactive oxygen species as indicated by NBT assay has been used by a few investigators to measure immunostimulation in shrimp. Song and Hsieh (1994) used the NBT assay to study the effect of treating shrimp hemocytes with glucan *in vitro*. Sung et al. (1996) noted enhanced production of reactive oxygen (O_2^-) in shrimp treated by immersion with the *Vibrio* bacterin and glucan. However, they noted that levels dropped to baseline levels within 12 h. In the present study, treatment was by the oral

Table 3. Vibriocidal activity in plasma and hemocytes of *P. monodon* fed with a combination of the *Vibrio* bacterin and yeast glucan.

Treatment (% bacterin + % glucan)	Percentage inhibition of *Vibrio harveyi*					
	Plasma			Hemocyte lysate supernatant		
	24h	48h	72h	24h	48h	72h
5.0+0.1	77.5	90.6	87.4	78.2	90.0	83.1
10.0+0.2	80.0	92.9	87.4	80.6	92.7	84.8
15.0+0.3	78.1	92.4	83.8	77.1	92.7	83.1
20.0+0.4	75.6	91.8	83.1	76.4	92.0	87.5

route and an enhanced response was observed in all groups, with a peak at 48 h (Table 4). Maximum response was noted at 5% bacterin and 0.2% glucan when they were used separately. Enhanced levels persisted even at 72h after the treatment. The longer duration of the response than that reported by Sung et al. (1996) could have been due to differences in the concentration of immunostimulants and/or the route of administration. Results in Table 4 further show that the treatment with a combination of 10% bacterin and 0.2% glucan resulted in much higher levels compared to individual immunostimulants. These observations show a partially additive effect in stimulating the generation of reactive oxygen in hemocytes.

Table 4. Respiratory burst activity in hemocytes of *P. monodon* measured by NBT assay after feeding with or without immuno-stimulants.

Treatment	OD_{630} at time post-treatment		
	24h	48h	72h
Nil	0.5	0.5	0.5
Bacterin			
5 %	1.1	2.2	1.9
10%	1.1	2.2	1.6
15%	1.6	2.2	1.8
20%	1.4	1.2	1.8
Glucan			
0.1%	0.8	1.2	1.1
0.2%	1.1	1.4	1.2
0.3%	1.2	1.4	1.4
0.4%	1.1	1.5	1.4
Bacterin+Glucan			
5 +0.1%	1.4	2.5	1.8
10+0.2%	1.5	2.8	2.0
15+0.3%	2.1	2.7	2.1
20+0.4%	1.9	2.6	2.0

The pro-phenol oxidase (proPO) system has been considered to play an important role in the defence system of crustaceans (Söderhäll & Cerenius 1992). Activation of the proPO system (measured in terms of the phenol oxidase activity) has been used by some investigators to measure immunostimulation in shrimp (Sung et al.1994 1996). The results in Table 5 show significant enhancement ($p < 0.05$) in PO activity in the hemocytes from all the treated batches. Peak activity in all the cases occurred 48h after treatment and the elevated levels persisted up to 72h after treatment. In the case of the bacterin treatment, highest activity (33.4 u/min/mg protein) was in the groups treated with 15% bacterin. Even at 72h after treatment, activity in this batch was 10 times greater than that of the untreated control. Glucan treatment also brought about increased PO activity and the highest activity (21.56 u/min/mg protein) was noticed with 0.2% glucan. In general, the activity elicited by the glucan was lower than that elicited by *Vibrio* bacterin. Sung et al. (1996) recorded PO activity in shrimp hemocytes treated by immersion with the *Vibrio* bacterin and glucan. They noted peak activity 3 h after treatment and a drop by 24 h. We found that treatment by the oral route resulted in a prolonged enhancement of PO activity. Further, our results showed that bacterin and the glucan in combination were most effective in eliciting PO activity. The highest level of 48.78 u/min/mg protein was obtained with 15% bacterin + 0.3% glucan. In these treatment groups the PO activity was higher than that of the untreated controls by a factor of 14, even 72 h after treatment.

Table 5. Phenol oxidase activity in hemocytes of *P. monodon* after feeding with or without immunostimulants.

Treatment	Phenol oxidase activity (u/min/mg protein)		
	24h	48h	72h
None	3.53	3.66	3.13
Bacterin			
5 %	22.21	25.33	22.62
10%	28.65	32.41	30.11
15%	28.71	33.38	31.12
20%	26.69	32.74	30.47
Glucan			
0.1%	12.98	17.12	13.93
0.2%	13.65	21.56	17.34
0.3%	12.71	19.18	15.23
0.4%	13.93	19.91	17.31
Bacterin+Glucan			
5 +0.1%	33.16	38.49	37.18
10+0.2%	37.46	46.51	43.46
15+0.3%	38.19	48.78	44.39
20+0.4%	36.93	47.65	43.56

The results of this study show that immunostimulation of shrimp, *P. monodon*, is possible through oral administration of *Vibrio* bacterin and the yeast glucan. The results also demonstrate that a combination of these two immunostimulants is much more effective than either of them used individually.

Acknowledgements. This research was supported by grants from the Department of Biotechnology, Govt. of India and this financial assistance is gratefully acknowledged.

LITERATURE CITED

Adams A (1991) Response of penaeid shrimp to exposure to Vibrio species. Fish & Shellfish. Immunol 1: 59-70.

Itami T, Takahashi Y, Tsuchihira E, Igusa H, Kondo M (1994) En-

hancement of disease resistance of Kuruma prawn *Penaeus japonicus* and increase in phagocytic activity of prawn hemocytes after oral administration of B 1-3 glucan (Schizophyllan) In: Chou LM, Munro AD, Lam TT, Chen TW, Leong LKK, Ding JK, Hooi KK, Khoo HW, Phang VPE, Shim KF C.H.Tan (eds) The Third Asian Fisheries Forum, Asian Fish.Soc. Manila, p 375-378.

Itami T, Takahashi Y, Nakamura Y (1989) Efficacy of vaccination against vibriosis in cultured Kuruma prawns *Penaeus japonicus*. J Aquat Animal Health 1: 238-242

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193 : 265 - 275

Söderhäll K , Unestam T (1991) Activation of serum prophenoloxidase in arthopod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of cray fish phenoloxidase. Can J Microbiol 25 : 406 - 414

Söderhäll K, Cerenius L (1992) Crustacean immunity. Ann Rev Fish Dis 2: 3-23

Song Y, Hsieh Y (1994) Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. Dev Comp Immunol 18: 201-209

Sung HH, Song YL, Kou GH (1991) Potential uses of bacterin to prevent shrimp vibriosis. Fish & Shellfish Immunol 1: 311-312

Sung HH, Kou GH, Song YL (1994) Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). Fish Pathol 29: 11-17

Sung HH, Yans YL, Song YLJ (1996) Enhancement of microbicidal activity in the tiger shrimp *Penaeus monodon* via immunostimulations. J Crustacean Biol 16: 278 -284

Waber E (1993) Non parametric methods. In : Delaunnois AL (ed) Biostatistics in Pharmacology - II. Pergamon Press Ltd. Oxford , p 937 - 942

Efficacy of Oral Administration of Fucoidan, a Sulfated Polysaccharide, in Controlling White Spot Syndrome in Kuruma Shrimp in Japan

[1]Yukinori Takahashi, [2]Kaori Uehara, [1]Rikio Watanabe, [3]Takekazu Okumura
[3]Tetsuro Yamashita, [3]Hiroshi Omura, [2]Toshikatsu Yomo, [2]Toshiro Kawano
[2]Akio Kanemitsu, [2]Hideo Narasaka, [1]Nobutaka Suzuki, [*1]Toshiaki Itami

[1]National Fisheries University, Shimonoseki, Yamaguchi, Japan
[2]Miyako Kagaku Co., Ltd., Tokyo, Japan
[3]Yakult Central Institute for Microbiological Research, Kunitachi, Tokyo, Japan

ABSTRACT: Sulfated polysaccharides were previously shown to inhibit virus adsorption to the surfaces of animal cells. In this paper, we report that oral administration of fucoidan, a type of sulfated polysaccharide, to shrimp can prevent white spot syndrome (WSS). Partially purified fucoidan (PPF) was prepared from the brown alga "Okinawa-mozuku", *Cladosiphon okamuranus*, and mixed with shrimp diet. PPF-mixed diet was fed to kuruma shrimp, *Penaeus japonicus*, at concentrations of 60 and 100 mg PPF/kg shrimp body weight/day for 15 days. Four days after the initiation of PPF feeding, the shrimp were challenged with WSS virus (WSSV) by immersion. Mortality was monitored for 10 days after the challenge. The average survivals of the PPF-fed shrimp were 77.0% in the 60-mg group and 76.2% in the 100-mg group. These two values were significantly higher than the values for the control groups (0%), which were fed with PPF-free diet ($P<0.01$). Therefore, we conclude that fucoidan inhibits the attachment of WSSV to kuruma shrimp cells, resulting in prevention of WSSV infection.

KEY WORDS: Penaeid acute viremia, PRDV, shrimp, virus, baculovirus, fucoidan, *Cladosiphon okamuranus*

INTRODUCTION

Penaeid acute viremia (PAV) has been causing serious problems to kuruma shrimp (*Penaeus japonicus*) farmers in Japan (Inouye et al, 1994; Takahashi et al, 1994). Its causative agent is called penaeid rod-shaped DNA virus (PRDV) (Inouye et al, 1996). Infected shrimp show white spots inside of the carapace and reddish discoloration of the body. Similar virus diseases were reported from other Asian countries (Chou et al, 1995; Huang et al, 1995; Wongteerasupaya et al, 1995; Lightner, 1996) and the USA, where the disease has been named white spot syndrome (WSS) (Durand et al, 1996). These diseases are commonly characterized by the presence of white spots inside of the carapace, by hypertrophied nuclei of infected cells and by the unique morphology of the ovoid-shaped virions. Therefore, these two viruses, PRDV and WSS virus (WSSV), are thought to be closely related, if not identical. Although PAV and PRDV are the standardized names of this infection in Japan, in this paper, the names of WSS and WSSV are used instead since they are recognized worldwide.

The inhibitory effects of sulfated polysaccharides (e.g., fucoidan and dextran sulfate) on the replication of enveloped viruses (herpes simplex virus, human cytomegalovirus and human immunodeficiency virus) have been proven (Baba et al, 1988) and they are potent prophylactic agents against infections of these viruses. The mechanism of action of these compounds has been attributed to the inhibition of virus adsorption to host cells (Baba et al, 1988). Of these sulfated polysaccharides, fucoidan can be extracted from the brown alga "Okinawa-mozuku", *Cladosiphon okamuranus*, by a simple process.

In this paper, we examined the efficacy of oral administration of fucoidan derived from Okinawa-mozuku for prevention of WSS in kuruma shrimp.

MATERIALS AND METHODS

Shrimp

Healthy juvenile kuruma shrimp were used for the feeding and challenge trails, and the experiments were done twice using different sizes of shrimp: 8.2 g in the first experiment and 12.3 g in the second experiment. The number of shrimp used in each group was 15-20. Water temperature in the holding tanks was 21-22°C.

Fucoidan

Fucoidan was extracted and partially purified from the brown alga "Okinawa-mozuku", *Cladosiphon okamuranus.*

Takahashi Y, Uehara K, Watanabe R, Okumura T, Yamashita T, Omura H, Yomo T, Kawano T, Kanemitsu A, Narasaka H, Suzuki N, Itami T (1998) Efficacy of oral administration of fucoidan, a sulfated polysaccharide, in controlling white spot syndrome in kuruma shrimp in Japan. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: itamit@fish-u.ac.jp

The concentration of fucoidan in this extract (partially purified fucoidan: PPF) was 71.3%. PPF was mixed with components of the shrimp diet and dry pellets were prepared. The dosages of PPF were 60 and 100 mg/kg shrimp body weight/day. PPF-mixed diet was fed to shrimp every day for 15 days. Four days after the initiation of feeding PPF diet, the shrimp were challenged with WSSV by the water-borne method. The same diet without added PPF was fed to the control groups.

Challenge trials

Three heads of WSS-infected shrimp stored at -80°C were used. Carapace, eyes and legs of the heads were removed and pooled heads were homogenized in 20 ml sterile sea water. The homogenate was diluted 1/8,000 with sea water. Shrimp were immersed in this diluted homogenate for 2 h at 22°C with aeration and then were transferred to holding tanks. Mortality was monitored for 10 days after the challenge and polymerase chain reaction (PCR) assays were carried out to detect the virus in dead shrimp (Takahashi et al, 1996). The statistical significance was determined using a χ^2 test.

RESULTS AND DISCUSSION

The survival rates of shrimp challenged with WSSV are shown in Fig. 1. The final survival rates in the first experiment (Fig. 1A) were 75.0% in the 60-mg PPF-fed group (n=20) and 70.0% in the 100-mg fed group (n=20). These

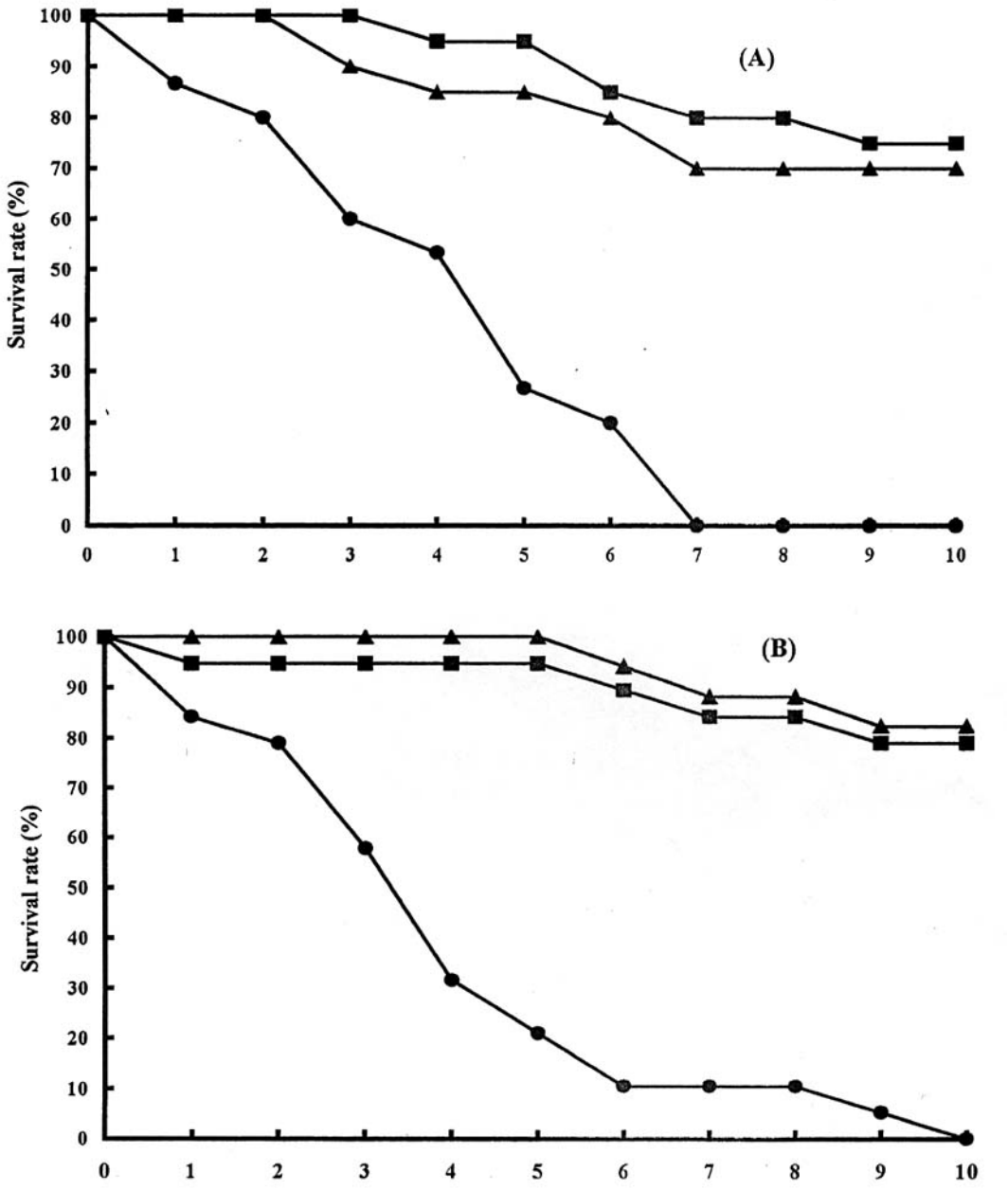


Figure 1. Survival of shrimp fed with partially purified fucoidan (PPF) compared with control shrimp after water-borne exposure to white spot syndrome virus. The experiments were repeated using different sizes of shrimp. (A) Survival of 8.2 g shrimp. (B) Survival of 12.2 g shrimp. ■ = fed 60 mg PPF per kg; ∇ = fed 100 mg PPF per kg; ● = control group.

values were significantly higher than the value in the control group (0%, n=15, $p<0.01$). In the second experiment (Fig. 1B), the final survival rates were 78.9% in the 60-mg PPF-fed group (n=19) and 82.4% in the 100-mg fed group (n=17). These values are significantly higher than the value in the control group (0%, n=19, $p<0.01$). There were no significant differences between the 60-mg PPF-fed group and the 100-mg fed group in either experiment. The shrimp that died 3-4 days post-challenge showed the typical signs of WSS, and WSSV was detected by PCR in all the dead shrimp. These results revealed that the oral administration of PPF was able to prevent WSS in shrimp, at as little as 60 mg/kg shrimp body weight/day.

In general, sulfated polysaccharides (e.g., fucoidan and dextran sulfate) inhibit the adsorption of enveloped viruses to host cells (Baba et al, 1988). The same mechanism of action of fucoidan is presumed to have occurred in the challenged shrimp when fucoidan was administered orally. Further investigations are required to demonstrate the mode of action of fucoidan in the digestive tract by *in vivo* and *in vitro* examination.

PPF, the preparation used in this experiment, was rather costly and would not be cost-effective for use in aquaculture. In order to minimize the production cost of fucoidan for application to aquaculture industries, the potency of the crude extract of this alga or other sources should be examined and evaluated. The optimum feeding schedule of the fucoidan might also be determined with a view to reducing the total cost. Our demonstration of the effectiveness of intermittent feeding of peptidoglycan of *Bifidobacterium thermophilum* (Itami et al. 1998) is an example of such a reduced and optimized feeding schedule. For long-term feeding, the effect on growth performance with fucoidan should also be clarified.

LITERATURE CITED

Baba M, Snoeck R, Pauwels R, de Clercq E (1988) Sulfated polysaccharides are potent and selective inhibitors of various enveloped viruses, including herpes simplex virus, cytomegalovirus, vesicular stomatitis virus, and human iumodeficiency virus. Antimicrobe Agents Chemother 32: 1742-1745

Chou H.-Y, Huang C-Y, Wang C-H, Chiang H-C, Lo C-F (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 23: 165-173

Durand S, Lightner DV, Nunan LM, Redman RM, Mari J, Bonami J-R (1996) Application of gene probes as diagnostic tools for white spot baculovirus (WSBV) of penaeid shrimp. Dis Aquat Org 27: 59-66

Huang J, Song X, Yu J, Yang C (1995) Baculoviral hypodermal and hematopoietic necrosis - study on the pathogen and pathology of the explosive epidemic disease of shrimp. Marine Fisheries Research 16: 1-10. (In Chinese)

Inouye K, Miwa S, Oseko N, Nakano H, Kimura T, Momoyama K, Hiraoka M (1994) Mass mortality of cultured kuruma shrimp *Penaeus japonicus* in Japan in 1993: Electron microscopic evidence of the causative virus. Fish Pathol 29:149-158

Inouye K, Yamano K, Ikeda N, Kimura T, Nakano H, Momoyama K, Kobayashi J, Miyajima S (1996) The penaeid rod-shaped DNA virus (PRDV), which causes penaeid acute viremia (PAV). Fish Pathol 31: 39-45

Itami T, Kubono K, Asano M, Tokushige K, Takeno N, Nishimura H, Kondo M, Takahashi Y (1998) Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. Aquaculture 164: 277-288

Lightner, D. V. (1996) A handbook of shrimp pathology and diagnostic procedures for diseases of culrured penaeid shrimp. World Aquaculture Society, Baton Rouge, Louisiana, USA. Section 3. 11

Maeda M, Itami T, Furumoto A, Hennig O, Imamura T, Kondo M, Hirono I, Aoki T, Takahashi Y (1998) Detection of penaeid rod-shaped dna virus (PRDV) in wild-caught shrimp and other crustaceans. Fish Pathol, in press

Takahashi Y, Itami T, Kondo M, Maeda M, Fujii R, Tomonaga S, Supamattaya K, Boonyaratpalin S (1994) Electron microscopic evidence of bacilliform virus infection in kuruma shrimp (*Penaeus japonicus*). Fish Pathol 29: 121-125

Takahashi Y, Itami T, Maeda M, Suzuki N, Kasornchandra J, Supamattaya K, Khongpradit R, Boonyaratpalin S, Kondo M, Kawai K, Kusuda R, Hirono I, T. Aoki (1996) Polymerase chain reaction (PCR) amplification of bacilliform virus (RV-PJ) DNA in *Penaeus japonicus* Bate and systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *Penaeus monodon* Fabricius. J Fish Dis 19: 399-403

Wongteerasupaya, C, Vickers JE, Sriurairatana S, Nash G.L, Akrajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat Org 21: 69-77

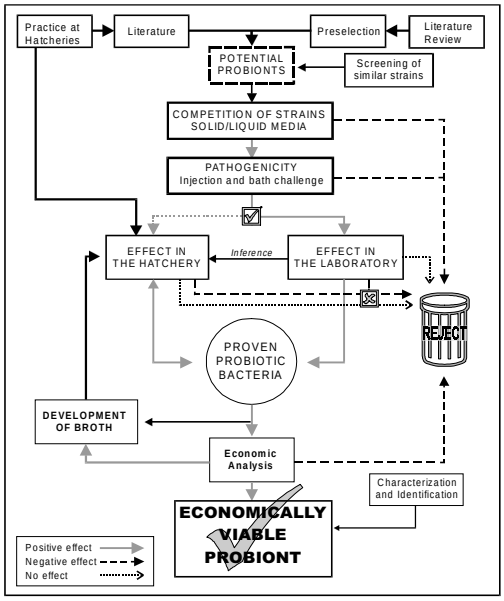
Selection of Probiotic Bacteria for Use in Aquaculture

Bruno Gomez-Gil, Ana Roque

CIAD Mazatlán Unit for Aquaculture and Environmental Management
AP. 711, Mazatlán, Sin. CP. 82000 Mexico

ABSTRACT: Selection of probiotic bacteria has usually been an empirical process based on limited scientific evidence. Many of the failures in probiotic research could be attributed to the selection of inappropriate microorganisms. Selection steps have been defined, but they need to be adapted for different host species and environments. General selection criteria are determined mainly by biosafety aspects, methods of production and processing, the method of administration of the probiotic and the location in the body where the microorganisms are expected to be active. 1. Background information. The first two steps for the selection of potential probiotic bacteria are a critical review of the literature available and the practice at hatcheries. 2. Acquisition of potential probiotic bacteria. For practical purposes the identification of the potential probiotic bacteria is not a primary objective; although proper identification is required for production quality control and patenting to protect commercial interests. Important aspects are viability of the strain, resistance to antibiotics and ability to outcompete other strains. 3. Evaluating the ability of potential probiotic bacteria to out-compete pathogenic strains. The crucial step in selecting a probiotic bacterium may be its ability to out-compete potentially pathogenic strains. This involves viability of the probiotic strain within larvae or in their environment, adherence to host surfaces, ability to colonise and to prevent the establishment of potentially pathogenic bacteria. If any of these features are absent it is difficult for a potentially probiotic strain to benefit the host for a reasonable period. Observing the growth of a selected potential probiont in a similar medium as the larval rearing environment can serve to predict its viability. 4. Evaluating the pathogenicity of selected strains. The next step would be to evaluate the pathogenicity of the strain for larvae. A bath challenge with larvae would be preferable, but such challenges are not yet reproducible. Alternative methods that can provide acceptable results are injection challenge of juvenile shrimp or bath challenge of disinfected *Artemia* nauplii. If reliable results can be obtained from a larval challenge and if a highly positive correlation is established between mortalities observed in *Artemia* nauplii trials and results with shrimp larvae, then the *Artemia* nauplii challenge might be used as a practical method to assess the pathogenicity of candidate strains. Nevertheless, one has to be aware that nothing can replace the actual challenge with the desired target organisms. 5. Evaluating potentially probiotic bacteria with larvae. A selected probiont must be tested with shrimp larvae. Tests can be carried out in an actual hatchery environment, but it is advisable first to perform laboratory trials. Positive results might be not only higher survival but an improvement in larval performance such as more rapid development, higher weight gains, improved resistance to stress and lower incidence of potentially pathogenic bacteria. 6. Economic evaluation. Finally it is necessary to perform an economic analysis to ascertain whethr implementation of the probiotic treatment would be worth the investment.

Figure 1. Flow diagram proposed to select probiotic microorganisms for use in the larval rearing of aquatic organisms.



KEY WORDS: Probiotics, aquaculture, shrimp

Gomez-Gil B, Roque A (1998) Selection of probiotic bacteria for use in aquaculture. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Probiotics in Aquaculture: A Case Study of Probiotics for Larvae of the Black Tiger Shrimp (*Penaeus monodon*)

*[1]Sirirat Rengpipat, [1]Sombat Rukpratanporn
[2]Somkiat Piyatiratitivorakul, [2]Piamsak Menasveta

[1]Department of Microbiology and [2]Department of Marine Science, Faculty of Science Chulalongkorn University, Bangkok 10330, Thailand
[2]Aquatic Resources Research Institute, Chulalongkorn University, Bangkok 10330, Thailand

ABSTRACT: The trend of using probiotics in aquaculture is increasing due to research results indicating their ability to increase production and prevent disease in farm animals. The development of suitable probiotics for biocontrol in aquaculture would result in less reliance on chemicals and antibiotics and result in a better environment. In this investigation, a Thai *Bacillus* isolate (strain S11) was used as a probiotic bacterium by passage through *Artemia* sp. fed to the black tiger shrimp (*Penaeus monodon*). It was found that black tiger shrimp larvae reared using the *Bacillus*-fortified *Artemia* probiotic as a feed had significantly shorter development times and fewer disease problems than larvae reared without the probiotic.

Key words: *Penaeus monodon*, black tiger shrimp larvae, probiotics

INTRODUCTION

Microbes and aquaculture

Microbes play both direct and indirect roles in aquaculture. They are important causes of diseases which may readily spread through water to aquatic animal hosts. As flora in soil and water, they also influence the aquatic environment by involvement in C, N, S and P cycles important for ecological balances. Other microbes live in and on aquatic plants and animals. These may be specific for individual organisms and important to their health. An inbalance in the microbial flora in the water or in these organisms often leads to pathogenesis. For example, an inbalance in *Vibrio* species in the rearing water or in the GI tracts of shrimp and fish can lead to pathogenesis (Rengpipat 1996).

Marine culture of shrimp, crabs, fish, oysters and mussels in Thailand provides good income for producers and products that are popular because of their good taste and reasonable price. The diminishing seafood from capture fisheries has paved the way to industrial scale aquaculture. In Thailand, particularly for the black tiger shrimp (*Penaeus monodon*), most farmers build large ponds and raise shrimp intensively. Even though the production has been good for the past 10 years (Thai Department of Business Economics 1996), the trend for the last 2-3 years has been diminished production due to viral diseases such as yellow head virus and white spot syndrome virus (WSSV) (also called systemic ectodermal and mesodermal baculovirus or SEMBV) and to luminescent bacterial disease (Rengpipat 1996). These agents can cause sudden death on a massive scale.

Probiotic microbes in aquaculture

For more than 50 years, beneficial microbes defined as probiotics (Fuller, 1989, 1992 and 1997) have been used successfully for raising healthy and disease-tolerant farm animals like swine (Baird 1977; Pollman et al. 1980) and chickens (Dilworth & Day 1978; Miles et al.1981). These probiotics are now widely used for enhancing production of land animals and they have gained acceptance as being better, cheaper and more effective in promoting animal health than administration of antibiotics or chemical substances.

More recently (within the past 10 years) researchers have sought beneficial microbes for aquaculture by attempting to isolate from seawater, sediments and GI tracts those capable of producing antibiotics and/or antimicrobial substances that can inhibit pathogens *in vitro* (Table 1). Bacteria and unicellular algae capable of inhibiting pathogenic bacteria have been found (Munro et al. 1995; Austin and Day 1990). In setting criteria for the most suitable probiotics in aquaculture, one must be concerned with indirect effects on ecosystem cycles and food chains.

Douillet and Langdon (1994) used a commercial probiotic bacterium (CA2) as a larval feed supplement to increase production in oysters. Other researchers have found that probiotics prevent diseases in salmon (Austin et al. 1992 and 1995), larvae of scallop (Requelme et al. 1997) and black tiger shrimp (Rengpipat et al. 1998 and unpublished data). Austin et al. (1992) stressed that probiotics control diseases by prophylaxis, and that they are not meant to be used as

Rengpipat S, Rukpratanporn S, Piyatiratitivorakul S, Menasveta P (1998) Probiotics in Aquaculture: A Case Study of Probiotics for Larvae of the Black Tiger Shrimp (*Penaeus monodon*). *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: sirirat@mail.sc.chula.ac.th

Table 1. Reduction of pathogens by microorganisms that possess probiotic properties at *in vitro*.

Aquatic animal	Microorganisms	Pathogens	References
Fish	Antibiotic-producing marine bacteria (Obligate halophilic bacteria)	*Aeromonas hydrophila* B-32 *A. salmonicida* ATCC 14174	Dopazo et al. (1988)
Prawns	*Tetraselmis suecica*(microalgae)	*Vibrio alginolyticus* *V. anguillarum* *V. parahaemolyticus* *V. vulnificus*	Austin and Day (1990)
Fish	*Planococcus*	*Serratia liquefaciens*	Austin and Billaud (1990)
Fish	Gram -ve rod Gram +ve rod	*V. anguillarum* HI 11345 *A. salmonicida* *A. hydrophila*	Westerdahl et al. (1991)
Fish (Turbot larvae)	*Flavobacterium* sp. *V. fluvialis* *V. natvigens* *Vibrio* spp.	*Pavlova lutheri* (unicellular algae)	Munro et al. (1995)

Table 2. Probiotics and feed supplements used in aquaculture.

Aquatic animal	Probiotic strain	Challenge test with	Results	References
Salmon	*Tetraselmis suecica*	*A. salmonicida* *A. hydrophila* *Lactobacillus* spp. *S. liquefaciens* *V. anguillarum* *V. salmonicida* *Yersinia ruckeri* type I	-good control of diseases by Prophylaxis	Austin et al. (1992)
Oyster (larval culture)	CA2	N.D.	-better yield	Douillet and Langdon (1994)
Salmon	*V. alginolyticus*	*A. salmonicida* *V. anguillarum* *V. ordalii*	-good control of disease	Austin et al. (1995)
Salmon	*L. plantarum* (lyophilized form) could inhibit *V. anguillarum*	*A. salmonicida*	-*Lactobacillus* colonized intestinal wall -could not control disease	Gildberg et al. (1995)
Scallop (larval stage)	*Vibrio* spp. *Pseudomonas* sp.	*V. anguillarum* related (VAR)	- good control of disease	Riquelme et al. (1997)
Black tiger shrimp	*Bacillus* strain S11	*V. harveyi*	- better yield - good control of disease	Rengpipat et al. (1998)
Black tiger shrimp	*Lactobacillus* spp.	*V. harveyi*	- better yield - good control of disease	Rengpipat et al. (unpublished data)

therapeutics. For example, *Lactobacillus plantarum* inhibitory to the salmon pathogen *Vibrio anguillarum* could not be used to treat fish infected with the lethal pathogen, *A. salmonicida.* However, lyophilized *Lactobacillus plantarum* fed to salmon was shown to be able to survive in the GI tract (Gildberg et al, 1995).

Probiotics and black tiger shrimp culture

Rengpipat et al. (1998) isolated *Bacillus* strain S11 from the GI tract of *Penaeus monodon* broodstock caught in the Gulf of Thailand. It inhibited the luminescent disease bacterium, *Vibrio harveyi*, 100% and could promote better yields of black tiger shrimp. Mixed *Lactobacillus* species isolated

from the GI tract of local Thai chickens was also used as a feed supplement to black tiger shrimp (Rengpipat et al., unpublished data) and also resulted in higher shrimp production (Table 2).

Probiotics can be freshly prepared and mixed with the shrimp diet as described by Rengpipat et al. (1998). However, this study was carried out to determine the effectiveness of feeding the *Artemia* encapsulated probiotic *Bacillus* strain S11 for enhancing growth and survival of shrimp larvae.

MATERIALS AND METHODS

The present study was conducted at the aquaculture laboratory, Department of Marine Science and at the Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.

Bacterial culture

Bacillus strain S11 (Rengpipat et al. 1998) was stocked on tryptic soy agar (TSA) (Difco) and cultured in tryptic soy broth (TSB) (Difco). Culture conditions were at 37°C in 2-L flasks for 24 h, after which the cells were centrifuged and washed in sterile normal saline solution (NSS) three times immediately before use.

Preparation of encapsulated probiotic *Artemia* sp.

Artemia cysts (Great Lake Artemia, Salt Lake City, Utah, U.S.A.) were hatched (1 g of cysts per liter gently aerated 30 ppt seawater) and harvested at 24 h. During harvesting the cysts and nauplii were seperated. The nauplii were then fed directly to the shrimp postlarvae or kept at 4°C for further use. For probiotic encapsulation, freshly prepared cell cultures of *Bacillus* strain S11 were added to *Artemia* cultures at the begining of the hatching process at a concentration of 10^4 cells/ml. The *Artemia* were subsequently harvested at 24 h and fed immediately to the shrimp postlarvae.

Experimental design

Penaeus monodon postlarvae-10 were bought from a backyard hatchery in Chonburi Province, Thailand. The postlarvae were from a single parent. After acclimatization at the laboratory for 5 days, uniform size postlarvae were selected for probiotic testing.

Experimental units for postlarvae rearing comprised 10-L cylindrical fiber glass tanks containing 7.5-L of 25 ppt seawater and an initital stocking of 50 postlarvae. Three replicates were used for a total of 150 postlarvae per treatment. Each rearing unit was a closed recirculating system with self-contained filtering unit consisting of sand and oyster shells. Water was continuously pumped through the filtering unit by an air lift system. A small amount of dechlorinated tap water was added every two days to compensate for evaporation and maintain a constant salinity.

Statistical comparisons of the control group (fed *Artemia* only) and the treated group (fed probiotic *Artemia*) were carried out using a student t-test. The experiment was monitored for two weeks. Shrimp were fed three times daily at 9:00, 13:00 and 18:00 h with an excess of *Artemia* nauplii. All materials used for each experimental unit were separated to avoid any cross contamination.

Lengths and weights of 15 randomly selected shrimp from each tank were recorded weekly. Shrimp survival was also determined in each tank at the end of the first and second weeks. Weekly water samples of 100 ml were collected from the center of each tank for two weeks. Water quality was monitored weekly and included the parameters of temperature, pH, salinity, dissolved oxygen, ammonium ion and phosphate ion measured using techniques described by Strickland and Parsons (1972).

Pathogen challenge test

After feeding for two weeks, shrimp were challenged with the luminous bacterium *V. harveyi* D331 which had been cultured and maintained using thiosulphate citrate bile salt TCBS broth and agar (Difco). Shrimp in the control and treated groups (85 shrimp per treatment) were immersed in a suspension of *V. harveyi* D 331 at ~ 10^7 CFUml^{-1} according to Austin et al. (1995). Shrimp survival was determined after 4 days of challenge. At the same time, three shrimp from each treatment were randomly sampled. Each whole shrimp was cut into small pieces using sterile surgical sciccors and transferred to a sterile tube. Bacterial determinations were then made using serial dilutions in NSS, followed by plating on TSA and TCBS agar. After 24-48 h of incubation at 37°C, colonies were counted and recorded. Microbial strains from TSA were re-examined using Gram staining, spore staining and selected biochemical tests as described by Sneath (1986). *V. harveyi* cultures isolated from shrimp were purified and identified using Gram staining, an oxidase test and motility test and they were compared with the original *V. harveyi* D331 culture. *V. harveyi* D331 culture was kindly provided by the Shrimp Culture Research Center, Charoen Pokphand Feedmill Co. Ltd., Samutsakorn, Thailand. We reconfirmed the identity of *V. harveyi* by following procedures described by Holt et al. (1986). Shrimp survival was determined for each treatment after 4 days of the challenge test.

RESULTS AND DISCUSSION

Bacillus strain S11 showed no inhibitory effect on *Artemia* hatching when compared to *Artemia* alone or *Artemia* fed with *Saccharomyces cerevisiae*. *Artemia* nauplii at ~$1.84x10^5$ g^{-1} were counted after hatching for 24 h. *Bacillus* strain S11 on *Artemia* nauplii were found to be ~$2x10^2$, ~ $6.4x10^4$ and ~$1x10^2$ CFU g^{-1} (wet weight) at 0, 4 and 8 h, respectively, after hatching.

The raising of black tiger shrimp postlarvae using *Artemia* encapsulated *Bacillus* strain S11 showed an increase in body weight and length (Table 3). No obvious effects of *Bacillus* strain S11 on water quality were found (Table 4). During the first week, however, ammonium increased to 1.67 mgL^{-1} in one control group, but later decreased to near zero. At two weeks, *Penaeus monodon* survival was significantly different between the control group (85%) and the treated group (89%)(Figure 1).

Table 3. Average live weight and length of *Penaeus monodon* cultured for 2 weeks in two feed treatments

Parameters	Control	Probiotics
Weight (mg)	26.0*	43.8*
Length (cm)	$1.71^{b} \pm 0.20$	$1.83^{a} \pm 0.31$

Control: shrimp with artemia; Probiotics: shrimp with *Bacillus* strain S11- fed artemia; *Total weight divided by a number of shrimp (43 shrimp); ^{b,a} Different superscripts in the same row significantly different

Table 4. Range of water quality values in shrimp culture water during 2 weeks of probiotic trial.

	Range of water quality values	
Parameter	Control	Probiotics
Temperature (°C)	29.5	29.5
pH	7.79 - 8.23	7.78 - 8.22
Salinity (ppt)	25	25
Dissolved oxygen (mg L^{-1})	7.9 - 8.1	8.0 - 8.1
Ammonium (mg L^{-1})	0 - 1.67	0 - 0.5
Phosphate (mg L^{-1})	3	3

Control : shrimp with artemia
Probiotics : shrimp with *Bacillus* strain S11- fed artemia

When challenged with the luminous disease bacterium *Vibrio harveyi*, the shrimp treated with probiotics showed a higher survival (13%) when compared to the control group (4%) (Figure 2). *Vibrio harveyi* was more virulent to younger stages of shrimp. High numbers of *Vibrio harveyi* were present in both the rearing water and the shrimp themselves on the fourth day of this experiment (Table 5). However, *Bacillus* strain S11 was also detected in significant numbers in the rearing water and the shrimp, clearly showing that *Artemia* was an effective probiotic carrier.

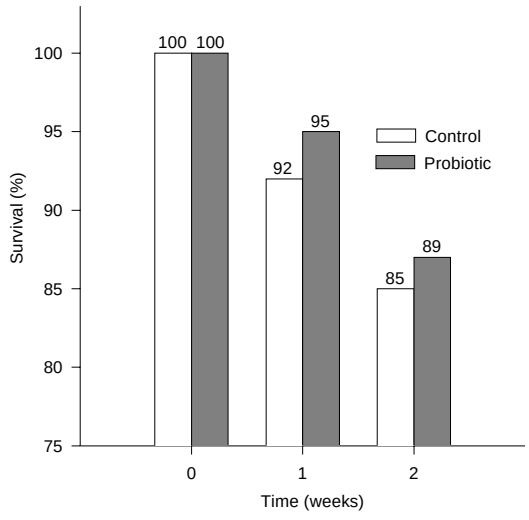


Figure 1. *Penaeus monodon* survival after culture for 2 weeks on control and probiotic feeds.

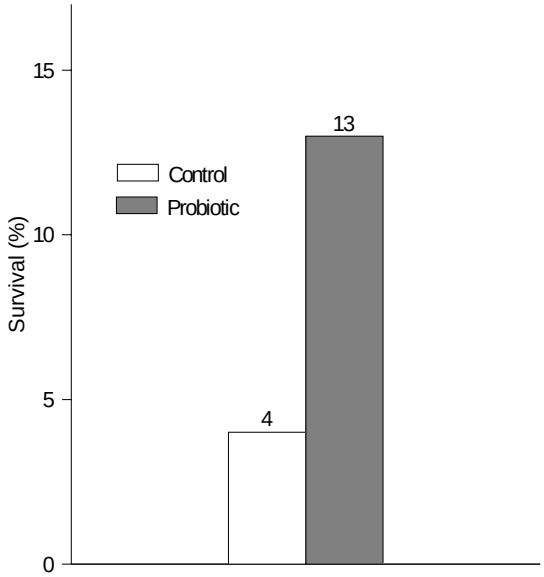


Figure 2. Percentage survival of *Penaeus monodon* after challenge with *Vibrio harveyi* D331.

Bacillus strain S11 is considered a saprophytic strain which is environmental-friendly and has been proven before as a probiotic bacterium for black tiger shrimp (Rengpipat et al., 1998) when mixed with shrimp feed. Therefore, this investigation supported the previous work and also showed that probiotics could be passed through *Artemia* which are routinely used to feed shrimp larvae. Our method may prove beneficial as an enhancement for hatchery postlarvae or for improvement of young shrimp survival at the initial stages of earthen pond culture.

Mechanisms of probiotic action in the host are not fully understood. However, a user may select strains that are suitable or specific for a particular host and environmentally safe. The purpose of their use in aquaculture is to reduce the dependence on antibiotics and chemicals, thus improving environmental safety. Use of local isolates is recommended for biosafety reasons and to avoid sudden changes in the microbial flora of the ecosystem.

ACKNOWLEDGEMENTS: The authors are grateful to Dr. Hunsa Punnapayak for proof reading. This research was supported by the Thailand Reseach Fund (TRF) and The Royal Thai Government Budget for Research.

LITERATURE CITED

Austin B, Billaud AC (1990) Inhibition of the fish pathogen, *Serratia liquefaciens*, by an antibiotic-producing isolate of *Planococcus* recovered from sea water. J Fish Dis 13:553-556

Austin B, Day JG (1990) Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture 90:389-392

Austin B, Baudet E, Stobie M (1992) Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. J Fish Dis 15:55-61

Austin B, Stuckey LF, Robertson PAW, Effendi I, Griffith DRW (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J Fish Dis 18:93-96

Table 5. Average bacterial counts in water and whole shrimp during challenged with *V. harveyi* D331 by immersion

		V. harveyi (CFU ml^{-1} or g^{-1})		*Bacillus* strain S11 (CFU ml^{-1} or g^{-1})	
		Control	Probiotics	Control	Probiotics
Water	- day 1	$1.28x10^7$	$1.31x10^7$	0	75
	- day 4	$1.07x10^6$	$3.64x10^5$	0	50
Shrimp	- day 1	$6.36x10^6$	$6.09x10^6$	0	$1.20x10^3$
	- day 4	TNTC	TNTC	0	$2.76x10^2$

Control : shrimp with artemia; Probiotics : shrimp with *Bacillus* strain S11- fed artemia
TNTC : Too numerous to count

Baird DM (1977) Probiotics help boost feed efficiency. Feedstuffs 49:11-12

Dilworth BC, Day EJ (1978) Lactobacillus cultures in brooder diets. Poultry Science 57:1101

Dopazo CP, Lemos ML, Lodeiros C, Bolinches J, Barja JL, Toranzo AE (1988) Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. J Appl Bacteriol 65:97-101

Douillet PA, Langdon CJ (1994) Use of a probiotic for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunberg). Aquaculture 119:25-40

Fuller R (1989) Probiotic in man and animals. J Appl Bacteriol 66: 365-378

Fuller R (1992) Probiotics: The scientific basis. 1st ed. London: Chapman & Hall 398 pp.

Fuller R (1997) Probiotics 2 Applications and practical aspects. 1st ed. London: Chapman & Hall 211 pp.

Gildberg A, Johansen A, Bogwald J (1995) Growth and survival of Atlantic salmon (*Salmo salar*) fry given diets supplemented with fish protein hydrolysate and lactic acid bacteria during a challenge trial with *Aeromonas salmonicida*. Aquaculture 138:23

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1986) Facultatively anaerobic Gram-negative rods. In: Bergey's manual of determinative bacteriology. ninth edition, William&Wilkins, Baltimore, pp 175-289.

Miles RD, Arafa AS, Harms RH Carlson CW Reid BL, Crawford JS (1981) Effects of a living non-freeze dried *Lactobacillus acidophilus* culture on performance, egg quality and gut microflora in commercial layers. Poultry Science 60;993-1004

Munro PD, McLean HA, Barbour A, Birkbeck TH (1995) Stimulation or inhibition of growth of the unicellular alga *Pavlova lutheri* by bacteria isolated from larval turbot culture systems. J Appl Bacteriol 79:519-524

Pollman DS, Danielson DM, Peo ER, (1980) Effect of microbial feed additives on performance of starter and growing-finishing pigs. J Anim Sci 51:577-581

Rengpipat S, (1996) Microbes and black tiger shrimp culture. Thai J Aquat Sci 3:42-51

Rengpipat S, Phianpak W, Piyatiratitivorakul S, Menasveta P (1998) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture (in press)

Rengpipat S, Phianpak W, Piyatiratitivorakul S, Menasveta P Utilization of *Lactobacillus* spp. as probiotics for feeding *Penaeus monodon*, black tiger shrimp. (unpublished data)

Riquelme C, Araya R, Vergara N, Rojas A, Guaita M, Candia M (1997) Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). Aquaculture 154:17-26

Sneath, PHA (1986) Endospore-forming Gram-positive rods and cocci. In: PHA Sneath, NS Mair, ME Sharp and JG Holt (Editors), Bergey's manual of systematic bacteriology. Vol 2 Baltimore:Williams&Wilkins, pp.1104-1105

Strickland JDH , Parsons TR (1972) A practical handbook of seawater analysis. 2nd edition. Fisheries Research Board of Canada Bull., 167. Alger Press, 310pp.

Thai Department of Business Economics (1996) Export value of Thai frozen shrimp 1986-1996. Shrimp Culture Newsletter 96:1-4

Westerdahl A, Olsson JC, Kjelleberg S, Conway PL (1991) Isolation and characterisation of Turbot (*Scophtalmus maximus*)-associated bacteria with inhibitory effects agianst *Vibrio anguillarum*. Appl Environ Microbiol 57:2223-2228

Use of By-9 as a Probiotic Agent in the Larval Rearing of *Penaeus monodon*

Ketut Sugama Haryanti, S. Tsumura

Gondol Research Station of Coastal Fisheries
PO. Box. 140 Sin-araja 8 I 101 Bali, INDONESIA

ABSTRACT: Gondol Research Station for Coastal Fisheries Indonesia, has been testing the inoculaiton of bacterial strains that promote the growth and survival of *P. monodon.* Batceria were required that would suppress the growth of other pathogenic bacteria, especially *Vibrio harveyi*, but would not kill or inhibit useful microflora. The present study reports the isolation, vibriostatic testing and mass culture of such bacteria, followed by application in the larval rearing of *P. monodon* to promote better growth and survival rates and also to protect the larvae from pathogens without the use of antibiotics. Of 123 coastal bacterial isolates collected in East Java and Bali and tested for vibriostatic activity, one strain coded BY-9 was confirmed to inhibit the growth of *V. harveyi*. BY-9 was produced on a large scale and several tests were conducted before it was used for mass production of *P. monodon* larvae. The latest test was conducted in 18 ton larval rearing tanks where its density was maintained at 10^6 cfu/ml. The larvae were reared up to PL-10. The results showed that BY-9 inoculation gave lower *Vibrio* density and a higher survival rate (46.11 %) than uninoculated controls (10.57%). These results suggested that BY-9 could be applied as a probiotic agent and as a biological control agent to promote better growth and survival in the larval rearing of *P. monodon*.

KEY WORDS: *Penaeus monodon*, Probiotic

Haryanti KS, Tsumura S (1998) Use of By-9 as a probiotic agent in the larval rearing of *Penaeus monodon*. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Will Microbial Manipulation Sustain the Ecological Balance in Shrimp (*Penaeus monodon*) Hatcheries?

***[1]C.R. Lavilla-Pitogo, [2]L.J. Albright, [1]M.G. Paner**

[1]Fish Health Section, Aquaculture Department, Southeast Asian Fisheries Development Center Tigbauan 5021, Iloilo, Philippines
[2]Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada

ABSTRACT: A shift in preferred methods employed to contain bacterial diseases in the hatchery phase of shrimp culture has resulted largely from the unsuccessful control by and deleterious effects of chemotherapy. Manipulation of hatchery microbial ecology has gained popularity, but for successful implementation, this niche-filling approach requires a thorough understanding of the epidemiology of bacterial diseases in the hatchery. This study examined the responses of *Vibrio harveyi* populations, (associated with luminescent vibriosis in shrimp larvae) to various physico-chemical factors and various hatchery components. Results showed that *V. harveyi* had a wider range of tolerance to environmental parameters than larvae of *Penaeus monodon,* such that control measures based on manipulation of these parameters might not be feasible. However, it was evident from the results that there were components in the shrimp hatchery environment that could be manipulated to control high populations of *V. harveyi*. The natural microflora of seawater, as well as the microbial flora associated with the diatoms *Skeletonema costatum* and *Chaetoceros calcitrans* negatively affected the survival of *V. harveyi* in experimental mixed cultures. The successful manipulation of such benign microbial components to compete with and exclude potential pathogens is necessary to sustain ecological balance in the shrimp hatchery environment.

KEY WORDS: *Vibrio harveyi*, luminescent vibriosis, shrimp hatchery, *Penaeus monodon*

INTRODUCTION

Luminescent vibriosis due to the bacteria *Vibrio harveyi* is one of the major causes of failure in shrimp, *Penaeus monodon*, larval rearing in the Philippines (Lavilla-Pitogo et al. 1990). Chemical control of the disease based on efficacy of available drugs appears to be limited because of the restricted tolerance of shrimp larvae to drugs and because of the possible development of resistant strains of bacteria (Baticados et al. 1990). Preventive measures are, therefore, necessary. The effect of chlorine as water treatment against *V. harveyi* was tested to establish effective levels for use in hatcheries (Baticados & Pitogo 1990). Furthermore, the sources of *V. harveyi* in shrimp hatcheries were investigated to unravel part of its epidemiology (Lavilla-Pitogo et al. 1992). A detailed analysis of the possible origin of luminescent bacterial disease outbreaks in the shrimp hatchery was reviewed recently (Lavilla-Pitogo & dela Peña 1998) and factors like shifting husbandry and feed practices leading to ecological imbalance were pointed out. From successful efforts in land-based animal production industries, it is generally recognized that environmental management to reduce disease impacts is more reliable for increasing profitability than "pathogen" hunts for most diseases (Stoskopf 1996).

Armed with this view and with growing evidence for feasible microbial manipulation in the larval rearing environment of various aquatic species (Dopazo et al. 1988, Nogami & Maeda 1992, Austin et al. 1995, Garriques & Arevalo 1995, Riquelme et al. 1997, Skjermo et al. 1997), some aspects in the larval rearing of *Penaeus monodon* were examined to give sound basis for effective control of bacterial pathogens in the shrimp hatchery.

The study measured the tolerance of *Vibrio harveyi* to various physico-chemical factors, determined the hatchery conditions and components that could control its growth, and tested its growth in mix cultures with diatoms and other bacteria. All these were aimed at identifying an ecologically sound preventive practice against luminescent vibriosis. This work was based on the premise that sound management of the microbial balance in the hatchery will prevent the creation of a niche for opportunistically pathogenic bacteria.

MATERIALS AND METHODS

Growth response of *Vibrio harveyi* to different salinity, pH and temperature

The growth response of *Vibrio harveyi* to various temperatures, salinity, and pH levels were studied using nutrient broth (NB) medium. Standardized bacterial inoculum contained an initial bacterial density of 10^1 colony forming units (cfu)/ml. For salinity and pH tolerance tests, cultures were incubated at room temperature (27-29°C). Inoculated NB

Lavilla-Pitogo CR, Albright LJ, Paner MG (1998) Will microbial manipulation sustain the ecological balance in shrimp (*penaeus monodon*) hatcheries? *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: celiap@seafdec.org.ph

for temperature tolerance tests was incubated in a temperature gradient incubator (Toyo Kagaku Sangyu Co. Ltd.) set at 10-39°C with minimum shaking. Viable cell counts were monitored by the spread plate method on nutrient agar (NA) with 1.5% NaCl. Monitoring of bacterial populations was done daily for 7 consecutive days.

Growth response of *Vibrio harveyi* to various hatchery components

The effect of various hatchery components on the survival of *Vibrio harveyi* was determined. This experiment was done in 3.5 l glass jars containing 2 l autoclaved seawater. A treatment using untreated seawater which was obtained directly from SEAFDEC's seawater line, was included to measure the effects of natural components of seawater. *Vibrio harveyi* was added to the following: (a) autoclaved seawater, (b) untreated seawater, (c) mixed diatoms (*Skeletonema costatum* and *Chaetoceros calcitrans*) in autoclaved seawater, (d) mixed diatoms and larvae in autoclaved seawater, and (e) larvae in autoclaved seawater. Diatoms were obtained from peak cultures and added into the vessels to obtain initial cell densities of 30,000 - 50,000 cells/ml. Larvae were washed gently to remove surface-attached bacteria and debris. Inoculated bacterial suspensions resulted to an initial density of 10^4 cells/ml. The jars were aerated and incubated under static conditions at room temperature. *Vibrio harveyi* populations were monitored daily for 7 days by the spread plate method using NA with 1.5% NaCl.

Determination of the bacterial load of *Skeletonema costatum*

One-liter samples from *Skeletonema costatum* cultures at different phases of growth were obtained, serially diluted and spread plated on NA with 1.5% NaCl to determine the total bacterial count and luminescent bacterial count. To determine the number of presumptive *Vibrio*, samples were plated on thiosulfate citrate bile sucrose (TCBS) agar, a selective medium for *Vibrio*. Plates were incubated at room temperature (28-30°C) and colonies were counted after 24 h.

Mixed culture tests

The growth pattern of *Vibrio harveyi* in various mixed cultures and in cell-free diatom filtrates was tested further to determine the factors that influence its population. *Vibrio harveyi* cultures used in all tests were grown overnight on nutrient agar with 2.0% NaCl, harvested, and suspended in sterile seawater. Initial densities in test containers were not more than 10^2 cfu/ml in all cases. The following mixed culture tests were done.

Skeletonema costatum and *Chaetoceros calcitrans* populations

Two levels of *Skeletonema* and *Chaetoceros* were used: peak cell cultures and feeding levels. The test was done using 500 ml sterile flasks with 250 ml of test mixture. Preparation of feeding densities was accomplished by adding portions of peak cell cultures to sterilized seawater to obtain a density of 30,000 - 50,000 cells/ml. Control flasks contained sterilized seawater only. Inoculation was done as described above.

Cell-free diatom filtrate

To know which component of the diatom culture had an influence on populations of *V. harveyi*, peak cultures of *C. calcitrans* and *S. costatum* were centrifuged to remove the cells. The supernatants were sterilized by filtration using 0.22µ Millipore filters. The filtrate was then distributed at 250 ml/flask. Control flasks contained sterile seawater only. *Vibrio harveyi* was inoculated as previously described.

Commercial bacterial products

The growth response of *V. harveyi* to the presence of two commercially available bacterial products was tested. Aqua Bacta Aid (ABA, Argent Laboratories) is a suspension reportedly containing 7 species of live complementary bacteria for use in hatcheries to improve water quality. NS Series HF is advertised as a scientifically blended concentration of selected, adapted and cultured bacteria formulated with enzymes and special buffers to help minimize problems in prawn hatcheries. Product ABA was available in liquid form while HF was available in granular form. Sterile seawater was used as the test medium and control. The manner of preparation of solutions and product concentration followed the manufacturers' directions. *Vibrio harveyi* was prepared and inoculated as previously described.

Mixed cultures were kept with minimum shaking throughout the tests. Bacterial counts was carried out upon inoculation of test containers and daily for 3 to 5 days thereafter, applying standard spread plate methods using nutrient agar with 1.5 % NaCl. All cultures were maintained at room temperature (28-30°C). Luminescent colonies of *V. harveyi* were counted in a dark room.

RESULTS

The growth patterns of *Vibrio harveyi* at various temperature, pH, and salinity levels are shown in Figs. 1, 2, and 3, respectively. Fig. 1 shows that *V. harveyi* can grow well at salinity levels of 5 to 70 parts per thousand (ppt), although it took two days for the bacteria to reach peak populations at 5 and 70 ppt. High numbers of colony forming units were obtained at various salinity levels up to and beyond 7 days. However, bacteria inoculated into NB without NaCl and those at 80 ppt died within one day of inoculation.

Results of the pH tolerance test are shown in Fig. 2. The growth patterns of *Vibrio harveyi* at pH 6 to 9 were very similar. However, at pH levels of 5 and 10, no colonies were recovered after one day. The result of the temperature tolerance test is shown in Fig. 3. The bacteria survived at temperatures of 14-37°C, but rapid growth occurred from 17-35°C (Fig. 3). High populations of bacteria remained viable in the tests even after 7 days of inoculation.

The growth patterns of *Vibrio harveyi* exposed to various components in the hatchery are shown in Fig. 4. Bacterial density in all treatments after inoculation was 10^4 cfu/ml. It is interesting to note that in the absence of competitors in sterilized seawater, *V. harveyi* sustained high populations of up to 10^6 cfu/ml, even after 7 days. By contrast, the population of *V. harveyi* inoculated into untreated seawater obtained directly from SEAFDEC's seawater line started to

Figure 1. Growth patterns of *Vibrio harveyi* at different NaCl concentrations.

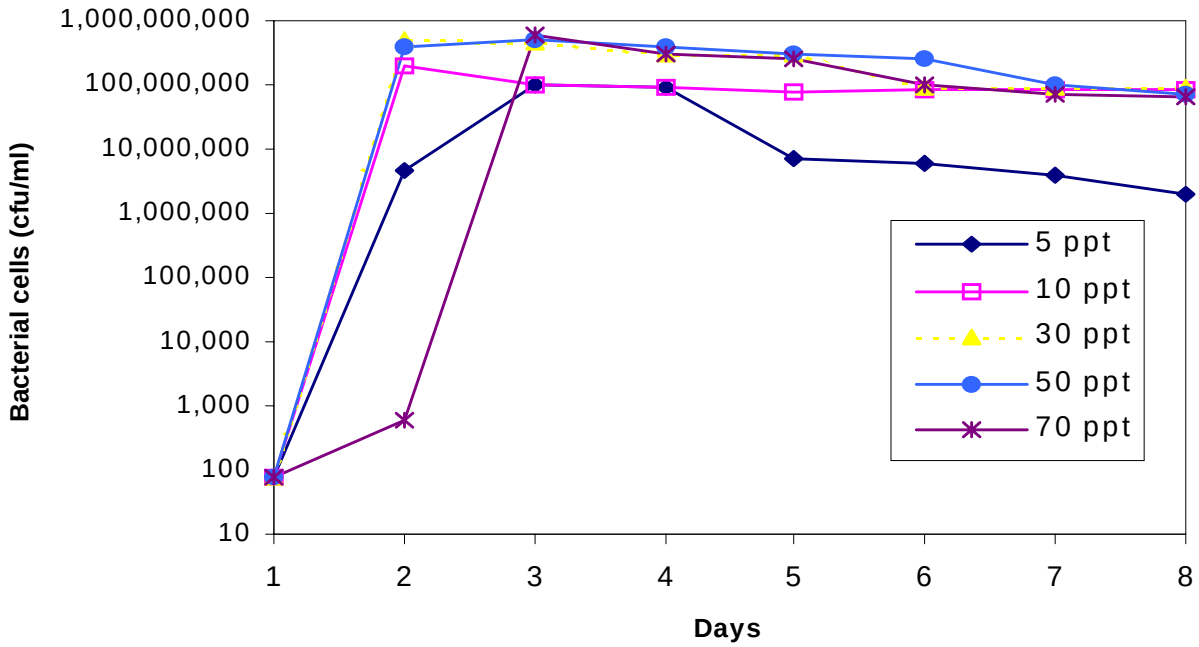
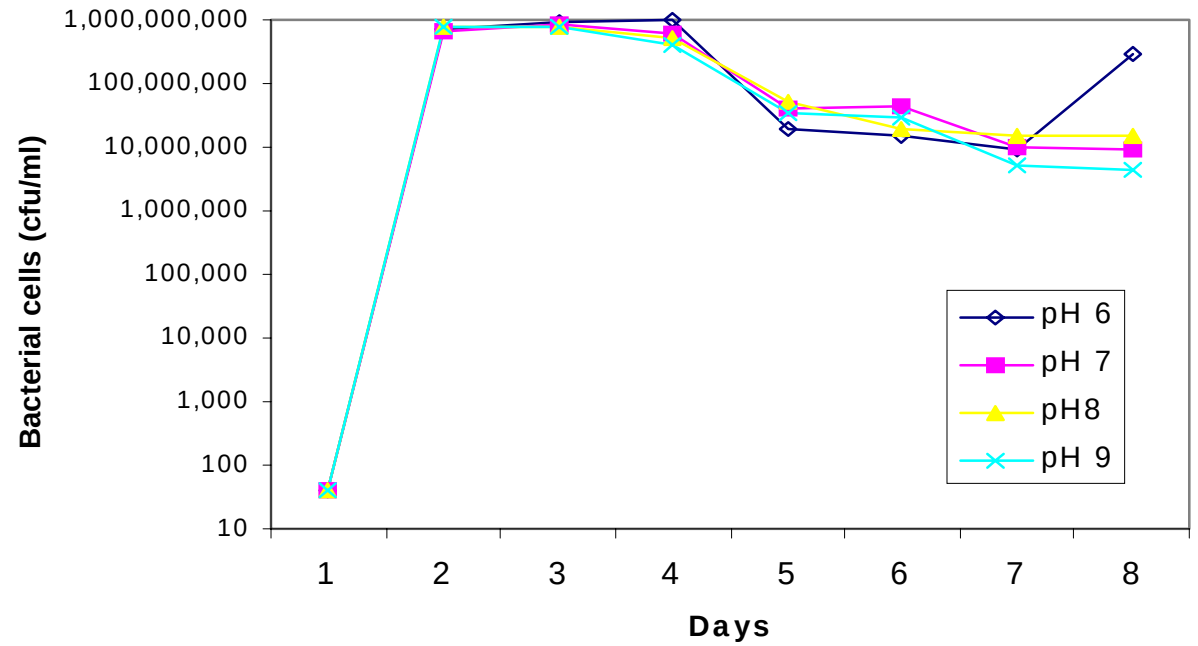


Figure 2. Growth patterns of *Vibrio harveyi* at various pH levels.



decline 3 days after inoculation and were undetectable after 7 days. *Vibrio harveyi* counts in treatments with diatoms, larvae or a combination of both, started to decrease after 3 days, finally reaching levels similar to those from near shore seawater populations after 5 days. In treatment combinations with larvae, *V. harveyi* populations were also reduced starting on the fourth day, but total larval mortality occurred within 3 days.

Table 1 shows the mean bacterial populations (cfu/ml) associated with *Skeletonema costatum* at various phases of culture. Total plate counts of bacteria on NA were constant at 10^4 cfu/ml throughout the diatom culture period, which normally lasts for three days. Presumptive *Vibrio* colonies decreased from 7.5% on the first day of diatom culture to 2% after three days. This period corresponds to the period when peak *Skeletonema* cell density is attained. A similar trend was observed for *Chaetoceros* (Table 1).

Figs. 5a and 5b show the reduction of *Vibrio harveyi* populations exposed to peak cell densities of *Skeletonema costatum* (922,500 cells/ml) and *Chaetoceros calcitrans* (2,700,000 cells/ml). Effective reduction of *V. harveyi* to levels below 10^2 cfu/ml were achieved in one day in both mixed

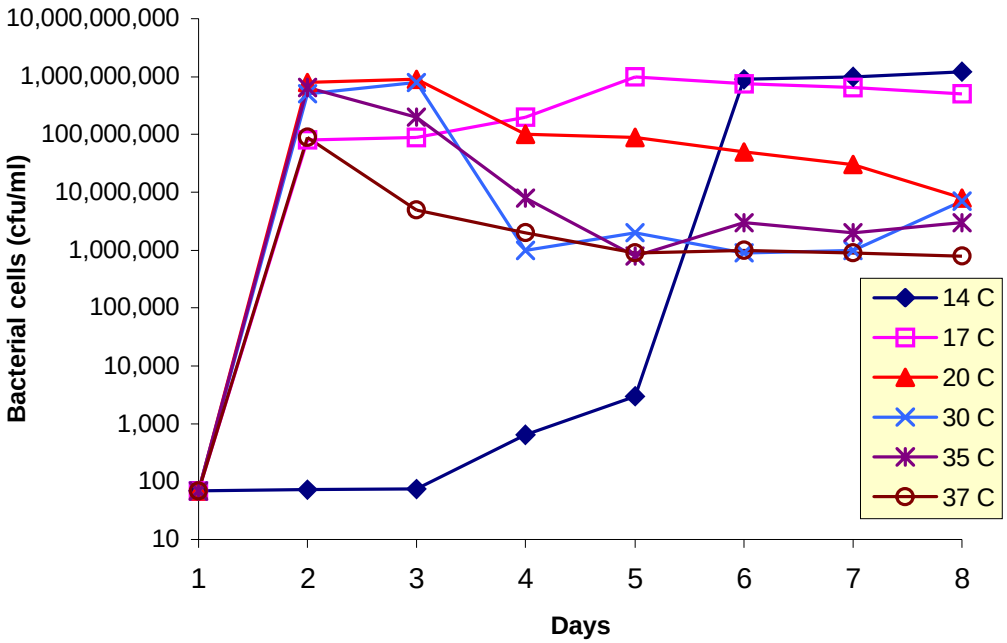


Figure3. Growth patterns of *Vibrio harveyi* at different temperatures.

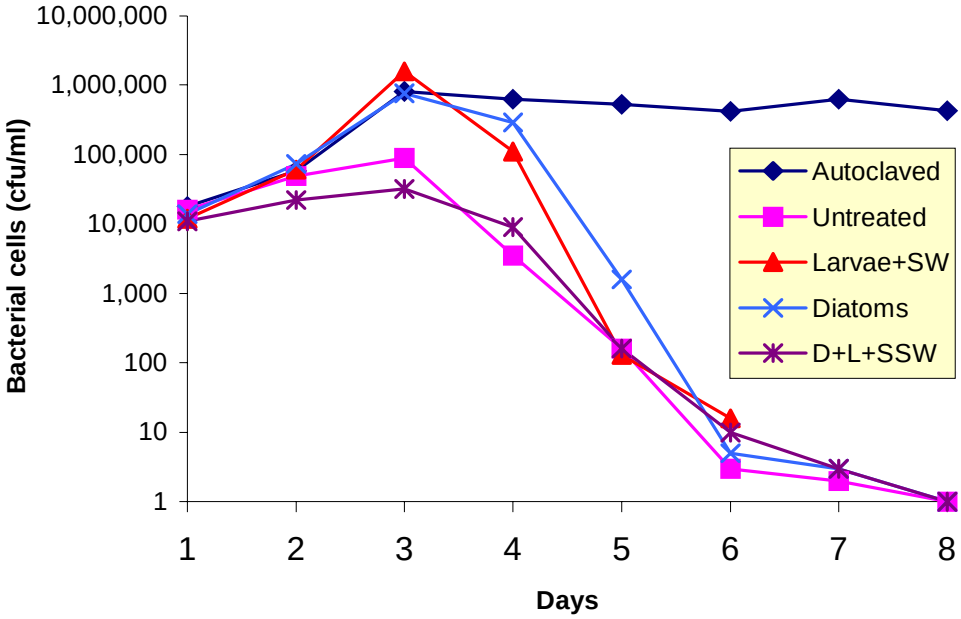


Figure 4. Reduction in *Vibrio harveyi* populations exposed to various hatchery components (Autoclaved = sterilized sea water or SSW; Untreated = untreated sea water; D = diatoms; L = larvae).

Table 1. Mean bacterial counts obtained from various phases of *Skeletonema costatum* and *Chaetoceros calcitrans* cultures. Data for *Chaetoceros calcitrans* was obtained from Lavilla-Pitogo et al. (1992) and presented here for comparison.

	Skeletonema			*Chaetoceros*		
Day	TPC*	% *Vibrio*	% *V. harveyi*	TPC*	% *Vibrio*	% *V. harveyi*
0	2.5 X 10^4	7.5	0	1.2 X 10^4	5.0	0
1	3.2 X 10^4	6.0	0	1.4 X 10^4	3.5	0
2	3.4 X 10^4	4.5	0	3.7 X 10^4	3.0	0
3	3.7 X 10^4	2.0	0	3.8 X 10^4	0.9	0

*Total plate count

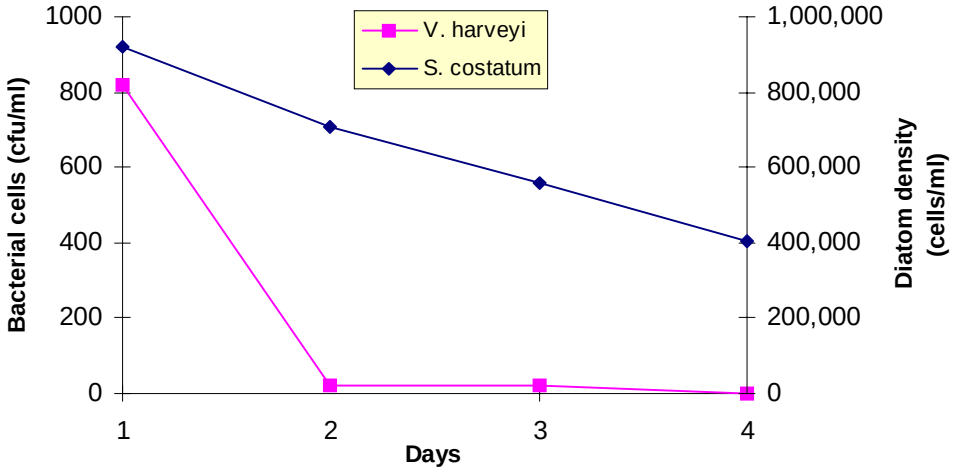


Figure 5a. Reduction of *Vibrio harveyi* population in mixed cultures with *Skeletonema costatim.*

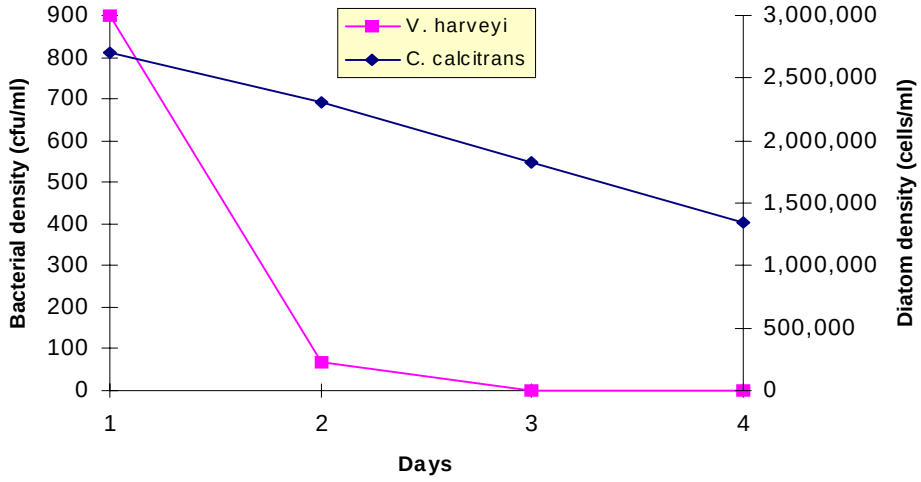


Figure 5b. Reduction of *Vibrio harveyi* population in mixed cultures with *Chaetoceros calcitrans.*

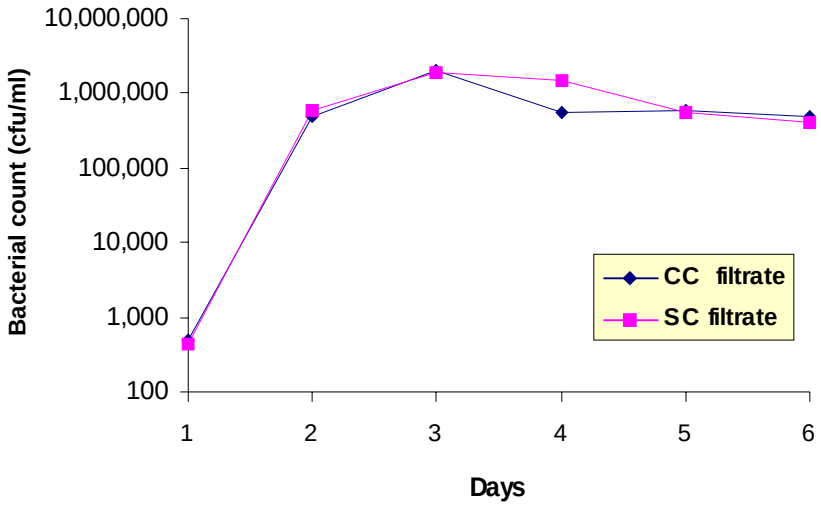


Figure 6. Growth patterns of *Vibrio harveyi* inoculated into cell-free diatom filtrates (CC = *Chaetoceros calcitrans;* SC = *Skeletonema costatum).*

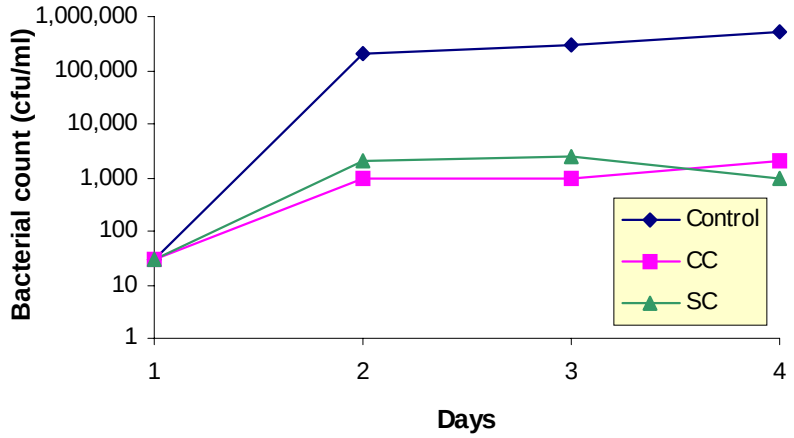


Figure 7. Growth patterns of *Vibrio harveyi* in mixed culture with diatoms at 30,000-50,000 cells/ml. (CC = *V. harveyi* with *Chaetoceros calcitrans;* SC = *V. harveyi* with *Skeletonema costatum).*

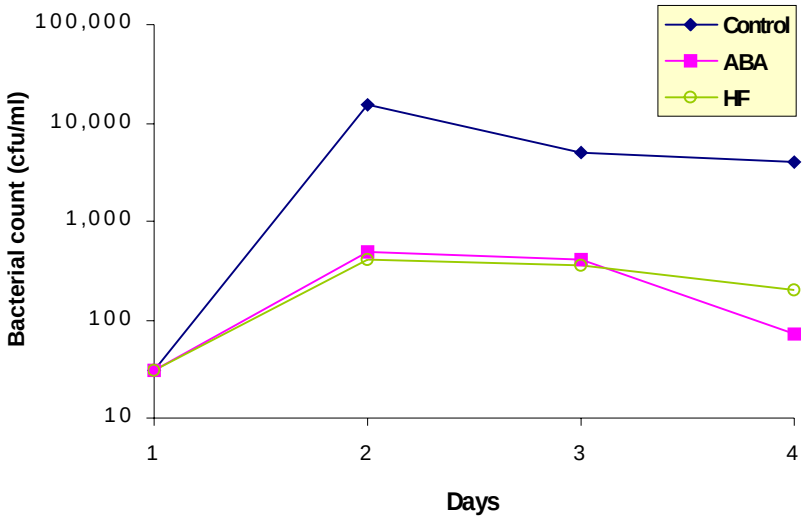


Figure 8. Growth patterns of *Vibrio harveyi* in mixed culture with commercially available bacterial products (ABA = *V. harveyi* with ABA; HF = *V. harveyi* with HF).

cultures. After 3 days with *C. calcitrans* and 4 days with *S. costatum*, *V. harveyi* reached undetectable levels. In cell-free filtrates of the same diatoms, however, the cells of *V. harveyi* maintained high populations even after five days (Fig. 6). In mixed cultures with feeding-density levels of diatoms, no reduction in *V. harveyi* population was obtained and the population maintained a plateau count at 10^3 cfu/ml for more than three days. *Vibrio harveyi* populations in the control reached more than 10^5 cfu/ml.

In mixed cultures with commercially available bacterial preparations, *Vibrio harveyi* populations were not reduced to levels lower than the initial inoculum of 10^1 cfu/ml after three days, but they were lower than the population of 10^4 cfu/ml reached in the controls (Fig. 8).

DISCUSSION

The responses of *Vibrio harveyi* to various salinity, pH and temperature levels conform to those reported for the type strain by Krieg and Holt (1984), and they show that there are no constraining environmental conditions that *Penaeus monodon* larvae can tolerate (Parado-Estepa et al. 1996). Control measures based on the manipulation of these parameters would therefore be unlikely or impossible in the hatchery. However, due to the halophilic nature of *V. harveyi*, luminescent vibriosis may not be expected in freshwater culture facilities.

In the absence of competitors in seawater, *Vibrio harveyi* sustained high populations for several days. This means that thorough cleaning and disinfection of rearing water may have negative effects, if added components like feed and larvae are contaminated. Since sources of the bacterium have been identified (Lavilla-Pitogo et al. 1992), care must be taken to prevent the introduction of grossly contaminated materials either during stocking, feeding, or water management, especially when water treatment has been carried out thoroughly to eradicate its microbial components. Re-population of dechlorinated seawater has been found to occur very rapidly (Baticados & Pitogo 1990). Hence, recontamination must be avoided, especially with known *V. harveyi*-carrying components. Reduction in *V. harveyi* populations exposed to untreated seawater after 96 h explains the benefits of aging seawater before use. Reported antibacterial activity of seawater has been ascribed to physico-chemical effects and biological factors, including predation, competition for nutrients, bacteriophage activity, and antibiotics produced by some marine organisms like the diatoms *Skeletonema*, *Chaetoceros*, *Nitzchia*, and *Licmophora*, among others

(Baslow 1969). The inhibition of bacterial pathogens by extracts and supernatants derived from spray-dried preparations of the microalga *Tetraselmis suecica* have been reported (Austin & Day 1990, Austin et al. 1992).

In the present study, effective reduction of *Vibrio harveyi* populations in mixed culture with diatoms was influenced mainly by the associated microflora rather than the diatoms or their metabolic products, as shown by the high bacterial population sustained in cell-free diatom filtrates. Sieburth (1968) found that high populations of the diatom *Skeletonema costatum* could inhibit *Vibrio*. Interestingly, however, the same study showed that dominant *Flavobacterium* associated with the diatom did not show inhibitory action against *Vibrio* in cross-streak plates. In another study (Rico-Mora & Voltolina 1995a), *Flavobacterium* was also among the five strains of bacteria (including *Plesiomonas*, *Aeromonas* and two strains of *Vibrio*) found in non-axenic cultures of *S. costatum*. In that study, however, bacteriostatic and antibiotic reactions among the strains were found. In another study by the same authors (Rico-Mora & Voltolina 1995b), it was shown that none of the *S. costatum*-associated bacteria were pathogenic to *Artemia franciscana* nauplii. Thorough analysis of bacterial associations is needed in order to exploit their potential. Aside from inhibitory activity, bacteria of choice should be benign to larval stages being cultured. In the present study, although bacterial populations associated with shrimp larvae also effectively reduced *V. harveyi*, they also caused mortality, thus limiting their potential as disease control agents.

Bacteria are among the most sought after marine organisms as potential sources of inhibitory compounds against fish diseases (Dopazo et al. 1988, Lemos et al. 1991, Nogami & Maeda 1992, Austin et al. 1995, Garriques & Arevalo 1995, Sugita et al. 1996, Gatesoupe 1997, Riquelme et al. 1997). Depending on how the bacteria or their inhibitory products are processed and applied, the action against target pathogens may be that of antibiosis, growth inhibition, competition for available nutrients, colonization leading to competitive exclusion, or habitat and rearing water conditioning. Probiotic application of defined or undefined bacterial populations has a long history of successful experimentation and implementation in the poultry industry and it is generally based on competitive exclusion of *Salmonella* (Stavric & D'Aoust 1993). Whether candidate probiotic marine bacteria can be be effective as feed additives or simply added to the water and whether they act as competitors or inhibitors should be studied further. There is no doubt that possible agents of control are already in the hatchery, but careful study and manipulation of their inherent control potential is needed to achieve the desired ecological balance in that system. The following items need consideration before bacteria or their products are applied as biological agents for disease control in the hatchery:

- The candidate bacteria must be benign
- They should provide antagonistic activity towards pathogens and maintain them at manageable levels in the rearing environment
- They should be able to re-establish rapidly in the rearing environment following regular water exchange
- To work as exclusion agents, they should be able to attach and divide on shrimp surfaces since *V. harveyi* is known to colonize and replicate on larval (Lavilla-Pitogo et al. 1990) and egg surfaces (Lavilla-Pitogo 1995)
- They should be stable under conditions for optimum larval rearing

The disease and pest problems that the aquaculture industry is facing are mirror images of the travails faced by other animal industries when they were starting. Thus, much can be learned from disease management protocols adapted by the poultry, cattle and hog industries. When formulating biological products and protocols for disease control, it is worthwhile to look beyond the present culture system and consider areas that will make its implementation successful and acceptable (Waage 1996).

Acknowledgements. We thank the International Development Research Centre (IDRC) of Canada for funding the research under Project 3-P-88-1053-02. Sheila Mae Buen provided excellent help with the graphs.

LITERATURE CITED

Austin B, Baudet E, Stobie M (1992) Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. J Fish Dis 15:55-61

Austin B, Day JG (1990) Inhibition of prawn pathogenic *Vibrio* spp. by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture 90: 389-392

Austin B, Stuckey LF, Robertson PAW, Effendi I, Griffith DRW (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida, Vibrio anguillarum* and *Vibrio ordalii*. J Fish Dis 18:93-96

Baslow MH (1969) Marine Pharmacology. The Williams and Wilkins Co., Baltimore, MD, USA, 286 p

Baticados MCL, Lavilla-Pitogo CR, Cruz-Lacierda ER, dela Peña LD, Suñaz NA (1990) Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* from diseased *Penaeus monodon* larvae and rearing water. Dis Aquat Org 9:133-140

Baticados MCL, Pitogo CL (1990) Chlorination of seawater used for shrimp culture. Israeli J Aquacult - Bamidgeh 42:128-130

Dopazo C, Lemos M, Lodieros C, Bolinches J, Barja J, Toranzo A (1988) Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. J Appl Bacteriol 65: 97-101

Garriques D, Arevalo G (1995) An evaluation of the production and use of live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador. In: CL Browdy, JS Hopkins (eds) Proceedings of the Special Session on Shrimp Farming. Aquaculture 95, World Aquaculture Society, Baton Rouge Louisiana, USA, p 53-59

Gatesoupe F-J (1997) Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. Aquat Living Resour 10: 239-246

Krieg NR, Holt JG (eds) (1984) Bergey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins Co., Baltimore, MD, USA, 964 p

Lavilla-Pitogo CR (1995) Bacterial diseases of penaeid shrimps: an Asian view. In: Shariff M, Arthur JR, Subasinghe RP (eds) Diseases in Asian Aquaculture II. Fish Health Section, Asian Fisheries Society, Manila, Philippines, p 107-121

Lavilla-Pitogo CR, Albright LJ, Paner MG and Suñaz NA (1992) Studies on the sources of luminescent *Vibrio harveyi* in *Penaeus monodon* hatcheries. In: Shariff M, Subasinghe RM, Arthur JR (eds) Diseases in Asian Aquaculture I. Fish Health Section, Asian Fisheries Society, Manila, Philippines, p 157-164

Lavilla-Pitogo CR, Baticados MCL, Cruz-Lacierda ER and de la Peña LD (1990) Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture 91:1-13

Lavilla-Pitogo CR, de la Peña LD (1998) Bacterial diseases in shrimp (*Penaeus monodon*) culture in the Philippines. Fish Pathol 33: (in press).

Lemos ML, Dopazo CP, Toranzo AE, and Barja JL (1991) Competitive dominance of antibiotic-producing marine bacteria in mixed cultures. J Appl Bacteriol 71:228-232

Parado-Estepa FD, Quinitio ET, Borlongan EL (1996) Prawn Hatchery Operations. Aquaculture Extension Manual No 19. SEAFDEC Aquaculture Department, Tigbauan, Iloilo, Philippines. 45 p

Nogami K, Maeda M (1992) Bacteria as biocontrol agents for rearing larvae of the crab *Portunus trituberculatus*. Can J Fish Aquat Sci 49: 2373-2376

Rico-Mora R, Voltolina D (1995a) Bacterial interactions in *Skeletonema costatum* Cleve (Bacillariophyceae) cultures. Rivista Italiana Acquacoltura 30:105-109

Rico-Mora R, Voltolina D (1995b) Effects of bacterial isolates from *Skeletonema costatum* cultures on the survival of *Artemia franciscana* nauplii. J Invert Pathol 66:203-204

Riquelme C, Araya R, Vergara N, Rojas A, Guaita M, Candia M (1997) Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). Aquaculture 154: 17-26

Seiburth JM (1968) The influence of algal antibiosis on the ecology of marine organisms. In: Droop MR and Ferguson Wood EJ (eds) Advances in Microbiology of the Sea. Academic Press, London, p 63-94

Skjermo J, Salvesen I, Oie G, Olsen Y, Vadstein O (1997) Microbially matured water - a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquacult International 5:13-28

Stavric S, D'Aoust J-Y (1993) Undefined and defined bacterial preparations for the competitive exclusion of *Salmonella* in poultry - a review. J Food Protect 56:173-180

Stoskopf MK (1996) Comparison of aquaculture and other animal production medicine strategies in the United States of America. In: Main K, Rosenfeld C (eds) Aquaculture Health Management Strategies for Marine Fishes. The Oceanic Institute, Honolulu, Hawaii, p 91-100

Sugita H, Shibuya K, Shimooka H, Deguchi Y (1996) Antibacterial abilities of intestinal bacteria in freshwater cultured fish. Aquaculture 145: 195-203

Waage J (1996) Yes, but does it work in the field - the challenge of technology transfer in biological control. Entomophaga 41: 315-332

Influence of LPS-Injection on Morphological, Antigenic and Functional Aspects of Hemocytes from *Penaeus monodon*

***C.B.T. van de Braak, W.P.W. van der Knaap**

Wageningen Institute of Animal Sciences, Fish Culture and Fisheries Group, Wageningen, The Netherlands

ABSTRACT: Mass culture of penaeid shrimp suffers severe losses caused by infectious diseases. Augmenting the internal defense capacities of shrimp may help limit such losses. One potential way to increase the shrimp defense potential may be immunostimulation, i.e., by confronting the defense system with pathogens or molecules derived from them. In the present study, we focused on the hemocytes of *Penaeus monodon*. Numerical, morphological and ultrastructural aspects of the three main hemocyte types (hyaline, semi-granular and granular hemocytes) were studied. With the aid of eight monoclonal antibodies, a finer discrimination was made of subpopulations, using light and electron microscopy and facs analysis. Functional defense capacities were measured by studying phagocytic activity, lysosomal enzyme activities, chemotactic responses, and mitotic activity of the hemocytes. All these parameters were studied in naive shrimp, and in shrimp at different time intervals after the injection of bacterial lipopolysaccharide (LPS).

KEY WORDS: *Penaeus monodon*, haemocytes, immunostimulation, lipopolysaccharide, monoclonal antibodies

van de Braak CBT, van der Knaap WPW (1998) Influence of LPS-injection on morphological, antigenic and functional aspects of hemocytes from *Penaeus monodon. In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: Karin.vandebraak@alg.venv.wau.nl

Evaluation of a Booster Diet for the Nursery of Penaeid Shrimp

Greet Merchie, Marleen Dehasque, Piet Verstraete, Pete Bridson, David Jones

INVE ASIA Ltd., Representative Office, 218/47-48, 4th Floor Geneva Building, Lakeview Condominium, Muang Thong Thani, Amphur Pakkred, Nonthaburi 11120, Thailand

ABSTRACT: In view of the disease problems that are currently facing the shrimp farming sector, optimal quality postlarvae is recommended for stocking in grow-out ponds. In this respect, the significance of an adequate diet in maintaining animal health is widely recognized. Stresspak, a dietary supplement produced by INVE (Thailand) Ltd. for the shrimp nursery and early grow-out, aims at fortifying the animals before and just after stocking in ponds. This specialty diet is enriched with vitamins (C, E, choline) and combined with immunostimulants to prepare shrimp to deal with stress conditions. The use of this booster diet as a partial replacement for a commercial diet was evaluated for the early postlarval stages of *Penaeus indicus* (PL1-PL15) in two consecutive lab-scale trials at the School of Ocean Sciences (University of Wales, Bangor-UK). In the first experiment, PL15-stage animals fed a 50% ration of Stresspak showed a significantly higher survival and faster growth rate than shrimp on the control feed. A 30-% substitution gave intermediate results. Length was similar for the three groups during the first week; from day 8 onwards both treatments receiving Stresspak displayed faster growth. In the second trial, a similar significant effect on growth was noticed when feeding 50% of the booster diet, although survival for both test and control treatments was similar. In addition, feeding 50% Stresspak resulted in improved stress resistance of the postlarvae when exposed to low salinities. A preliminary verification in a commercial tiger shrimp (*P. monodon*) operation demonstrated the practical relevance of the booster diet. Stresspak was used in the hatchery for the molt of mysis to PL, again 24h prior to transfer of the PL7 to hatchery nursery ponds (25% of the diet), and again 24-48h before transfer of PL12 from hatchery nursery ponds to nursery ponds at a grow-out farm (10-15% of the diet). According to the production manager at the grow-out farm, the quality score of the postlarvae upon arrival at the farm increased from an average of 100 out of 150 to 120 out of 150 when using Stresspak (score determined following internal farm procedures).

KEY WORDS: *Penaeus indicus*, *Penaeus monodon*, Stresspak, larvae, post larvae, immunostimulant

Merchie G, Dehasque M, Verstraete P, Bridson P, Jones D (1998) Evaluation of a booster diet for the nursery of penaeid shrimp. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Bacterial Diseases and Toxins

Standardisation of Three Techniques for Experimental *Vibrio* Infections in the Marine Shrimp *Penaeus vannamei*

[*1]Ana Roque, [1]Bruno Gomez-Gil, [2]Ana Luisa Guerra Flores

[1]Centro de Investigacion en Alimentacion y Desarrollo
Sabalo Cerritos SN Estero del Yugo CP 82010 AP 711 Mazatlán, Sin. MEXICO
[2]Facultad de Ciencias Marinas de la Universidad Autonoma de Sinaloa
Paseo Centenario CP 82000 Mazatlan, Sin. MEXICO

ABSTRACT: The need of a reproducible experimental challenge technique for shrimp bacterial diseases has long been felt. In this study, two challenge techniques were standardised: bath and oral challenges and compared with the technique of injection challenge. The uses of each of these techniques is discussed.

KEY WORDS: *Penaeus vannamei*, *Vibrio* sp., challenge

INTRODUCTION

A reproducible experimental challenge technique for shrimp bacterial diseases is required for a number of reasons such as assessing the virulence of bacterial isolates and testing chemicals to be used for disease treatment or prevention. It would also help to determine which environmental factors affect the onset of vibriosis in cultured shrimp and to give a better understanding of the relationship between environmental stressors and bacteria in the aetiology of disease. In this study, challenge techniques by injection, bath and oral adminstration were standardised.

INJECTION CHALLENGE

Experimental system

The experimental system used was a small recirculating system with 16 experimental units (15 L glass tanks). The tanks were individually aerated through an airline with airstones all connected to a high volume air blower. The water conditions were: temperature 25 ± 1° C, salinity 35 ‰, pH 8.0 ± 0.1.

Origin of bacterial isolates and inoculum preparation

The *Vibrio* isolates used in this work were collected at shrimp farms from the state of Sinaloa, Mexico, either from the sea water or from shrimp. They all were identified with the help of the BIOLOG system (Biolog inc., Hayward, USA). They were preserved in Protect Bacterial Preservers (Technical Service Consultant, Ltd Heywood, Lancs, UK) at -70° C and resuscitated into tryptone soya broth (TSB, Difco laboratories, Detroit, USA) with 2 % sodium chloride (NaCl) at the beginning of each experiment. They were grown in 10 ml of TSB + NaCl at 30° C for 24h and then centrifuged twice at 3500 rpm, 10°C for 15 min. Resuspensions were made in sterile saline solution (2.5% NaCl). Before being injected, the inoculum was brought to an optical density of 1.0 at 610 nm using a spectrophotometer (HACH DR 2010) and then diluted 1:1000. Positive control *E.coli* (ATCC 25922) was preserved and prepared for injection in the same manner as the *Vibrio* isolates.

Bacteriology

The density of inoculum used was monitored by the plate count method in both TCBS (thiosulphate citrate bile sucrose agar, Difco Laboratories, Detroit, USA) and tryptone soya agar (TSA, Difco) with 2.5% NaCl (Austin 1988). TSA plates inoculated with *E. coli* (positive control) and with sterile saline solution were also made to verify a similar density of injected *E. coli* and the sterility of the saline solution.

Challenge protocol

In these experiments three treatments were used: 1) injection with *Vibrio* HL57, 2) injection with *Vibrio* C14, 3) injection with *E. coli*, and 4) injection with sterile saline solution (2.5% NaCl). Each treatment had four randomly allocated replicates and each tank contained 10 shrimp.

Experimental procedure

The experimental system was set up with UV sterilised sea water. After 1 day for stabilization, ten shrimp (approximately 0.7 g or 4-6 weeks old) were introduced to each tank. They were allowed to adapt to this system for another three days before trials were initiated. The shrimp from each tank were removed and injected with 20 ml of the desired inoculum. A record of daily mortalities for each tank was kept for five days. The experiment was performed several times to verify whether there consistently similar levels of mortalities.

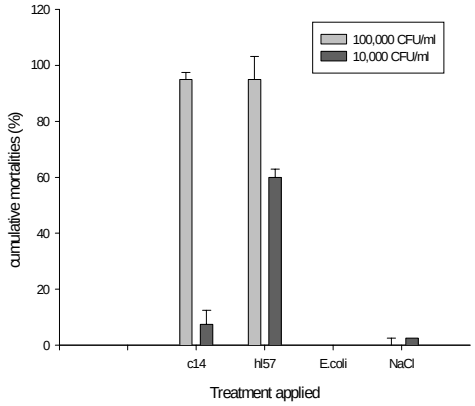
Roque A, Gomez-Gil B, Guerra Flores AL (1998) Standardisation of three techniques for experimental *Vibrio* infections in the marine shrimp *Penaeus vannamei*. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: roque@victoria.ciad.mx

Results

Figure 1 shows the results of an experiment in which the same bacteria were injected into shrimp on two different occasions. In Trial 1 all the bacteria were injected with a density of 10^5 CFU/ml and in trial 2 they were injected with 10^4. For Trial 1, 100% of the shrimp injected with the two *Vibrio* species died, whereas in Trial 2 the mortality levels for the two *Vibrio* species were significantly different.

Figure 1. Final mortalities after bacterial injection at 2 densities. Error bars represent the standard error of 4 replicates.



Discussion

Although this technique is not new, it has not been standardised. In our facilities *E. coli* was used as a positive control. This is an important feature to the experiment, because the results from Trial 1 might otherwise have been misleading if the deaths were solely attributed to cellular shocks. It is evident from both experiments that *Vibrio* HL57 is potentially more pathogenic to shrimp than *Vibrio* C14.

In all trials most mortalities occurred during the first 48 h after injection. This may indicate that the shrimp were unable to eliminate the inoculated bacteria before they were able to multiply and produce lethal doses of toxins. The rapid clearance of bacteria by the defence mechanisms of the shrimp has been shown by Adams (1991), who demonstrated that more than 99% of heat killed *Vibrio alginolyticus* were cleared from the haemolymph of injected *P. monodon* within 4h.

The results presented in Fig. 1 suggest that this technique is only reliable to assess the virulence of an isolate if preliminary studies are performed to investigate what bacterial density causes mortality due to pathogenicity and not due to excessive numbers of injected cells. In Trial 1 the shrimp seem to have died from cellular shock, as previously suggested in other studies (Adams 1991, Roque 1995). Conversely, it may be possible that too few injected bacteria would be easily and rapidly handled by shrimp. Another important necessity is to define the volume of injection accordingly to the size of the shrimp used. Even though reproducible, work done with this technique is limited due to its artificiality and that is why 2 other techniques were developed.

BATH CHALLENGE

Experimental system

The experimental system used was a static system consisting of 24, 10 L glass tanks. Each tank was individually aerated by an airstone. The water conditions were: temperature 25 ± 1° C, salinity 35 ‰, pH 8.0 ± 0.1.

Origin of Vibrio isolates and inoculum preparation

The *Vibrio* isolate used for this series of experiments was isolated from the haemolymph of a diseased juvenile shrimp (*P. vannamei*) collected at a farm from the state of Sinaloa, Mexico. The shrimp from that pond presented gross signs of reddish coloration, anorexia and lethargy. With the help of the BIOLOG system (Biolog Inc., Hayward, USA) the isolate was identified as *Vibrio parahaemolyticus*, hereafter referred to as HL58. HL 58 was preserved and resuscitated in the same manner as the isolates used in the injection challenges. It was grown in 10 ml of TSB + NaCl at 30° C for 24h and then transferred to 60 ml of TSB + NaCl for a further 24 h before being used in the experiments.

Wounding technique

In order to facilitate the induction of vibriosis, some of the shrimp were artificially wounded. To achieve this, the shrimp were individually netted and a small incision (approximately 2 mm long) was made through the cuticle and into the muscle of the third abdominal segment by pushing a scalpel against the carapace until it penetrated.

Bacteriology

The concentration of inoculum used was also monitored daily through the plate count method (Austin 1988). Daily *Vibrio* spp. total counts from the water of each tank were made using TCBS.

Challenge protocol

During these series of trials four different treatments were applied to the shrimp: 1) wounding and then challenge with HL58 ("wound+vibrio"), 2) wounding only ("wound"), 3) challenge with HL58 only ("vibrio") and 4) neither wounding nor challenge ("control"). Each treatment comprised six randomly allocated replicates.

Experimental procedure

The tanks were filled with 3.0 L of UV-sterilised sea water. The following day, ten shrimp (approximately 1 g or 6-8 weeks old) were introduced to each tank. They were left to adapt to this system for another two days before the trial was initiated. On the first day of challenge 75% of the water in each tank was replaced with UV sterilised sea water. The "vibrio" tanks were inoculated with 1.5 ml/L of HL58 cultured in TSB with 2% NaCl. The "wound" shrimp were removed from their tanks, wounded as described above and replaced. For the "wound+vibrio" treatment the shrimp were

removed from the tanks and the tanks were inoculated the same volume of HL58 as the "vibrio" treatment tanks. At the same time, the shrimp were wounded and placed back in the tanks. On the following days, 75% of the water in each tank was replaced with UV-sterilised water and then the "vibrio" and "wound+vibrio" tanks were re-inoculated with 1.5 ml/L of HL58 culture. A record of daily mortalities for each tank was kept for five days. This trial was repeated four times to verify the reproducibility of the technique.

Results

The average bacterial numbers recorded on TCBS during the trials differed for treatments where HL58 was inoculated ("wound+vibrio" and "vibrio") (10^5 CFU/ml) and was not inoculated ("wound and control") (10^2 CFU/ml) (T=155, n=10, P<0.0001 for all the trials). The types of colonies also differed between the treatments. For the treatments "wound+vibrio" and "vibrio", most of the colonies were green and their macroscopic features matched those of HL58 (4 mm diameter colonies with complete borders, convex elevation and a bright, smooth texture). Some yellow colonies also appeared, but they represented less than 20% of the total count. For the treatments "wound" and "control" most of TCBS colonies were yellow

All the mortalities occurred within four days of initial challenge. Cumulative mortalities for each trial are presented in Fig 2. For the "wound+vibrio" treatments, mortalities varied from 32-52%; for "wound" from 12-22%; for "vibrio" from 0-13% and for "control" from 0-3 %. The cumulative mortalities for the treatments "wound+vibrio" were significantly different from all the other treatments in each trial (P < 0.001).

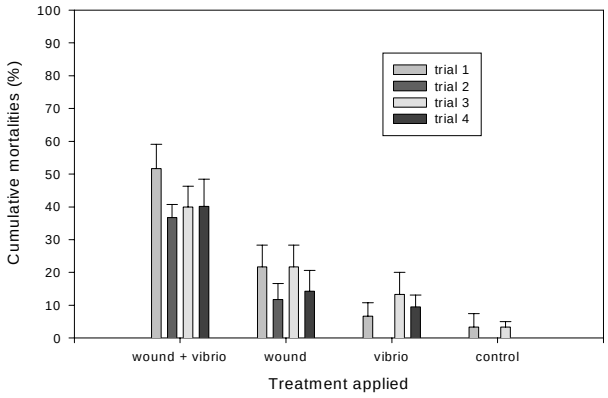


Figure 2. Final shrimp mortalities after bath challenge with bacteria. Each bar corresponds to one trial. The error bars represent the standard error of the mean for 6 replicates.

Discussion

The objective of this study was to develop a bath challenge technique which would mimic, as far as possible, the conditions of a natural outbreak of vibriosis in a farm situation.

Flegel *et al*. (1992) speculated that bacteria continually invade shrimp. If this were so, high numbers of bacteria in the water could overwhelm the shrimp haemocytes and even if only a small proportion of the total count consisted of pathogenic *Vibrio* sp., disease would result. Based on this hypothesis, the challenge model developed in this series of trials used daily inoculation of bacteria in an attempt to maintain high levels of bacteria.

Because *Vibrio* spp. are opportunists, a stressor was considered necessary to induce disease. Wounding was thought to be an appropriate stressor since physical damage has been referred as a primary risk in the development of vibriosis (Lightner 1988, Ruangpan & Kitao 1991). In addition to stressing the shrimp, physical damage may provide a route of access for bacterial invasion.

In shrimp it has been shown that wounds require approximately 16 days before they are completely sealed (Fontaine & Lightner 1973). By 48 h hours, there is a haemocytic concentration at the ends of the wound. By 96 h the epidermis begins to migrate into the wound, using the haemocytic network as a basal support. Thus, at this stage the wound is already closed although not totally repaired. These observations may explain why the mortalities ceased after 4 days, since after that time there was no easy bacterial access route from the water to the shrimp body. The brief duration of mortality may suggest either that vibriosis occurs only when the shrimp are continually invaded by bacteria and/or that they rapidly die or recover from any established infection.

The specific cause of death was not verified in these experiments, but inferred by comparison with the controls. The dead shrimp would have been invaded by any bacteria in the water, invalidating bacterial analyses. In addition, autolysis would have rendered them unsuitable for histology. It may have been possible to sample moribund animals for specific diagnoses, but this would have required continuous monitoring of the tanks. The same trial was repeated four times to ensure the reproducibility of this technique and results suggested that it was a reliable bath challenge technique which could be used for a variety of studies.

ORAL CHALLENGE

Experimental system

This static system employed six glass tanks divided into two experimental units of 5 L capacity each. Each experimental unit was individually aerated, and the water conditions were kept at 28 ± 0.5 °C, salinity 35 ‰ and pH 8.4 ± 0.1.

Artemia

Sterile *Artemia* nauplii were obtained following the procedure described by Sogerloos *et al.* (1977) and modified by Gomez-Gil *et al.* (1998).

Bacterial isolate

The *Vibrio* species used in developing this technique was the same as that used for the bath challenge (HL58). At the beginning of each experiment, the isolate was resuscitated in TSB + 2% NaCl. This culture was centrifuged at 4000g at 20°C for 8 min. The supernatant fluid was discarded and the bacterial cells were rinsed in sterile saline solution (2.5% NaCl). These operations were repeated twice to ensure the cells were free of extracellular particles or exotoxins. The

bacteria were resuspended in sterile saline water (2.5% NaCl) and the absorbance of the suspension adjusted to 1.00 at 610 nm. Bacterial counts of the suspension were carried out on TCBS at each preparation.

Bioencapsulation of the HL58 in *Artemia franciscana*

Artemia nauplii were incubated with the HL58 for 2 h (Gomez-Gil *et al*. 1998) After this period, 50 *Artemia* nauplii were collected in 1 ml of suspension water for the determination of total *Vibrio* spp. counts on TCBS.

Challenge protocol

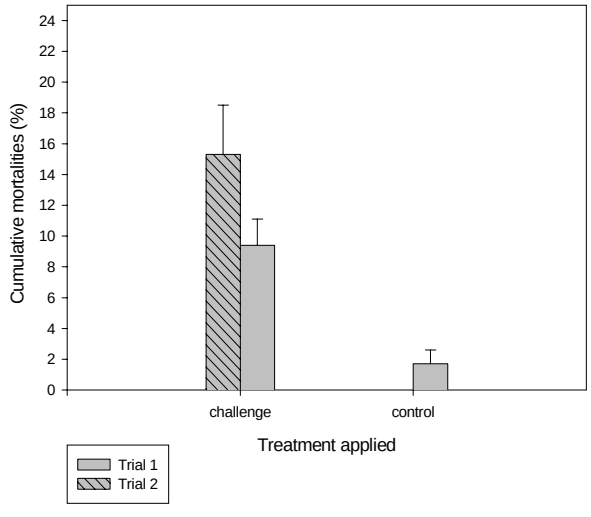
The experimental system was set up with 2.5 L of sterile sea water in each of the 12 experimental units. After one day for stabilization, 30 postlarvae (PL8) were placed in each experimental unit. In six units the PL were fed *A. franciscana* which had been incubated with HL58 while in the other six, the PL were fed with sterile *A. franciscana*. The PL were supplied with an average of 150 nauplii per postlarva, every 24 h for seven consecutive days. The type of diet fed to each of the experimental units was assigned randomly and a daily record of mortalities was kept.

Results

The density of HL58 in the treated nauplii was approximately 10^2CFU/nauplius for both trials, while no *Vibrio* sp. was isolated from the sterile *A. franciscana*. These values were calculated from a sample of 50 *Artemia* nauplii in 1 ml of sterile saline solution.

During the first trial, an average of 9.5% of the HL58 treated shrimp died, while only 1.6% of the control shrimp died (Fig. 3). In the second trial, an average of 15% of the HL58 treated shrimp died and none of the control died (Fig. 3). For both trials the U-Mann and Whitney test showed a significant difference between treatments ($U= 57.0$, $p<0.005$, $n=6$).

Figure 3. Final shrimp mortalities after oral challenge with bacteria. Each bar corresponds to one trial. The error bars represent the standard error of the mean of 6 replicates.



Discussion

Neither of the first two techniques described herein were appropriate for shrimp larvae and postlarvae. Therefore a different technique was required to study vibriosis at these stages. Accordingly, we undertook to improve available challenge techniques basing our approach on the following considerations: 1) *Artemia* sp. has recently been suggested for the delivery *Vibrio* spp. as potential probiotics (Gomez-Gil *et al*. 1998) and 2) it has been suggested that systemic vibriosis in larval and postlarval stages arises from oral exposure (Jiravainichpaisal *et al*. 1993).

Although not a natural way of shrimp infection, the technique of bioencapsulation has the advantage of ensuring that bacterial cells gain intimate contact with the inside of shrimp. In addition, mortality is not immediate, so testing of treatments or preventive measures is possible.

No histological study was carried out, and therefore we could not relate mortalities to possible tissue damage caused by vibriosis. As with the bath challenge tests, the oral model involved continuous challenge at a constant level of exposure. It was found that the shrimp started to die only after 4 d of being fed with HL58-colonised *A. franciscana*. There are two possible explanations for this: 1) the inoculum administered had a much lower density than that used for the other 2 techniques and 2) the administration route might have had some influence on the results since the hepatopancreas is a physical barrier for bacteria (Alday-Sanz 1994).

The same trial was done twice to verify its reproducibility and since both experiments showed significantly different levels of mortalities between the treatments, the technique used should be appropriate for testing the effect of new products for the treatment or prevention of vibriosis.

GENERAL DISCUSSION

The main objective of this study was to compare the different challenge techniques developed or standardised in CIAD (Centro de Investigacion en Alimentacion y Desarrollo) for shrimp disease studies. All the techniques described were repeated and proved to give reproducible results. Each has limitations and therefore each is best suited for different purposes. In the case of the injection challenge (the most commonly used method for pathogenicity studies), its artificiality limits its usefulness. Although it is apparently very simple and straightforward, its use demands extensive preliminary work to determine the best inoculum volumes and densities. The use of a positive control is needed to remove confusion that might arise in classifying bacteria as pathogenic when they really kill the shrimp by cellular shock. We found the technique useful mainly for quick determination as to whether bacteria were potentially pathogenic or not. The bath challenge proved quite efficient, provided that some details are observed. These include the use of sterile sea water, the provision of TSB with the inoculum and the wounding of shrimp as a stressor. The fact that the maximum cumulative mortalities achieved were 50% makes this a promising technique not only for comparing pathogenicities but also for verifying the influence of different

Table 1. Summary of main features and results for the techniques studied.

	Challenge Route		
Feature	Injection	Bath	Oral
Inoculum (CFU/ml)	10^4- 10^5	10^5	10^2
Challenge frequency	once	daily	daily
Time to last death (d)	96 h	120 h	168 h
With stress	no	yes	no
Repeatibility	yes	yes	yes
Maximum cumulative mortality/tank (%)	100	70	25
Uses	quick pathogenicity tests; virulence factor tests	water quality parameter tests; treatment tests; prophylaxis tests in juveniles	water quality parameter tests; treatment tests; prophylaxis tests in mysis and postlarvae
Innovative changes	volume according to shrimp; importance of inoculum positive control density	daily challenge; sterile seawater; wound as stressor; death induction reproducibility	sterile artemia; completely new technique

water quality parameters on the onset of vibriosis. The fact that the mortalities took longer than 48 h to occur also makes feasible studies on the the use of antibiotics or other potential treatments, either therapeutic or prophylactic. The technique was useless for larviculture studies because it is extremely difficult to wound very small shrimp. Therefore, the oral challenge technique was developed to serve the same purposes as the bath challenge. Table 1 summarises the main features of each of the techniques used in this study.

LITERATURE CITED

Adams A (1991) Response of penaeid shrimp to exposure to *Vibrio* species. Fish and shellfish immunology, 00: 1-12

Alday-Sanz MV (1994) Studies on the pathogenicity of Vibrio sp. in Penaeus monodon Fabricius. PhD thesis, Institute of Aquaculture, University of Stirling. UK.

Austin, B. (1988). Aquatic Microbiology. Cambridge press, 222 pp.

Flegel TW, Fegan DF, Kongsom B, Vithikornudomkit D, Sriurairatana S, Boonyaratpalin S, Chanachookin C, Vickers JE, Macdonald OD (1992) Occurrence diagnosis and treatment of shrimp diseases in Thailand In: W. Fulks, KL Main (eds) Diseases of Cultured Penaeid Shrimp in Asia and the USA. Oceanic Institute Honlulu Hawaii. pp 57-112.

Fontaine CT, Lightner DV (1973) Observations on the process of wound repair in penaeid shrimp. Journal of Invertebrate pathology, 22:23-33

Gomez-Gil B, Herrera Vega MA, Abreu Gorbois FA, Roque A, 1998 Bioencapsulation of two strains of *Vibrio* spp. in *Artemia* nauplii. Applied and Environmental Microbiology, 64: 2318-2322

Jiravanichpaisal P., Miyasaki T., Limsuwan C. and Somjetlerdchalern A. 1993. Comparative histopathology of vibriosis in black tiger prawn Penaeus monodon. Diseases of Asian Aquaculture II, Asian Fisheries Society, Fish Health Section.

Lightner DV (1988) Vibrio disease. In: CJ Sinderman, DV Lightner (eds) Disease diagnosis and control in North American marine aquaculture , Developments in Aquaculture and fisheries science, 17:42-47. Sci. Publishing inc. New York USA

Roque A, 1995- The experimental induction of *Vibrio* spp. infection in *Penaeus monodon* (Fabricius). PhD thesis, Institute of Aquaculture, University of Stirling United Kingdom.

Ruangpan L, Kitao T (1991) *Vibrio* bacteria isolated from black tiger shrimp *Penaeus monodon* Fabricius Journal of fish diseases, 14: 383-388

Sorgeloos P, Bossuyt E, Lavina E, Baeza-Meza M, Persoone G (1977) Decapsulation of *Artemia* cysts: a simple technique for the improvement of the use of brine shrimp in aquaculture. Aquaculture, 12: 311-315.

Luminous Bacteria Associated with Shrimp Mortality

Lila Ruangpan

Marine Shrimp Research and Development Center
Pawong, Muang Songkhla 90100, Thailand

ABSTRACT : Luminous disease caused by luminous bacterial infection is currently a significant problem among the Asian countries where marine shrimp is the main aquaculture product. Luminous bacteria isolated from the shrimp farm environment include *Vibrio fischeri*, *V. harveyi*, *V. cholerae* biotype *albensis*, and *Photobacterium leiognathi*. Of these, *V. harveyi* is claimed to be the causative agent associated with shrimp mortality. In hatcheries, its pathogenicity is correlated with the developmental stage of the shrimp larvae. However, successful prophylactic measures have been established. In grow-out ponds, luminous disease frequently causes mortality with 2-3 month old stock of *Penaeus monodon* and a bacteroiphage of *V. harveyi* has been implicated in mortality of such shrimp exhibiting tea-brown gill syndrome (TBGS). Chemotherapeutics are more or less effective for controlling the disease but are not effective for curing it. Recent *in vitro* studies have demonstrated growth inhibition of this bacteria using biological control and the information gained paves the way for further investigations.

Key words : Luminous bacteria, Shrimp mortality, *Vibrio harveyi*

INTRODUCTION

Widespread interest in shrimp culture in Thailand has developed only in the past 15 years. During that period, intensive farming systems have been introduced and the areas of shrimp farms number of farms has rapidly expanded. In order to reduce the introduction of high organic matter into the coastal environment and thus avoid severe damage to the production system (Nygaard et al. 1992), either closed pond systems or recycle systems are necessary. These closed and recycle systems increase the problems associated with bacterial pathogens. It is recognized that damage to shrimp stocks is frequently associated with bacterial diseases that are mostly caused by luminous bacteria (Ruangpan 1987, Songserm et al. 1990, Ruangpan et al. 1997). Luminous bacterial diseases have also been reported to cause economic losses to the shrimp industry in the Philippines (Fernandez & Mayo 1994), Vietnam (Nguyen & Le Trong 1994), India (Raju 1994), and Indonesia (Sulasmi et al. 1994, Taslihan & Wijayati 1994). The problem seems to be common among the Asian countries where shrimp farming is the main aquaculture activity. This presentation describes the species composition (incidence and intensity) of luminous bacteria in shrimp farms and the coastal environment in Thailand, their pathogenicity and recent studies on the chemotherapy and biological control. It is hoped that the information will contribute towards further investigations leading to successful resolution of the luminous disease problem.

SPECIES COMPOSITION

Preliminary studies on luminous bacterial species in Thailand have been conducted using numerical taxonomic analysis (Ruangpan et al. 1995). Media used for isolation included thiosulfate citrate bile salts sucrose (TCBS, Difco) agar and modified sea water complete (MSWC) media. A total of 180 luminous and nonluminous vibrios were isolated from diseased *P. monodon* cultivated in several areas of Thailand. Each strain was tested for 98 characters. Luminescence was observed on MSWC medium 18-24 h after isolation using the technique of Furniss et al. (1978). Based on unweighed average linkage analysis at 80% similarity, 15 phenotypic clusters were obtained. Among those, luminous vibrios represented 4 clusters, according to their similarity in phenotypic characters, in guanine-cytosine (G + C) contents and to reference cultures. Members in the first cluster comprised 21 strains identified as *V. harveyi*. The second cluster containing 2 strains identified as *V. fischeri*. The last 2 clusters containing 7 strains which could not be identified as any known species. However, their characters and G+C contents were very close to either *V. harveyi* or *V. cholerae* non-01 reference cultures ATCC 14126 and NCTC 8021, respectively (Fig. 1).

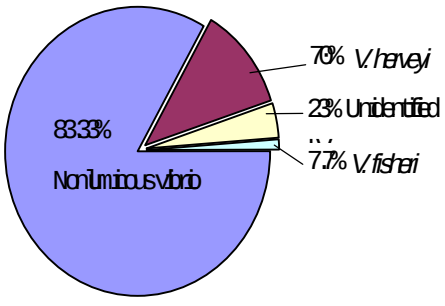


Figure 1. Species composition of luminous and nonluminous vibrios from cultured black tiger shrimp. The exploded portion of the pie (16.7% of total strains) are subdivided into groups by percentage of isolates.

Ruangpan L (1998) Luminous bacteria associated with shrimp mortality. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

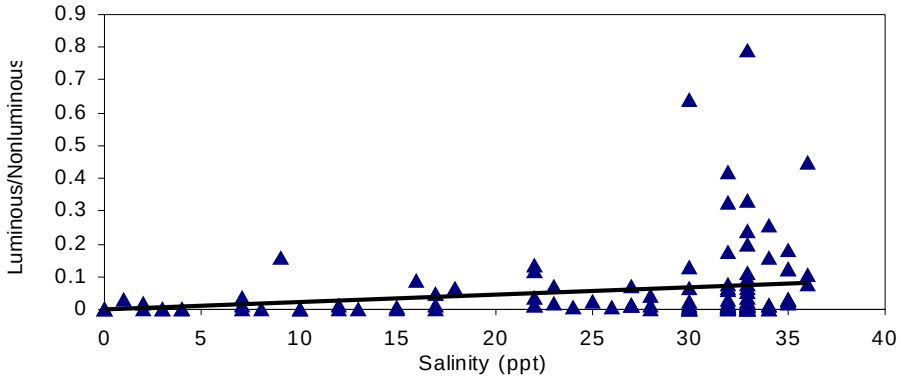


Figure 2. Variation in ratio of liminous/nonluminous bacteria at various salinities.

In 1996, identification of luminous bacteria isolated from the coastal water of Thailand was carried out by Sodthonkong (1996)(Table 1). Water samples were obtained from 128 stations in 21 provinces located along the Andaman and Siam coasts. Based on 49 phenotypic characters, a total of 210 representative isolates were identified to *V. harveyi*, *V. cholerae* biotype *albensis* and *Photo-bacterium leiognathi*.

According to the results most of the *V. harveyi* isolates exhibited green to yellow-green colonies on TCBS. Fifty seven percent of these isolates grew on media supplemented with 6% NaCl. All isolates had the ability to emit light when cultured in media containing 0.5-6% NaCl, although, 10% of them also grew and emitted light in media containing 8% NaCl.

V. cholerae biotype *albensis* exhibited yellow colonies on TCBS. All of its isolates grew on media without added NaCl or with NaCl up to 3%. Forty seven percent grew on media containing 6% NaCl while only 7% grew on media containing 8% NaCl. They gave high light when on media containing 0-6% NaCl but low light with 8% NaCl.

Table 1. The percentage incidence and intensity of luminous bacteria found in the coastal environment (shrimp ponds, pond effluent and hatcheries).

Sources	Incidence (%)	Intensity (cfu/ml)
Water : coastal environment	75	0.2 x 10 - 1.4 x 10
: shrimp ponds	84	9.1 x 10 - 1.2 x 10^2
: effluent from ponds	92	1.5 x 10 - 1.0 x 10^2
: hatcheries	67	0.2 x 10 - 2.9 x 10^3
: effluent from hatcheries	44	1.2 x 10 - 3.8 x 10^2
Sediment : shrimp pond	80	ND

Sources : Sodthongkong 1996, Ruangpan et al. 1997c

P. leiognathi gave green colonies on TCBS. All its isolates grew and emited light in the media containing 0.5-3% NaCl but only 50% grew in 6% NaCl and no growth was found in the media containing 8% NaCl. Ruangpan et al. (1995) indicated that *V. fischeri* exhibited deep green colonies on TCBS, 100% of the isolates grew and emited light on the media containing 1-8% NaCl.

INCIDENCE AND INTENSITY

Results from the investigation of luminous and nonluminous bacteria in Thai coastal water (Sodthongkong 1996) revealed that luminous bacteria are normal components of the microbial flora of estuaries and brackish water (Table 1). This finding is supported by farmer reports of luminous bacteria in the water and sediments of shrimp ponds, as well as the inlet and outlet water of mariculture operations which depend totally on water supplied from the coastal environment (Sae-Oui et al. 1987, Songserm et al. 1990, Ruangpan et al. 1995 and 1997c).

The incidence of isolation from environmental water samples was found to differ for species and sampling area. Isolates of *V. harveyi* were detected from 96 sampling stations with 75% incidence. The incidence of *V. cholerae* biotype *albensis* was 43% while that of *P. leiognathi* was 9%. Figure 1 demonstrates the correlation between salinity and luminous bacteria or vibrios. Interestingly, high incidences and intensities of both types were manifested at salinities between 30 and 33 ppt. However, the ratios of these bacteria are shown for estuarine water in the range of 1 ppt (Fig. 2).

Table 2. Incidence of *V. harveyi* infection at different developmental stages of *P. monodon* larvae.

Shrimp stock	% Incidence of infection			
	Zoea	Mysis	Postlarvae	Total
A	3.33	5.33	3.00	10.66
B	4.00	4.66	2.66	11.33
C	2.66	5.33	4.00	11.99
D	5.33	6.00	3.00	13.33
E	5.33	6.66	3.00	13.99
Total	20.65	27.98	15.66	61.30

Source : Songserm et al. 1990

Low intensities of luminous bacteria (0-4.2x10 cfu/ml) were found in the sampling stations located far from aquaculture ponds and communities. The highest intensity ($2.9x10^3$ cfu/ml) was recorded from aquaculture effluent water (Sodthongkong 1996). The incidence of *V. harveyi* isolation from shrimp pond sediment was reported to be 80%, although its intensity was not mentioned (Prapassorn 1995). Very little information is available regarding its incidence and intensity in shrimp. Most studies have focused on virulent isolates. Songserm et al. (1990) reported on the incidence from naupliar to postlarval stages of *P. monodon* (Table 2). Other investigators have reported *Vibrio* bacteria which included luminous and non-luminous isolates. Ruangpan et al. (1995) attempted to investigate the incidence of luminous bacterial species found in diseased shrimp. The

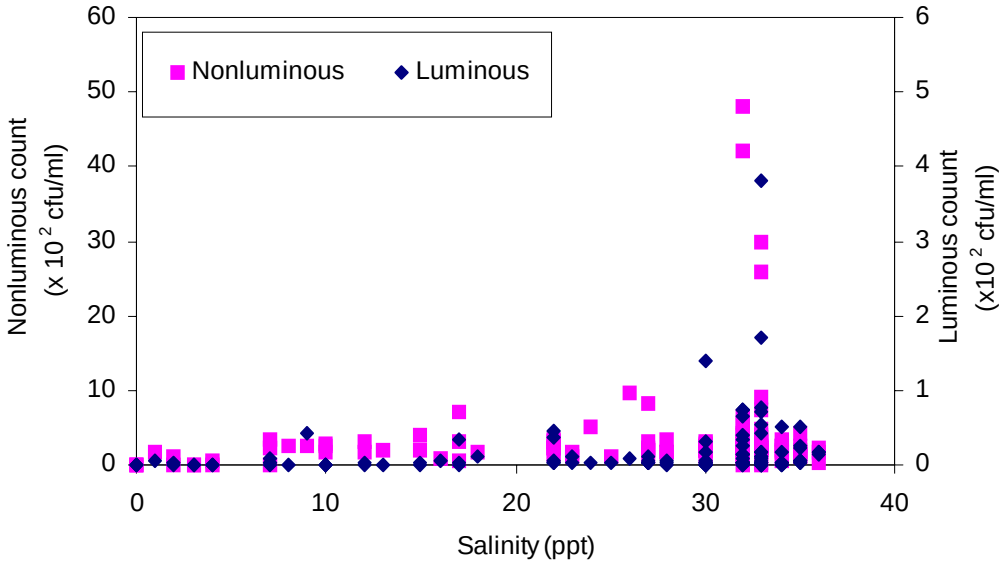


Figure 3. Distribution of luminous and nonluminous vibrio bacteria at various salinities sampled from 21 provinces located along the coast of Thailand.

results indicated that *V. harveyi* was predominant, accounting for 70% of the isolates while *V. fischeri* and other luminous species accounted for 6.7 and 23.3%, respectively (Fig. 3).

PATHOGENESIS AND HOST SUSCEPTIBILITY

Among the luminous bacterial species reported from shrimp ponds, hatcheries and moribund shrimp, only *V. harveyi* has been confirmed to cause mortality of shrimp. The disease is widely known as "luminous disease" or "Kung ruangsang" in Thai. Epizootics of this disease occur several times a year and are expanding throughout shrimp farming areas along the coast. A number of shrimp farmers have suffered severely from damage of long duration caused by this disease. Because of similar problems throughout Asia, scientists from many countries including the Philippines, Indonesia, Malaysia, India, Thailand and Taiwan are becoming increasingly interested in this disease.

The first report on shrimp luminous disease in Thailand was published in 1987 by Sae-oui and collaegues. The report indicated an outbreak of the disease in shrimp hatcheries in the central part of the country, which used to be the largest productive area for marine shrimp. The bacterial pathogenesis resulted in mortalities up to 100% for naupliar to zoeal stages of *P. merguiensis*. Living and dead shrimp larvae and even the sea water in disease outbreak areas were luminescent in dim light. Other gross features of the diseased shrimp were milky white bodies, weakness, swimming disorders and loss of appetite, eventually leading to death. Using luminous media (LM) (Baumann & Baumann 1981), luminescent bacterial colonies could be isolated from the diseased specimens as well as from hatchery water. Based on 47 phenotypic characters, the causative agents were identified to *V. harveyi*. Experimental trials for pathogenicity gave 100 and 95% mortality for naupliar and zoeal stages, respectively (Table 3), but no mortality for the larvae that reached the mysis stage.

Table 3. Pathogenicity of *V. harveyi* CHA 86021 to *P. merguiensis* larvae infected by bath exposure for 24 h.

	% Mortality		
	control	Treatment (cfu/ml)	
Shrimp stages		10^7	10^8
Nauplius	70	100	100
Zoea	60	ND	95

Source : Sae-Oui et al. 1987

Table 4. Mortality of different developmental stages of *P. monodon* exposed to V. harveyi at various concentrations.

	% Mortality				
	Control	Treatment/(cfu/ml)			
Shrimp stages		10^8	10^6	10^5	10^4
Nauplius	35	100	100	ND	100
Zoea	25	100	100	75	100
Mysis	25	75	70	ND	53
Postlarva	20	50	50	45	35

Source : Limsuwan 1989, Songserm et al., 1990

In *P. monodon*, luminous disease was also reported initially from larvae at naupliar to mysis stages (Ruangpan 1987). Since then, a rising incidence of luminous disease that can cause 80-100% mortality to the larval stocks has occurred in hatchery areas all over the country (unpublished results). In 1990, Songserm et al. reported that luminous disease in *P. monodon* larvae was also caused by *V. harveyi*. Results from experimental bath infections confirmed that zoea and mysis stages were more susceptible than postlarvae (PL). Prayinto & Latchford (1995) reported similar results with experimental infections of *P. monodon* larvae using suspensions of luminous bacteria related to *Photobacterium* and

Vibrio. Mortalities of zoea and postlarvae, after 48 h exposure, were 75 and 45%, respectively. Furthermore, Limsuwan (1989) has found that larvae beyond PL3 and 4 developmental stages resisted luminous bacterial infection (Table 4). Subsequently, various reports have indicated that luminous bacteria are also associated with shrimp mortality in grow-out ponds. In a survey of rearing water from numerous shrimp farms in southern Thailand from April to August 1996, Ruangpan et al. (1997a) found large numbers of the luminous bacteria. They also noted that massive mortality occurred in some farms within 3 to 4 days after the first appearance of moribund shrimp exhibiting tea-brown colored gills or tea-brown gill syndrome (TBGS). Isolations of bacteria from the moribund shrimp revealed that *V. harveyi* was dominant. Based on results from electron microscopy and injection trials with naturally and experimentally infected TBGS shrimp, it was suggested that a bacteriophage might be implicated in shrimp mortality by mediating toxigenesis of *V. harveyi*.

Disease induced by *V. harveyi* has been investigated by experimental infection of various crustacean species under stress conditions. It has the ability to induce disease and cause mortality in *P. monodon*, *P. indicus*, and *Artemia* nauplii (Prayinto & Latchford 1995) and *P. merguiensis* (Sae-Oui et al. 1987). No significant mortality occurred in *Macrobrachium* species (Kasornchan 1987, Prayinto & Latchford 1995) and banacles (Prayinto & Latchford 1995).

PREVENTION AND CONTROL

Prevention and control of luminous disease in shrimp are generally achieved by chemical treatment. Chemotherapeutants may be applied in the feed or in the water. Usually antibiotics are applied in the feed while disinfectants (and sometimes antibiotics as well) are applied in the water (Ruangpan 1987, Ruangpan et al. 1997c).

To prevent the luminous disease in hatchery operations, water should be filtered through a biofilter system prior to chemical treatment with 60% active chlorine at 10-30 g/ton or commercial grade formalin at 50-100 ml/ton. During treatment aeration is necessary. In addition, *in vitro* trials have been conducted on the inhibition of luminous bacterial growth using several disinfectants. Table 7 shows the effective doses for growth inhibition of *V. harveyi* treated with benzalkonium chloride, formalin and povidone iodine. Antibiotics treatment via the water is a common practice during rearing the early larval stages (nauplius to postlarvae 3-4). Application of antibiotics at 3-5 g/ton into the rearing water, especially during periods of metamorphosis has been found to greatly reduce luminous bacteria and stock mortality (Chayarat 1997). Oxytetracycline (OTC) was the main drug used for several years in all areas of the country. The effectiveness of OTC gradually declined after repeated application for long periods due to the development of bacterial resistance (Ruangpan et al. 1997c). Recently, various drugs have been tested against luminous and nonluminous vibrios, including resistant strains (Tables 5 and 6). Therefore, alternative drugs can be recommended based on sensitivity tests. After harvesting, treatment of the larval rearing facilities by flushing or vigorous spraying with freshwater has been suggested as a routine practice.

The major disinfectants used to reduce bacterial numbers in grow-out ponds are formalin and chlorine. When the *Vibrio* count in a pond rises to 10^3 to 10^4 cfu/ml, addition of 5 to 20 liters of disinfectant (per 1 ha pond) has been recommended. Benzalkonium chloride and povidone iodine have also been found to be effective chemicals to inhibit growth of *V. harveyi* (Table 7). At present, these chemicals are seldom used due to their high cost.

Antibiotics are usually applied in grow-out pond by addition to pelleted feed at approximately 3-5 g/kg administered to the shrimp for 7-10 days. Oxytetracycline and oxolinic acid were commonly used in the past. Recently they appear to be of limited value due to the development of bac-

Table 5. Minimal inhibitory concentration (MIC) ranges and inhibitory concentration (IC) of 6 drugs against 60 strains of luminous bacteria.

Drugs	MIC ranges (ug/ml)	IC s (ug/ml) 50% *1	90% *2
Oxytetracycline	6.25 - 100	15.00	69.20
Oxolinic acid	12.5 - 100	28.20	56.70
Norfloxacin	0.8 - 100	65.50	100
Chloramphenicol	3.13 - 50	8.75	45.20
Trimethoprim	3.13 - 25	9.37	15.00
Kanamycin	100 -> 100	>100	>100

*1 and *2 : Concentration inhibiting 50% and 90% of the strains, respectively. Source: Ruangpan et al. 1997c

Table 6. Drug resistant strains of luminous and nonluminous vibrios isolated from cultured shrimp *P. monodon* and the farm environment.

	% of drug resistant strains/year					
Drugs	1989	1990	1992	1994	1995	1996
Trimethoprim	0	23.4	25.5	ND	ND	20
Chloramphenicol	0	2.92	5.1	14.8	47.1	20
Oxolinic acid	90	0	0	11	20.5	45
Oxytetracycline	ND	32.2	36.4	45.9	71.4	0.4
Norfloxacin	ND	22.9	7.3	41.5	87.6	90
Kanamycin	0	ND	14.5	100	100	100
Sulfamonomethoxine	0	61.95	100	ND	ND	ND

Sources : Ruangpan and Kitao 1992, Sangrungruang et al. 1993, Prapassorn 1995, Sudthongkong 1996, Ruangpan et al. 1997

Table 7. Summary of *in vitro* trials for control of *V. harveyi*.

Agents **Biological control**	**Application**	**Dosage**	**Effectiveness**	**Reference**
Bacillus S11	Probiotic/feeding	ND	74 % RPS	Phianphak et al. 1997
Lactobacillus casei *L. acidophilus*	Probiotic/feeding	ND	Growth-inhibition	Jiravanichpaisal et al. 1997
Vibrio alginolyticus	Concomitant culture	1 : 10 cfu	Growth inhibition 0 to 100%	Ruangpan et al. (in press)
Chlorella sp.	Concomitant culture	1000 : 1 cell/efu	Growth inhibition	Direkbusarakom et al. 1997
Skeletonema costatum	Concomitant culture	500:1 cell/cfu	Growth inhibition 35 to 100 %	Panichsuke et al. 1997
Guava leaf extract	Feeding	MIC 1250 µg/ml	Growth inhibition	Direkbusarakom et al. 1997
Fresh water	Spray or bathe containers and equipment	Several times	Growth inhibition	Unpublished data
Chemical control				
Benzalkonium chloride	Water treatment	40% MIC 64 ppm MBC 64 ppm	Growth inhibition	Lin and Nash 1996
Formalin	Water treatment	40-50 ppm	Growth inhibition	Lin and Nash 1996
Providone iodine	Water treatment	1-3 ppm MBC 64 ppm MIC 1024ppm	Growth inhibition	Areechon 1990

ND = No data; MIC = Minimal inhibitory concentration; RPS = Relative percent of survival
MBC = Minimal bactericidal concentration

terial resistance (Ruangpan et al. 1997c). Several drugs claimed to be effective against luminous bacteria are supplied in commercial grade although many show very low bacterial growth inhibition (Ruangpan et al. 1997b).

Governmental guidelines to restrict the use of antibiotics in shrimp farms have been issued. To prevent problems regarding drug residues, the guidelines recommend that antibiotics should not be used beyond 3 months in shrimp grow-out ponds.

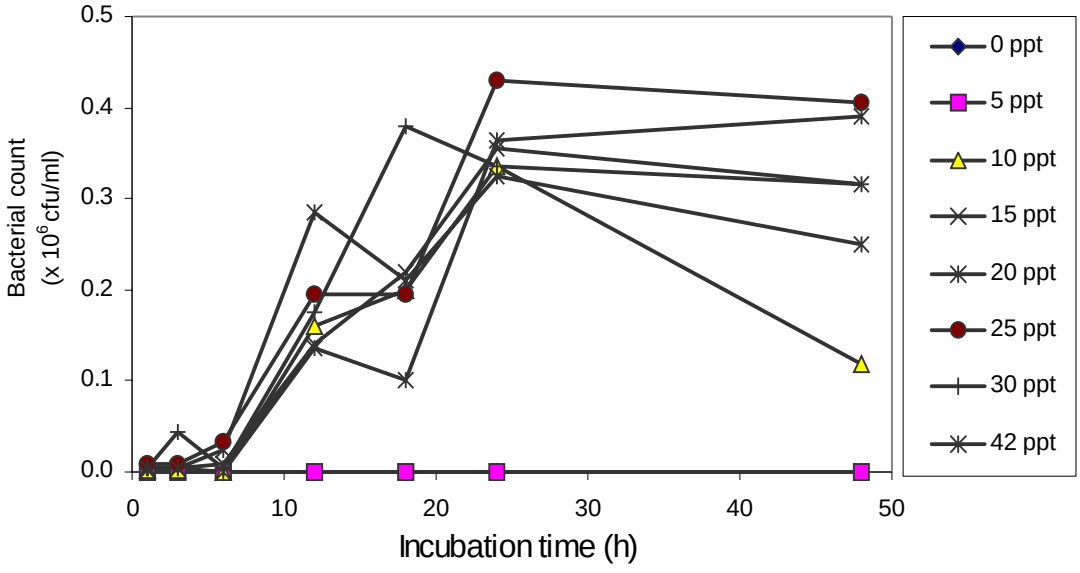


Figure 4. Number of luminous vibrios cultured at various salinity levels.

Experiments on preventing and controlling luminous disease using alternative methods such as probiotics or biological control have been attempted (Table 7). Phianpark et al. (1997) successfully used *Bacillus* sp. as probiotic feed in laboratory scale tests to achieve 74% relative survival after challenge with *V. harveyi*. *Lactobacillus casei* and *L. acidophilus* have also been claimed to inhibit the growth of *V. harveyi* when used as a probiotic feed (Jiravanichpaisal et al. 1997). Beneficial microorganisms found to be effective for growth inhibition of *V. harveyi* on the laboratory scale include *V. alginolyticus* (Ruangpan et al. 1998) *Chlorella* sp. (Direkbusarakom et al. 1997) and *Skeletonema costatum* (Panichsuke et al. 1997). Sodthongkong (unpublished data) studied the multiplication of *V. harveyi* at various salinity levels and found that the number of bacteria was reduced from $7.5x10^3$ to $8.9x10^2$, $4.0x10^2$, 1.3x10 and 0 cfu/ml in 1, 6, 12 and 18 h, respectively, after transfer from 25 ppt to 5 ppt. By contrast, bacteria transferred to 10 ppt decreased to $2.0x10^3$ and $32.x10^2$ cfu/ml after 1 and 6 h, respectively, but then increased to $1.6x10^5$ and $3.4x10^5$ after 12 and 24 h, respectively. At salinities between 15 and 42 ppt, numbers of *V. harveyi* increased gradually from 1-48 h of cultivation (Fig. 4). The results suggested that shrimp culture in seasons of low water salinity may experience less risk of luminous disease. Further studies are needed to ascertain the efficacy of using various microorganisms to control luminous bacteria in shrimp operations. They may prove a better alternative than antibiotics in overcoming problems.

CONCLUSIONS

Thailand is now one of the top of shrimp producing countries and highly developed culture systems have been established and expanded in many shrimp farming areas. However, more attention should be focused on how to develop shrimp culture as a sustainable industry. Disease problems still exist and are associated with serious crop losses. The survey data on luminous bacteria has shown that they are members of the natural estuarine and brackishwater environment. The studies also suggest that they induce disease more frequently in dry season than the rainy season and that their is related to salinity levels and effluent from farming operations. Only *V. harveyi* has been confirmed to be a pathogenic agent associated with shrimp mortality. In hatcheries, luminous disease can be controlled by chemical treatment with disinfectants and antibiotics, while in grow-out ponds, effective treatment and control methods are still uncertain. Some experiments indicate effective growth inhibition of *V. harveyi* by probiotics and biological treatments. However, effectiveness has not yet been proven in field trials. Although luminous pathogens exist naturally in the water, they certainly need carbon and introgen (C+N) for growth and multiplication. Thus, the first action to take in reducing their numbers is to be careful not to overfeed the shrimp and thus supply plentiful nutrients in the pond and the adjacent environment.

LITERATURE CITED

Areechon N (1990) Bactericidal activity of povidone iodine against *Aeromonashydrophila* and *Vibrio harveyi*. Thai Fish. Gazette 43:199-204 (In Thai)

Baumann P and Baumann L (1981) Marine genera *Photobacterium, Bencekea, Alteromonas, Pseudomonas, Alcaligenes*. In : Mortmer PS et al. (eds) The prokaryotes. Vol. 2 New York Spinger - Verlag p 1302-1331.

Chayarat C (1997) Correlation on seed production of *Penaeus monodon* and bacterial number in nursing pond. Tech. paper No. 24/1997 Coastal Aquaculture Div. DOF, Bangkok 11 p

Direkbusarakom S, Herunsalee A, Yoshimizu M, Ezura Y, Kimura T (1997a)Efficacy of guava (*Psidium quajava*) extract against some fish and shrimp pathogenic agents. In : Flegel TW and MacRae IH (eds), Disease in Asian Aquaculture I I I Fish Health Section, Asian Fisheries Society, Manila p 359-363

Direkbusarakom S, Pechmanee T, Assavaaree M, Danayadol Y (1997b) Effect of *Chlorella* on the growth of *Vibrio* isolated from diseased shrimp. In : Flegel TW, MacRae IH (eds) Disease in Asian Aquaculture III. Fish Health Section, Asian Fisheries Society, Manila p 355-358

Fernandez RD, Mayo S (1994) Current status of prawn farming and prawn health management in the Philippine. In : Turnbull J et al. (eds) Procs. of the SEAADCP, Prawn health management workshop. Aquatic Animal Health Research Institute, DOF Bangkok, Thailand p 61-89

Furniss AL, Lee JV and Donovan TJ (1978) The vibrios. Public Health Lab. Service Monograph Series No. 11 HMSO London

Jiravanichpaisal P, Chuaychuwong P, Menasveta P (1997) The use of *Lactobacillus* sp. as the probiotic bacteria in the giant tiger shrimp (*Penaeus monodon* Fabricius) In : Abstract of poster session of the 2nd Asian-Pacific Marine Biotechnology conference, Phuket thailand p 16

Kasornchandra J, Boonyaratpalin S, Jadesadakraisorn U (1987) Luminescent disease in giant prawn larvae. Tech. paper No. 67 National Inland Fisheries Ins. Bangkok 9 p (In Thai with English abstract)

Limsuwan C (1989) Common disease found in cultured shrimp in Thailand. Asian shrimp News 1 : fourth quarter 1989

Lin CK, Nash GL (1996) Asian shrimp news. Collected volume 1989-1995, Asian shrimp culture council, Bangkok 312 p

Nguyen H T, Le Trong K (1994) Report on shrimp diseases in Vietnam. In: Turnbull J et al. (eds) Procs. of the SEAADCP, Prawn health management workshop. Aquatic Animal Health Research Institute, DOF Bangkok, Thailand p103-106

Nygaard K, Lunested BT, Hekton H, Berge JA, Hormazabal V (1992) Resistance to oxytetracycline, oxolinic acid and furazolidone in bacteria from marine sediments. Aquaculture 104:31-36

Panichsuke P, Ruangpan L, Pechmanee T (1997) Inhibitory effect *of Skeletonema costatum* on the growth of *Vibrio harveyi* In : Abstract of poster session of the 2nd Asian-Pacific Marine Biotechnology conference Phuket Thailand p 122

Phianphak W, Piyatiratitivorakul S, Menasveta P, Rengpipat S (1997) Use of probiotics in *Penaeus monodon* In : Abstract of poster session of the 2nd Asian-Pacific Marine Biotechnology Conference, Phuket Thailand p 18

Prapassorn S (1995) Bacterial flora in the sediment of black tiger shrimp pond with the emphasis in *Vibrio* spp. and their resistance to antimicrobial agents. Msc Thesis in Fisheries Science, Faculty of Fisheries, Kasetsart University, Bangkok p 74

Prayinto SB and Latchford JW (1995) Experimental infections of crustaceans with luminous bacteria related to *Photobacterium* and *Vibrio* : Effect of salinity and pH on infectiosity. Aquaculture 132: 105-112

Raju GA (1994) Shrimp aquaculture in India. In : Turnbull J et al. (eds) Procs. of the SEAADEP, Prawn health management workshop. Aquatic Animal Health Research Institute, DOF Bangkok, Thailand p 39-42

Ruangpan L (1987) Diseases of cultured marine shrimp in Thailand : *Penaeus monodon* and *P. merguiensis*. In : Limsuwan C et al.(eds) Tech. paper for the 1st seminar on Marine Shrimp Culture, Fisheries Science Club. Kasetsart Univ. Bangkok p 46-62 (In Thai)

Ruangpan L, Kitao T (1992) Minimal inhibitory concentration of 19 chemothera-peutants against *Vibrio* bacteria of shrimp *Penaeus monodon.* In : Shariff M et al. (eds) Diseases in Asian Aquaculture I. Fish Health Section. Asian Fisheries Society Manila p 135-142

Ruangpan L, Tabkaew R, Yoshida T, Kawatsu H and Saitanu K (1995) Numerical taxonomy of *Vibrio* spp. isolated from black tiger shrimp, *Penaeus monodon*, cultured in Thailand. In : Shariff M et al. (eds) Fish Health Section, Asian Fisheries Society, Manila p 131-140

Ruangpan L, Danayadol Y, Direkbusarakom S, Siurairatana S and Flegel TW (1997a) Bacteriophage implicated in mortality of cultivated *Penaeus monodon* exhibiting tea-brown gill syndrome (TBGS). Tech. paper present in Shrimp Biotechnology seminar 13 Dec. 1997 NSTDA Bangkok p 91-96

Ruangpan L, Direkbusarakom S, Danayadol Y and Srikaew Y (1997b) Efficiency of commercial drugs for control disease in black tiger shrimp on the growth inhibition of luminous and vibrio bacteria. Tech. paper No. 19/1997 National Institute of Coastal Aquaculture, Songkhla 10 p (In Thai with English abstract)

Ruangpan L, Sodthongkong C, Srikaew Y (1997c) Luminous bacteria in shrimp aquaculture area of Songkhla. Procs. of DOF seminar 1997. p 65-74

Ruangpan L, Na-anan P, Direkbusarakom S (1998) Inhibitory effect *of Vibrio alginolyticus* on the growth of *V. harveyi*. Fish Pathology (in press)

Sae-Oui D, Tunsutapanit A, Ruangpan L (1987) *Vibrio harveyi*, a causative agent of marine shrimp *Penaeus merguiensis* larvae. In : Limsuwan C et al. (eds) "Shrimp Disease and Chemotherapy" Kasetsart Univ. Press, Bangkok p 1-8

Sangrungrueng K, Aoki T, Yoshida T (1993) Minimal inhibitory concentration of chemotherapeutants and drug resistance against vibrio bacteria isolated from shrimp (*Penaeus monodon*) in Thailand. In : Snidvong A et al. (eds) NRCT-JSPS seminar on marine science, Chulalongkorn Univ. Bangkok p 221-226

Sodthongkong C (1996) Identification and drug resistance of luminous bacteria in coastal water of Thailand. Msc Thesis in Fisheries Science, Faculty of Fisheries, Kasetsart University Bangkok 109 p (In Thai with English abstract)

Songserm M, Suksringam B, Srisopaporn P (1990) Study on the bacterial luminescent disease in jumbo tiger shrimp (*Penaeus monodon*) Srinakarin-tarawiroj Research and Development Journal 4(1): 15-24 (In Thai with English abstract)

Sulasmi S, Naibaho L, Dewi E (1994) Shrimp farming in north Sumatra province, Indonesia. In : Turnbull J et al. (eds) Procs. of the SEAADCP, Prawn health management workshop. Aquatic Animal Health Research Institute, DOF Bangkok, Thailand p 53-59

Taslihan A, Wijayati A (1994) Diseases in Indonesia shrimp (*P. monodon*) culture. In : Turnbull J et al. (eds) Procs. of the SEAADCP, Prawn health management workshop. Aquatic Animal Health Research Institute, DOF Bangkok, Thailand p 45-52

Luminous *Vibrio harveyi* Associated with Tea Brown Gill Syndrome in Black Tiger Shrimp

[1]Tirasak Pasharawipas, [2]Siriporn Sriurairatana, [3]Sataporn Direkbusarakom [3]Yaowanit Donayadol, [4]Surayut Thaikua, [3]Lila Ruangpan, [4]T.W. Flegel

[1]Microbiology dept, Faculty of Science, Rangsit university, Paholyathin Rd., Pathumthani, Thailand, 12000
[2]Pathology Dept, Faculty of Medicine, Ramathibodi hospital, Mahidol university Rama VI Rd, Bangkok, Thailand, 10400.
[3]National Institute of Coastal Aquaculture, Koaseng Rd., Muang District, SongKhla 90000, Thailand
[4]Biotechnology Department, Faculty of Science, Mahidol University, Rama VI Rd, Bangkok 10400, Thailand

ABSTRACT: Luminous *Vibrio harveyi* (VH1039) isolated from tea brown gill syndrome (TBGS) black tiger shrimp, *Penaeus monodon*, was examined as a lysogenic bacteriophage host. Temperate phages found were morphologically identified as siphoviruses of various sizes (i.e., heads of 20-100 nm diameter and tails of 20-200 nm in length). The colonies of VH1039 were highly variable upon extended cultivation. This variability might be caused by transposon like activity of these phages. Lysogenic VH1039 caused no disease symptoms in the shrimp. This study also showed that luminescence was influenced by temperature and pH conditions but that it was not critical to shrimp pathogenicity. The relationship of the phage to the TBGS shrimp is being studied.

KEY WORDS: *Vibrio harveyi*, luminescence, tea brown gill syndrome, TBGS, bacteriophage, *Penaeus monodon*

INTRODUCTION

Vibrio harveyi (VH) is one of many luminous bacteria like *V. fisheri*, *Photobacterium leiognathi* and *P. phosphoreum*. It is found abundantly in marine and estuarial habitats (Hastings *et al*. 1981, Jiravanichpaisal *et al*. 1994). Some studies have reported that VH is a devastating pathogen of penaeid larvae, especially those of the black tiger shrimp (Baticados *et al*. 1990, Lavillla-Patogo *et al*. 1992). However, previous studies have also reported that it is a normal constituent of the non-pathogenic flora of marine animals (O'Brien & Sizemore 1979, Ruby & Morin JG 1979). All these luminous bacteria, whether normal flora or pathogens, can exhibit luminescence in marine animals. A recent study found that luminous VH isolate and a bacteriophage could be found in black tiger shrimp exhibiting tea brawn gill syndrome (TBGS). A bioassay study showed that intramuscular injections of this VH strain into juvenile shrimp causes no symptoms, but that the combination of bacteriophage and VH caused shrimp death (Ruangpan *et al*. 1998). *Vibrio* species, including VH, are major hosts of marine bacteriophages (Proctor 1997). Most bacteriophages are temperate phages and they have been reported to cause diversity in bacterial hosts by acting as transposon-like elements. It is well known that some temperate phages in bacterial hosts play major roles in causing diseases in humans (Cheetham & Katz 1995, Reid & Mekalanos 1995). In marine shrimp, however, there is still no confirmed evidence that temperate phages lead to disease.

In the present paper we report highly variable characteristics of *Vibrio harveyi* VH1039 isolated from TBGS black tiger shrimp. This bacterial strain is lysogenic and luminous. We report the optimal physical conditions for its growth and luminescent expression. The temperate phage was isolated and morphologically identified. Moreover, there was evidence to show that a transposon (Tn3) might also reside in this strain.

MATERIALS AND METHODS

Cultivation and identification of VH1039

TBGS in black tiger shrimp was first reported in 1998 (Ruangpan *et al*. 1998). A luminescent bacterial strain was isolated from moribund TBGS shrimp and it was later identified as VH1039 by biochemical tests. The bacterium was cultured on TCBS agar, on tryptic soy agar (TSA) and in tryptic soy broth (TSB). It was incubated at various temperatures (from room temperature to 37 °C), in media of various percentages of NaCl (between 0.5-8%), and various alkalinity (pH 5-12). Under these physical conditions, the strain grew well and colony morphology and luminescence production were investigated. The bacterium was also subjected to confirmatory biochemical tests and antibiotic sensitivity tests were carried out using BBL Sensi-Discs (BBL).

Isolation and examination of temperate phages

To investigate the possibility that VH1039 was infected with temperate phages, it was cultured in TSA with 3% NaCl

Pasharawipas T, Sriurairatana S, Direkbusarakom S, Donayadol Y, Thaikua S, Ruangpan L, Flegel TW (1998) Luminous *Vibrio harveyi* associated with tea brown gill syndrome in black tiger shrimp. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

for 18-20 hours at temperatures between 30°C and 33°C. Bacterial colonies were then fixed in a solution of 0.5% glutaraldehyde in 4% paraformaldehyde, stained with 2% phosphotungstic acid and examined by transmission electron microscopy.

To stimulate the production of higher quantities of phage particles, VH1039 was cultured in TSB with 3% NaCl at 30-33°C for 18-20 hours to reach OD 0.6-0.7 at 590 nm. The medium was then divided into portions containing different concentrations of mitomycin-c (from 1-250 ug/ml) and allowed to incubate at 37°C for another 18-20 hours. These suspension were also examined by transmission electron microscopy. To concentrate and purify phage particles, bacterial cells were precipitated by adding NaCl to a final concentration of 1 M followed by immersion in an ice bath for 1 hour before centrifugation at 12,000 g for 20 minutes. After this, bacterial debris was eliminated from the supernatant by repeated centrifugation under the same conditions. Bacteriophage suspended in the supernatant was then precipitated by adding polyethylene glycol 8000 at a concentration of 10% followd by immersion in an ice bath for 1 hour before pelleting by centrifugation at 12,000 g for 20 minutes. The pellet was resuspended in phage buffer (20 mM Tris-HCl, pH 7.5 contianing 10 mM $MgSO_4$). The solution was finally filtered through a 0.22 m filter membrane before examination by the transmission electron microscopy.

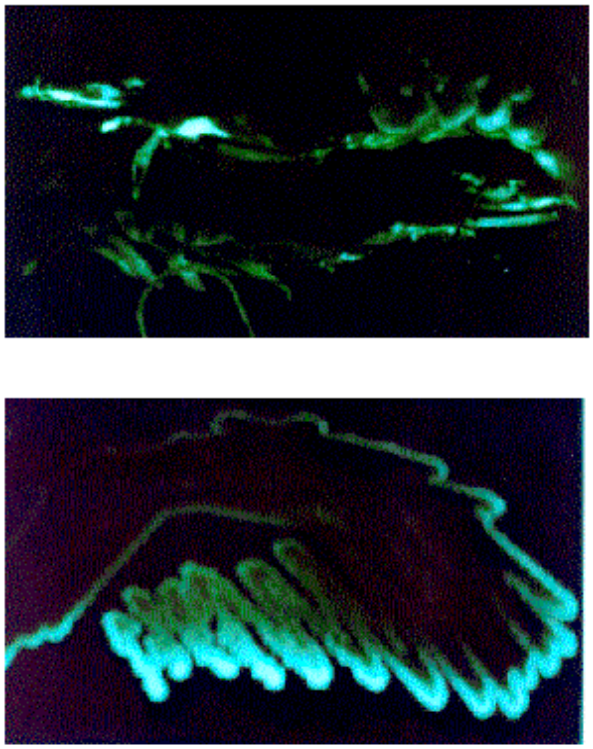


Figure 1. Luminescence of VH infected *P. monodon* (top) and VH on TSA with 3% NaCl (bottom).

RESULTS

Optimal condition for culture of VH1039

VH1039 grew well from room temperature (28-30°C) to 33°C, but the best temperature for growth was 33°C. It tolerated but did not grow at 35°C or above. At higher temperatures like 37°C, it exhibited strong luminescence, but little or none at temperatures from room temperature to 33°C. However, luminescense weakened after incubation for 4-5 days, regardless of the incubation temperature. The luminescense could be reactivated in approximately 6 hours after switching the cultures from a lower to a higher temperature (e.g., 30°C to 37°C). If the temperature was lowered once again, the luminescence faded.

Salinity also influenced the growth of VH1039. It grew well in 2-3% NaCl but also persisted in percentages of NaCl from 1% up to 6.5%. It grew well between pH 7-9, but not at pH lower than 5, and slowly at any pH above 9. Also, at pH 7-9 it exhibited stronger luminescence than at lower pH. The luminescent shrimp and luminescent VH are shown in Fig. 1.

Antimicrobial sensitivity and biochemical tests

VH1039 was tested with antimicrobial agents to examine for the possibile presence of transposons. Among the antimicrobial agents tested were neomycin, kanamycin, streptomycin, trimethoprim, tetracyclin and ampicillin. It was resistant to only ampicillin, a characteristic of Tn3. In biochemical tests, it expressed an unusual character for *Vibrio* species by producing H_2S in 24-48 hours in triple sugar iron (TSI) medium.

Variation in colony morphology

When VH1039 was sub-cultured in 3% NaCl TSA and TCBS, it presented variable colonial morphology. The appearance of the colonies was diverse in size, shape and color (Fig. 2). In spite of these diverse forms, tests identified each colony as biochemically identical. This variation appeared only in solid culture media incubated for periods from 3 to 5 days or longer. The variation was not evident upon daily subculture.

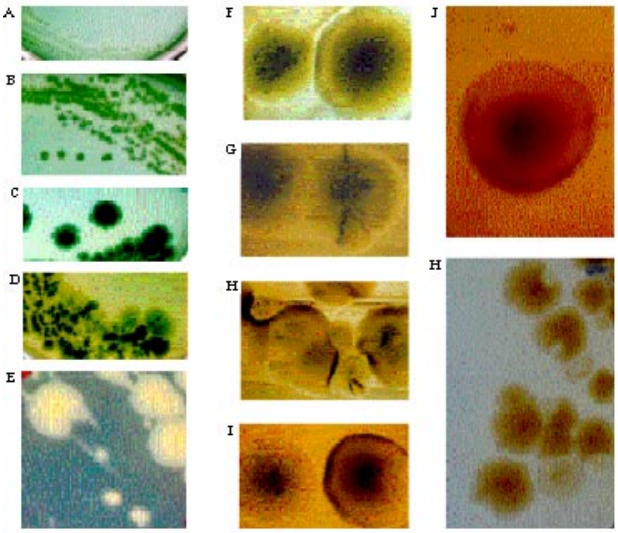


Figure2. Various colony types of VH 1039; (A)clear and (B) clouded colonies; (C-D) colonies with black pigments on TCBS agar, some small and rough (C), others big and smooth (D); (E) margins of big and small colonies on the same plate; (F-J) other shapes and colors of colonies; (K) colony partly digested.

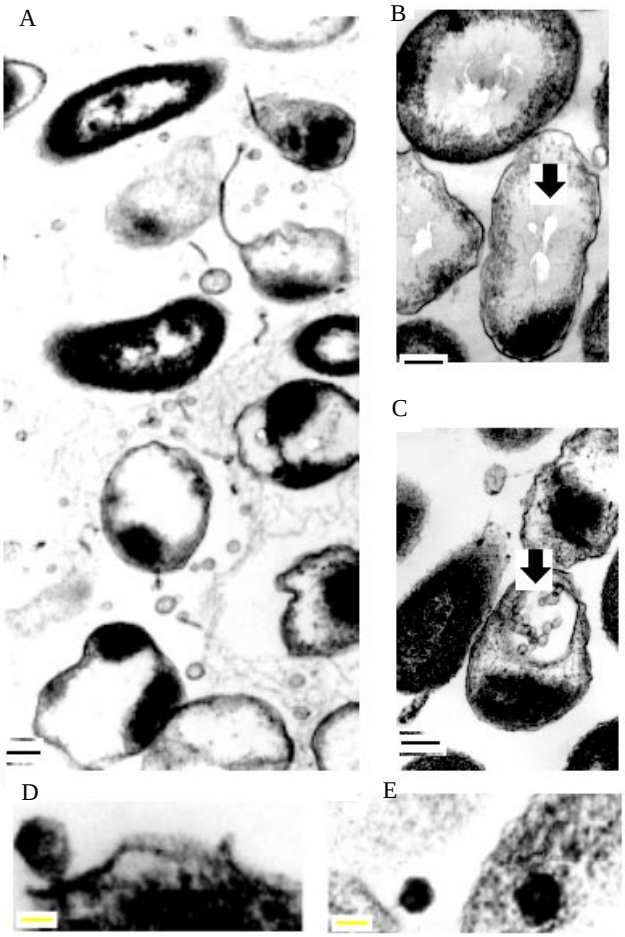


Figure 3. TEM of various sizes of bacteriophages with VH 1039. (A) Phage particleoutside VH 1039; (B) phage particle inside VH 1039 (arrow); (C) unidentified particle inside VH 1039 (arrow); and (D-E) phage adhered to VH 1039 (bars 100 nm).

Detection of temperate phages in VH1039

Studies using the electron microscopic showed that VH1039 could produce a siphovirus or λ-like phage particles with icosahedral heads and a filamentous tails. The sizes of the phages produced varied, with head diameters of 20 – 100 nm and a tail lengths of 20-200 nm. The sizes of the head and the tails were not related (Figure 3 and 4). Induction of phages (using mitomycin-C) and extraction (i.e., using the NaCl/PEG technique) resulted in higher density yields. However, the use of mitomycin-C (optimal at 5 µg/ml) generally resulted in the formation of separate heads and tails (Figure 4).

DISCUSSION

Bacterial luminescense is reportedly autoinduced, with each genus or species of luminous bacteria producing a different autoinducer. However, the major autoinducer of VH has been reported to be a long chain aliphatic aldehyde. Autoinducers are accumulated during bacterial growth and their synthesis triggers lux gene expression. The electron transport proceeds by reaction of luciferase enzyme which catalizes the reaction amongst reduced flavin mononucleotide ($FMNH_2$), oxygen and a long chain aliphatic aldehyde to produce flavin mononucleotide (FMN) and an aliphatic carboxylic acid which emits the light (Fisher *et al*. 1995). Our results showed that temperature may also influence expression of luminescence. The temperature may either stimulate luciferase activity and/or the production and/or function of the autoinducer. Logically, since VH1039 does not grow at higher temperatures like 37°C, it would seem unlikely that autoinducer production would be involved and it is more likely that temperature affects enzyme or autoinducer activity. We also found that alkalinity affected luminescence expression. Higher than optimal-pH media gave strong lumi-

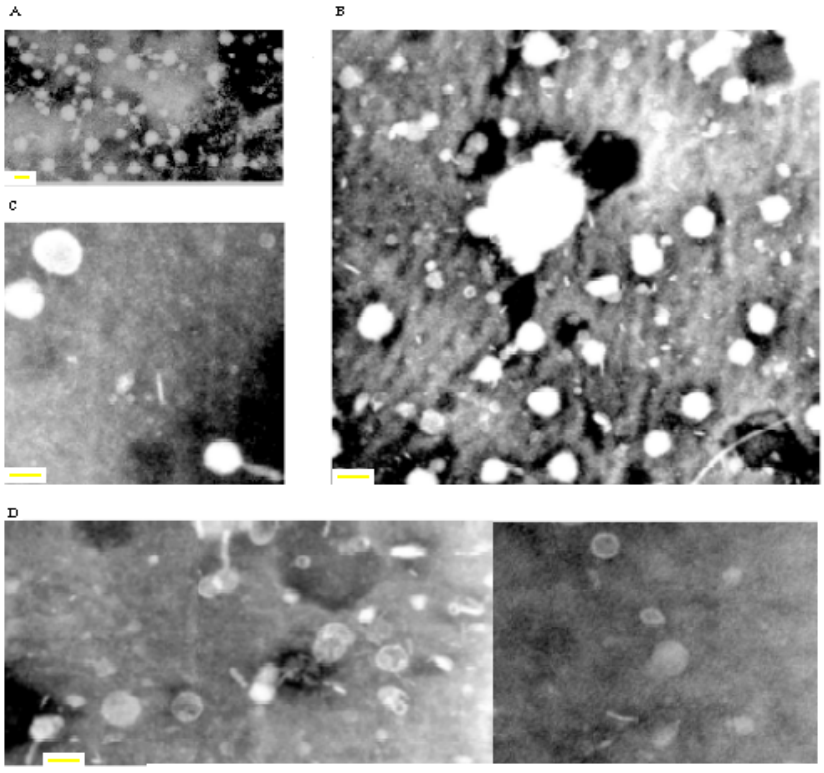


Figure 4. Temperate phage induced with mitomycin-C and isolated by NaCl/PEG. (A-B) mostly phage heads; (C) complete phage particle; (D) phage tails are seperated from the heads (bars 100 nm).

nescence. This combination (temperature and alkalinity) might find some application in manipulation of VH strains for higher production of poly-3-hydroxybutyrate (PHB), a raw material of interest in the plastic industry due to its properties of thermoplasticity, water resistance and biodegradability. It was previously reported that the production of PHB is related to luminous expression and that it is controlled by the lux autoinducer (Sun et al. 1994).

The variable morphology of VH1039 colonies is of some interest. This variability might involve the fact that it is a lysogenic host of a temperate phage(s). Indeed, the variability of other bacteria has also been reported to be due to transposon-like behavior of bacteriophages (Reidl & Makalanos 1995, Belas *et al*. 1984). This may be the best explanation for variable morphology of bacterial colonies since the biochemical tests did not change for each variety.

To study the possibility of transposon element integration, we checked for antibiotic drug sensitivity and found that VH1039 was resistant to ampicillin. This might mean that it contains a Tn3 element. We have recently isolated another luminescent *Vibrio* strain (VHN1) which acts similar to VH1039 in that it causes shrimp mortality in the laboratory only when combined with a bacteriophage partner. This new strain can resist ampicillin, neomycin, kanamycin, and streptomycin. Thus, in addition to Tn3, this *Vibrio* strain may also contain Tn5 (known to carry resistance to neomycin, kanamycin and streptomycin) and Tn 10 (known to carry resistance to tetracycline). This strain must be carefully handled due to its antibiotic resistance and its potential for pathogenicity. To confirm the presence of Tn3 in VH1039, we are currently using PCR and sounthern blot hybridization tests.

According to this work and the previous work of Ruangpan et al. (1998), VH1039 cannot induce TBGS on its own, even though it contains a temperate phage(s). The phage partner that cooperates with VH1039 in causing TBGS has still not been isolated and characterized. Although TBGS shrimp exhibit tea brown pigment at their gills, the only obvious histopathological changes evident are devastation of the hepatopancreas which contains both bacteria and phage. It is possible that the tea brawn pigment in the gills is a coincident symptom, and not the main cause of shrimp death. For an explanation of the tea brown color in TBGS shrimp, the unusual characteristic of H_2S production by VH1039 may be considered. It is possible that H_2S produced by the bacterium in the shrimp could react with Fe^{++} in the shrimp pond water to produce FeS which would precipitate in shrimp gills to produce a tea brown color.

The phage partner of VHN1 is a lytic phage that was isolated from shrimp pond water in screening tests for lytic phages of *Vibrio*. The combination of VHN1 with this phage can cause shrimp death in several hours after intra muscular injection while neither the phage nor the bacterium alone cause mortality. Nor is there any mortality when this phage is combined with VH1039. The situation appears to be complex, with the probable interaction of several bacterial strains and specific bacteriophage partners. They can apparently combine under appropriate circumstances to cause shrimp mortality.

Acknowledgements. This work was supported by the National Science and Technology Development Agency through the Thai National Center for Genetic Engineering and Biotechnology (BIOTEC).

LITERATURE CITED

Baticados MCL, Lavilla-Pitogo CR, Cruz-Lacierda ER, De La Pena LD, Sunaz NA. (1990) Studied on the chemical control of luminous bacteria Vibrio harveyi and Vibrio splendidus isolated from diseased Penaeus monodon laevae and rearing water. Dis Aquat. Org. 9: 133-139.

Belas R, Mileham A, Simon M, Silverman M. (1984). Transposon mutagenesis of marine *Vibrio spp*.. 158 (3): 890-896.

Cheetham BF, Katz ME. (1995). A role of bacteriophages in the evolution and transfer of bacterial virulence determinants. Molecular Microbiology, 18(2), 201-208.

Hastings JW, Nealson K (1981). The symbiotic luminous bacteria. In M. Starr, H. Trueper, A Balows, H. Schlegel(eds), The prokaryotes. A handbook on habitats, isolation and identification of bacteria. Spriger- Vrlag KG, Berlin, p 1322-1345.

Fisher AJ, Raushel FM, Baldwin TO, Rayment I (1995) Three dimensional structure of bacterial luciferase from *Vibrio harveyi* at 2.4 A° resolution. Biochemistry, 34, 6581-6586,.

Jiravanichpaisal P, Miyazaki T, Limsuwan C (1994) Histopathology, biochemistry and pathogenicity of Vibrio harveyi infecting black tiger prawn *Penaeus monodon*. J Aquat Anim Health; 6: 27-35)

Lavillla-Patogo CR, Albright LJ, Paner MG, Sunaz NA (1992) Studies on the sources of luminescent Vibrio harveyi in *Penaeus monodon* hatcheries,. In Shariff IM, Subasinghe RP, Arthur JR (eds) Diseases in Asian aquaculture I. Fish Health section. Asian Fisheries Society. Manila. The Philippines. p. 157-164

O'Brien CH, Sizemore RK (1979) Distribution of the luminous bacterium *Beneckea harveyi* in a semitropical environment. Appl Environ Microbiol 38: 928-933

Proctor LM (1997) Advances in the study of marine viruses. Microscopy research and techniques 37:136-161.

Reidl J, Mekalanos J (1995) Characterization Of *Vibrio cholerae* bacteriophage K139 and use of a novel mini-transposon to identify a phage- encoded virulence factor. 18 (4), 685-701.

Ruby EG, Morin JG (1979) Luminous enteric bacteria of marine fishes: a study of their distribution. Density and dispersion. Appl. Environ. Microbiol. 38: 406-411.

Reidl J, Mekalanos J (1995) Characterization of Vibrio cholerae bacteriophage K139 and use of a novel mini-transposon to identify a phage-encoded virulence factor. Molecular Microbiology. 18(4), 685-701.

Ruangpan L, Danayadol Y, Direkbusarakom S, Sriurairatana S, Flegel TW (1998) Bacteriophage implicated in mortality of cultivated Penaeus monodon exhibiting tea-brawn gill syndrome. Summitted

Sun W, Cao JG, Teng K, Meighen EA.(1994) Biosynthesis of poly-3-hydroxybutyrate in the luminescent bacterium, *Vibrio harveyi*, and regulation by the lux autoinducer, N-(3-hydroxybutanoyl) homoserine lactone. J of Biological Chemistry. 269 (32): 20785-20790

Wolk CP, Cai Y, Panoff JM (1991) Use of a transposon with luiferase as a reporter to identify environmentally responsive genes in a cyanobacterium. Proc. Natl. Acad. Sci. USA. 88: 5355-5359.

Genetic Diversity of Luminous *Vibrio* Isolated from Shrimp Larvae

*[1]Antonius Suwanto, [2]Munti Yuhana, [2]Emi Herawaty, [2]Sri Lestari Angka

[1]Department of Biology, Faculty of Science and Mathematics
[2]Department of Aquaculture, Faculty of Fisheries
Bogor Agricultural University, Bogor 16144, Indonesia.

ABSTRACT: A collection of 58 luminous vibrio isolated from shrimp farms in Java island, Indonesia were characterised and grouped by ribotyping and macrorestriction fragment-length polymorphisms (MFLP) or schizotyping employing pulsed field gel electrophoresis (PFGE). Restriction endonuclease *Not*I (5'-GCGGCCGC-3') digestion of luminous vibrio genomes yielded small numbers of DNA fragments with distributions that were readily interpreted for strain comparison. Several schizotypes (19) were distinguished following digestion of total genomic DNA with *Not*I and subsequent separation of DNA fragments using PFGE. The reproducibility of this method was 100%. Biochemical and physiological studies indicated that all of the isolates (except one which belonged to *Vibrio fischeri*) were identified as strains of *Vibrio harveyi*. Schizotype similarity was examined by cluster analysis, and four main groups with 19 different schizotypes were found. There was no correlation between schizotypes and geographical location of the shrimp farms or sample collection. Schizotyping was found to be a simple and reliable method for differentiating luminous vibrios. In addition, this method alone was able to reveal a high degree of genetic diversity within *V. harveyi* isolates from shrimp farms and larvae.

KEY WORDS: luminous, *Vibrio harveyi*, shrimp, ribotyping, schizotyping

INTRODUCTION

Tiger shrimp (*Penaeus monodon*) culture in Indonesia has become more intensive and extensive because of high demand and economic value of this export commodity. In fact, in the last five years, shrimp export has yielded the highest Indonesian income from the agricultural sector. Intensive aquaculture began to expand rapidly in the early 1980's with the acquisition of new technology. More than half of approximately 300,000 ha of shrimp ponds were located in Java island. Overall, Indonesia is currently the second largest producer of cultured shrimp, just behind Thailand (Winarno 1995). However, the exponential growth of shrimp culture is not supported by a sufficient supply of healthy fry, due to many complicated and inter-related problems in this area. Bacterial diseases have been implicated to be one of the most devastating diseases which can completely destroy hatchery productivity for extended periods (Lavilla-Pitogo et al. 1990).

Significant larval mortalities in Asian shrimp hatcheries, including Indonesia, are often associated with luminescent vibriosis which is caused by *Vibrio harveyi* or *V. splendidus* (Sunaryanto & Mariam 1986; Shariff & Subasinghe 1992). Midgut contents of broodstock shed into the water almost simultaneously with the eggs during spawning are suspected to be the main source of luminous vibrios (Shariff and Subasinghe 1992). In addition, the nearshore sea water may also be a major sources of infection (Lavilla-Pitogo et al. 1990).

Control of luminous vibrios by supplementation of antibiotics has become less effective due to the occurrence of bacterial resistance to a number of antibiotics. Tjahjadi et al. (1994) reported that most luminous vibrios isolated from shrimp hatcheries in Kalianget, East Java were resistant to a number of antibiotics tested except to rifampicin (50 mg/ml). Use of excessive antibiotics has also been implicated in shrimp growth retardation, abnormal morphogenesis and rejection of the exported shrimp due to the residuals.

Integrated control of luminous vibrios in tiger shrimp hatcheries requires more information on the their numbers, diversity, distribution, association with shrimp disease, population in nearshore sea water and larva-rearing water, and route of infection. The aims of this study were to investigate genetic diversity and relationships among luminous vibrios isolated from shrimp hatcheries and their nearby seawater environments in Java, Indonesia. DNA profiling employing rare-cutting restriction endonucleases and PFGE, designated as schizotyping (Suwanto and Kaplan 1992) as well as ribotyping was employed to reveal the genotypes of a number of luminous vibrios isolated.

MATERIALS AND METHODS

Bacterial isolates and growth media

A total of 55 luminous *Vibrio* strains were isolated from selected shrimp farms and sea water in coastal areas of

Suwanto A, Yuhana M, Herawaty E, Angka SL (1998) Genetic Diversity of Luminous *Vibrio* Isolated from Shrimp Larvae *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail address:asuwanto@indo.net.id

Table 1. Isolates of luminous *Vibrio* used in this study.

No	Code	Source material	Location	Luminescence
1	Gc1B	Shrimp gut	Besuki, E Java	Luminous
2	Gc2B	Shrimp gut	Besuki, E Java	Luminous
3	GC3B	Shrimp gut	Besuki, E Java	Luminous
4	GC4B	Shrimp gut	Besuki, E Java	Luminous
5	GC5B	Shrimp gut	Besuki, E Java	Luminous
6	GC6B	Shrimp gut	Besuki, E Java	Luminous
7	U7.2B	Shrimp HP	Besuki, E Java	Luminous
8	P1.1B	PL1	Besuki, E Java	Luminous
9	P1.2B	PL1	Besuki, E Java	Luminous
10	P1.5B	PL1	Besuki, E Java	Luminous
11	P6.1B	PL6	Besuki, E Java	Luminous
12	P6.2B	PL6	Besuki, E Java	Luminous
13	P6.3B	PL6	Besuki, E Java	Luminous
14	P6.5B	PL6	Besuki, E Java	Luminous
15	W1.1B	Sea water	Besuki, E Java	Luminous
16	W1.3B	Sea water	Besuki, E Java	Luminous
17	W1.5B	Sea water	Besuki, E Java	Luminous
18	W1.6B	Sea water	Besuki, E Java	Luminous
19	W1.7B	Sea water	Besuki, E Java	Luminous
20	W1.8B	Sea water	Besuki, E Java	Luminous
21	W1.1P	Sea water	Puger, E Java	Luminous
22	S14.3B	Sediment	Besuki, E Java	Luminous
23	S16.8B	Sediment	Besuki, E Java	Luminous
24	S9.4P	Sediment	Puger, E Java	Luminous
25	W3.2B	Sea water	Besuki, E Java	Luminous
26	W3.4B	Sea water	Besuki, E Java	Luminous
27	W3.7B	Sea water	Besuki, E Java	Luminous
28	HHT	PL	Besuki, E Java	Luminous
29	Dan 10	PL	Besuki, E Java	Luminous
30	VTM1	PL	Besuki, E Java	Luminous
31	VTM2	PL	Besuki, E Java	Luminous
32	VTMR	PL	Besuki, E Java	Luminous
33	VhBk	PL	Balitkanwar, W Java	Luminous
34	B2H	Shrimp HP	Besuki, E Java	Luminous
35	AU2	Shrimp HP	Besuki, E Java	Luminous
36	BU2	Shrimp HP	Besuki, E Java	Luminous
37	BU3	Shrimp HP	Besuki, E Java	Luminous
38	P1.3L	PL1	Labuhan, W Java	Luminous
39	P2.1L	PL2	Labuhan, W Java	Luminous
40	P2.4L	PL2	Labuhan, W Java	Luminous
41	P5.3L	PL5	Labuhan, W Java	Luminous
42	M3.2L	Mysis	Labuhan, W Java	Luminous
43	M3.3L	Mysis	Labuhan, W Java	Luminous
44	M3.4L	Mysis	Labuhan, W Java	Luminous
45	Mys2	Mysis	Besuki, E Java	Luminous
46	Mys3	Mysis	Besuki, E Java	Luminous
47	Mys4	Mysis	Besuki, E Java	Luminous
48	Mys5	Mysis	Besuki, E Java	Luminous
49	Mys6	Mysis	Besuki, E Java	Luminous
50	Mys9	Mysis	Besuki, E Java	Luminous
51	Mys10	Mysis	Besuki, E Java	Luminous
52	HB3	Sea water	Pacitan, E Java	Luminous
53	TR1	Sea water	Pacitan, E Java	Luminous
54	PT3	Sea water	Pacitan, E Java	Luminous
55	AT2.3B	Shrimp HP	Besuki, E Java	Non luminous

Java island, Indonesia (Table 1). Several shrimp farms were sampled repeatedly at 2-4 month intervals, with samples comprising pond water, coastal water, sediment, shrimp, and shrimp larvae from nearby hatcheries. Luminous vibrios were isolated employing sea water complete (SWC) agar (750 ml sea water, 250 ml distilled water, 5 g bactopeptone, 1 g yeast extract, 3 ml glicerol, and 15 g agar).

Isolation of luminous vibrios from shrimp larvae was conducted by spreading suspensions of hepatopancreas or direct inoculation of shrimp larvae onto thiosulfate citrate bile-Salt sucrose agar (TCBS-agar, Oxoid). Characterization and identification of luminous vibrios were done as described by Baumann et al. (1984). This included *V. harveyi* B392 (ATCC 33843), *V. harveyi* B356, and *V. orientalis* ATCC 33934 for strain comparison. Isolated colonies were stored in 10% glycerol at -60°C.

Ribotyping

Total genomic DNA of vibrios was isolated as described for *E. coli* (Sambrook et al. 1989) with slight modification. DNA digestion with restriction enzymes and Southern blots were performed as described previously (Sambrook et al. 1989). For rDNA probes, a DNA fragment of approximately 7.5 kb containing the 16S ribosomal RNA gene of *E. coli* was isolated from pKK3535 (Brosius et al. 1981). DNA fragments were isolated from agarose gel using a Gene Clean kit (Bio 101, La Jolla, CA) and labelled with biotin using Bionick System (GIBCO-BRL). Labeled probes were purified employing column chromatogaphy (Sephadex G50). Detection of hybridized DNA was performed using the Photogene Detection System (GIBCO-BRL) as described by manufacturer.

Preparation of intact genomic DNA and restriction digestion

Purified isolates of luminous vibrios were grown in SWC agar for 24 hours at room temperature (28-31°C). Isolated colonies (3-4) were picked and suspended in sterile PIV solution (10 mM Tris-Cll pH 7.5, 1 M NaCl) such that the final cell concentration was approximately 2 x 10^9 cell/ml.

Intact genomic DNA isolation was performed by imbedding the bacterial cells in low melting point agarose blocks as described previously (Suwanto & Kaplan 1989). Digestion of intact genomic DNA using restriction endonucleases was performed as described by Suwanto and Kaplan (1989) as follows: restriction endonuclease digestions were performed in 150 ml of appropriate restriction buffer in a 1.5 ml micro tube for each piece of gel plug or gel insert. The gel plugs were equilibrated for 15 minutes on ice, the buffer was changed for new buffer and10 units of enzyme were added. The mixture was incubated on ice for 10-15 minutes to allow the enzymes to diffuse into the agarose plugs prior to digestion at 37°C. Digestions were performed in a shaking water bath for at least 4 hrs. After digestion, the buffer was aspirated and then 150 ml of ES solution was added. The mixture was incubated at 55°C for 10-15 minutes, and then the ES solution was removed by aspiration. The gel plug was dialyzed for at least 10 minutes by adding 1.5 ml 1x TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) before placing the gel plug into the wells of the running gel.

Molecular size markers

Rhodobacter sphaeroides 2.4.1 genomic DNA digested with *Ase*I (Suwanto & Kaplan 1989) was routinely used as a PFGE molecular size marker in this study. *1 Kb-DNA Ladder* (GIBCO-BRL) or a biotinylated Lambda DNA *Bst*EII digest (NEB, Beverly, MA) were used as DNA migration references in ribotyping studies.

DNA fragment separation by PFGE

CHEF-DR II (BioRad, Richmond, CA) was used to separate DNA fragments throughout these studies. We used a

1% (w/v) agarose gel (Pharmacia) or SeaKem GTG agarose (FMC Corp.) and various pulse times depending on the range of resolution needed. For *Not*I digestion, gels were typically run in 0.5x Tris-Borate-EDTA (TBE) buffer, 175 volt (0.06 - 0.09 mA), 14°C with a ramping pulse time from 10 - 80 seconds for 20 hrs.

Statistical Analysis

A matrix was constructed as the basis for determining the presence or absence of ribotyping or schizotyping bands at a given position over the size range from 1 to 20 kb for ribotyping and from 30 to 500 kb for schizotyping. A cluster analysis was carried out using the unweighted pair group method with arithmetic means (UPGMA clustering with simple matching coefficients) of similarity coefficients for all pairs of strains and a dendrogram was generated using a computer based taxonomy program (Numerical Taxonomy System, NTSYS-PC version 1.60 (Rohlf et al. 1990).

Materials

Bionick translation kits and the Photogene Detection System were purchased from GBCO-BRL. The Gene Clean kit for DNA purification was obtained from BIO 101 Inc. (La Jolla, CA), Proteinase K from Boehringer-Mannheim or SIGMA Chemicals, St. Louis, and X-ray film from Eastman Kodak Co., Rochester, N.Y. All chemicals were of reagent grade purity and were used without further purification.

RESULTS AND DISCUSSION

Physiological and biochemical characterisation of *Vibrio* isolates

A total of 55 isolates of luminous *Vibrio* were isolated from shrimp farms in Java, mainly from the Besuki area in East Java. There were 39 isolates from shrimp larvae with symptoms of luminous disease. Three isolates were from sea sand near shrimp farms, and 13 isolates from sea water in Besuki (East Java), Puger (East Java), Pacitan (East Java), and Labuhan(West Java). Designations for the bacterial isolates, source materials, and names of coastal sampling sites in Java are presented in Table 1.

With the exception of AT2.3B, that was isolated from a shrimp with symptom of tail erosion ("udang ekor gripis" in Indonesian), all isolates were luminous on either SWC or TCBS agar. All of the isolates, except *V. harveyi* B392 and B356, failed to ferment sucrose in the biochemical tests or were unable to produce acid from sucrose and so produced green colonies on TCBS agar. Therefore, in terms of sucrose fermentation, all of Java isolates have characteristics of *V. harveyi*. Moreover, all the luminous vibrios had similar morphological and physiological characteristics (i.e., Gram-negative, short rods, fermented glucose, oxidase and catalase positive, motile, produced H_2S and indole, did not grow in peptone at 4°C or 55°C, gave green colonies on TCBS at 28-37 °C, did not ferment lactose and sucrose, utilized L-serine and acetate as carbon sources, did not produce arginine dihydrolase, liquefied gelatin, produced amylase, and utilized L-tyrosine, glycine, and citrate as sole sources of carbon) (Table 2). According to these biochemical or physiological characteristics, all of the luminous *Vibrio* isolates, except W1.1P, were identified as *Vibrio harveyi* isolates. W1.1P was found to be an isolate of *Vibrio* (*Photobacterium*) *fischeri* (Table 2).

Schizotyping analysis

Restriction endonucleases *Apa*I (5'-GGGCCC-3'), *Eag*I (5'-CGGCCG-3'), *Not*I (5'-GCGGCCGC-3'), *Ase*I (5'-ATTAAT-3'), *Spe*I (5'-ACTAGT), and *Sma*I (5'-CCCGGG-3') were used in the preliminary experiment to screen for the most suitable restriction enzyme for PFGE analysis of genomic DNA. As shown in Fig. 1., *Not*I digested Gc1B genomic DNA into the fewest fragments ranging in size from approximately 33 kb to more than 410 kb.

Since *Vibrio* sp. genomic DNA contains low mol (G+C) (Logan 1994), restriction endonuclease *Ase*I which recognizes high A+T sequences might be expected to digest *Vibrio* genomes very frequently. Meanwhile, our studies indicated that *Sma*I and *Apa*I could be classified only as medium rare-cutter enzymes. Therefore *Not*I was chosen as the restriction endonuclease to be used in subsequent PFGE analysis of all *Vibrio* isolates (Fig. 1).

*Not*I macrorestriction fragment length polymorphisms (MFLP) or schizotyping (Suwanto and Kaplan 1992) of a number of isolates clearly demonstrated different genomic DNA profiles (Table 3), either in the number of DNA fragments generated or in the distribution of the fragments. *Not*I digested total genomic DNA into 6 - 19 resolvable fragments ranging in size from 18 - 1050 kilo base pairs (kb) (Fig. 2.). From analysis of a total 53 isolates, 19 different genomic DNA profiles were obtained by *Not*I schizotyping. These DNA profiles were unique and represented DNA fingerprints for a particular isolate or group of isolates (Fig. 3).

Table 3. Genomic DNA profiles of 58 *Vibrio* isolates by *Not*I schizotype.

No	Schizotype profile	Isolates with similar schizotype
1	*V. harveyi* B392	B392, B356
2	*V. orientalis*	*V. orientalis*, S14B, M3.2L, M3.3L, M3.4L, P1.3L, P2.1L, P2.4L, P5.3L
3	HHT	HHT
4	P6B / P1B	P1.1B, P1.2B, P1.5B, P1.6B, P6.1B, P6.2B, P6.3B, P6.5B
5	Mys	Mys2, Mys3, Mys4, Mys5, Mys6, Mys9, Mys10
6	VTM	VTM1, VTM2, VTMR
7	VhBk	VhBk
8	GcB	Gc1B, Gc2B, Gc3b, Gc4B, Gc5B, Gc7B, U7.2B
9	Dan10	Dan10
10	B2H	B2H, AU2, BU2, BU3
11	W1B	W1.1B, W1.2B, W1.5B, W1.6B, W1.7B, W1.8B
12	W3B	W3.2B, W3.4B, W3.7B
13	S16B	S16.8B
14	W1.1P	W1.1P
15	S9.4P	S9.4P
16	HB3	HB3
17	TR1	TR1
18	PT3	PT3
19	AT2.3B	AT2.3B

Table 2. Biochemical and physiological characteristics of the *Vibrio* isolates.

Characteristics	GcB	B2H	P1B	HHT	VTM	VhBk	MYS	AT23B	Dan10	W1B	W3B	S14B
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-
Glucose fermentation	F	F	F	F	F	F	F	F	F	F	F	F
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Indole formation	+	+	+	+	+	+	+	+	+	+	+	+
H2S formation	+	+	+	+	+	+	+	+	+	+	+	+
Luminescence	+	+	+	+	+	+	+	-	+	+	+	+
Gelatin liquifaction	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	-	-
Amylase production	+	+	+	+	+	+	+	+	+	+	+	+
Colony color on TCBS	G	G	G	G	G	G	G	G	G	G	G	G
Fermentation to acid												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Growth at												
4oC	-	-	-	-	-	-	-	-	-	-	-	-
28oC	+	+	+	+	+	+	+	+	+	+	+	+
37oC	+	+	+	+	+	+	+	+	+	+	+	+
55oC	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl at												
0%	-	-	-	-	-	-	-	-	-	-	-	-
0.50%	+	+	+	+	+	+	+	+	+	+	+	+
1%	+	+	+	+	+	+	+	+	+	+	+	+
3%	+	+	+	+	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+	+	+
7%	+	+	+	+	+	+	+	+	+	+	+	+
9%	+	+	+	+	+	+	+	+	+	+	+	+
10%	-	-	-	-	-	-	-	-	-	-	-	-
11%	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of												
Citrate	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Glycine	+	+	+	+	+	+	+	+	+	+	+	+
L-Arginine	+	+	+	+	+	+	+	+	+	+	+	+
L-Tyrosine	+	+	+	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+
Identification	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh	Vh

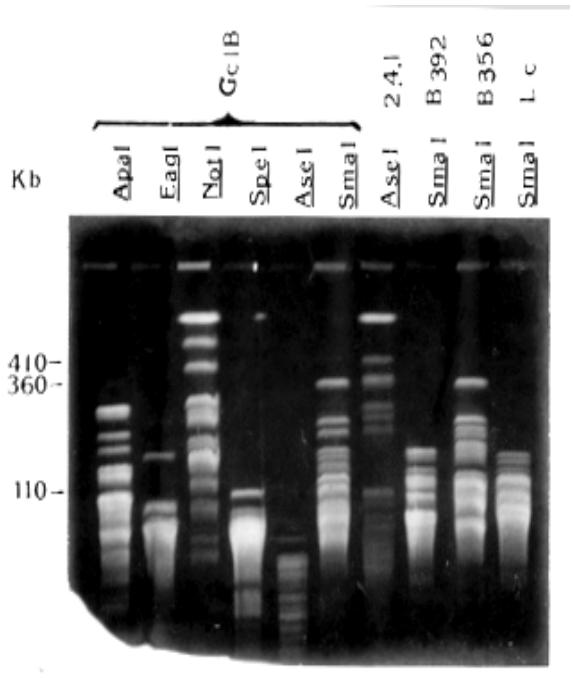


Figure 1. Pulsed-field gel electrophoresis of *Vibrio* isolates Gc1B, digested with *Apa*I, *Eag*I, *Not*I, *Spe*I and *Sma*I. 2.4.1 = R. sphaeroides 2.4.1 total genomic DNA digested with *Ase*I used as a molecular size marker. Running conditions: pulse time ramping 5-48 sec, running time 17 hr, 175 V (0.06-0.09 mA), running buffer temperature 14±1°C.

Table (cont'd). Biochemical and physiological characteristics of the *Vibrio* isolates.

Characteristics	S16B	PL	W11P	S94P	HB3	PT3	B356	B392	Vo	Vs1	Vs11	Vp
Gram stain	-	-	-	-	-	-	-	-	-	-	-	-
Glucose fermentation	F	F	F	F	F	F	F	F	F	F	F	F
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Indole formation	+	+	+	+	+	+	+	+	+	+	+	+
H2S formation	+	+	-	+	+	+	-	+	+	-	+	+
Luminescence	+	+	+	+	+	+	+	+	+	-	-	-
Gelatin liquifaction	+	+	-	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	+	-	-
Amylase production	+	+	-	+	+	+	+	+	+	+	+	+
Colony color on TCBS	G	G	G	G	G	G	Y	Y	G	Y	G	G
Fermentation to acid												
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Growth at												
4oC	-	-	-	-	-	-	-	-	-	-	-	-
28oC	+	+	+	+	+	+	+	+	+	+	+	+
37oC	+	+	+	+	+	+	+	+	+	+	-	-
55oC	-	-	-	-	-	-	-	-	-	-	-	-
Growth in NaCl at												
0%	-	-	-	-	-	-	-	-	-	-	-	-
0.50%	+	+	+	+	+	+	+	+	+	+	-	-
1%	+	+	+	+	+	+	+	+	+	+	-	+
3%	+	+	+	+	+	+	+	+	+	+	+	+
5%	+	+	+	+	+	+	+	+	+	+	+	+
7%	+	+	+	+	+	+	+	+	+	+	+	-
9%	+	+	+	+	+	+	+	+	+	+	+	-
10%	-	-	-	-	-	-	-	-	-	-	-	-
11%	-	-	-	-	-	-	-	-	-	-	-	-
Utilization of												
Citrate	+	+	-	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-
Glycine	+	+	-	+	+	+	+	+	+	+	+	+
L-Arginine	+	+	-	+	+	+	+	+	+	+	+	+
L-Tyrosine	+	+	-	+	+	+	+	+	+	+	+	+
L-Serine	+	+	+	+	+	+	+	+	+	+	+	+
Acetate	+	+	+	+	+	+	+	+	+	+	+	+
Identification	Vh	Vh	Vf	Vh	Vh	Vh	Vh	Vh	Vh	Vs1	Vs11	Vp

Remarks: + = positive; - = negative; G = green; Y = yellow; Vh = *V. harveyi*; Vf = *V. fischeri*; VsI = *V. splendidus* I; VsII = *V. splendidus* II; Vp = *V. penaecida*.

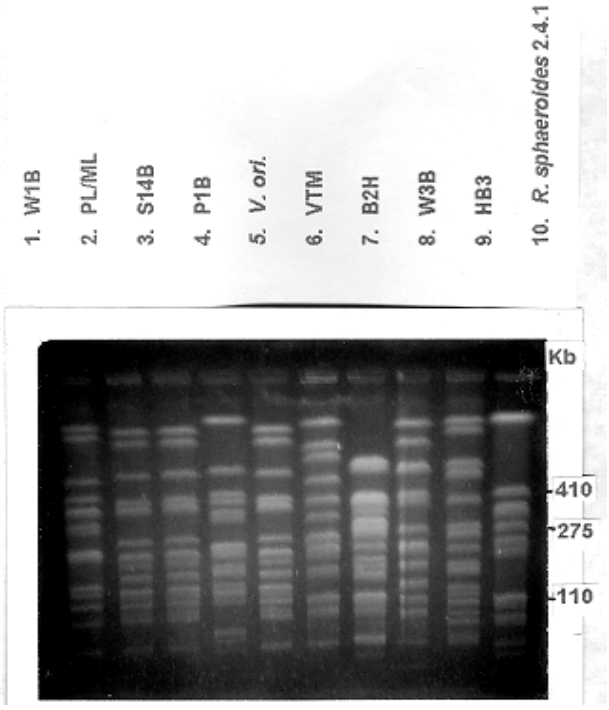


Figure 2. Pulsed-field gel electrophoresis of *Not*I digested genomic DNA from a number of *Vibrio* isolates. *Rhodobacter sphaeroides* 2.4.1 DNA digested with *Ase*I was used as the molecular size marker. Pulse time ramping 10-80 sec, running time 20 hr, 175 V (0.06-0.08 mA), running buffer temperature 14±1°C.

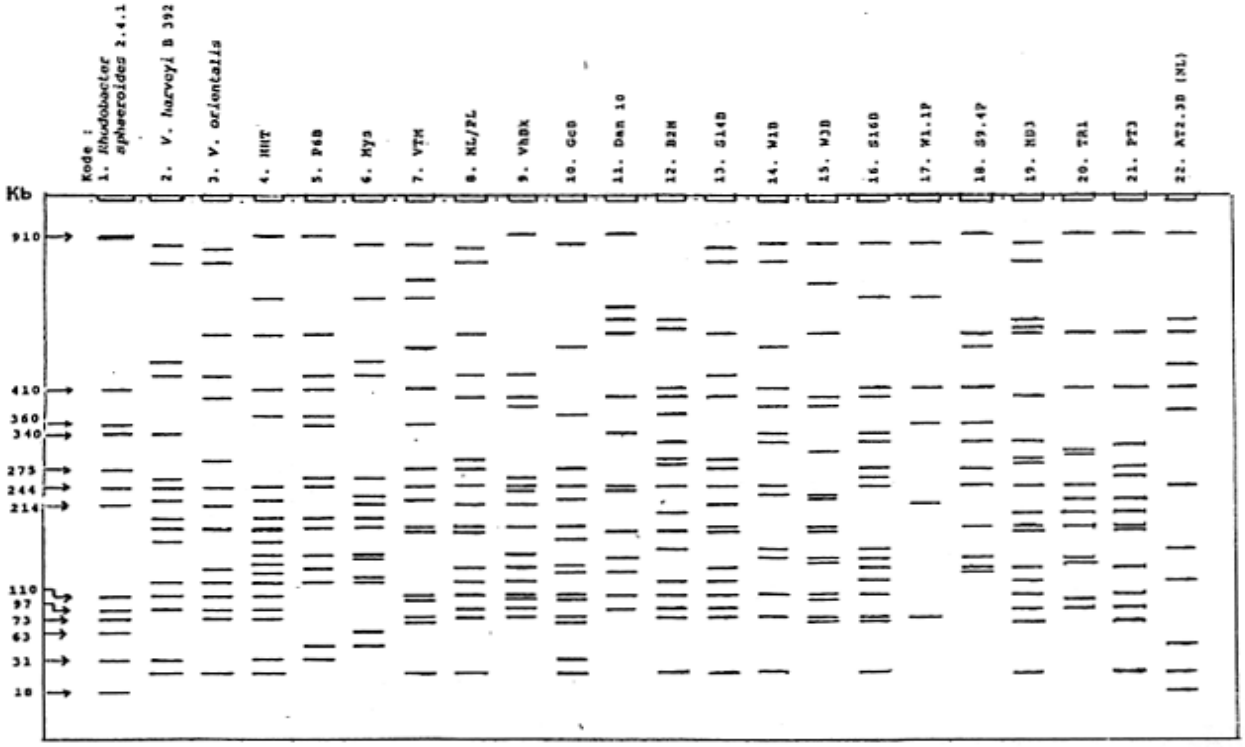


Figure 3. Idealized gel band plot of *Not*I digested genomic DNA of the *Vibrio* isolates together with estimated sizes.

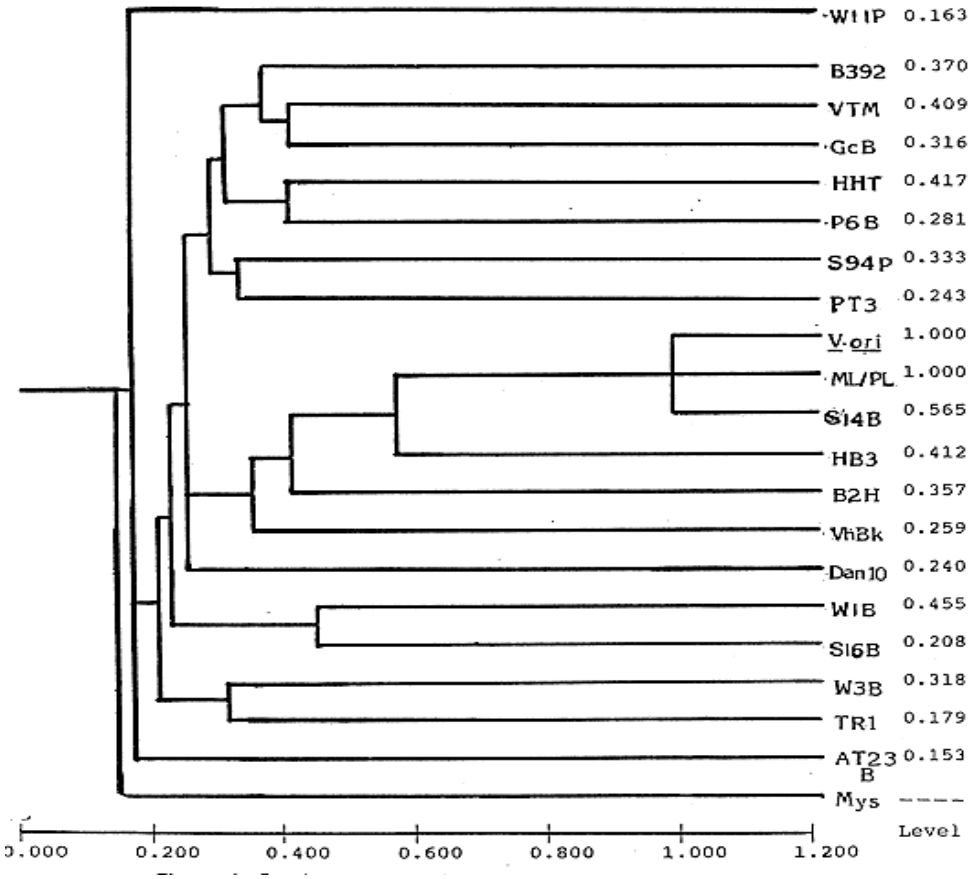


Figure 4. Dendrogram showing the relationships amongst *Vibrio* isolates based on PAGE analysis of genomic restriction fragments generated by *Not*I. similarities were calculated using Dice's coefficient, and clustering was achieved by UPGMA. A total of 55 isolates from shrimp farms and seashore areas on Java Island were studied together with 3 reference isolates from ATCC.

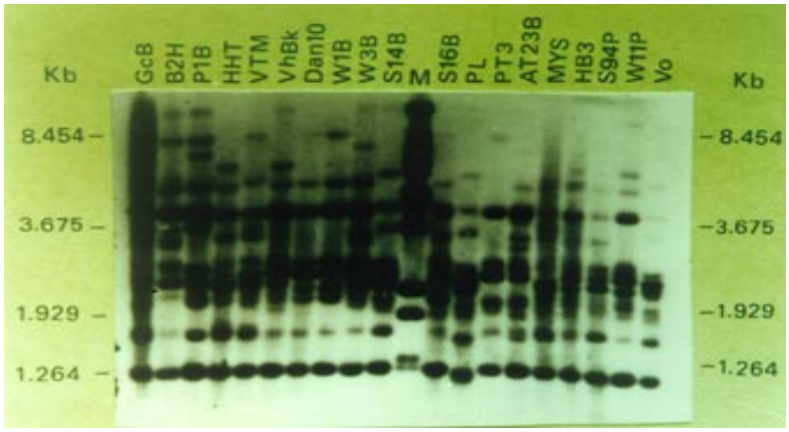


Figure 5. A representative gel of ribotypes for the *Vibrio* isolates.

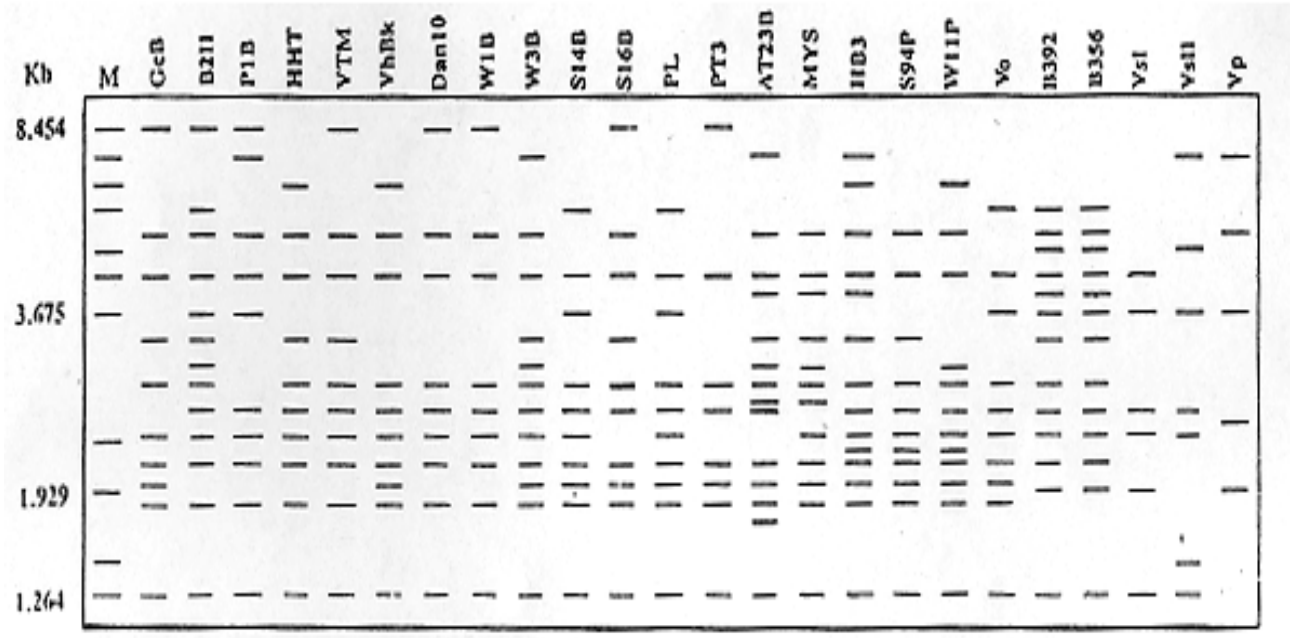


Figure 6. Interpretation diagram for all of the ribotypes of the *Vibrio* isolates studied.

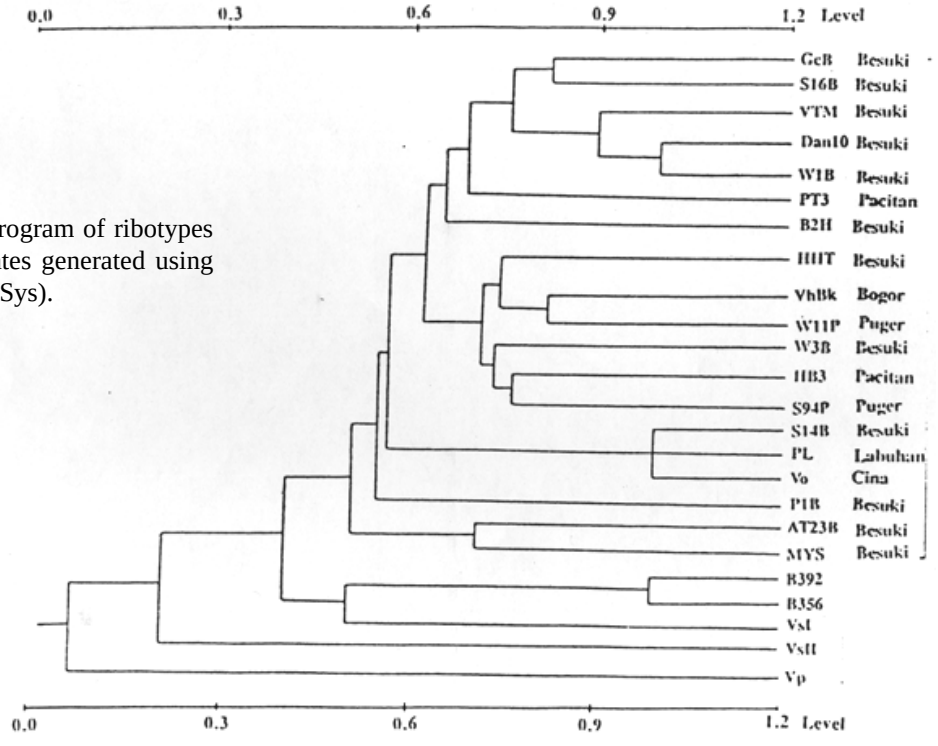


Figure 7. A dendrogram of ribotypes of *Vibrio* isolates generated using UPGMA (NT-Sys).

Determination of similarity coefficients and clustering by UPGMA of the 55 *Vibrio Not*I schizotypes and the reference strains suggested that there was no correlation between cluster membership and geographical distribution (Fig. 4.). For example, isolates S14B, ML/PL, and *V. orientalis* showed identical *Not*I schizotypes, although they were isolated from East Java (Besuki), West Java (Labuhan), and China (Yellow Sea), respectively. These results suggested that isolates of marine vibrios might have high mobility and be widely distributed as a consequence of their aquatic habitat or migration through the use of broodstock from other locations. This realization has consequences for shrimp farmers, because recognition of clonal lineages that conform to regional geographic boundaries is necessary for the formulation of disease prevention programs. Clearly, the fluctuation and widespread genetic diversity reported here may help explain the difficulty in obtaining broodstock resistant to luminous bacterial disease. In addition, the genetic diversity of luminous vibrios may cause difficulty in generating shrimp vaccines directed to a particular strain of luminous *Vibrio*. Therefore, the development of biocontrol or bioconditioners in shrimp farms should be an effective alternative approach to the prevention of disease, especially if it is combined with other practices such as low input sustainable agriculture.

Ribotyping

Fourteen isolates of *Vibrio*, including the non-luminous *V. penaeicida* (Ishimaru et al., 1995) were analysed using ribotyping. A representative result is depicted in Fig. 5., while an interpretation diagram from all of ribotypes is presented in Fig. 6. The dendrogram generated using UPGMA (NT-Sys) is shown in Fig. 7. The results indicated that strains of luminous vibrios (i.e., *Vibrio harveyi*) isolated from the sea and shrimp farms in Java were genetically heterogenous, as was demonstrated using *Not*I schizotyping. However, the degree of similarity in ribotyping analysis is relatively higher than the corresponding similarity using schizotyping. Interestingly, a number of isolates that were shown to be identical according to *Not*I schizotyping were also identical by ribotyping analysis (i.e., Dan10, W1B, S14B and PL/ML; *V. orientalis*, B392 and B356; VhBk and W1.1P). All the *V. harveyi* isolates showed distance relationship to *V. penaeicida*, a pathogen of Kuruma prawn isolated in Japan (Ishimaru et al., 1995).

In this study, we have been able to show that although ribotyping is not as highly discriminative as *Not*I schizotyping, it has sufficient discriminatory power to differentiate many genotypes of *V. harveyi* from Java island. This might be due to the fact that all of the members of the Enterobacteriaceae described to date carry seven rRNA operons. In contrast, bacteria such as *Xanthomonas campestris* pv. *glycines* with a similar chromosome size has only three rRNA operons. Strains of *X. campestris* pv. *glycines* could not be distinguished significantly using ribotyping. In other words, schizotyping is much more powerful than ribotyping in differentiating *X. campestris pv. glycines* isolates (Wahidin 1996, Rukayadi 1995).

The results of ribotyping analysis unambiguously demonstrated the enormous diversity of *Vibrio harveyi* isolates which might be important indicator for the occurance of shrimp and shrimp fry diseases in South East Asia. The ribotyping results support the conclusion obtained from *Not*I schizotyping, and altogether suggest that the development of microorganisms which can suppress the growth of *V. harveyi* might be a promising alternative for preventing luminous disease in shrimp

Acknowledgements. We thank PT Tirta Mutirva Makaur, Besuki, for all of the facilities provided during sample collection for the isolation of luminous vibrios. This work was supported by the International Foundation for Science (IFS), Sweden, Grant No. A/2207-1.

LITERATURE CITED

Brosius J, Ullrich A, Raker MA, Gray A, Dull TJ, Guttell RR, Noller HF (1981) Construction and fine mapping of recombinant plasmid containing the rRNA operon of *E. coli*. Plasmid 6:112-118.

Baumann P, Furniss AL, Lee JV (1984) Facultatively anaerobic gram negative rods. *In* Krieg, NR (Ed) Bergey's Manual of Systematics Bacteriology. Williams and Wilkins. Baltimore, MA. Vol.I:1513-1523.

Ishimaru K, Matsushita M, Muroga K (1995) *Vibrio penaeicida* sp. nov., a pathogen of Kuruma prawns (*Penaeus japonicus*). Internat. J Systematic Bacteriol 45:134-138

Lavilla-Pitogo CR, Baticados MCL, Cruz-Lacierda ER, de la Pena LD (1990) Occurrence of luminous bacterial disease of Penaeus monodon larvae in the Phillipines. Aquaculture 91:1-13

Logan NA (1994) Bacterial Systematics. Blackwell Scientific Publication, London

Rohlf CL, Yeo CC, and L Tay 1990 NT-Sys-pc, Numerical Taxonomy and Multivariate Analysis System, Version 1.60. Exeter Software, New York

Rukayadi Y (1995) Genomic DNA profile analysis of a number of *Xanthomonas campestris* pv. *glycines* employing pulsed field gel electrophoresis. M.Sc. Thesis, Bogor Agricultural University, Bogor, Indonesia

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A Laboratory Manual. 2nd edition. Cold Spring Harbor Laboratory Press, New York

Sunaryanto A, Mariam A (1986) Occurrence of a pathogenic bacteria causing luminescence in penaeid larvae in Indonesian hatcheries. Bull. Brackish Water Aqua Devl Center 8:64-70

Shariff M, Subasinghe RP (1992) Major diseases of culture shrimp in Asia: An overview. *In* Fulks, W. and K.L. Main. Diseases of cultured Penaeid shrimp in Asia and the United States. The Oceanic Institute, Honolulu, Hawaii

Suwanto A Kaplan S (1989) Physical and genetic mapping of *Rhodobacter sphaeroides* 2.4.1 genome: Genome size, fragment identification, and gene localization. J Bacteriol 171:5840-5849

Suwanto A Kaplan S (1992) Chromosome transfer in *Rhodobacter sphaeroides*: Hfr formation and genetic evidence for two unique circular chromosomes. J Bacteriol 174:1135-1145

Tjahjadi MR, Angka SL, Suwanto A (1994) Isolation and evaluation of marine bacteria for biocontrol of luminous bacterial disease in tiger shrimp larvae (*Penaeus monodon*, Fab). AsPac J Mol Biol Biotechnol 2:347-352

Wahidin (1996) Schizotypes and ribotypes comparison of a number of *Xanthomonas campestris* pv. *glycines*. Sarjana Thesis. Bogor Agricultural University, Bogor, Indonesia

Winarno B (1995) Shrimp aquaculture in Indonesia. *In* CL Browdy and JS Hopkins (Eds) Swimming through troubled water. Proceedings on shrimp farming, Aquaculture '95. World Aquaculture Society, Baton Rouge, Lousiana, USA

Vibrionaceae Associated with the Larvae and Larval Rearing System of *Macrobrachium rosenbergii* : Systematics and Pathogenicity

Sarita G. Bhat, *I.S. Bright Singh

Environmental Microbiology Laboratory, School of Environmental Studies
Cochin University of Science and Technology, Fine Arts Avenue, Cochin 682 016, India.

ABSTRACT: Systematics of the family Vibrionaceae associated with the larvae of *Macrobrachium rosenbergii* in the hatchery has been worked out based on the principles of numerical taxonomy and the mole per cent G+C content of DNA. Altogether 313 isolates of Gram negative, motile/nonmotile, fermentative, Kovac's oxidase positive rods comprising 204 isolates from moribund larvae and 109 from apparently healthy larvae were subjected to the study along with 20 type strains. They were further divided as sensitive or resistant to 2,4 -diamino,6,7 di-isopropyl pteridine (0/129 compound) at 150 µg ml^{-1}. The operational taxonomic units were tested for 233 unit characters and the data thus generated were analysed using simple matching coefficient (Ssm) and unweighted average linkage by applying TAXAN. On completing the analysis, it was surprising to find that none of the type strains integrated with any of the isolated strains. Represntative strains from each phenon were segregated and the percentage G+C ratio was determined based on *Tm* values. Irrespective of the sensitivity of the phena to 0/129, the majority had G+C ratios falling within the range of those for *Vibrio* species while a few fell within the range of those for *Aeromonas* and *Photobacterium*. Suggested affiliations based on G+C ratios contrasted with gross phenotypic dissimilarities. Several strains had G+C ratios out of the range of Vibrionaceae and the newly constructed family Aeromonadaceae, even though they exhibited certain phenotypic traits of these families. It is postulated that the isolates studied here were either phenotypic variants or new species of the families mentioned above. This has to be further substantiated by DNA homology comparisons with existing type species. In any case, this study proved that sensitivity to 0/129 could not be used as a core character differentiating *Vibrio* and *Photobacterium* from *Aeromonas*. The study broadens the base of the familyVibrionaceae and Aeromonadaceae and opens up avenues for developing new taxonomic schemes for tropical isolates. Pathogenicity of 47 representative strains was determined based on the extent of larval mortality upon challenge with revitalized cultures as compared to mortality in positive and negative controls. With all the the test organisms, there was significantly higher larval mortality, indicating that the families Vibrionaceae and Aeromonadaceae as a whole are pathogenic to the larvae of *Macrobrachium rosenbergii.*

KEY WORDS: Vibrionaceae, Aeromonadaceae *M. rosenbergii*, Hatchery, Virulence, Taxan

Bhat SG, Singh ISB (1998) Vibrionaceae associated with the larvae and larval rearing system of *Macrobrachium rosenbergii* : systematics and pathogenicity. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Detection of *Vibrio parahaemolyticus* in Shrimp Haemolymph by DNA Hybridization and PCR Amplification

[1]Prasert Rojlorsakul, [2]Vichai Boonsaeng, [1]Watanalai Panbangred
[3]Orasa Suthienkul, [4]Tirasak Pasharawipas, *[1]T.W. Flegel

[1]Department of Biotechnology and [2]Department of Biochemistry, Faculty of Science
Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
[3]Department of Microbiology, Faculty of Public Health, Mahidol University
Ratchawithi Road Bangkok 10400, Thailand
[4]Department of Microbiology, Facutly of Science, Rangsit University
Paholyothin Road Pathumthani 12000, Thailand

ABSTRACT: A DNA probe and a PCR method were developed for detection of penaeid shrimp pathogenic *Vibrio parahaemolyticus*. From a genomic library, a probe named pVPA7 was selected and subcloned. It contained a 1.5 Kb species-specific DNA fragment which was selected by hybridization techniques for use as a DNA probe. This probe gave positive hybridization for all of the *V. parahaemolyticus* strains tested (including 141 strains of *V. parahaemolyticus* from clinical and environmental sources) but gave negative hybridization with a wide range of other *Vibrio* species and with bacterial species representative of other genera. The probe sensitivity limit for *V. parahaemolyticus* was 10^5 CFU/ml by dot blot hybridization. After sequencing the 1.5 Kb fragment, primers VPAFOR3 and VPAREV1 were designed and showed good specificity for *V. parahaemolyticus*, yielding a single 285 bp fragment in PCR amplification tests. The specificity was 100% for the bacterial strains tested and sensitivity as 100 fg DNA and $4x10^3$ CFU/ml (i.e., crude lysate equivalent to 20 CFU/ reaction). Southern blot hybridization of amplified products improved detection to as low as 0.1 fg DNA. PCR amplification was completely inhibited by $2x10^{10}$ CFU/ml (i.e., 10^8 CFU/reaction). The lowest concentration of cells that could be detected in shrimp haemolymph was $4.0x10^3$ CFU/ml (i.e., 20 CFU/reaction). However, *V. parahaemolyticus* cells at 10^8 did not inhibit amplification in the haemolymph samples. Nor was there any interference from addition of *E. coli* at equal concentrations up to $2x10^9$ CFU/ml (i.e., 10^7 CFU/reaction). Furthermore, *E. coli* additions up to $2x10^9$ CFU/ml (i.e., 10^7 CFU/reaction) did not affect detection of *V. parahaemolyticus* at $4x10^3$ CFU/ml (i.e., 20 CFU/reaction). Dot blot hybridization of amplified products improved detection to as low as 20 CFU/ml of *V. parahaemolyticus*. No hybridization occurred with PCR amplification reaction mixtures using other bacteria, native haemolymph or diluted anticoagulant/haemolymph as templates. The lowest concentration of *V. parahaemolyticus* cells that could be detetected in haemolymph by PCR combined with chromatographic capture detection using a Gene Comb kit was $2x10^3$ CFU/ml (i.e., 10 CFU/ reaction). Other *Vibrio* species gave negative results.

KEY WORDS: *Vibrio parahaemolyticus*, DNA probe, PCR, shrimp, haemolymph, detection

INTRODUCTION

Vibriosis is a serious problem in the majority of penaeid shrimp culture operations. *Vibrio* species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1988). However, some more recently occurring disease syndromes of penaeid shrimp have been caused by *Vibrio* species which behave more like true pathogens than opportunistic invaders (Lightner *et al.*, 1992). In Thailand, vibriosis is the main cause of production loss due to bacterial disease in penaeid shrimp farms (Nash *et al.*, 1992). The major *Vibrio* species isolated from diseased shrimp are *V. parahaemolyticus, V. harveyi, V. alginolyticus* and *V. vulnificus*, while *V. damsela, V. anguillarum* and *V. fluvialis* have been reported less frequently (Leangphibul *et al.*, 1985; Lightner, 1988; Ruangpan and Kitao, 1991, Nash *et al.*, 1992; Jiravanichpaisal *et al.*, 1994). Vibriosis causes mortality in larvae, postlarvae, juveniles, subadults and also adults. At times, outbreaks cause mortality up to nearly 100% of affected populations (Lighner 1983). The gross signs of localized infection in the cuticle or subcuticle are called shell disease or black or brown spot disease and these superficial infections can develop into systemic infections under some circumstances. It is the systemic infections that cause mortality.

The usual method for diagnosis of *Vibrio* infections in farmed shrimp is based on identification of single colony isolates by traditional nutritional and biochemical tests (Baumann and Schubert, 1984, Anderson et al., 1988). However, this process can take several days to complete and it may be too slow for practical management decisions by

Rojlorsakul P, Boonsaeng V, Panbangred W, Suthienkul, Pasharawipas T, Flegel TW (1998) Detection of *Vibrio parahaemolyticus* in shrimp haemolymph by DNA hybridization and PCR amplification. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: sctwf@mahidol.ac.th

shrimp farmers. By contrast, DNA diagnostic techniques can be used to speed up diagnosis to only a few hours, without loss in reliability of identification (Dalsgaard *et al.,* 1996). The diagnosis of Vibriosis is usually performed by presumptive diagnosis based on gross signs, followed by histological and confirmatory diagnosis (Lightner, 1996). DNA probes for the identification of clinical strains of *V. parahaemolyticus* have been in use for over a decade. This is because *V. parahaemolyticus* can cause acute gastroenteritis in humans, often after the consumption of contaminated seafood (Spite *et al.,* 1978). These probes are based on virulence factor genes called *tdh* and *trh* which are usually found in strains isolated from clinical specimens. By contrast, these genes are rarely found in strains isolated from the environment (Miyamoto *et al.,* 1969). Recently, a fragment called pR72H was identified and developed as a DNA probe (Lee *et al.,* 1995a). However, colony hybridization gave very faint signals (brownish) with non-*V. parahaemolyticus* strains. Thus, densitometer measurements were needed to read color density and a cut-off value had to be set to distinguish *V. parahaemolyticus* from other *Vibrio* species (Lee *et al.,* 1995a). The DNA fragments used in these hybridization assays have also been used to develop PCR assays (Tada *etal.,* 1992a, Lee *etal.,* 1995b). The PCR primers VP33 and VP32 based on pR72H and described by Lee *et al.* (1995b) have been applied to PCR detection of *V. parahaemolyticus* in fish and shellfish. They have been shown to be helpful in detecting biochemically atypical strains of *V. parahaemolyticus* (Karunasagar *et al.,* 1997). PCR based on the *tdh* and *trh* gene probe sequences has also been described and used in non-isotopic microtitre plate-based assays that can easily be automated (Tada *et al.,* 1992b). However, as with the hybridization probe, these assays would not work with environmental strains lacking *tdh* or *trh.*

The aim of this study was to develop methods for the easy and rapid detection of *V. parahaemolyticus* in shrimp haemolymph by DNA hybridization and PCR techniques using species specific DNA sequences that would give positive assay results with both clinical and environmental isolates of *V. parahaemolyticus*.

MATERIALS AND METHODS

Bacterial strains and culture media

The bacterial strains used in this study are shown in Table 1. Generally, they were grown on LB medium at 37°C. Marine bacterial substrains were enriched on MHA agar or marine broth 2216 medium at 30°C and then cultured in TSB (tryptic soy broth, Difco) supplemented with 1-3% NaCl at 30°C. *V. parahaemolyticus* strains were selectively grown on TCBS agar at 42°C and then cultured on TSB supplemented with 3% NaCl at 30°C.

Preparation of DNA

Chromosomal DNA was extracted following the procedure of Miura (1962), and plasmid DNA was prepared by the rapid alkaline lysis method (Sambrook *et al.,* 1989).

Recombinant plasmid digestion with resriction enzymes

Recombinant plasmids that had been screened by colony hybridization followed by confirmatory Southern blot hybridization were extracted and digested with restriction enzymes according to the manufacturer's instructions. The sizes of inserted fragments could be determined by comparison with an l *Hin*dIII DNA size marker.

Agarose gel electrophoresis

Agarose was prepared between 0.7-1.5% in 1xTBE (tris borate buffer) containing 89 mM Tris-HCl pH 8.3, 89 mM Boric acid and 2.5 mM EDTA. It was melted and then cooled to 55-60°C before being poured into a casting tray with a well comb. After hardening, the gel was placed in an electrophoresis chamber and 1xTBE solution was added until the gel was submerged. DNA samples were mixed with BJII loading dye solution (40% sucrose, 0.25% bromophenol blue) in a ratio of 1:3 and loaded into the wells. Electrophoresis was carried out at a constant voltage (100 volts) for 30 min. Gels were stained with 1 mg/ml ethidium bromide solution for 5 min and destained with distilled water. The DNA patterns in the gel were visualized using a UV transluminator (Spectrolin, Spectronic Corporation, USA) and photographed with a Polaroid camera (FCR-10, Fotodyne, USA).

DNA probe from a *V. parahaemolyticus* recombinant clone

Shotgun cloning was used to find a species-specific DNA fragment from *V. parahaemolyticus* for use as a DNA probe. *V. parahaemolyticus* BG26 was partially digested with *Sau*IIIAI. The digest fragments were ligated into the *Bam*HI site of pGEM7Zf+ vector and the ligated products were transformed into *E.coli* DH5a. Approximately 400 white colonies resulted and these were screened with digoxigenin-labeled genomic DNA of *V. parahaemolyticus* BG26 and closely related *P. shigelloides* DNA (negative control). Clone pVPA22 showed high intensity signals when hybridized with genomic DNA of *V. parahaemolyticus* BG26 DNA but not with DNA of *P. shigelloides* (Fig. 14). Specificity of this clone was confirmed by digoxigenin-labeled Southern blot hybridization with genomic DNA of *V. parahaemolyticus* BG26 (Fig. 15A lane 3, 4, 5) but not with DNA of other bacteria. It was then subcloned to yield a 1.5 Kb *Eco*RI/*Sac*I digest fragment with the same specificity. This was named pVPA7 (Fig. 16 lane 9).

DNA Blotting

For dot blots, a positively charged nylon membrane (S&S) was soaked in double distilled water for 30 min followed by 10x SSC solution for 30 min. Target DNA solutions (500 ng) were denatured by boiling for 10 min and transfered to ice for 5 min before being loaded onto the membrane using a dot blot apparatus (Biodot, Biorad, USA). The membrane was then placed in two changes of denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 min each followed by neutralizing solution (0.5 M Tris-HCl pH 7.0, 1.5 M NaCl) and 2xSSC. After air drying, the target DNA was immobilized using UV crosslinking. For dot blot hybridization of bacterial strains without DNA extraction, 100 ml culture broth at

10^9 CFU/ml was loaded on a prewetted membrane using a dot blot apparatus. To lyse the cells, the membrane was placed twice for 5 min on a filter paper soaked with 10% SDS. The membranes were then denatured and neutralized and DNA was immobilized using UV crosslinking as already described.

For Southern blots, agarose electrophoresis gels were first photographed and DNA patterns were then transferred to nylon membranes following the protocol of Sambrook *et al.*, 1989 using a sandwich blotting device (Hybaid, UK).

Digoxigenin labeling, hybridization and detection using the DNA probe

All of these procedures were performed according to the methods described in the Boehringer Mannheim kit manual. The probe pVPA7 was labeled with digoxigenin-11-dUTP using a random prime labeling method followed by hybridization and detection of the Dig-labeled probe by colorimetric means.

To test specificity, *V. parahaemolyticus* strains, others *Vibrio* species and other bacteria were used in dot blot and Southern blot hybridizations with pVPA7 probe as described above.

To test sensitivity, *V. parahaemolyticus* BG26 was cultured at 10^9 CFU/ml in TSB supplemented with 3% NaCl and then serially diluted in steps of 1:10 using 0.85% NaCl. The resulting diluted suspensions were used in dot blot hybridization tests to determine the sensitivity of the DNA probe. Samples were also spread on TSA and incubated overnight at 30°C to verify viable counts. Moreover, *V. parahaemolyticus* BG26 was also tested in mixtures with *E.coli* cells using haemolymph as the diluent to test sensitivity in the presence of other bacteria.

PCR amplification

After sequencing of pVPA7 (BSU, Bioservice Unit, National Science and Technology Development Agency, Thailand) and CEL (Central equipment laboratory, Institute of Science and Technology for Development, Mahidol University), primers VPAFOR3 (5'-GTT AGC CAC AGA TGC GAC AT-3') and VPAREV1 (CTT GTG GAT TGG ATT CTC GC-3') were designed using DNASIS 7 and Oligo 4.1 programs (Fig. 1). Standard PCR amplification was then performed for 40 cycles in a final volume of 50 ml per reaction. The mixture contained 1xPCR buffer II, 200 mM of dNIPs, 200 nM of each primer, 100 ng of DNA template and 1.25 units of AmpliTaq DNA polymerase. For PCR amplification (Gene Amp PCR system 2400, Perkin Elmer, USA), the temperature conditions were denaturing at 94°C for 1 min, annealing at 65°C for 1 min and primer extension at 72°C for 1 min followed by incubation at 72°C for 10 min at the last cycle. PCR products were analyzed by agarose gel electrophoresis as described above.

PCR specificity and sensitivity

Purified DNA from *V. parahaemolyticus* strains, other V*ibrio* species and other bacteria (100 ng) were tested using standard PCR conditions. To test sensitivity, *V. parahaemolyticus* BG26 DNA was quantified and then 10 fold serially diluted from100 pg, to 0.01 fg. For sensitivity and detection tests using cell samples, culture broth was diluted in 0.85% NaCl to make serial dilutions verified by viable counts as described above. Dilutions were kept at -20°C until after viable counts were determined. Then cell suspensions were adjusted to $2x10^{10}$ CFU/ml and serially diluted 1:10 down to 20 CFU/ml using double distilled water. The diluted suspensions were then lysed by boiling for 15 min and 5 ml was added to the PCR reagent mixture for PCR amplification. The amplified PCR products were then tested by Southern blot hybridization using a VPAFOR3, REV1 (285 bp) labeled probe.

Detection of *V. parahaemolyticus* in haemolymph

Detection of *V. parahaemolyticus* BG26 was also tested in mixtures with *E. coli* cells serially diluted and lysed by boiling. These lysed cell suspensions (5 ml) were mixed with 5 ml lysed *V. parahaemolyticus* cells at the same dilution and also with low *V. parahaemolyticus* but high *E. coli* concentrations before PCR amplification. First, haemolymph was drawn from black tiger shrimp (*Penaeus monodon)* into shrimp salt solution (SSS) anticoagulant contained in a syringe at the ratio of 1:1. This diluted haemolymph sample was then used as the serial diluent with *V. parahaemolyitcus* and *E. coli* cell suspensions. The mixtures were boiled for 15 min and then centrifuged at 5000 rpm for 5 min to pack cell debris and denatured protein. A supernatant sample (5 ml) was then subjected to PCR amplification. PCR products were detected by ethidium bromide staining of 1.5% agarose electrophoresis gels.

Detection *V. parahaemolyticus* PCR product using Gene Combâ

A Gene Combâ kit (Biorad, USA) was used to test for sensitivity and specificity to the 285 bp PCR amplicon (10 ml from each PCR reaction tube) along with incorporated negative and positive controls. Biotin labels were incorporated into amplicons by inclusion of biotinilated VPAREV1 primer (5'-biotin-CTT GTG GAT TGG ATT CTC GC-3') (GIBCOBRL, USA) in the PCR reaction mix. To test senstivity, *V. parahaemolyticus* cells were adjusted to $2x10^7$ CFU/ml and serially diluted 1:10 down to 20 CFU/ml using double distilled water. To test specificity, template DNA from *V. parahaemolyticus* and other *Vibrio* species were tested. Prior to the assay, a capture probe homologous to a portion of the 285 bp amplicon but excluding the labeled primer region was commercially synthesized and spotted onto the teeth of the comb. It was called VPA1 (5'-CTA ATG CAC GAC GAG TCC CTT CAA-3'). Subsequent assay steps for DNA hybridization and visualization followed the Universal Gene Comb test kit protocol. Negative controls included the standard kit negative control and PCR reaction product without template while positive controls consisted of the kit positive control and labeled pVPA7.

RESULTS

Specificity and sensitivity of probe pVPA7

In Southern blot hybridization tests with extracted DNA, labeled pVPA7 hybridized only with *Eco*RI/*Bam*HI digested *V. parahaemolyticus* strain AQ4613 at fragment length 6.8

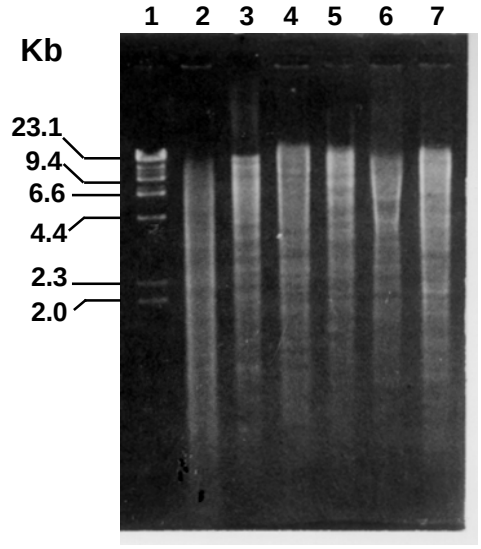


Figure 1. Agarose gel electrophoresis of digested chromosomal DNA from various sources with combined *Eco*RI/*Bam*HI restriction enzymes. Lane 1, λ/*Hin*d III DNA markers; lane 2, *A. sobria* DNA; lane 3, *P. shigelloides* DNA; lane 4, *V. alginolyticus* DNA; lane 5,*V. cholerae* DNA; lane 6, *V. parahaemolyticus* AQ4613 DNA; lane 7, *V. parahaemolyticus* BG26.

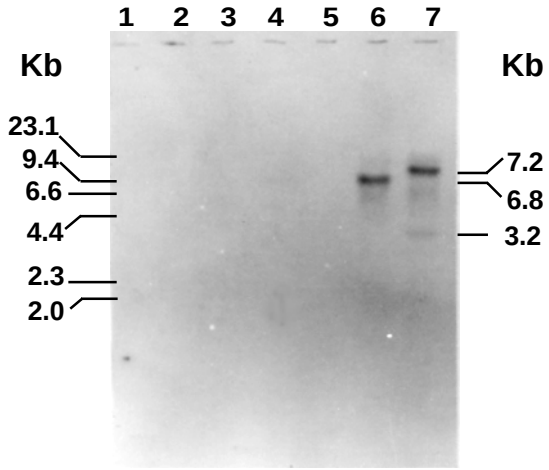


Figure 2. Southern blot hybridization of various digested chromosomal DNA preparations from the gel in Fig. 1 (above) using the digoxigenin labeled 1.5 Kb fragment derived from pVPA7. All lanes are as described in Fig. 1.

Kb (Fig. 1 and 2) and strain BG26 at two fragment lengths of 7.2 and 3.2 Kb (Figs. 1 and 2). It did not hybridize with closely related species such as *V. alginolyticus* and *P. shigelloides* or with other *Vibrio* species or other bacterial species tested in Southern blot or dot blot hybridization tests. The lowest number of cells that could be detected was 10^5 cells per dot. All the *V. parahaemolyticus* sea food (environmental) isolates tested (126 strains) gave positive hybridization signals with pVPA7 probe in such tests. The highest sensitivity for detection of *V. parahaemolyticus* cells in shrimp haemolymph was also 10^5 cells per dot with or without the presence of equal numbers of cells of *E. coli* (Fig. 3).

PCR specificity and sensitivity

Using VPAFOR3 and VPAREV1 primers (Fig. 4), a PCR product of 285 bp occurred with all *V. parahaemolyticus*

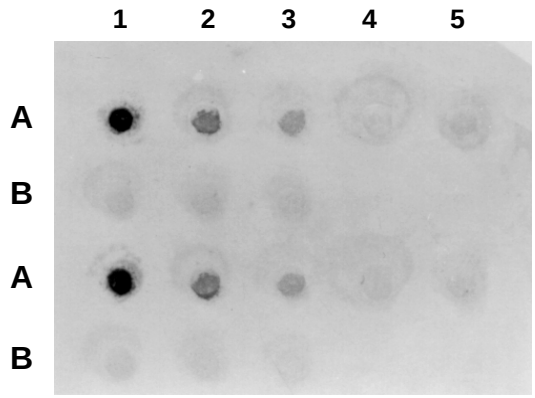


Figure 3. Sensitivity test of pVPA7 probe using dot blot hybridization with serial dilutions of *V. parahaemolyticus* BG26 cells and equal numbers of *E. coli* cells (negative control). A1 to 5 = $7.5x10^7$, 10^6, 10^5, 10^4, 10^3 cells *V. parahaemolyticus*. B1 to 3 = $7.5x10^2$, 10^1, *E. coli* DH5α (negative control).

Figure 4. Primers and annealing positions in the pVPA7 sequence. The primer sequences are labeled and underlined.

```
                                                    50
GAATTCGCCATCTCCGACTCAAAACAATGGCTAGATGTNACCAACCCGGC
                                                   100
AACCAACGAAGTGATTGCGCAAGTACCTTGTGCCACGGAATCGGAAATGA
                                                   150
ATGCGGCGATCGAACTTCTAGATGACCTAATTAACCAAAACGTGAAAGTG
                                                   200
CAAGGAACGCCTCCTGAAGANCAAAGCTTGCTTGGCACAATTTTCATTTC
                                                   250
TTGGTTCCCAATGATCGTGACAGCCTTTGCACTTTGCACAGGAAACTGGT
                                                   300
GGTCATATTTCGATTGATAACGTATCGGATAATCCTCTTGGATTGGAAGA
                                                   350
ATGGCAGCAAGCTTTGGTTCACGTACGTGAAATGGGCTGGTCAGAAGGTC
                                                   400
AAGAAGGTTACACGACAGGTTGTGGTCAGAATGACTGGCAAAACAAACGT
                                                   450
TGGCCATGTGAACCGGGACAAGGTTACTTTGGACGTGGTGCTAAACAGCT
                                                   500
TTCTTACCACTTTAACTATGGCGCGTTCTCTGAAGTGATGTTTGATGGCG
                                                   550
ANGCATCTGTATTGCTGAAAAATCCAAGTTTGGTTGCGGATTCCTGGCTG
                                                   600
AACTTGGCATCCGCTATCTGGTTCTTCCTAACCCCACAGCACCTAAACCA
                                                   650
GCAATGCTACATGTTATTGAACGTACTTGGGTTCCTTCNCAACNTGAACT
                                                   700
TGATGCAGCATTGGTTACGCTTCCGTACCACTATCAACGTGATCGACAAA
                                                   750
CAATTTTGATGGATGTTGAGGTTAATTCCCTGACTTTGCGAAAAGTGTTC
                                                   800
CGGATCGATGAGCCTGAAACCAGACCCGATAGACCAGATGCGACAACGGC
                                                   850
GGCTTTAGCTGGGCCACCTTTCATGTGACCAAGTAGAGAAAATGCGACTT
                                                   900
TAATGAAGTAAGCACCCGCACCCGCACGCTCTANCATCGCGCCAAACAGT
                                                   950
ACAAACAAGAATACGAAAGAAGTGGATACACCTAACGCAACACCAAATAC
                                                  1000
CCCTTCGGTGGTTAGCCACAGATGCGACATCGCTTTACCTAAGCTTGCGC
                    VPAFOR3→                      1050
CTTTGTGGGCTATGACATCTGGCATGTATGGGCCAGCAAACGTGTTAGTC
                                                  1100
NGGAACACGGCGGCCACAACCATTAGCGGAGGCCCTAATGCACGACGAGT
                                                  1150
CCCTTCAAGTAGCAATATCATACCAACGACCGCAACCACAATATCCGCAG
                                                  1200
TGGTGGGCGCGCCGGAACTCCTGCGAGTTCGGTGTAGAAAAGGTAGATAT
                                                  1250
AGGCAGCCGAAAAGCTACCCGCTAACGCGAGAATCCAATCCACAAGAGGA
                              ←VPAREV1      1300
ATTGATCTTAACGACTGGCACAACGATACTGGCAACAAAAATAATAATGG
                                                  1350
CGATACCATACATTCCGCTTCTGACCAAAGACGCAACACCAGAGAAAATC
                                                  1400
GTATCTTCAAGGCGTTGACCGTTGGTTATCAAAATCGAAATAGGAATAAG
                                                  1450
GTTAGCAGGAAAAATCGCGATAGTAGCCGCGACTAGGTAAGCCCACGTTT

TTTGAATCGAGTTTGGCTTACGATGATAGAGCTCC
```

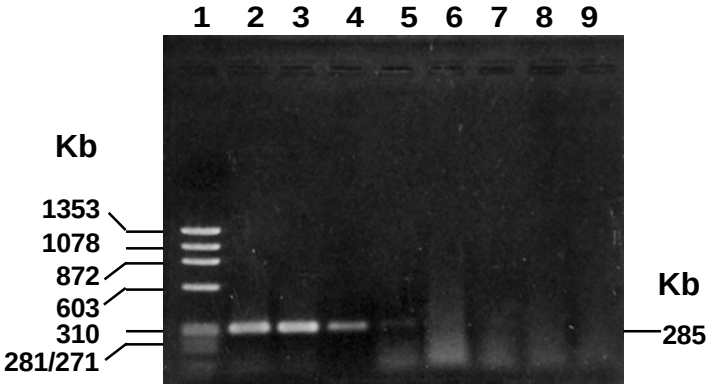


Figure 5. Test of PCR sensitivity. *V. parahaemolyticus* BG26 DNA serially diluted and subjected to PCR amplification. Lane 1, ∅X174/*Hae*III DNA markers; lane 2 to 9,*V. parahaemolyticus* BG26 DNA at concentrations of 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg, and 0.01fg.

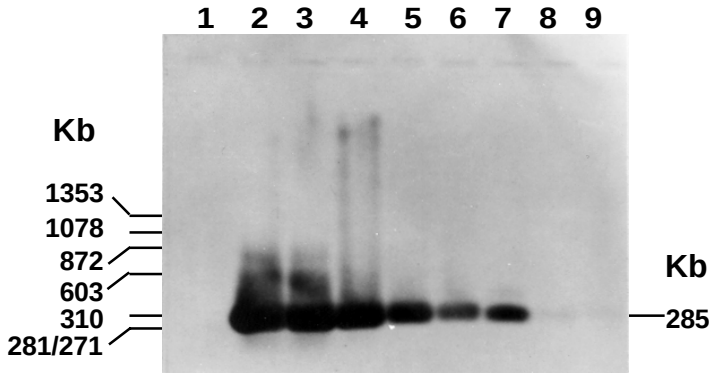


Figure 6. Southern blot hybridization of PCR amplified products from gel in Fig. 5. Lane 1, ∅X174/*Hae*III DNA markers; lanes 2 to 9, *V. parahaemolyticus* DNA at concentrations of 100 Pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg and 0.01 fg.

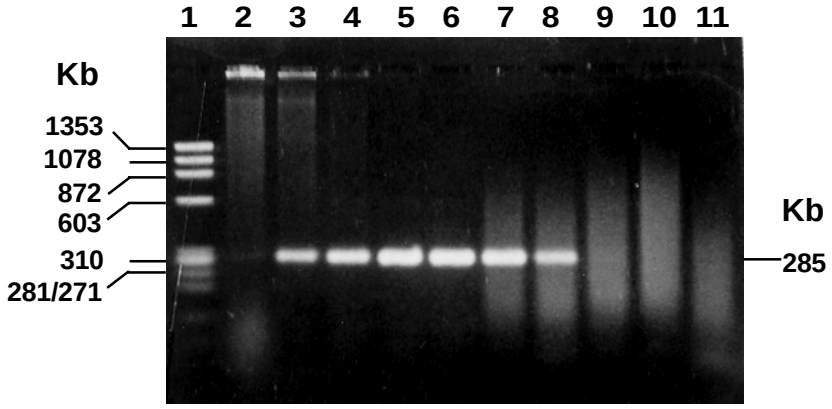


Figure 7. Test of PCR sensitivity using *V. parahaemolyticus* BG26 cultured cells as the template. Lane 1, ∅X174/*Hae*III DNA markers; lane 2 to 11, cell concentrations at $2x10^{10}$, 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3,10^2, 10^1 CFU/ml.

strains tested (91 strains) and no bands were generated from other bacterial DNA templates tested. Amplicon bands were generated at a primer concentration as low as 50 nM, but a concentration of 200 nM was chosen for routine PCR analysis. The lowest template DNA concentration that gave an amplified visible product in agarose gel electrophoresis was 100 fg (Fig. 5). However, by Southern blot hybridization a faint hybridization signal could be seen at as low as 0.1 fg DNA. (Fig. 6). With boiled cell suspensions, a faint PCR product band could be visualized at the equivalent of $4x10^3$ CFU/ml (i.e., 20 CFU/reaction). Amplification was greatly inhibited when the number of cells was $2x10^{10}$ CFU/ml (i.e., 10^8 CFU/reaction) (Fig. 7) and it was partly inhibited at $2x10^9$ CFU/ml (i.e., 10^7 CFU/reaction). Suspensions of all 75 strains of *V. parahaemolyticus* isolated from seafood (i.e., environmental isolates) gave positive amplicons in these tests.

Detection *V. parahaemolyticus* BG26 in shrimp haemolymph

As with the broth culture suspensions, bacterial cell suspensions in shrimp haemolymph gave a faint band at $4x10^3$ CFU/ml (i.e., 20 CFU/reaction) (Fig. 8). By contrast to the broth result, however, there was no inhibition of the PCR reaction at cell concentrations up to $2x10^{10}$ CFU/ml haemolymph (i.e., 10^8 CFU/reaction) (Fig. 9). Nor was there any interference from addition of *E. coli* at equal concentrations up to $2x10^9$ CFU/ml (i.e., 10^7 CFU/reaction) (data not shown). Furthermore, *E. coli* additions up to $2x10^9$ CFU/ml (10^7 CFU/reaction) did not affect detection of *V. parahaemolyticus* at $2x10^4$ CFU/ml (i.e., 100 CFU/reaction) or $4x10^3$ CFU/ml (i.e., 20 CFU/reaction) as bands were similar to those seen for the same concentrations in Fig. 8 (i.e., lanes 2 and 10, respectively).

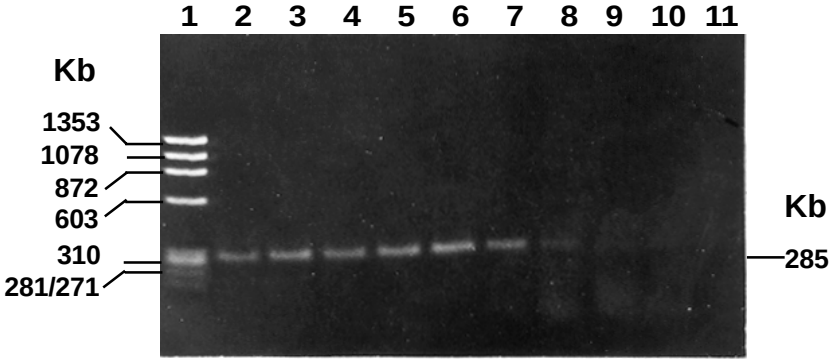


Figure 8. Testing PCR sensitivity of *V. parahaemolyticus* BG26 cells in shrimp haemolymph. Culture broth of *V. parahaemolyticus* BG26 was serially diluted with haemolymph. Lane 1, ∅X174/*Hae*III DNA markers; lanes 2 to 11, cell concentrations of $2.0x10^4$, $1.8x10^4$, $1.6x10^4$, $1.4x10^4$, $1.2x10^4$, $1.0x10^4$, $0.8x10^4$, $0.6x10^4$, $0.4x10^4$, $0.2x10^4$ CFU/ml.

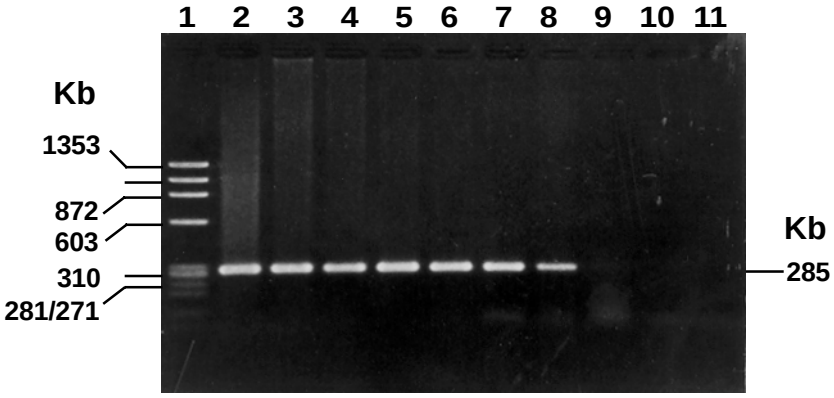


Figure 9. Test of PCR sensitivity for *V. parahaemolyticus* cells in shrimp haemolymph. Culture broth of *V. parahaemolyticus* BG26 was serially diluted with haemolymph. Lane 1, ∅X174/*Hae*III DNA markers; lanes 2 to 11, concentrations of cells of $2x10^{10}$, 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 CFU/ml.

The PCR products from these haemolymph experiments were confirmed by dot blots to hybridiaze with pVPA7 probe and sensitivity was the same as with the broth tests, in that a faint positive hybridization signal could be observed at a concentration of 20 CFU/ml (similar to Fig. 3). No hybridization signals were obtained with PCR amplification reaction mixtures using other bacteria, native haemolymph or diluted anticoagulant/haemolymph as templates.

Detection *V. parahaemolyticus* amplicons using the Gene CombÒ kit

The lowest concentration of *V. parahaemolyticus* cells in haemolymph that could be detected by PCR and the Gene CombÒ kit was $2x10^3$ cfu/ml or 10 cells per reaction (Fig. 10). Other *Vibrio* species gave negative results.

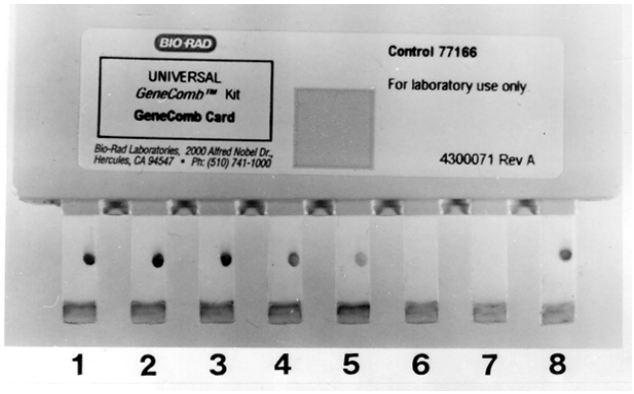


Figure 10. Results from use of a Gene Comb kit for detection of amplcons from templates of various concentrations of *V. parahaemolyticus* in haemolymph. Teeth 1 to 7, cell concentrations at $2x10^7$, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 per ml; tooth 8 = control, with negative and positive control spots.

DISCUSSION

Testing for the specificity and sensitivity of pVPA7 probe

Probe pVPA7 gave good sensitivity and specificity for detection of *V. parahaemolyticus* strains by dot blot and Southern blot hybridization. At 100% specificity for 141 tested strains (clinical and environmental), it may be more useful than probe pR72H (Lee *et al.*, 1995a) which gave 98% specificity for *V. parahaemolyticus*. Interestingly, double hybridization bands occurred only in Southern blots with digested chromosomal DNA of *V. parahaemolyticus* BG26. This showed that there may be sequence variation in the pVPA7 target region for some strains. It is possible that this variation with pVPA7 could be developed for use in subspecies characterization for *V. parahaemolyticus*.

By dot blot, the sensitivity of pVPA7 probe was 10^5 cells of *V. parahaemolyticus*. Lee *et al.* (1995a) reported that pR72H required a minimum of 10 ng purified *V. parahaemolyticus* DNA for detection. Based on rough calculations where 1 bacterial genome was estimated as having a mass of 2-5 fg (Hunt and Persing, 1993), 10 ng of DNA would correspond to approximately $4x10^6$ cells. Thus, probe pVPA7 might be considerd to be approximately 10 times more sensitive than pR72H in dot blot assays, although comparisons are difficult since Lee *et al.* (1995a) used purified DNA and we used lysed cells.

Probe pVPA7 showed no significant homology with any DNA sequence in international databases (Genbank and EMBL databases) or with any amino acid sequence from the SWISS PROTEIN database.

PCR specificity and sensitivity

Using VPAFOR3 and VPAREV1 primers, PCR specificity was 100% for the strains tested and the lowest quantity of bacterial DNA that could be detected was 100 fg by agarose gel electrophoresis. This constituted higher sensitivity than that reported for PCR detection using *tdh* and *trh* gene sequences (400 fg DNA corresponding to approximately 100 cells) (Tada *et al.*, 1992a). However, sensitivity was less than that reported for PCR detection by Lee *et al.* (1995b) at 2.6 fg DNA (equivalent to 1 cell), although we could increase detection sensitivity to 0.1 fg DNA by using Southern blot hybridization. This was more sensitive than detection of *tdh* and *trh* amplicons by Southern blot hybridization (lowest 40 fg DNA) (Tada *et al.*, 1992a) and by non-isotopic microtitre plate-based assay (lowest 28 fg DNA) (Tada *et al.*, 1992b).

For cells in broth culture, a PCR product could be visualized at as low as $4x10^3$ CFU/ml which was equivalent to 20 CFU crude bacterial lysate per PCR reaction tube. This was less sensitive than the 10 cells reported by Lee *et al.* (1995b). However, their method involved preparation of crude DNA extracts from bacterial lysates, while the experiments described herein employed the total lysis solution treated by proteinase K digestion followed by boiling. This was felt to be more appropriate for rapid and convenient processing of samples by industry. However, it is possible that sensitivity could be increased if DNA was extracted before PCR assays were performed.

PCR amplification was completely inhibited when *V. parahaemolyticus* reached concentrations of 10^8 cells per PCR reaction (i.e., $2x10^{10}$ CFU/ml of culture), and product was reduced at 10^7 cells per reaction (i.e., 10^9 CFU/ml of culture). This corresponded with the report of Lee *et al.* (1995b) who also found complete PCR inhibition at 10^8 cells. It also agrees with the generally accepted fact that PCR can be inhibited and product formation stopped by the presence of high numbers of target or non-target cells (Olive, 1989; Brooks *et al.*, 1992a). Accumulation of cellular debris is also known to inhibit PCR amplification (Saiki *et al.*, 1990). By contrast, *V. parahaemolyticus* cells at 10^8 in haemolymph did not inhibit PCR amplification as they did in broth experiments. It is possible that inhibiting substances in the crude bacterial lysates were trapped in the mass of coagulated protein that formed when the shrimp haemolymph lysates were boiled and centrifuged.

The lowest concentration of *V. parahaemolyticus* that could be detected in shrimp haemolymph was $4x10^3$ CFU/ml (i.e., 20 CFU/reaction). This was the same detection limit as in culture broth and it was not affected by the presence of even large numbers of added *E. coli*. These results also corresponded to those of Lee *et al.* (1995b) and Giesendorf *et al.* (1992) who found that high levels of contaminating bacteria did not affect the specificity of the PCR culture assays for detection of *V. parahaemolyticus* and *Campylobacter* spp., respectively.

Dot blot hybridization of PCR amplified products gave a weak hybridization signal at as low as 20 CFU/ml and no hybridization was found with PCR reaction mixtures from other bacteria or from samples containing hemolymph only. Thus, Southern blot and dot blot hybridization detection was many times more sensitive than that based on visualization of bands in agarose gels. However, these techniques are somewhat labor intensive and time consuming, especially for large numbers of samples. To speed up detection assays using simplified equipment, we tested detection of amplicons using a Gene Combâ kit. This proved equally reliable (100% specificity) to agarose gel detection, but sensitivity was increased by about two times (as low as $2x10^3$ CFU/ml or 10 CFU/reaction). This corresponded with a report on non-radioisotopic PCR-based methods for detection of human immunodeficiency virus type 1 at 10 preamplification gene copies (Whetsell *et al.*, 1992). The technique would have advantages over electrophoresis of increased sensitivity of detection and more rapid and convenient processing for many samples.

To prove its utility, the pVPA7 DNA probe and PCR amplification based on it will need to be tested in field trials. It is hoped that application will increase the accuracy and speed up the process for detection of *V. parahaemolyticus* infections in shrimp ponds by non-destructive testing of haemolymph. The information obtained may be useful in the development of programs for the prevention and treatment of vibriosis. The reagents may also find application for the detection of *V. parahaemolyticus* in contaminated, frozen seafood.

Acknowledgements. The authors would like to thank the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand, for partial support to carry out this work.

LITERATURE CITED

Anderson IG, Shamsudin MN, Shariff M, Nash G (1988) Bacterial septicemia in juvenile tiger shrimp, *Penaeus monodon*, cultured in Malaysian brackishwater ponds. Asian Fish Sci 2:93-108

Baumann P, Schubert RHW (1984) Family II *Vibrionaceae*. In: Krieg NR, Holt JG (eds) Bergey's Manual of Systemic Bacteriology, vol 1. Williams&Wilkins, Baltimore, p 516-538

Brooks JL, Moore AS, Patchett RA, Collins MD, Kroll RG (1992a) Use of the polymerase chain reaction and oligonucleotide probes for the rapid detection and identification of *Carnobacterium* species from meat. J Appl Bacteriol 72:294-301

Dalsgaard A, Dalgaard I, Hoi L, Larsen JL (1996) Comparison of a commercial biochemical kit and an oligonucleotide probe for identification of environmental isolates of *Vibrio vulnificus*. Lett App Microbiol 22:184-188

Giesendorf 1992 BAJ, Quint WGV, Henkens MHC, Stegeman H, Huf FA, Niesters HGM (1992) Rapid and sensitive detection of *Campylobacter* spp. in chicken products by using the polymerase chain reaction. Appl Environ Microbiol 58:3804-3808

Hunt JM, Persing DH (1993) Bacterial detection. In: Keller GH, Manak MM (eds) DNA Probes. Stockton Press, New York, p 525-564

Jiravanichpaisal P, Miyazaki T, Limsuwan C (1994) Histopathology, biochemistry and pathogenicity of Vibrio harveyi infecting black tiger prawn Penaeus monodon. J Aquat Anim Health 6:27-35

Karunasagar I, Nayak BB, Karunasagar I (1997) Rapid detection of *Vibrio parahaemolyticus* from fish by polymerase chain reaction (PCR). In : Flegel TW, MacRae IH (eds) Diseases in

Asian Aquaculture III. Asian Fish Soc, Bangkok, p 119-122

Leangphibul P, Nilakul C, Sornchai C, et al (1985) Investigation of pathogenic bacteria from shrimp farms. J kasetsart Sci 7:171-199

Lee CY, Chen CH, Chou YW (1995a) Characterization of a clon pR72H probe for *Vibrio parahaemolyticus* detection and development of a nonisotopic colony hybridization assay. Microbiol Immun 39:177-183

Lee CY, Pan SF, Chen CH (1995b) Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with the PCR. App Environ Microbiol 61:1311-1317

Lightner DV (1983) Disease of culture penaeid shrimp. In: McVey J (ed) CRC Handbook of Mariculture, vol.1, Crustacean Aquaculture. CRC Press, Boca Raton, FL, p 289-320

Lightner DV (1988) Vibrio disease of penaeid shrimp. In: Sindermann CJ, Lightner DV (eds) Disease Diagnosis and Control in North American Marine Aquaculture. 2nd. ed. Elsevier, Amsterdam, p 42-47

Lightner DV, Bell TA, Redman RM, Mohney LL (1992) A review of some major diseases of economic significance in penaeid prawns/shrimps of the Americas and Indopacific. In : : Shariff M, Subasinghe RP, Arthur JR (eds) Disease in Asian Aquaculture I. Asian Fish Soc, Manila, p 57-80

Lightner DV (1996) A Handbook of Shrimp Pathology and Diagnostic Procedures for Disease of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, LA, p 4-1-4-27

Miyamoto Y, Kato T, Obara Y, Akiyama S, Takizawa K, Yamai S (1969) *In vitro* hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation with human pathogenicity. J bacteriol 100:1147-1149

Nash G, Nithimathachobe C, Tungmandi C, et al (1992) Vibriosis and its control in pond-reared *Penaeus monodon* in Thailand. In: Shariff M, Subasinghe RP, Arthur JR (eds) Disease in Asian Aquaculture I. Asian Fish Soc, Manila, p 143-155

Olive DM (1989) Detection of enterotoxigenic Escherichia coli after polymerase chain reaction amplification with a thermostable DNA polymerase. J Clin Microbiol 27:261-265

Ruangpan L, Kitao T (1991) *Vibrio* bacteria isolated from black tiger shrimp, *Penaeus monodon* Fabricius. J Fish Dis 14:383-388

Saiki RK (1990) Amplification of genomic DNA. In: Innis MA, Gelfand DH, Sninsky JJ, White (eds) PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA, p 13-20

Spite GT, Brown DF, Twedt RM (1978) Isolation of an enteropathogenic Kanagawa-positive strains of *Vibrio parahaemolyticus* from seafood implicated in acute gastroenteritis. Appl Environ Microbiol 35:1226-1227

Tada J, Ohashi T, Nishimura N, Shirasaki Y, Ozaki H, Fukushima S, Takano J, Nishibuchi M, Takeda Y (1992a) Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. Mol Cell Probes 6:477-487

Tada J, Ohashi T, Nishimura N, Ozaki H, Fukushima S, Takeno J, Nishibuchi M, Takeda Y (1992b) Non-isotopic microtitre plate-based assay for detecting products of polymerase chain reaction amplification: apllication to detection of the *tdh* gene of *Vibrio parahaemolyticus.* Mol Cell Probes 6:489-494

Whetsell et al (1992) Comparison of three nonradioisotopic polymerase chain reaction based methods for detection of human immunodeficiency virus type1 J Clin Microbiol 30:845-853

Successful use of Bacterial Bioremediation to Control *Vibrio* Populations in Shrimp Ponds

[1]Jason J. David, [2]C.T. Chu, [1]S. Santhana Krishnan

[1]MarineTechnologies, 43-13 M.G. Road, Sastri Nagar, Chennai-600 041, India
[2]Team Aqua Corporation, HF-4, No.82, SCC.3, Pel-11sin Road, Hsintien, Taipei 11sicn, Taiwan, ROC

ABSTRACT: Bioremediation in shrimp aquaculture has not been well understood by farmers. Despite its vagaries and its responses which largely rest on individual pond conditions, there have been some reports highlighting its techno-economic advantages. In our study of 29 ponds, we found that *Vibrio* populations (yellow, green and luminescent colonies) modulated depending on the use of the bacterial bioremediation product WUNAPUO-15. In WUNAPUO pre-application ponds yellow *Vibrio* colony numbers went up by 3 orders within 8 days of culture in both the water and sediment. Water intake seemed to be the initial *Vibrio* introduction route. After the WUNAPUO probiotic formulation was added to the pond there was a one fold reduction in bacterial numbers in the water and sediment. Around 65 days of culture (DOC) onwards, green *Vibrio* colonies started to show up in the culture ponds. Controls indicated that their numbers would have been an order higher and would have posed a threat of further multiplication had WUNAPUO not been used. Although the green *Vibrio* colonies were not eliminated, there was an apparent suppression in their numbers until the culture was completed at DOC 130 since they did not increase in proportion to the accumulated feed / organic matter in the pond. Neither the yellow nor the green *Vibrio* colonies increased to levels that were considered even slightly alarming throughout the culture cycle. Moreover the onset of luminescent *Vibrio* after 75 DOC was controlled effectively by suspending WUNAPUO in bag nets in the water column.

KEY WORDS: Shrimp culture, probiotics, Wunapuo-15, *Vibrio*

David JJ, Chu CT, Santhana Krishnan S (1998) Successful use of bacterial bioremediation to control *Vibrio* populations in shrimp ponds. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Ultrastructure of a Mollicute Associated, Gut-node Disease of Penaeid Shrimp (*Penaeus chinensis*)

Yang Jifang

Second Institute of Oceanography, SOA, PO Box 1207, Hangzhou, Peoples Republic of China

ABSTRACT: Infection by a mollicute occurred in midgut epithelial cells of cultured penaeid shrimp *(Penaeus chinensis*). Grossly, the midgut of moribund shrimp was partly swollen with 1-3 red, knob like to sausage shaped areas. The knob diameters were approximately 2mm and consisted of many granules. Examination by transmission electron microscopy (TEM) revealed heavy infections of a filamentous mollicute-like bacterium in cytoplasm and perinuclear spaces of epithelial cells. Bacterial shape varied from spherical (0.12 to 1.2mm in diameter) to slender branched filaments of uniform diameter (approximately 0.09mm) and ranging from 0.25 to 14mm in length. In the perinuclear space they were generally spherical (0.12 to 0.16mm in diameter). There were no mollicutes in the nuclear substance. Both mollicute forms possessed no cell wall and were bounded only by a plasma membrane. Highly electron dense particles were situated on the membrane, and there were lucent vacuoles in the centre of some cells. The filamentous form branched, and branch ends expanded into spherical knobs. Some host nuclei were obviously changed in shape. Such mollicutes are rarely reported in crustaceans.

KEY WORDS: Penaeid shrimp, *Penaeus chinensis*, gut node, mollicute, aquaculture, transmission electron microscopy

INTRODUCTION

In this paper, we report on the ultrastructure and the pathological changes caused by a mollicute-like bacterium in diseased penaeid shrimp (*Penaeus chinensis*). In the summer of 1991 and 1992, gut-node disease of shrimp (GND) broke out in shrimp culture-ponds on the east coast of Zhejiang province in China. We examined the hepatopancreas and diseased areas of the midgut of moribund shrimp by TEM and found an unusual bacterium that was tentatively described as a mollicute in the cytoplasm and perinuclear spaces of gut cells.

Many species of bacteria infect and cause mortality in cultured penaeid shrimp (Sindermann & Lightner 1988). The class mollicutes includes pleomorphic mycoplasmas (Razin & Freundt 1984). Spiroplasma, the only reported helical form of mycoplasma (Davis et al. 1972), has been found in arthropods and plants (Whitcomb & Tully 1989). Other mollicutes have been reported from several marine and freshwater organisms. However, few of them have been cultivated and isolated. Harshbarger et al. (1977) reported a mycoplasma in the goblet cells of the American Oyster (*Crassostrea virginica*). Mycoplasma-like organisms have been associated with the coiling stunt disease of the brown algal sea tangle (*Laminaria japonica*) (in Hackett & Clark 1989) and from the marine bryozoans *Watersipora cucullata* (Zimmer & Woollacott 1983) and *W. arcuata* (Boyle et al. 1987). A mycoplasma has also been recovered from a freshwater fish, the European tench (*Tinca tinca*) (Kirchhoff et al. 1987). Krol et al. (1991) first reported a mollicute-like bacterium associated with hepatopancreatic disease of penaeid shrimp (*Penaeus vannamei*). Yang Jifang (1992) also found a mollicute-like bacterium in the gill epithelial cells of penaeid shrimp (*P. chinensis*) with black gill disease. However, no one has successfully isolated mollicutes from shrimp. Here the morphology and pathogenesis of a mollicute-like bacterium in midgut cells of *P. chinensis* are described.

MATERIALS AND METHODS

Shrimp

When mortalities began to occur, representative specimens (7-9 cm in length) were sent to the Second Institute of Oceanography (SOA) for examination.

Electron microscopy

Heart, gill, hepatopancreatic and midgut tissues were removed from twenity moribund shrimp and prepared for transmission electron microscopy (TEM). All tissues were fixed in 3% glutaraldehyde in sodium cacodylate buffer, postfixed in 1% Osmium tetroxide, and processed in a graded series of alcohols. Specimens for TEM were embedded in epoxy resin, sectioned, stained with uranyl acetate and lead citrate, and examined with a JEOL-1200EX TEM.

RESULTS

Shrimp (7-9 cm in length) began dying about 90 days after introduction into outdoor aquaculture ponds on the east coast of Zhejiang province in China. The mortality rate was

Jifang Y (1998) Ultrastructure of a mollicute associated, gut-node disease of penaeid shrimp (*Penaeus chinensis*). *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

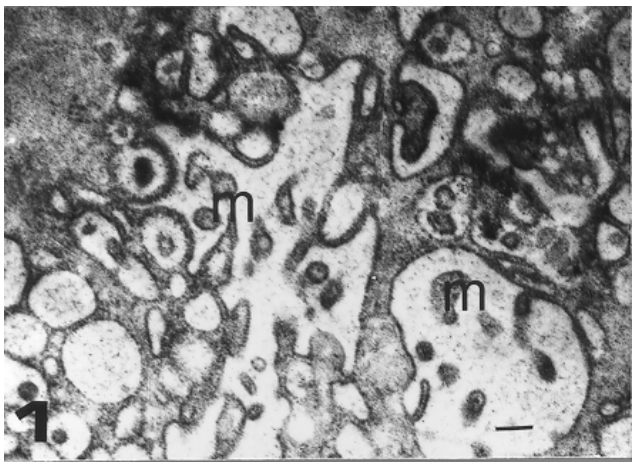


Figure 1. Transmission electron micrograph of cytoplasma of gut epithelium cells infected by a filamentous mollicute (M). Bar=200nm.

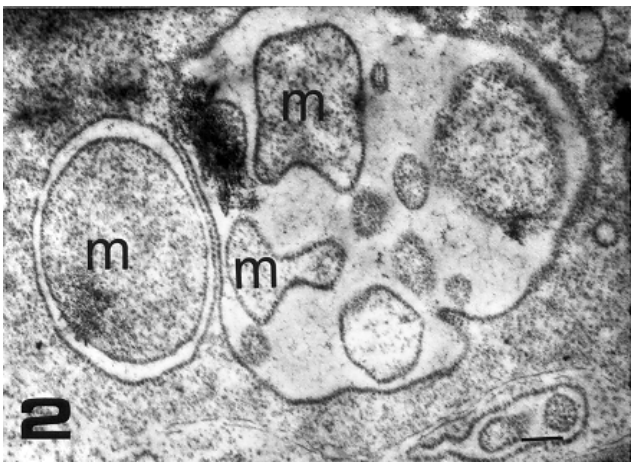


Figure 2. Transmission electron micrograph of a pleomorphous mollicute (M) in the cell cytoplasma. Bar=200nm.

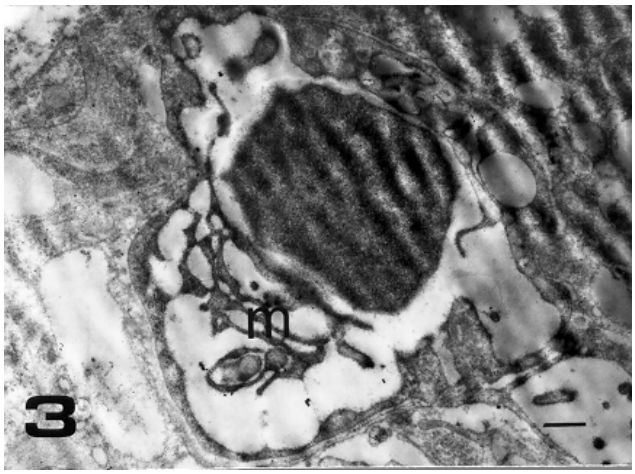


Figure 3. Transmission electron micrograph of a filamentous mollicute (M) in the perinuclear space of gut cell. Bar=500nm.

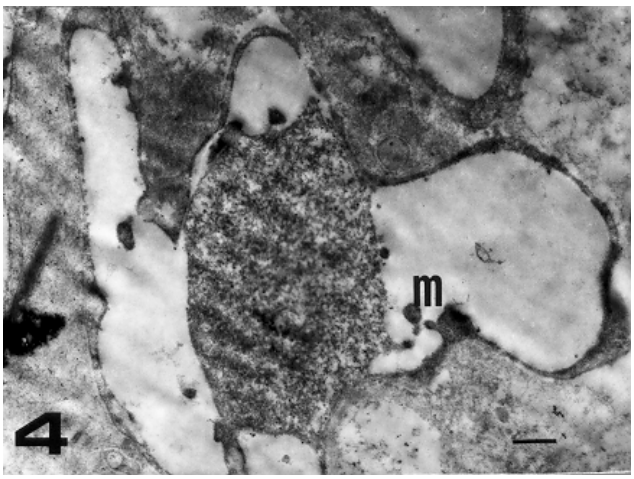


Figure 4. Transmission electron micrograph of a mollicute (M) in the expanding extremely perinuclear space. Bar=500nm.

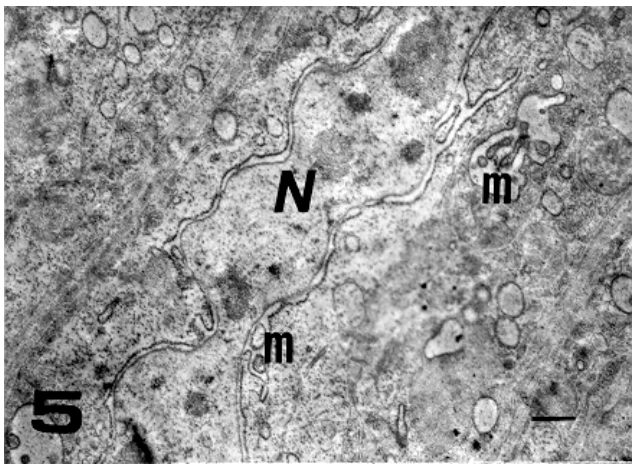


Figure 5. Transmission electron micrograph of host cell nucleus (N) changed in shape with multi-trace-strangle. Bar=500nm.

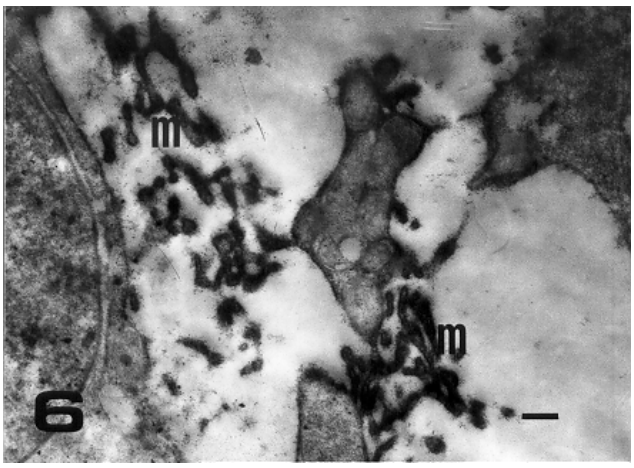


Figure 6. Transmission electron micrograph of cell cytoplasma severed by the mollicute filamentous (M). Bar=200nm.

about 80%. On gross examination, the most obvious indication of disease was 1-3 red, knob like to sausage shaped swellings in the midgut of some moribund specimens. The midgut was also the only tissue which exhibited evidence of disease at the TEM level. Midgut epithelial cells of all the shrimp examined by TEM were infected with a filamentous mollicute (Figs. 1, 2). The mollicute varied in shape from spherical (0.12 to 1.2 μm in diameter) to slender branched filaments of uniform diameter (about 0.09 μm), ranging in length from 0.25 to 14 μm. It was located in the cytoplasm

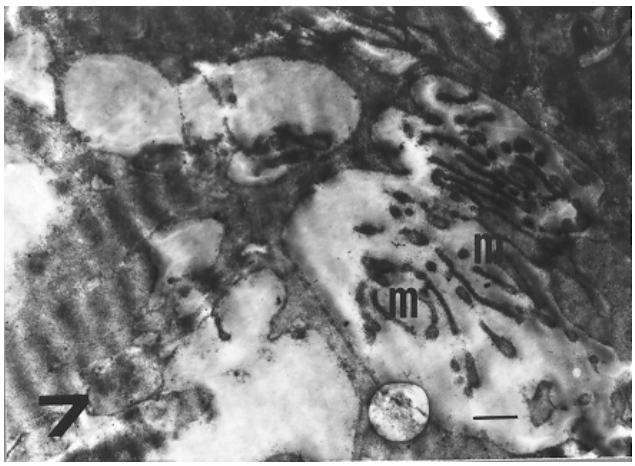


Figure 7. Transmission electron micrograph of many filamentous mollicute (M) in the host cell cytoplasma. Bar=500nm.

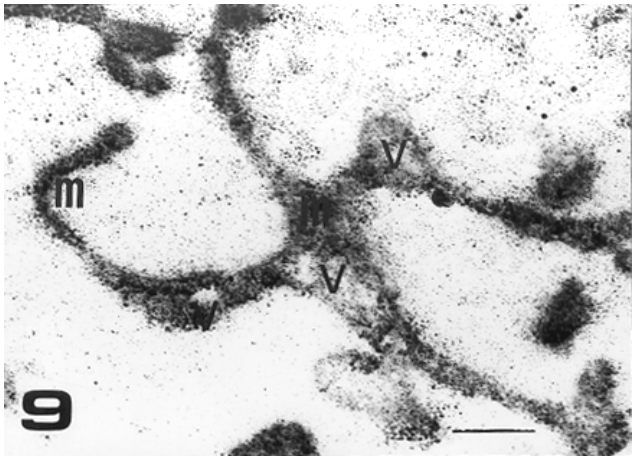


Figure 9. Transmission electron micrograph of a branched mollicute (M) with a electron-lucent Vacuole (V) Bar=200nm.

of host cell (Figs. 2, 7, 8, 9). Those in the perinuclear space were generally spherical (0.12 to 0.16 µm in diameter) (Figs. 3, 4). The mollicute had no cell wall or envelope and was bounded only by a plasma membrane. Many highly electron dense particles were situated on the membrane. Spherical dilations occurred in some of the filamentous mollicute forms (Fig. 8) and terminal knobs were seen in some of the branched, filamentous forms. There were electron lucent vacuoles in the terminal knobs (Fig. 9). There was no evidence of phagocytosis of any of the mollicutes within cells. Organelles were destroyed (Fig. 1). Cytoplasm of the host cell was depleted (Fig. 6). Some host cell nuclei were clearly changed in shape, being elongated with an irregular outline and an inflated nuclear membrane (Fig. 5).

DISCUSSION

Whereas definitive diagnosis must await bacterial culture, tentative identification of the bacterium in *P. chinensis* can be based on morphology in preliminary electron micrographs. These intracellular, filamentous organisms were considered to be mollicutes because they lacked cell walls and were pleomorphic. They may also be a type of mycoplasma.

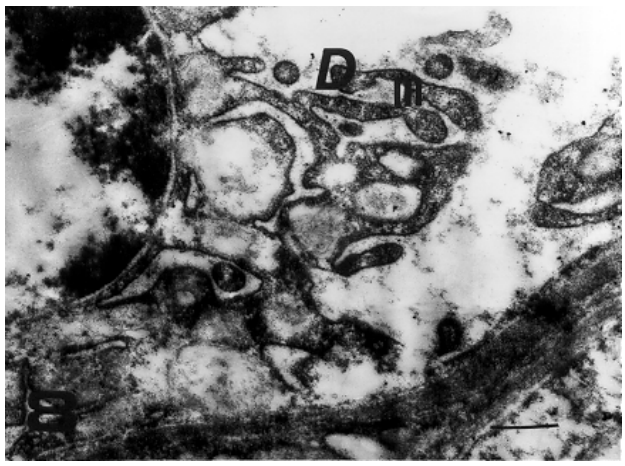


Figure 8. Transmission electron micrograph of a branched filamentous mollicute (M) with a spherical dilation (D) in the end. Bar=500nm.

On the other hand, some spiroplasmas have been reported to lose their helicity in an intracellular environment (Williamson et al. 1989). Mollicutes as pathogens of penaeid shrimp were first reported by Krol et al. (1991) as infecting the cytoplasm of hepatopancreatic cells in both helical and filamentous form. The ultrastructural features of the filamentous mollicute reported herein are consistent with those reported Krol et al. (1991), except that the filamentous mollicute in *P. vannamei* apparently did not invade the perinuclear space of hepatopancreatic cells as the mollicute here did in epithelial cells of the midgut in *P. chinensis*. Yang Jifang (et al. 1992) also reported a mollicute invading the cytoplasm and perinuelear space of gill epithelial cells of *P. chinensis* with black gill disease. However, there are differences in ultrastructural features for that mollicute and the one reported herein associated with GND. Whether these organisms are related or identical will require further investigation.

Yang Jifang et al. (1992) considered that the gill mollicute would enter the perinuclear space when the membrane of mollicute in the cytoplasma began to interact with the membranes in the perinuclear space. The spherical nature and size of the mollicute in the perinuclear space may result from nutritional factors or environmental parameters there (Luscombe et al. 1971, Muse et al. 1976, Razin 1973).

The obvious pathological signs of mollicute infection in *P.chinensis* indicated that death may occur when gut cell swelling and necrosis have become sufficiently extensive. Further analysis will be necessary to determine the identity of the bacterium and to understand its pathogenesis in cultured shrimp.

Acknowledgments. I thank the International foundation for science (IFS), grant No. A/2199-1 for financial support to carry out this work.

LITERATURE CITED

Boyle PJ, Maki JS, Mitchell R (1987) Mollicute identified in novel association with aquatic invertebrate. Curr Microbiol 15: 85-89

Davis RE, Worley JF, Whitcomb RF, Ishuima T, Steere RL (1972) Helical filaments produced by a mycoplasma-like organism associated with corn stunt disease. Science 176: 521-523

Hackett KJ, Clark TB (1989) Ecology of spiroplasmas. In "The Mycoplasmas", Vol. 5, "Spiroplasmas, Acholeplasmas, and Mycoplasmas of Plants and Arthropods" (R F Whitcomb and JG Tully, Eds), Academic Press, San Diego pp. 113-120

Harshbarger FC, Chang SC, Otto SV (1977) Chlamydiae (with phages), Mycoplasmas, and rickettsiae in chesapeake Bay bivalves. Science 196: 666-668

Kirchhoff H, Beyene P, Fischer M, Flossdorf J, Heitmann J, Khattab B, Lopatta D, Rosengarten R, Seidel G, Yousef C (1987) Mycoplasma mobile sp. nov., a new species from fish. INT. J Syst Bacteriol 37: 192-197

Krol RM, Hawkins WE, Overstreet RM (1991) Rickettsial and mollicute infection in Hepatopancreatic cells of cultured pacific white shrimp (Penaeus Vannamei). J Inverbr Pathol 57: 362-370

Luscombe BM, TRGGray 1971 Effect of Varying growth rate on the morphology of Arthrobacter. J Gen Microbiol 69: 433-434

Muse KE, Powell DA, Collier AM (1976) Mycoplasma pneumoniae in hamster tracheal organ culture studied by scanning electron microscopy. Infect Immun 13: 229-237

Razin S (1973) Physiology of mycoplasmas. Adv Microb Physiol 10: 1-80

Razin S, Freundt EA (1984) The Mycoplasmas. In "Bergey's Manual of Systematic Bacteriology" (NR Krieg JG Holt Eds) Vol. I, pp. 740-742. Williams & Wilkins, Baltimore

Sindermann CJ, Lightner DV (eds) (1988) "Disease Diagnosis and Control in North Americal Marine Aquaculture. "Elsevier: New York

Whitcomb RF, Tully JG (eds) (1989) "The Mycoplasmas," Vol. 5, "Spiroplasmas, Acholeplasmas, and Mycoplasmas of Plants and Arthropods." Academic Press, San Diego

Williamson DL, Tully JG, Whitcomb RF (1989) The genus spiroplasma. *In* "The Mycoplasmas," Vol.5, Spiroplasmas, Acholeplasmas, and mycoplasmas of plants and Arthropods (Whitcomb RF, Tully JG (eds) Academic Press, San Diego, pp. 71-111

Yang JF and Wu YL (1992) Electron microscope study on mycoplasmas-like of penaeid shrimp (*Penaeus Chinensis*). Donghai Marine Science, Journal of the Zhejiang Society of Oceanography & the Second Institute of Oceanography, SOA. Vol. 10, No. 4, 63-67

Zimmer RL, Woollacott RM (1983) Mycoplasma-like organisms: Occurrence with the Larvae and adults of a marine bryozoan. Science 220: 208-209

Toxiciological Studies with Juveniles of the Marine Shrimp *Penaeus vannamei* Using a Flow-Through System

[1,2]Miguel Betancourt-Lozano, [2]Donald J Baird, [1]Luz Maria Garcia-de al Parra [3]Ferdenando Gonzalez-Farias, [3]Sylvia Morales-Gonzalez

[1]CIAD Mazatlán Unit for Agriculture and Environment Management, AP. 711, CP 82000, Mazatlán, Sin, Mexico
[2]Environment Group, Institute of Aquaculture, University of Stirling, Scotland K9 4LA, UK
[3]ICMY Unit for Agriculture and Environment Management, AP 811, CP 82000, Mazatlán, Sin, Mexico

ABSTRACT: The State of Sinaloa located in the northwest of Mexico has numerous agricultural activities working side by side with shrimp farms in an area where a large number of wild species occur. It is well known that more than 100 agrochemicals are currently being used in this region, including herbicides, acaricides, insecticides, fungicides and nematicides. Toxicological data on the effects of these chemicals upon local species is scarce or non-existent. The marine shrimp *Penaeus vannamei* was selected as a test organism considering its high sensitivity to a broad range of pollutants. Moreover, *P. vannamei* plays a very important ecological role in estuarine environments along the Pacific coast of Mexico, as it supports one of the most important fisheries and is the principal marine shrimp used in aquaculture. A flow-through systen (FTS) was designed to carry out toxicity experiments with shrimp juveniles ranging from 0.3 to 2 g. The FTS is a simple apparatus that uses a 16 channel peristaltic pump, which delivers the test solution from the reservoirs into the exposure chambers. The flow rate can be adjusted in relation to the loading of organisms and the volume of the chamber, so that it replaces 90% of the water every 8 to 12 hours under constant room temperature (28 ± 1°C) on a 12/12 h dark/light photoperiod. The FTS has proved to be efficient in maintaining the organisms in good condition during long term experiments, making it suitable for toxicological studies, in particular those assesssing sublethal responses like growth, behaviour, moult frequency, histological and morphological effects, and biomarkers.

KEY WORDS: *Penaeus vannamei*, shrimp, toxicology, pollutants, agrochemicals, flow-through system

Betancourt-Lozano M, Baird DJ, Garcia-de al Parra1 LM, Gonzalez-Farias F, Morales-Gonzalez S (1998) Toxiciological studies with juveniles of the marine shrimp *Penaeus vannamei* using a flow-through system. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Viral Diseases

Active Viral Accommodation: A New Concept for Crustacean Response to Viral Pathogens

***[1]T.W. Flegel, [2]T. Pasharawipas**

[1]Dept. Biotechnology, Faculty of Science, Mahidol UniversityRama 6 Road, Bangkok 10400
[2]Dept. Microbiology, Faculty of Science, Rangsit Unviersity, Pathumthani 12000, Thailand

ABSTRACT: A radically new concept of active accommodation is proposed to explain the lack of inflammatory response to viral pathogens in crustaceans. It is proposed that crustaceans have developed a viral binding and recognition system that is distinct from that involved in binding for infection, and that the function of this system is to actively accommodate viral pathogens. We propose that active accommodation results from an initial viral binding step by the host and that this results in specific memory, such that simultaneous or subsequent viral binding for infection (by different receptors) does not trigger cellular apoptosis that leads to host death. Since the initial binding and subsequent recognition steps are distinct from the binding required for viral infection, they may occur in the absence of infection. However, once initiated for a particular virus, the binding and memory processes enable the host to tolerate its active infection without mortality. As a corollary, absence of memory due to lack of prior binding in a "naïve" host would result in death by viral triggered apoptosis. This concept is supported not only by the lack of visible defense reactions against viral pathogens, but also by recent information from shrimp farming systems indicating that different viral infections in various shrimp species have rapidly become innocuous after an initial period of catastrophic mortality. Based on shrimp biology, farming practice and the nature of geographical spread, these innocuous infections could not be explained in terms of induced or genetically selected resistance on the part of the shrimp, nor in terms of genetically selected, lowered virulence on the part of the viruses. The innocuous infections could be explained by invoking the concept of accommodation. The concept is also supported by the fact that high levels of viral replication by some viruses cause mortality for some species but not other species, or cause mortality for a single species under some circumstances but not others. We further propose that viral accommodation is a successful, alternative evolutionary development to resistance and that it arose from the interaction of crustacean (and perhaps other arthropod) hosts with their viral pathogens. Accommodation is characterized by the presence of an active mechanism to tolerate a pathogen and by the absence of an active defense against it. Thus, it contrasts with the crustacean response (i.e., active defense or resistance) to bacterial and fungal pathogens. From this viewpoint, the crustacean response to pathogens could be described as divided. Active accommodation may have evolutionary advantages in that the absence of host resistance pressure may slow the development of virulence and accelerate progression towards mutual host/virus existence.

KEY WORDS: shirimp, crustacean, immunity, defense, tolerance, viral accommodation, apoptosis

INTRODUCTION

In an earlier review of the viral diseases of cultivated shrimp in Thailand (Flegel 1997), scattered pieces of evidence were presented which indicated that shrimp had an unknown mechanism to adapt to viral pathogens. During manuscript processing and in the few months following its publication, more scattered evidence came to light and a number of exciting discussions took place. Although the data are still patchy, enough new pieces have fallen into place for us to perceive a startling vision and to resolve it into a single testable concept for crustacean response to viral pathogens. This concept could be used to explain curious factual phenomena occurring with cultivated shrimp. Although the concept (the forest) is clear and its predictions precise, the details of its workings (the trees) are indistinct. This is a weakness, but it should not discourage us from the first task of assessing the worth of the concept by testing its predictions. Through such tests, the concept may be pursued, modified or discarded, but the knowledge gained in the process may eventually lead us to the real explanation for phenomena we are observing. By describing the path that led to our concept, we aim to introduce it and persuade the reader to consider it potentially viable. In failing to do this, we hope that the ideas generated will lead to new directions in research on shrimp viral pathogens.

Lack of "inflammatory" response in crustacean viral infections

In 1988, at a microbiology seminar given by one of us on shrimp diseases, a physician commented on the curious fact that there was no inflammatory response in any of the presented slides showing viral infected shrimp tissues (Fig. 1). This contrasted sharply with the slides of bacterial infections and responses to toxins (Fig 2) which were characterized by massive haemocytic aggregation, often leading to encapsulation and granuloma formation (Flegel *et al.* 1992,

Flegel TW, Pasharawipas T (1998) Active viral accommodation: a new concept for crustacean response to viral pathogens. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: sctwf@mahidol.ac.th

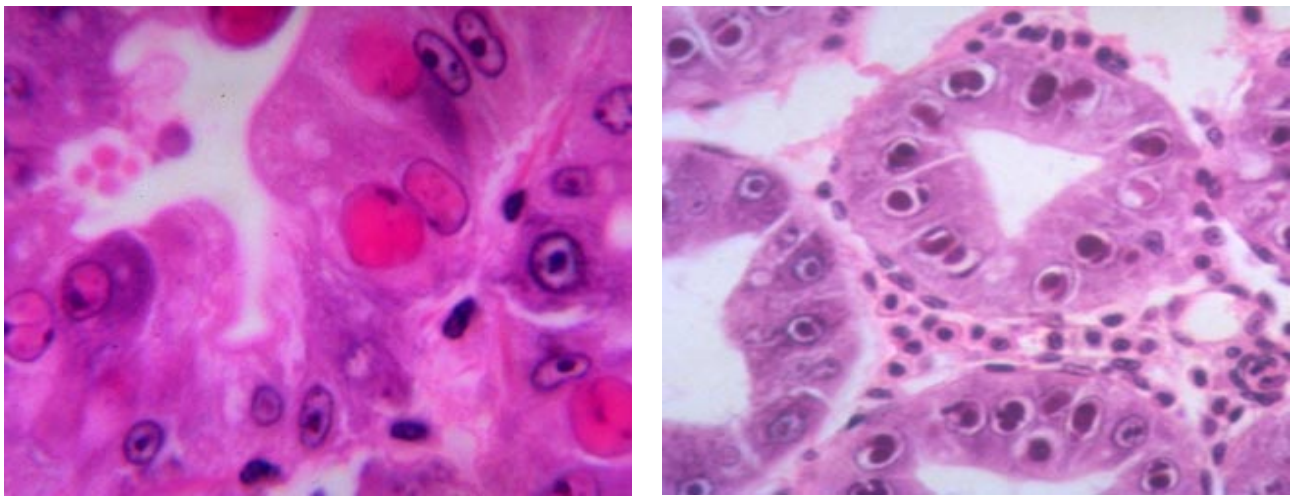


Figure 1. Examples of viral infetion in the hepatopancreas of *P. monodon*. On the left is tissue infected with monodon baculovirus (MBV) and on the right is tissue infected with hepatopancreatic parvovirus (HPV). Note the lack of host response in spite of the heavy infections and compare this with the same tissue type infected with bacteria (Fig. 2).

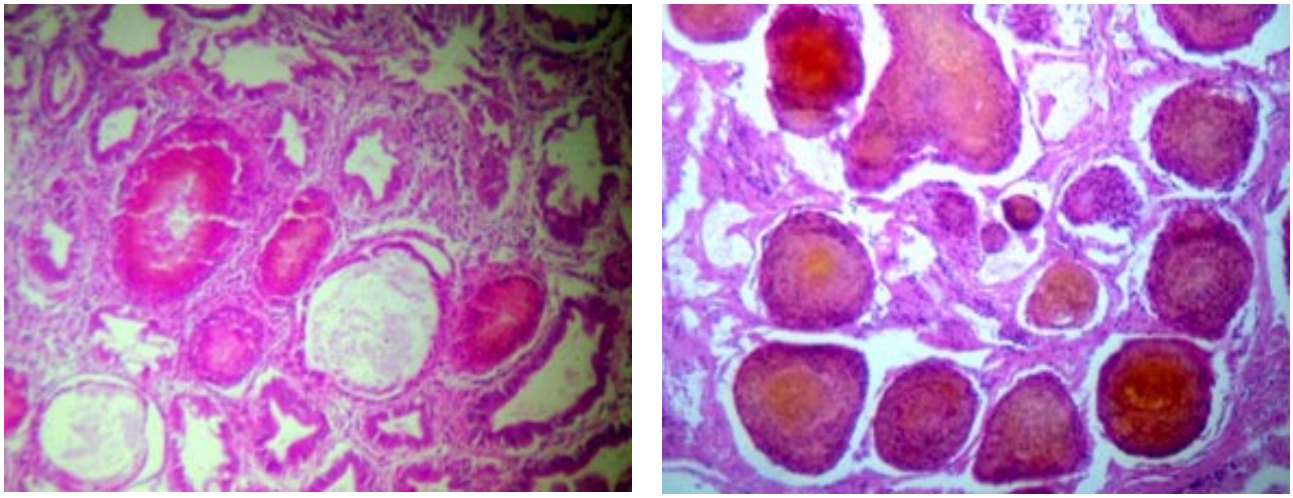


Figure 2. Examples of bacterial infection in the hepatopancreas of *P. monodon*. In the photomicrograph on the left, note the aggregation of haemocytes in rings around the infected tissue. On the right, the rings of haematocytes have condensed into melanized masses. contrast this response to the lack of response to viruses as seen in Fig. 1.

Lightner 1996). The questioner commented that this differed from his general experience with mammals, and he asked for an explanation. None could be given. Understandably, those were early days in the burgeoning shrimp industry in Thailand, and knowledge of shrimp defense mechanisms was (and still is) relatively poor. Even so, the question has remained unanswered over the past ten years. However, experience with recent viral epizootics in cultivated shrimp has led us to the startling conclusion that crustaceans may actively accommodate viral pathogens.

How we arrived at this conclusion is outlined in the following paragraphs. Basically, it was inspired by incidences in which farmed shrimp rapidly acquired what we initially called tolerance to formerly lethal viral infections (Pasharawipas *et al.* 1997). We called it acquired tolerance rather than resistance, because the shrimp had active but innocuous infections. Our initial description of these infections as "latent infections" (Pasharawipas *et al.* 1997) was inappropriate, because it inferred that the viruses were somehow transiently inactive. However, that was not the case. They were in the process of active replication. Thus, the situation with the shrimp was fundamentally different from acquired resistance in vertebrates which involves antigen/antibody reactions and normally results in pathogens being cleared from the host. Also, latent viral infections of vertebrates are usually quiescent, and long-term chronic infections (persistent, active infections) are the exception rather than the rule. By contrast, active, but innocuous, persistent infections seem to be the rule rather than the exception in crustaceans, and we eventually concluded that this predominance of persistent infections might be the manifestation of a general crustacean mechanism for accommodation of viral pathogens.

Acquired "tolerance" to viral infections

In 1989 our group (Fegan *et al.* 1991) first found in Thailand large numbers of polyhedral occlusion bodies of

monodon baculovirus (MBV) in the hepatopancreas of post larvae of *Penaeus monodon*, the major penaeid shrimp cultivated there and in the rest of Asia (Rosenberry 1997). We were frightened by the discovery of this virus in Thai hatcheries, since it had been implicated in the crash of the Taiwanese shrimp culture industry in 1987-1988 (Liao *et al.* 1992). However, we soon discovered that although MBV was ubiquitous in our hatcheries, shrimp farm production was not negatively affected, so long as cultivation conditions were ideal (Fegan *et al.* 1991). Indeed, shrimp larvae with very heavy MBV infections (i.e., large numbers of infected cells with large numbers of occlusion bodies and virions) showed no inflammation, were active, were apparently healthy, and grew normally. We subsequently visited Taiwan in the early 1990's and found that MBV was common in *P. monodon* hatcheries there. We assumed that the Taiwanese had mis-identified MBV as the cause of their shrimp industry crash in 1988. Later, from 1992 to 1994, we had the opportunity to work on MBV with Dr. Joan Vickers from Australia, and she was surprised that the larvae in Thailand were so unaffected by a virus that was such a severe pathogen in Australian shrimp hatcheries. At the time, we assumed that this difference was based on differing virulence for geographical strains of MBV.

Our work on MBV in Thailand was interrupted by the need to focus attention on a serious viral epizootic caused by yellow-head virus (YHV) (reviews, Flegel 1997; Flegel *et al.* 1995, 1997). This RNA virus caused losses of approximately 40 million US dollars to Thai shrimp farmers in 1992. However, in 1994 while preparing positive and negative YHV samples for work on a cDNA diagnostic probe, we stumbled upon the fact that the "negative control" ponds we had sampled were actually populated by high percentages of shrimp with innocuous YHV infections. Such ponds were widespread. In other words, within approximately 1.5 years of the catastrophic appearance of YHV in southern Thailand, most farmers were obtaining good harvests in spite of the presence of YHV in their ponds (Pasharawipas *et al.* 1997). We would have overlooked this phenomenon, had we not sampled the relevant ponds as sources of "negative controls" for our probe development program. We stumbled upon the innocuous infections because we were using the electron microscope to examine healthy shrimp for pathogens; a very unusual activity.

Attempting to explain the rather sudden and high incidence of innocuous YHV infections (Pasharawipas *et al.* 1997), we first considered the obvious possibilities of resistant shrimp and reduced virulence of YHV. We had no argument for acquired resistance, because crustaceans do not have antibodies (Mendosa & Faye 1996). The argument for genetically selected resistance was also weak, because the generation time of the shrimp was approximately 2 years, because broodstock for fry production were not recycled from cultivation ponds but always newly sourced from the wild, and because ponds were usually stocked with a mixture of larvae derived from several different captured females. Nor was there any indication of complaints from shrimp fishermen that could suggest reduced harvests from a catastrophic pan-epizootic underway in the wild shrimp population. In spite of all these factors, the shrimp in each pond responded in unison to the presence of the virus, either succumbing with almost total mortality (lethal infections) or giving a good harvest (innocuous infections). Thus, it appeared that the response of infected shrimp in a pond depended on an unknown shared feature(s) they had acquired, at some time during the rearing process.

Considering the possible occurrence of a less virulent form of YHV, we also had difficulty conceiving a reasonable selection mechanism. Selection arguments were weakened by many of the husbandry practices outlined above, by the lack of evidence for a pan-epizootic in wild shrimp and by the relatively rapid occurrence of the phenomenon over a wide geographical area. We also had evidence that a YHV epizootic had occurred on the southeast coast of the Gulf of Thailand in 1990 where it also dissipated within about 1.5 years after its first catastrophic appearance (Limsuwan 1991). In spite of this dissipation, YHV subsequently spread, as an extremely virulent agent, to more northerly farms and then southwards along the west coast of the Gulf until it reached the far south in 1992. Each regional outbreak was characterized by an initial period of catastrophic mortality followed by subsidence.

Failing to come up with any other rational explanation for what was happening, we proposed that shrimp had a specific "recognition mechanism" by which they could acquire "tolerance" to a new viral pathogen during their larval development (Pasharawipas *et al.* 1997). We called it the "tolerance hypothesis", and proposed that acquisition of tolerance for a particular virus would lead to innocuous rather than lethal infections upon subsequent challenge. According to the hypothesis, aquisition of tolerance involved an initial viral binding step that led to specific memory which was required for the subsequent development of innocuous infections. Also according to the hypothesis, the initial viral binding was independent of the binding required for viral infection and could occur in the absence of infection. Tolerance acquisition for each virus would be specific and not extendible to other viruses (i.e., there was no evidence for cross tolerance). For example, Thai shrimp were tolerant to MBV, but the tolerance did not extend to YHV when it first appeared. If the tolerance concept was correct, it would open up the possibility for development of "tolerines" that could be used in the same manner as vaccines. These reagents would help shrimp farmers avoid catastrophic mortality caused by viral infections, even though the infections would still occur.

With regard to the most appropriate interval for tolerine administration, we were/are not certain. However, it may be that initial exposure at the first zoeal stage would be most appropriate. This is the interval when (unpublished observation) *P. monodon* begins to produce haemocytes, and it may be that the development of tolerance involves processes related to self, non-self recognition. We also leave open the question as to whether booster exposures would be necessary to maintain a tolerant state over very long intervals in the absence of infection.

Using the hypothesis, we explained the YHV situation in Thailand in the following way. We reasoned that YHV first came to Thailand from an external source or as a newly mu-

tated form of a previously innocuous virus. This new virus hit the "naïve" Thai shrimp farm population like a storm, causing widespread, catastrophic mortality. However, during this explosive interval, the virus spread widely in the shrimp rearing environment, such that most batches of larvae in shrimp hatcheries were exposed to it during early stages of development (perhaps when their defenses were distinguishing self from non-self). Later work showed (Flegel *et al*. 1997) that early larval stages were refractory to YHV infection, but we reasoned that they had still bound the virus and acquired the memory necessary to develop innocuous infections during later exposure. By this reasoning, the first YHV epizootic occurred on the southeast coast of the Gulf of Thailand and then spread sequentially from there to the naive populations in other geographical locations. Each introduction started catastrophically but quickly subsided. The subsidence period of approximately 1.5 years is a puzzle, but was probably a fuzzy point in a gradual decline in numbers of outbreaks to the point that shrimp farmers no longer considered the problem to be "very serious".

The concept predicted that any severe viral epizootic of shrimp would lead to tolerance within approximately two years of the outbreak. It also provided an explanation for the history of MBV infections in various locations. In Thailand, it could be argued that MBV was ubiquitous and that larvae became exposed and infected early in the hatchery. Thus, almost all shrimp would be tolerant, and the virus would be innocuous there. In Taiwan, it is possible that MBV arrived in 1987 and caused catastrophic mortality to a naïve shrimp population, which subsequently became tolerant as the virus became ubiquitous, as it was in Thailand. In Australia, it appears that MBV is rarely encountered in geographically dispersed hatcheries, so that exposed shrimp are usually naïve and outbreaks are disastrous. The concept could also be applied to explain what happened to the stocks of *P. stylirostris* that arose in Tahiti with apparent "resistance" to infectious hypodermal and hematopoeitic necrosis virus (IHHNV). It could be argued that continuous rearing of *P. stylirostris* in the presence of IHHNV led to the development of tolerance, and that the shrimp eventually became innocuously infected. Indeed, when these stocks were subsequently placed with "naïve" *P. stylirostris*, the naïve shrimp died from severe IHHNV infections, indicating that the "resistant" shrimp were actually carriers of a virus that was still as virulent as ever (Lightner, personal communication).

Control of apoptosis: a mechanism for tolerance

The tolerance concept was developed to explain field observations with YHV and it appeared to do this in a way that was consistent for experience with MBV and IHHNV as well. However, finding a rational mechanism by which tolerance could arise in shrimp was problematic. In retrospect, it is unfortunate that we used the word "tolerance" to describe this phenomenon, because it invited comparison to an immunological phenomenon in vertebrates that is also described as "tolerance". In fact, there is little, if anything, in the two situations that can be compared. In the case of mammalian "tolerance", death sometimes occurs as a result of host immune defense reactions. By contrast, death in the crustaceans resulted in the absence of defense reactions. Thus, we had no reasonable mechanism to explain how naïve shrimp would be killed by a virus while tolerant ones would not. Inspiration came during the advent of the next serious viral epizootic in Thailand.

The white-spot baculovirus (WSBV) epizootic in Thailand followed soon after the YHV epizootic (reviews, Flegel 1997, Flegel *et al.* 1997). It caused even more serious losses for shrimp farmers, and it was largely responsible for a drop in production from 225,000 metric tons in 1995 to 160,000 tons in 1996 (Rosenberry 1997). At US$8 per kg, this was equivalent to approximately 500 million US dollars. The disease is apparently pan-Asian at this time, and the total value of lost production may be in the range of 3 billion dollars per year (Lunden, 1997).

Again, Thai scientists were drawn away, this time from YHV, to carry out the more urgently needed work on WSBV. It soon became apparent that WSBV was capable of infecting a large number of different crustaceans, including all the cultivated penaeid shrimp and many different species of crabs and lobsters (review, Flegel 1997). It was surprising to discover that many of the "carrier" species such as the crabs had active viral infections with many infected cells and large numbers of virions but showed no mortality (i.e., they had active, innocuous infections) (Supamataya et al. 1998; Kanchanaphum 1998). In retrospect, this was also true for some of the carriers of YHV (Flegel et al. 1995). Obviously, high viral production, in itself, did not guarantee death. This led us to question whether the moribund shrimp might not be dying from generalized programmed cell death (apoptosis), triggered by viral infection (Flegel 1997). Apoptosis, in turn, gave us a potential operational mechanism for our concept of tolerance. Simply put, shrimp and probably other crustaceans, would die from viral infections only if those infections induced the innate cellular mechanism of apoptosis. We could then propose that tolerance developed in situations where an early viral binding step resulted in specific memory that supressed apoptosis upon subsequent or concurrent viral infection.

To comply with our concept, the early binding system would have to be capable of specific memory for a wide variety of ever changing viral proteins. As such, it would probably be complex and sophisticated; perhaps no less so than the system for variable antibody response in vertebrates. It would also be largely membrane and membrane transduction based, so that any role for humoral factors like antibodies would be minimal or absent. Perhaps that is why antibodies have not been found in crustaceans and insects. On the other hand, membrane bound, evolutionarily related molecules have been found, and they might be involved (Mendosa & Faye 1996). Complex membrane-based recognition systems involving molecules evolutionarily related to antibodies are found in plants (Baker *et al.* 1997).

The idea of apoptosis involvement was attractive, especially because inhibition of apoptosis (IAP) genes are known to occur in the baculovirus group to which WSBV probably belongs or is related (Clem *et al.* 1996). It is significant that the existence of these viral genes proves that insect cells can

respond to viral infections by initiating apoptosis, at least under some circumstances. In addition, the complexity of the apoptotic pathway would help to explain phenomena that are known to occur widely with shrimp viral diseases. For example, we know that MBV infections in Thailand are mostly innocuous, but can become fatal, if shrimp are exposed to sufficient stress. We also have unpublished information that innocuous YHV and WSBV infections can be induced to full blown lethal infections by application of a sufficient level of appropriate stress. In some cases the type of stress is important. For example, lethal YHV infections can he induced in innocuously infected shrimp by low dissolved oxygen or a sudden pH change, but not by salinity shock. These phenomena indicate that the degree of protection from death offered by tolerance has limits and that shrimp with innocuous infections are still at risk. They are also consistent with the fact that the apoptotic pathway is complex, involving several routes of induction or suppression.

Although apoptosis is a key process in tissue and organ development for multicellular organisms, this must have been a secondary development in evolution. If apoptosis first arose in unicellular organisms it must have had advantages in limiting pathogen spread in a population. However, once it was recruited for differentiation in multicellular organisms, its role in defence had to be substantially modified. Indeed, it may be advantageous or detrimental for an individual vertebrate host during interaction with pathogens (Finlay & Cossart 1997). In our model, viral induced apoptosis in widely dispersed, wild crustaceans would be advantageous for the crustacean population and disadvantageous for the inducing viral strain. From this, one might interpret that crustaceans use apoptosis to defend against viruses and that viruses develop IAP genes to overcome this host defense mechanism. That is certainly the traditional viewpoint, but we now believe not the correct one. Even so, in a wild, dispersed population, a host that drops on the spot is unlikely to spread a pathogen far. In the shrimp farming system, by contrast, animals are raised at unnaturally high densities at all stages, and highly virulent viral strains can spread rapidly. An advantage for the wild shrimp population is a disadvantage for the shrimp farmer.

No viral resistance: an evolutionary alternative

In attempting to rationalize the phenomenon of accommodation in terms of crustacean survival, we have come to the conclusion that it may be the manifestation of a distinct response to viral pathogens that has arisen in the crustacean (and perhaps arthropod) evolutionary line. It appears to be characterized by active viral accommodation rather than viral resistance. This is fundamentally different from the resistance response that has developed in the vertebrate line, and even from the response of crustaceans themselves to bacterial and fungal pathogens. All the evidence indicates that the crustacean response to bacteria and fungi is based on active cellular resistance similar to that which occurs in vertebrates. On close inspection, a response of viral accommodation may offer some advantages to a viral host, in terms of limiting the development of pathogen virulence. Selection for increased virulence requires the pressure of host resistance. Removing that pressure should slow its development.

Let us imagine a population of crustaceans facing a new viral pathogen or a new virulent mutant, but armed with the capability for accommodation. The first appearance of the virus could cause widespread mortality. However, the presence of large quantities of the virus in the environment would quickly lead to exposure of young developing stages whose cells would bind and remember it. Widespread innocuous infections would then develop and the incidence of massive mortality would decrease. At the same time, there would be no pressure on the virus population for the selection of mutants with higher virulence. In fact, mutants with increased virulence would actually be at a disadvantage to the existing strain, since they would tend to limit their own spread by causing mortality. The situation would enter a new, long term phase of selective host/virus adaptation during which genetic variations in the host and virus would be favored if they resulted in better mutual accommodation (i.e., reduction in any negative effects of infection on growth and reproduction, especially when exposed to various stress factors). All this would occur without resistance pressure on the pathogen. With time, the host and the pathogen could reach a point of almost total compatibility. Perhaps the sequence homology between baculovirus IAP genes and host genes (Clem 1996) is an indication of some aspect of this mutual interaction. There is some evidence that a high degree of compatibility has already been achieved with shrimp viruses, like MBV for *P. monodon* in Thailand.

Penaeus monodon in Thailand appears to have an even higher degree of compatibility with IHHNV than with MBV. Lightner *et al.* (1991) have argued convincingly that IHHNV is endemic to *P. monodon* and that it was imported to the western hemisphere with living experimental stocks that were not properly quarantined. It subsequently infected *P. stylirostris* with devastating consequences. In spite of the endemic nature of IHHNV in *P. monodon*, it has never been reported as the cause of widespread mortality in shrimp ponds in Thailand. Indeed, on one occasion when we found clear lesions in the antennal gland only, the shrimp were normal and appeared to suffer no ill effects. However, upon examination by *in situ* hybridization with a DNA diagnostic probe, these shrimp were found to have very heavy infections of IHHNV and in many more tissues than indicated by the few visually apparent lesions (Flegel 1997). Obviously these shrimp were able to tolerate a high level of viral production with no ill effects.

It is not possible, nor is it our intention here to compare the evolutionary merits of viral accommodation versus resistance. We consider them to be alternative developments from a common origin, just as the widely different optical apparatuses in different animal lines have probably developed from a common ancestral photoreceptor system. It would be unwise to suggest that one extant development is "better" or "more primitive" than the other. For the same reasons, we cannot speculate why a divided response to pathogens might have arisen in the crustaceans. In addition, any evolutionary speculation would have to take into account that shrimp/viral interaction that took place in the natural

environment is radically different from that which takes place in farming systems

In conclusion, we hypothesize that tolerance to viral infections in crustaceans is the manifestation of an active system for accommodation that is based on membrane binding involving specific memory, leading to supression of viral triggered apoptosis and to persistent innocuous, infections. This hypothesis can be tested easily. It predicts that shrimp (other crustaceans and perhaps other arthropods too) will show evidence of extensive apoptosis when they are dying from viral infections. It also predicts that exposure of young larval stages to inactivated viral particles or subunit viral proteins ("tolerines" as opposed to vaccines), followed by subsequent challenge with active virus, would result in innocuous infections rather than mortality. This could be of great significance to shrimp farmers. Almost all of the massive losses suffered by the world's shrimp farmers over the past decade have arisen from viral epizootics. Other pathogens are manageable by appropriate husbandry or therapy. Any avenue of work with potential to solve the viral problem should be avidly pursued.

Acknowledgements. We would like to thank the National Center for Genetic Engineering and Biotechnology for their continued support for our research on shrimp biotechnology. Thanks also to the many colleagues who read drafts of this manuscript and made valuable comments that helped us in clarifying our arguments.

LITERATURE CITED

Baker B, Zambryski P, Staskawicz B, and Dinesh-Kumar SP (1997) Signalling in plant-microbe interactions. Science 276: 726-733

Clem RJ, Hardwick JM, Miller LK (1996) Anti-apoptotic genes of baculoviruses. Cell Death and Differentiation 3: 9-16

Fegan DF, Flegel TW, Sriurairatana S, and Waiakrutra M (1991) The occur-rence, development and histopathology of monodon baculovirus in *Penaeus monodon* in Southern Thailand. Aquaculture 96: 205-217

Finlay BB, and Cossart P (1997) Exploitation of mammalian host cell functions by bacterial pathogens. Science 276: 718-725

Flegel TW (1997) Special topic review: major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J Microbiol Biotechnol 13: 433-442

Flegel TW, Fegan DF, Kongsom S, Vuthikornudomkit S, Sriurairatana S, Boonyaratpalin S, Chantanachookhin C, Vickers JE, and Macdonald OD (1992) Occurrence, diagnosis and treatment of shrimp diseases in Thailand. Diseases of penaeid shrimp. In Fulks W, Main KL(eds) Diseases of cultured penaeid shrimp in Asia and the United States, Oceanic Institute, Honolulu, Hawaii, p. 57-112

Flegel TW, Sriurairatana S, Wongteerasupaya C, Boonsaeng V, Panyim S, Withyachumnarnkul B (1995) Progress in characterization and control of yellow-head virus of *Penaeus monodon.* In Browdy C, Hopkins S(eds) Swimming through troubled water; Proceedings of the special session on shrimp farming, Aquaculture '95, San Diego, February, 1995, World Aquaculture Society, Baton Rouge, LA, p. 76-83

Flegel TW, Boonyaratpalin S, Withyachumnarnkul B (1997) Current status of research on yellow-head virus and white-spot virus in Thailand. In Flegel TW' MacRae IH (eds) Diseases in Asian Aquaculture III. Asian Fisheries Society, p. 285-296

Liao IC, Su MS, Chang CF (1992) Diseases of *Penaeus monodon* in Taiwan: a review from 1977 to 1991. In W. Fulks and K.L. Main (eds). Diseases of cultured penaeid shrimp in Asia and the United States. Oceanic Institute, Honolulu, Hawaii, USA, p. 113-137

Kanchanaphum P, Boonsaeng V, Panyim S, Wongteerasupaya C, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1998) Experimental transmission of white-spot syndrome virus (WSSV) from crabs to shrimp (*Penaeus monodon*). Dis. Aquatic Org, in press

Lightner DV (ed) (1996) A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Society, Baton Rouge, LO, USA

Lightner DV, Bell TA, Redman RM (1990) A review of the known hosts, geographic range and current diagnostic procedures for the virus diseases of cultured penaeid shrimp. Actes de Colloque 9: 113-126

Lundin CG (1997) Global attempts to address shrimp disease. Proceedings to the Second Asia-Pacific Marine Biotechnology Conference, Phuket, Thailand, 7-10 May, 1997, National Center for Genetic Engineering and Biotechnology, Bangkok, in press

Mendoza HL, Faye I (1996) Immunoglobulin superfamily proteins in invertebrates. In K. Soderhall, S. Iwanaga and G.R. Vasta (eds) New directions in invertebrate immunology. SOS Publications, Fair Haven, N.J. pp. 285-302

Pasharawipas T, Flegel TW, Sriurairatana S, Morrison DJ (1997) Latent yellow-head infections in *Penaeus monodon* and implications regarding disease tolerance or resistance. In T.W. Flegel, P. Menasveta and S. Paisarnrat (eds). Shrimp Biotechnology in Thailand. National Center for Genetic Engineering and Biotechnology, Thailand, 45-53.

Rosenberry Bob (1997. World shrimp farming 1996) Shrimp News International, San Diego, CA

Supamattaya K, Hoffmann RW, Boonyaratpalin S, Kanchanaphum P (1998) Experimental transmission of white spot syndrome virus (WSSV) from black tiger shrimp *Penaeus monodon* to the sand crab *Portunus palagicus*, mud crab *Scyla serrata* and krill *Acetes* sp. Dis. Aquat. Org. 32: 79-85

Screening for Shrimp Viruses in the Philippines

***[1]J.D. Albaladejo, [2]L.M. Tapay, [2]V.P. Migo, [2]C.G. Alfafara, [1]J.R. Somga**
[1]S.L. Mayo, [1]R.C. Miranda, [2]K. Natividad, [2]F.O. Magbanua
[3]T.Itami, [4]M. Matsumura, [5]E.C.B. Nadala, Jr., [5]P.C. Loh

[1] Fish Health Section, Bureau of Fisheries and Aquatic Resources
Quezon City, Philippines
[2] National Institute of Molecular Biology and Biotechnology
University of the Philippines, Los Banos, Laguna, Philippines
[3] Department of Aquabiology, National Fisheries University
Shimonoseiki, Yamaguchi, Japan
[4] Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Japan
[5] Department of Microbiology, University of Hawaii, Honolulu, HI 96822 U.S.A.

ABSTRACT: Cases of shrimp mortalities in the Philippines were previously associated with luminous vibrosis. Lately, however, increasing incidences of possible virus-associated syndromes have also been reported. Consistent observation of eosinophilic intra-nuclear inclusions in the mid-gut of affected shrimp led us to use molecular biological tools such as SDS-western blot-enzyme immunoassays (EIA), polymerase chain reaction assays (PCR) and *in situ* hybridisation assays to determine whether a virus(es) was involved in generating these inclusions. Of 250 shrimp sampled, 41 were positive for yellow head virus (YHV) using EIA. Samples were negative for WSSV using EIA and PCR. Moreover, 3 of 10 representative samples were positive by *in situ* DNA hybridisation for spawner-isolated mortality virus (SMV). These results suggest that viruses along with luminous bacteria, may be involved in many cases of shrimp mortality in the Philippines. These seem to be moderately virulent viral diseases which are expressed and cause outward clinical signs and mortality when enhanced in mixed infections with the opportunistic bacteria related to shrimp culture. The results suggested that the shrimp disease syndromes in the Philippines may be different from those of other shrimp producing areas in Southeast Asia, India, China, Japan and the USA but may be similar to those affecting Australia.

KEY WORDS: Shrimp viruses, polyclonal antibodies, Polymerase Chain Reaction, *in-situ* hybridisation

INTRODUCTION

Diseases have emerged as a major constraint to the sustainable growth of shrimp culture in the Asia-Pacific region. Many of these diseases are associated with infectious agents, environmental deterioration and stress due to intensification of culture practices.

One of the most devastating diseases of shrimp in the Philippines today is the so-called "luminous vibriosis". The disease used to be a problem in many hatcheries throughout the country, but lately it has become a problem in grow-out farms as well. These past few years, results from challenge experiments testing luminous vibriosis as the primary cause of the disease outbreaks in the Philippines have remained inconclusive. New epidemiological data from the field have suggested that other disease entities might be involved in epizootics. Results of screening of shrimp samples from clinical cases have consistently shown intra-nuclear inclusion bodies related to viral infection (BFAR unpublished data). Cell and tissue tropisms are similar to other reported shrimp virus disease cases (Fraser & Owens 1996, Owens 1997). The observations of pathological lesions in our present samples concur with the findings from other countries.

The emergence of a viral agents in shrimp disease syndromes in the Philippines required a shift from conventional disease diagnosis to the present approach, which includes observation of clinical signs and lesions attributed to virus infections. Protocols used in other countries in screening of shrimp samples for viruses have been adapted.

The aim of this study was to screen for the probable presence of virus(es) in shrimp stocks in the Philippines using histopathology, combined with SDS PAGE-Western Blot-Enzyme Immunoassay (EIA) using polyclonal antibodies against white spot syndrome virus (WSSV) and yellow head virus (YHV) (Lu *et al.* 1996, Nadala *et al.* 1997) and PCR and *in situ* hybridisation assays using a DNA probe for spawner mortality syndrome virus (SMV) (Owens *et al.* 1998).

MATERIALS AND METHODS

Sampling Protocol

Shrimp samples of varying age were collected from selected farms in identified shrimp producing areas (i.e., Batangas, Negros Occidental, Cebu, Bohol, Misamis Occidental, Misamis Oriental, Butuan and General Santos).

Albaladejo JD, Tapay LM, Migo VP, Alfafara CG, Somga JR, Mayo SL, Miranda RC, Natividad K, Magbanua FO, Itami T, Matsumura M, Nadala ECB Jr., Loh PC (1998) *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail:juanda@vlink.net.ph

Hemolymph, gills, lymphoid organ and heart were aseptically removed from live shrimp and processed for polymerase chain reaction (PCR) and combined SDS-Western Blot-Enzyme Immunoassay (EIA) (Tapay et al., 1996). Whole shrimp samples from the same lots were fixed in Davidson's fixative for histopathology.

Histology

Fixed samples were processed using routine histological procedures. Tissue sections were stained with H&E and Feulgen DNA stain.

Combined SDS-Western Blot-Enzyme Immunoassay

Hemolymph, 10% homogenates from gill tissues (in TNE buffer, pH 7.4), lymphoid organ and heart pooled from individual shrimp or pooled whole fry samples were analysed for the presence of YHV and/or WSSV by Western blot protocol (Tapay 1996 and Nadala *et al.* 1997) .

Polymerase chain reaction (PCR)

Individual samples of the lymphoid organ and heart were homogenised using DNAzol (Gibco/BRL). Samples were precipitated using absolute ethyl alcohol and washed twice with 95% ethyl alcohol. Excess alcohol was drained and DNA was used as a template for PCR. After amplification the PCR product was run in agarose gel along with DNA of known molecular weights as standards. The separated bands were examined using ethidium bromide stain and observed under UV light for target band expression. To check for transient infection or low-grade infection, samples were also subjected to 2-step nested PCR.

In-situ Hybridisation

The in situ hybridisation method used was based on that described by Owens et al. (1998).

RESULTS AND DISCUSSION

From over 250 shrimp samples collected from field cases, gross manifestations were non-specific. No typical clinical signs associated with known shrimp viral diseases were observed. Cumulative mortalities ranged from 40-80% and assumed acute or chronic forms. Two syndromes were observed, one noted between 30-45 days of culture (DOC) with acute mortalities while another during mid-culture assumed either acute or chronic forms. Survivors usually showed poor growth performance without a drop in feed demand.

To determine involvement of viruses, preliminary screening was done using histopathology. Examination revealed a variety of virus-related inclusions and occlusion bodies in 90% of the samples (see Table 1). Observed eosinophilic intranuclear occlusion bodies are mainly attributed to MBV infection. As for the inclusion bodies, all appeared intranuclear and stained either as eosinophilic or basophilic. In all samples, intranuclear eosinophilic inclusion bodies present in the midgut cecae cells and apical regions of hepatopancreatic tubules were the most predominant cell lesion found. Similar cytopathology in lymphoid organs was observed from some tissue samples. This is a first report of distinct inclusion bodies in Philippine shrimp. These inclusions did not conform to the pathognomonic viral effects observed from other published reports of WSSV (Wongteerasupaya *et al.* 1995).

Moderate infections of gills and cuticular epithelial cells with intranuclear basophilic inclusion bodies dispersed in the cell cytoplasm were also noted from the histological sections. These inclusions were similar to those reported for cells infected with YHV (Boonyaratpalin *et al.* 1993 and Chantanachookin *et al.* 1993).

The described inclusions were seldom observed in mixed infection together with known shrimp viral infection agents such as HPV and LOVV. On the other hand, most of the tissue sections showing viral-associated lesions also showed typical lesions attributed to luminous vibriosis.

To confirm the viral entities involved, combined SDS-Western Blot-Enzyme Immunoassay was employed. Out of 175 samples examined, 41 yielded positive results for the presence of YHV. Protein analysis of these samples exhibited a band of approximately 135 kDa which has been reported as the putative G protein of YHV which has been associated with Rhabdoviruses (Nadala *et al.* 1997). Similar tests with the same samples but using polyclonal antibodies against WSSV produced negative results. To confirm this finding, the same samples were subjected to PCR assay, which also gave negative results, even with 2-step nested PCR designed to unmask transient WSSV infections.

The inability of the molecular biological tools used to detect the virus associated with eosinophilic intranuclear in-

Table 1. Number of shrimp diagnosed with infections of viral agents and *Vibrio*. A total of 175 specimens were examined using various techniques.

	Number of shrimp affected by each pathogenic agent						
Diagnostic tool used	MBV	HPV	LOVV	YHV	WSSV	SMV	*Vibrio*
Histology	51	23	27	58		70	183
Polyclonal Ab to YHV WSSV				41*	 All (-)		
PCR	ND	ND	ND	ND	All (-)	ND	ND
in situ hybridisation	ND	ND	ND	ND	ND	3**	ND

ND – not done * a total 175 shrimp examined ** ten shrimp examined

clusion bodies in the midgut suggested that we might be dealing with a new WSSV variant or a totally new virus. On the other hand, similar clinical observations were reported by shrimp health workers from James Cook University (L. Owens pers. comm.). They suggested that the syndrome reported in the Philippines resembled cases of a viral infection reported in Australia as spawner-isolated mortality virus (SMV) (Owens 1997, Owens *et al*. 1998). To confirm relatedness to SMV, representative tissue samples were sent to Australia and were subjected to *in situ* hybridisation using a probe for SMV. Three out 10 samples produced positive results.

These results suggested that a virus along with luminous bacteria might be involved in most cases of shrimp mortality in the Philippines. It seems that we are dealing with a moderately virulent viral disease the expression of which is enhanced by mixed infection with opportunistic bacteria. The combination produces the outward clinical signs and mortality during shrimp culture. The presence of YHV in a number of samples suggests that it could also be one of the partners in such mixed infections. The fact that SMV has not been reported from other shrimp producing areas in Southeast Asia, India, China, Japan and the USA but instead from Australia and the Philippines, suggests that the latter two areas may share some common features that are different from those in other shrimp producing areas.

Acknowledgements. The research was supported by grants from the Philippine Bureau of Fisheries and Aquatic Resources and the Australian Center for International Agricultural Research (ACIAR) under the Project No. 9411. We wish to thank Dr. Leigh Owens of James Cook University, Australia for the *in situ* hybridization results.

LITERATURE CITED

Boonyaratpalin S, Supamattaya K, Kasornchandra J, Direkbusaracom S, Aekpanithanpong U, Chantanahookin C (1993) Non-occluded baculo-like virus, the causative agent of yellow-head disease in the black tiger shrimp (*Penaeus monodon*). Fish Pathol 28: 103-109

Chantanachookin C, Boonyaratpalin S, Kasornchandra J, Direkbusarakom S, Aekpanithanpong U, Supamattaya K, Sriuraitana S, Flegel TW (1993) Histology and ultrastructure reveal a new granulosis-like virus in Penaeus monodon affected by yellow-head disease. Dis Aquat Org 17: 145-157

Fraser C A, Owens L (1996) Spawner-isolated mortality virus from Australian *Penaeus monodon*. Dis Aquat Org 23: 141-148

Lu Y, Tapay L M, Loh PC (1996) Development of a nitrocellulose-enzyme immunoassay for the detection of yellow-head virus from penaeid shrimp. J Fish Dis 19: 9-13

Nadala ECB, Tapay LM, Cao S, Loh PC (1997) Detection of yellow-head virus and Chinese baculovirus in penaeid shrimp by the Western blot technique. J Virol Mthd 69: 39-44

Owens L (1997) Special topic review : The history of the emergence of viruses in Australian prawn industry. World J Microbiol and Biotech 13: 427-431

Owens L, Hamooleh G, McElnea C, Coelen R (1998) Spawner-isolated mortality virus associated with mid-crop mortality ssyndrome in farmed *Penaeus monodon* from Northern Australia. Dis Aquat Org (in press)

Tapay L, (1996) Studies on penaeid shrimp viruses and cell cultures PhD dissertation University of Hawaii

Tapay L, Lu Y, Gose R, Nadala E C, Brock J, and Loh P J Virol Methods 64:37-41

Wongteerasupaya CJE, Vickers S, Sriurairatana GL, Nash A, Akarajamorn V, Boonsaeng S, Panyim A, Tassanakajon B (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn, *Penaeus monodon*. Dis Aquat Org 21: 69-77

Recent Developments in Immunologically-Based and Cell Culture Protocols for the Specific Detection of Shrimp Viral Pathogens

[1]Philip C. Loh, [1]E. Cesar, B. Nadala Jr., [3]Lourdes M. Tapay, [2]Yuanan Lu

[1]Department of Microbiology, [2]Retrovirology Laboratory, University of Hawaii, Honolulu, Hawaii, 96822, U.S.A.
[3]National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, Philippines

ABSTRACT: A Western blot protocol capable of detecting YHV and CBV (WSSV) in the hemolymph of infected shrimps was developed. This protocol was highly specific and rapid, and sensitive enough to detect the presence of the viruses before the appearance of overt symptoms. It was also useful in demonstrating the growth of both viruses in primary lymphoid cell cultures. A flow chart for routine shrimp virus pathogen detection and identification employing the more convenient but less specific NC-EIA as a presumptive test followed by the highly reliable WB protocol as a confirmatory assay was advanced. The combined protocols, together with the primary shrimp cell culture system, have been successfully used in field surveillance of shrimp hatcheries and farms.

KEY WORDS: Shrimp virus; Pathogen; Detection; Western blot; Enzyme immunoassay; cell culture.

INTRODUCTION

Shrimp viral diseases have seriously impacted the sustainability and economic success of the shrimp aquaculture industry worldwide. Among several of the recent viral pathogens which have caused massive mortalities in cultured shrimp are included yellow-head virus (YHV) and white spot syndrome virus (WSSV) (also called Chinese baculovirus [CBV] or systemic ectodermal and mesodermal baculovirus [SEMBV]). Both YHV and CBV (WSSV) have been reported by our laboratory to be highly pathogenic for *Penaeus stylirostris* (blue shrimp) and *P. vannamei* (white shrimp), the two principal penaeid species commercially cultured in Hawaii and the Western Hemisphere (Lu *et al.* 1997). They are thus potentially serious problems particularly to the broodstock industry.

A major problem for the control and prevention of shrimp viral diseases is the lack of relatively simple and cost-effective technologies for the early detection and diagnosis of viral infections, particularly asymptomatic infections. Control and eradication of the virus problem would be much more expeditious with an efficient detection/diagnostic test to identify infected animals long before they showed clinical signs. This is particularly true for asymptomatic broodstock carriers. In this paper, we report the successful development of a less invasive, combined Western blot and enzyme immunoassay protocol for the early detection of both YHV and CBV (WSSV) in experimentally infected animals, before the appearance of clinical signs of disease. Primary shrimp cell cultures were also used for the detection of both viruses. These protocols were successfully employed for the detection and isolation of both CBVand YHV from field samples.

MATERIALS AND METHODS

Preparation of anti-YHV and anti-CBV antibodies

Polyclonal antibody against YHV and CBVwere prepared in New Zealand white rabbits (7-8 lbs) using purified virus as antigen. Immunoglobulin G was purified from the antisera using recombinant bacterial protein-G columns (Gamma-Bindä). In order to remove antibodies that cross-reacted with normal shrimp antigens, the IgG was adsorbed onto acetone-dried, ground shrimp muscle tissue as well as shrimp hemolymph.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done according to the method of Laemmli (1970). Briefly, samples were suspended in loading buffer (0.5 mM Tris-Cl, pH 6.8, 2.5 ml; 10% sodium dodecyl sulfate, 4 ml; glycerol, 2 ml; ß-mercaptoethanol, 1 ml; and deionized distilled water, 0.5 ml), boiled at 95°C for 5 min, loaded into the wells of 5% (YHV) and 10% (CBV) SDS-PAGE, and electrophoresed at 200 V.

Western blotting

The electrophoresed gel was blotted onto a nitrocellulose membrane (pore size, 0.1 mm) in blotting buffer (3.03g

Loh PC, Cesar E, Nadala BJr., Tapay LM, Lu Y (1998) Recent developments in immunologically-based and cell culture protocols for the specific detection of shrimp viral pathogens. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Tris base, 14.4g glycine, & 200ml methanol per liter) at 100 V for 1 hr. The membrane was then rinsed in PBS (pH 7.4), soaked in 5% skim milk (in PBS) for 1 hr, and rinsed in PBS for 5 min. The membrane was treated with 1:1000 dilution of the primary antibody (either a polyclonal hyperimmune anti-YHV IgG or anti-CBV IgG) for 1 hr, rinsed 3X with PBS for 5 min, and then treated with 1:2500 dilution of the secondary antibody (goat anti-rabbit IgG-horseradish peroxidase conjugate [Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD]) for 1 hr. The membrane was again rinsed 3X with PBS for 5 min and then treated with the substrate (3,3',5,5'-tetramethylbenzidine or TMB [Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD]) until a bluish purple color developed. The reaction was stopped by soaking the membrane in distilled water. All of the incubations were done at room temperature (r.t., 25°C ± 2°C) unless specified otherwise.

Time-course infectivity experiments in whole animals

Ten *Penaeus vannamei* (40-60 gram) were injected intramuscularly in the second segment of the abdomen with either 5% w/v of head soft tissues from CBV -infected shrimp or 10% w/v head soft tissues from YHV-infected shrimp at 0.2 ml per shrimp. At specific periods after injection, 0.1 ml hemolymph was collected from the hemocoel of at least 2 randomly selected shrimps using a 1 ml syringe with 26G needle. The hemolymph were diluted in equal volume of 20% citrate buffer and then stored at -80°C until needed.

For Western blotting, 200 µl aliquots of hemolymph samples were clarified at 8,000 x g for 5 min and then pelleted at 140,000 x g for 5 min. The pellets were resuspended in 100 ml 2x loading buffer and heated to 95°C for 5 min. Only 10 µl of the treated samples were loaded per well of the SDS-PAGE gel.

Time-course infectivity experiments in primary shrimp lymphoid cell cultures

Forty-eight hr primary shrimp lymphoid cell cultures in 25 cm^2 Primaria™ flasks were inoculated with 0.5 ml of 100-fold dilution of CBV or YHV filtrate (clarified and filtered 10% w/v virus-infected head soft tissues). Adsorption was carried out at r.t. for 1.5 hr after which the excess filtrate was removed and replaced with 5 ml of growth medium (2X Leibovitz Medium-15 supplemented with 8% shrimp head extract, 5% fetal bovine serum, 6 ml salt solution, 100 I.U./100 mg/ml penicillin/streptomycin). The flasks were incubated at r.t. and were sampled at various days post-infection (p.i.) by freezing at -80°C. An uninoculated flask was frozen at 7 days and used as control. The flasks were then thawed and collected into each of sterile conical tubes for centrifugation at 1800 x g for 15 min at 5°C. The supernatant was stored at -80°C until needed.

For western blotting, the samples were clarified by centrifuging 200 µl aliquots at 8,000 x g for 5 min and then pelleting them at 140,000 x g for 5 min. The pellets were resuspended in 20 µl 2x loading buffer and heated to 95°C for 5 min. All 20 µl of the treated samples were loaded in the well of the SDS-PAGE gel.

RESULTS

Detection of viral proteins by NC-EIA

The nitrocellulose-enzyme immunoassay (NC-EIA) protocol when used for the detection of viral proteins was found to have a sensitivity of 0.4 ng for YHV and 1 ng for CBV. The polyclonal antisera used also exhibited strong cross-reactions with hemolymph and normal shrimp tissues. Such cross-reactivities however, could be significantly reduced by prior extensive adsorption with hemolymph. Since residual cross-reactivity could not be completely eliminated, the adsorbed antisera for both YHV and CBV were then employed in the Western blot format where virus-specific proteins were clearly dilineated from normal hemolymph and tissue proteins.

Detection of YHV proteins in the hemolymph and in primary cell cultures

Protein analysis of purified virus preparations by SDS-PAGE revealed four major bands with the following estimated molecular sizes (kDa): 175, 135, 67 and 22 (Tapay *et al*. 1997). These bands probably corresponded to the large (L), glycoprotein (G), nucleocapsid (N) and matrix (M) proteins of rhabdoviruses. Western blot analysis of these bands using polyclonal anti-YHV IgG showed strong reactivity of the antibody with the putative G protein band, as well as the L and N bands.

Western blot analysis of YHV-containing hemolymph samples obtained from experimentally-infected shrimp showed that, despite some cross-reaction with normal hemolymph proteins, the putative G protein (135kDa) could be specifically distinguished. Figure 1 shows that the viral protein first became detectable in the hemolymph 56 hours p.i.. Overt symptoms in infected animals were not observed until 72 hours p.i.

Western blot analysis of YHV-infected primary lymphoid cell cultures had similar results. The putative G protein of the virus was easily distinguished from background staining, and was detected as early as 4 days p.i. (Fig. 2).

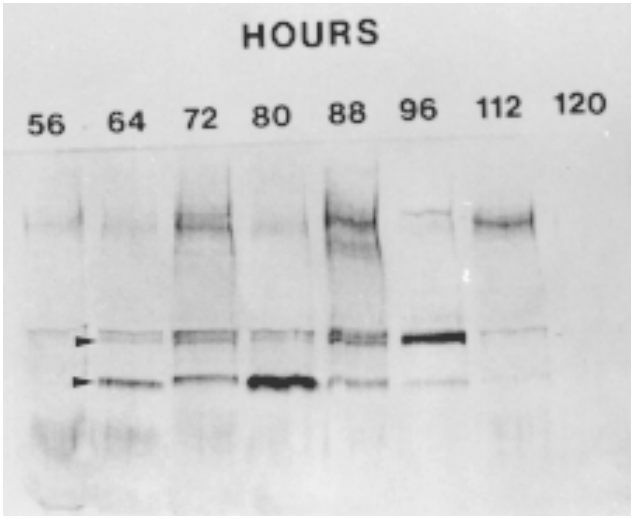


Figure 1. Western blot of YHV proteins (135 & 170 kDa, arrowheads) in hemolymph samples collected at various periods from shrimp (*P. vannamei*) experimentally infected with YHV.

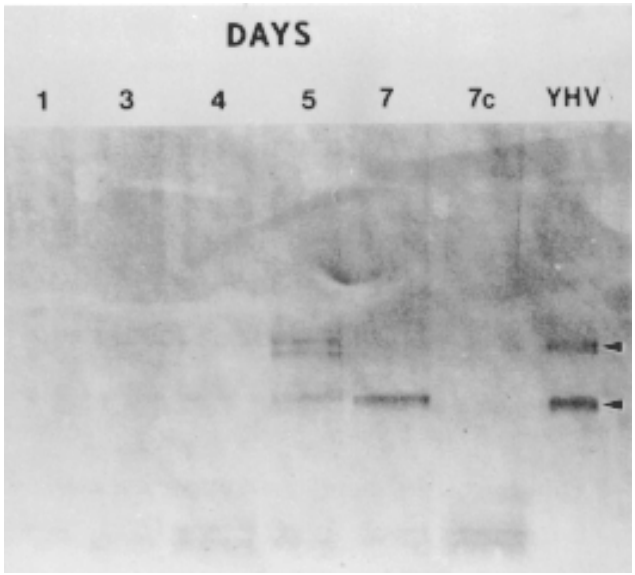


Figure 2. Western blot of YHV proteins (135 & 170 kDa, arrowheads) in cell culture lysates collected at various periods from primary shrimp lymphoid cell cultures infected with YHV. 7c = control uninfected cell culture lysate after 7 days.

Detection of CBVproteins in the hemolymph and in primary cell cultures

SDS-PAGE analysis of purified CBVrevealed, among several protein bands, three prominent bands with the following molecular sizes (kDa): 19, 23.5 and 27.5 (doublet band). The same bands were observed in Western blots of purified CBVusing polyclonal anti-CBVIgG. Preliminary studies indicate that the 27.5 and 19 kDa protein bands are associated with the envelope component of the virus. The 23.5 kDa protein is identified with the capsid structure (Nadala and Loh, 1998).

Western blot analysis of CBV-containing hemolymph and gill samples obtained from time-course experiments using experimentally-infected shrimps showed the appearance of the three prominent CBV-nucleocapsid bands 43 hours p.i. and 41 hours p.i., respectively (Fig. 3). The infected animals did not show overt symptoms of disease until 72 hours p.i..

Western blot analysis of CBV-infected primary lymphoid cell cultures showed similar results. The three prominent bands of the virus were easily visible as early as 4 days post-infection (Fig. 4).

Field Studies

Table 1 shows some of the results obtained when random samples of cultured shrimp from various hatcheries and farms in the Pacific, S.E. Asia and the U.S. were examined for CBV and YHV by the WB and NC-EIA methodologies.

The limited samples from the Pacific (New Caledonia, Tahiti), examined thus far were negative for both viral pathogens. On two occasions, samples from Indonesia and the U.S. were positive for CBV. The recent occurrence of CBVin the U.S. has aroused concern, particularly since this is the second incident reported and also involved a new species of penaeid shrimp indigenous to the east coast of north and central Americas (*P. setiferus*). On one occasion the animals were positive by WB for YHV.

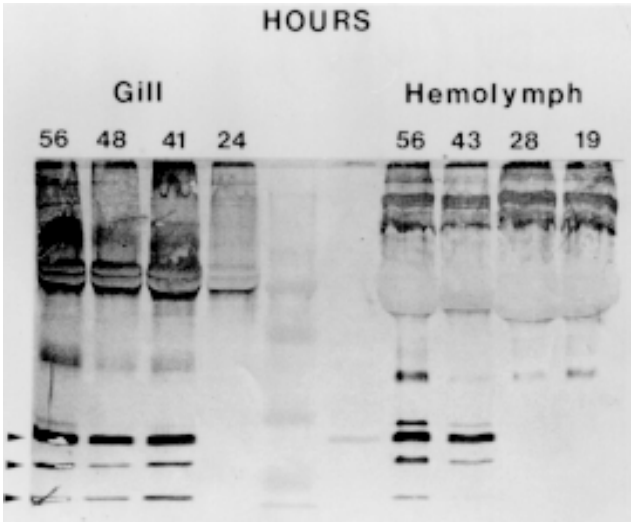


Fig. 3. Western blot of CBVproteins (arrowheads) in gill and hemolymph samples collected at various periods from shrimp (*P. vannamei*) experimentally infected with CBV.

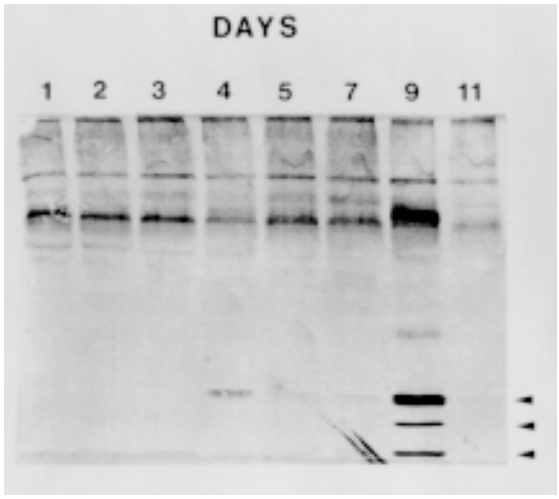


Fig. 4. Western blot of CBVproteins (arrowheads) in cell culture lystes collected at various periods from primary shrimp lymphoid cell cultures infected with CBV.

Table 1. YHV and CBVdetection results for different shrimp farms.

Farm Location	Sampling Date	YHV	CBV
US	9/23/96	-	-
New Caledonia	11/15/96	-	-
Tahiti	11/23/96	-	-
Indonesia	11/8/96	-	-
	12/4/96	-	-
	1/25/97	-	+
US	3/25/97	-	-
	5/6/97	-	+
	5/23/97	+	-

DISCUSSION

The production of high titer, specific anti-CBV and anti-YHV polyclonal sera enabled the development of a Western blot protocol which could detect two highly virulent and infectious viral pathogens. The protocol was able to demon-

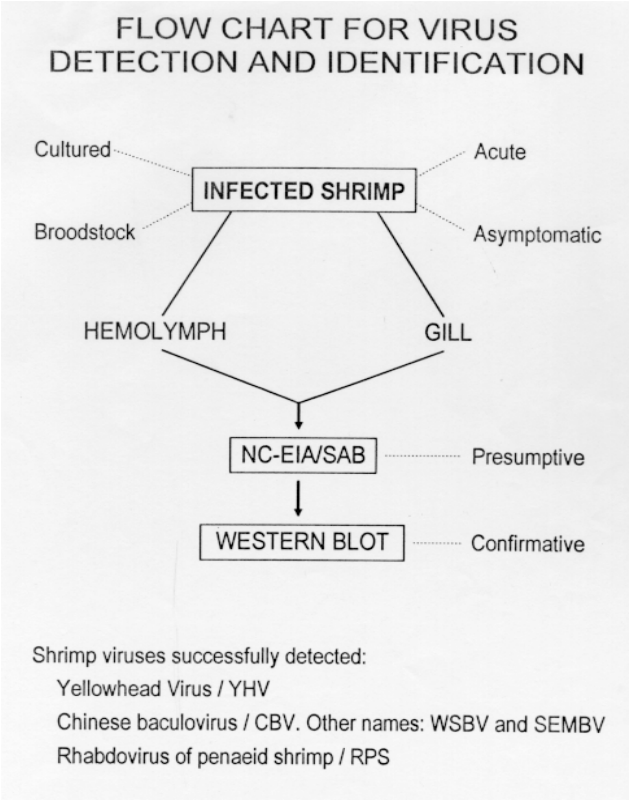


Fig. 5. Flow chart for penaeid shrimp virus detection.

strate the presence of specific viral proteins in the hemolymph of experimentally-infected animals as early as 43 hours post-injection and at least one day before clinical signs of the disease appeared.

The use of the hemolymph as material for detection of the virus allows for a method of sample collection that does not require the sacrifice of the animal or any part of the animal. This is particularly important when monitoring highly valuable broodstock populations for these pathogens. If necessary, both the gill tissues and lymphoid (Oka) organ may serve as highly satisfactory alternate sources of viral antigen.

The Western blot protocol also enabled us to detect specific viral proteins in primary lymphoid cell cultures inoculated with either YHV- or CBV-containing filtrates. This verified previous data showing that both viruses were able to replicate and cause CPE in primary lymphoid cell cultures (Tapay *et al*., 1996).

Immunological as well as nucleic acid hybridization assays often suffer from poor specificity, especially when using tissue extracts or biological fluids as samples. This is mainly due to either cross reactions with non-target proteins (EIA) or nucleic acids (hybridizations) or simply non-specific molecular interactions. The Western blot protocol has a distinct advantage over these other detection protocols in that it separates antigens on the basis of size, in addition to the specific antigen-antibody interaction. Therefore, a stricter criterion is applied in the identification of positive samples. This makes the Western blot protocol a good confirmatory assay for the less specific but more convenient enzyme immunoassay protocols.

The early detection of CBVand YHV, especially in asymptomatic latent carriers among shrimp populations, would forewarn shrimp farmers not only of the presence of the infectious agents but also of imminent potential disease outbreaks. This would allow for implementation of contingency management stategies that would prevent or at least minimize losses. Furthermore, a rapid, sensitive and specific detection protocol would enable quarantine officials to restrict importation of infected animals, especially into geographic areas free of them.

Both the NC-EIA and WB detection protocols are currently being evaluated in field studies of shrimp samples obtained from various hatcheries and farms in S.E. Asia and the U.S.A. The initial results indicated that the protocols can yield highly reliable results when used in a combined format. The less specific but more convenient NC-EIA served as a presumptive test to be followed by the WB as a confirmatory assay (Fig. 5). Thus far, the results have been highly accurate and reproducible.

*Acknowledgments***.** This study was supported in part by grants from the University of Hawaii Sea Grant and College Program, Institutional Grant No. NA36RG0507, UNIHI-SEAGRANT-CP-98-11, and the Aquaculture Development Program, Department of Land and Natural Resources, State of Hawaii, Contract No. 38066.

LITERATURE CITED

Chang PS, Wang YC, Lo CF, Kou GH (1996) Infection of white spot syndrome associated non-occluded baculovirus in cultured and wild shrimps. Second International Conference on the Culture of Penaeid Prawns and Shrimps, SEAFDEC, Iloilo City, Philippines, May 14-17, 1996

Durand S, Lightner DV, Nunan LM, Redman RM, Mari J, Bonami JR (1996) Application of gene probes as diagnostic tools for white spot baculovirus (WSBV) of penaeid shrimp. Dis Aquat Org 27:59-66

Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680-685

Lo CF, Leu J-H, Ho C-H, Chen C-H, Peng S-E, Chen Y-T, Chou C-M, Yeh P-Y, Huang C-J, Chou H-Y, Wang C-H, Kou G-H (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25: 133-141

Lu Y, Tapay LM, Loh PC (1996) Development of a nitrocellulose-enzyme immunoassay (NC-EIA) for the detection of yellowhead virus (YHV) from penaeid shrimp. J Fish Dis 19:9-13

Lu Y, Tapay LM, Loh PC, Brock JA, Gose R (1997) The pathogenicity of a baculo-like virus isolate from diseased penaeid shrimp obtained from China for cultured penaeid species in Hawaii. Aquaculture Internatl 5:277-282

Mari J, Bonami RJ, Lightner DV (1993) Partial cloning of infectious hypodermal and haematopoietic necrosis virus, an unusual parvovirus pathogen of penaeid shrimps; diagnosis of the disease using a specific probe. J Gen Virol 74: 2637-2643

Mari J , Lightner DV, Poulos BT, Bonami JR (1995) Partial cloning of the genome of an unusual shrimp parvovirus (HPV): use of gene probes in disease diagnosis. Dis Aquat Org 22: 129-134

Nadala ECB Jr, Lu Y, Loh PC, Brock JA (1992) Infection of *Penaeus stylirostris* with a rhabdovirus isolated from *Penaeus* spp. Fish Pathol 27: 143-147

Nadala ECB Jr, Tapay LM, Loh PC (1998) Characterization of a non-occluded baculovirus-like agent pathogenic to penaeid shrimp. Dis Aquat Org, Accepted for publication

Poulos BT, Mari J, Bonami JR, Redman RM, Lightner DV (1994) Use of non-radioactively labeled DNA probes for the detection of a baculovirus from *Penaeus monodon* by *in situ* hybridization on fixed tissue. J Virol Meth 49: 187-194

Tapay LM, Lu Y, Gose RB, Nadala ECB Jr, Brock JA, Loh PC (1997) Development of an *in vitro* quantal assay in primary cell cultures for a non-occluded baculo-like virus of penaeid shrimp. J Virol Meth 64:37-41

Tapay LM, Nadala ECB Jr, Cao S, Loh PC (1997) The specific detection of shrimp viral pathogens by Western blot. Oceanology Internatl 97-Pacific Rim 2:35-44

Genome Organization and Detection of Hepatopancreatic Parvovirus (HPV) from *Penaeus monodon* in Thailand

[1]Wasana Sukhumsirichart, [1]Chainarong Wongteerasupaya, [2]Vichai Boonsaeng
[2]Sakol Panyim, [3]Siriporn Sriurairatana
[4]Boonsirm Withyachumnarnkul, [5]T.W. Flegel

[1]Dept. Biochemistry, Faculty of Medicine, Srinakharinwirot University, Sukhumvit 23, Bangkok 10110, Thailand
[2]Dept. Biochemistry, [4]Dept. Anatomy, [5]Dept. Biotechnology, Faculty of Science, Mahidol University
Rama VI Road, Bangkok 10400, Thailand
[3]Dept. Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol Univerisyt
Rama VI Road, Bangkok 10400, Thailand

ABSTRACT: Hepatopancreatic parvovirus (HPV) causes disease in several species of penaeid shrimp. Heavy infections may rresult in poor growth and consequently reduce shrimp production. To characterize HPV genome organization and to develop detection methods for it in *P. monodon*, approximately 900 shrimp individuals were randomly sampled from a growout pond in which 30% of the shrimp were infected with the virus. HPV was charaterized by normal histology and by ultrastructure before it was purified by urografin gradient ultracentrifugation from hepatopancreatic homgenates. Nucleic acid was extracted and characterized. A genomic HPV DNA library was constructed and clones containing HPV-specific fragments were selected for sequencing. Based on one sequence, a specific primer set was designed for *in vitro* amplification of HPV DNA by PCR. The nucleotide sequence of HPV genomic DNA was determined and its genome organization was then analyzed. Negative staining of the purified HPV showed unenveloped, icosahedral viral particles 22-24 nm in diameter. The designed HPV-specific primers amplified an expected 156 bp product only in the presence of HPV DNA and they were able to detect at least 1 fg of purified DNA. No fragment was obtained using nucleic acid templates extracted from healthy *P. monodon* and other shrimp pathogens. This PCR assay is now ready for use as a rapid, specific, and sensitive method for diagnosis of HPV infection and for screening of suspected carriers.

KEY WORDS: Hepatopancreatic parvovirus, *Penaeus monodon*, PCR, genome, DNA

Sukhumsirichart W, Wongteerasupaya C, Boonsaeng V, Panyim S, Sriurairatana S, Withyachumnarnkul B, Flegel TW (1998) Genome organization and detection of hepatopancreatic parvovirus (HPV) from *Penaeus monodon* in Thailand. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

The Emergence of Yellow Head-Related Viruses in Australia

Peter J. Walker, Jeff A. Cowley, Kirsten M. Spann, Christine M. Dimmock

CRC for Aquaculture, CSIRO Tropical Agriculture, PMB 3, Indooroopilly 4068, Australia

ABSTRACT: Gill-associated virus (GAV) is a rod-shaped-enveloped virus which has been responsible for successive outbreaks of disease and associated mass mortalities in juvenile *P. monodon* farmed along the east coast of Queensland. Morphologically, GAV is similar to YHV from Thailand and to lymphoid organ virus (LOV) which is common in healthy *P. monodon* in Australia. Natural or experimental GAV infection causes lethargy, loss of appetite, red body colouration, erratic swimming and characteristic histopathology in the lymphoid organ and gills. All 4 species of farmed penaeid species in Australia are susceptible to experimental GAV infection, but *P. japonicus* displays an age/size-related resistance. Experimental transmission has been possible only by injection or by feeding on moribund prawns. Sequence analysis of the GAV genome has indicated that it is a (+) RNA virus with features characteristic of arteri-, toro- and coronaviruses. These include: a 7941 nucleotide ORF which encodes a 1b polyprotein containing characteristic polymerase, helicase and zinc finger motifs; an overlapping ORF which encodes an 1a-like polyprotein containing a 3C-like protease domain; a ribosomal frameshift site and associated stem-loop pseudoknot structure in the ORF1a-1b overlap; conserved intergenic sequences; and a putative S glycoprotein gene. In conjunction with colleagues at Mahidol University, PCR primers prepared from the GAV polymerase gene were used to amplify the corresponding region of YHV. Sequence analysis of the 618 bp product indicated that the viruses are closely related but distinct, sharing approximately 85% nucleotide and 96% amino acid sequence identity. GAV and YHV are likely to be classified as the first invertebrate viruses in the Order *Nidovirales*. GAV PCR primers were also used to screen wild and farmed crustaceans from various locations in Queensland. Although 96% of healthy *P. monodon* tested positive for LOV, there was no evidence of natural infection in any other species. Sequence analysis of the PCR products from a range of LOV and GAV isolates has indicated that LOV is a non-pathogenic variant of GAV. It is proposed that GAV outbreaks occur as a result of simple mutation in the LOV genome which results in dramatically more aggressive gill histopathology and subsequent gill invasion. This will have important implications for the implementation of disease control and prevention strategies.

KEY WORDS: *Penaeus monodon*, *Penaeus japonicus*, yellow head virus, gill associated virus, lymphoid organ virus, *Nidovirales*, (+) RNA virus

Walker PJ, Cowley JA, Spann KM, Dimmock CM (1998) The emergence of yellow head-related viruses in Australia. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Multiplex PCR for Detection of Yellow-Head Virus and White Spot Syndrome Virus in *Penaeus monodon*

[1]Chainarong Wongteerasupaya, [3]Vichai Boonsaeng
[2]Wansika Tongchuea, [2]Nusra Sitidilokratana, [3]Panan Kanchanaphum
[3]Ratchanee Klinputsorn, [3]Sakol Panyim

[1]Dept of Biochemistry, Faculty of Medicine, Srinakharinwirot University
Soi 23 Sukhumvit Road, Bangkok 10110, Thailand
[2]National Center for Genetic Engineering and Biotechnology, Rama 6 Road, Bangkok 10400, Thailand,
[3]Dept of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand

ABSTRACT: The polymerase chain reaction (PCR) applied in diagnostic and epidemiologic investigations has the very useful advantages of sensitivity, specificity and rapidity. We have developed a method for the simultaneous detection of both genomic RNA and DNA for two different species of shrimp viruses, yellow-head virus (YHV) and white-spot syndrome virus (WSSV), which are currently the cause of very serious and widespread losses in the shrimp industry in Thailand and in Asia. We describe a method which allows rapid diagnosis by performing a single step of extraction for both RNA and DNA using Trizol™ reagent. The co-extract was then used for successful nucleic acid amplification in a multiplex RT-PCR reaction employing the SuperScript One-Step RT-PCR process with primers 10F-144R for YHV and primers WSV4.2F-WSV4.2R3 for WSSV. This multiplex RT-PCR assay system is convenient and sensitive and should be useful to shrimp aquaculturists for simultaneous detection of YHV and WSSV in post-larvae and broodstock of *P. monodon* and in other asymptomatic carriers.

KEY WORDS: Multiplex PCR, *Penaeus monodon*, yellow-head virus, white spot syndrome virus

Wongteerasupaya C, Boonsaeng V, Tongchuea W, Sitidilokratana N, Kanchanaphum P, Klinputsorn R, Panyim S (1998) Multiplex PCR for detection of yellow-head virus and white spot syndrome virus in *Penaeus monodon*. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Tissue distribution of white spot syndrome virus (WSSV) in shrimp and crabs

***Guang-Hsiung Kou, Shao-En Peng, Ya-Lin Chiu, Chu-Fang Lo**

Department of Zoology, National Taiwan University, Taipei, Taiwan, ROC

ABSTRACT: White spot syndrome virus (WSSV) has caused mass mortalities of cultured shrimp and crab. In this study, polymerase chain reaction (PCR) was used to determine the pattern of WSSV virus multiplication in tissues of shrimp and crabs. Tested specimens were divided into three groups based on WSSV PCR results. Group I comprised specimens whose tissues were all 2-step WSSV PCR negative; Group II comprised lightly infected specimens which had at least some tissues positive after re-amplification; Group III comprised heavily infected specimens whose tissues tested mostly 1-step WSSV PCR positive. In very lightly infected specimens (Group II), WSSV was particularly prevalent in gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, stomach, pleopods, heart, midgut, integument, pereiopods, eyestalk and the hepatopancreas. (This order, which is based on a larger sample size, is slightly different to the order reported in our previous paper). Also, because of a problem with false negatives, WSSV is likely to be more prevalent in the eyestalk than its position in this list would imply. WSSV tissue distribution in crabs, *Charybdis feriatus*, *Charybdis natator*, *Portunus pelagicus*, and *Portunus sanguinolentus*, was also investigated. Almost all the tested tissues or organs of the heavily infected crabs (Group III) were one-step PCR positive, while tissues or organs of the lightly infected crabs were positive only after re-amplification. In lightly infected specimens the prevalence of WSSV was particularly high in the gills, pereiopods and hemolymph, followed in order of decreasing prevalence by the stomach, eyestalks, maxillipeds, heart, integument, reproductive organs, midgut, abdominal muscle, nervous tissue and hepatopancreas. We therefore suggest that the best sources for PCR template preparation during non-destructive screening of asymptomatic carrier brooders, would be (a small piece of) the gills or (a small aliquot of) the hemolymph. There is also some evidence to suggest that an ablated eyestalk might be a good alternative, provided that the compound eye is removed before use.

KEY WORDS: WSSV in shrimp and crabs; tissue distribution; PCR

INTRODUCTION

White spot syndrome (WSS) is one of the most important shrimp diseases. It is of global occurrence and it affects most of the commercially cultured shrimp species (Inouye et al. 1994, Cai et al. 1995, Chou et al. 1995, Lightner 1996, Flegal 1997, Lotz 1997, Spann & Lester 1997). The clinical signs of this disease include white spots in the exoskeleton and epidermis, lethargy, a pink to reddish-brown coloration, the gathering of affected shrimp around the edges of ponds throughout the day and a rapid reduction in food consumption. The causative agent of WSS is an enveloped, non-occluded, rod-shaped DNA virus known as white spot syndrome virus (WSSV) (Wang et al. 1995, Lightner 1996). WSSV is also referred to as white spot syndrome baculovirus (WSBV) (Lo et al. 1996a, 1996b, 1997), and it is apparently identical or closely related to penaeid rod-shaped DNA virus (PRDV) (Inouye et al. 1994, 1996), hypodermal and hematopoietic necrosis baculovirus (HHNBV) (Cai et al. 1995), and systemic ectodermal and mesodermal baculovirus (SEMBV) or white spot virus (WSV) (Wongteerasupaya et al. 1995, 1996). Because these viral agents appear to be very similar in morphology, histopathology and genome structure (Lo et al. 1998), they have been regrouped and are now often collectively referred to as WSSV (Lightner 1996).

In cultured shrimp, WSSV infection is characterized by a wide range of target tissues, rapid disease onset and high mortality. During the viremic phase of infection, the virus is present in many organs. We previously conducted a combined study using currently available nucleic acid diagnostic tools and conventional histological observations using light (LM) and transmission electron (TEM) microscopy to examine the sites for virus multiplication in *Penaeus monodon* (black tiger shrimp). Sixteen parts excised from shrimp specimens were examined: pleopods, gills, stomach, abdominal muscle, hemolymph, gut, heart, pereiopods, lymphoid organs, epidermis, nervous tissue, hepatopancreas, testes, ovaries, spermatophores, and eye stalks. All these tissues/organs were found to support WSSV replication (Lo et al. 1997). In lightly infected specimens, WSSV was particularly prevalent in pleopods, followed in order of decreasing prevalence by gills, hemolymph, stomach, abdominal muscle, reproductive organs, midgut, heart, periopods, lymphoid organ, integument, nervous tissue, and the hepatopancreas (Lo et al. 1997). WSSV tissue distribution data from lightly infected specimens helps to suggest the most appropriate tissue source for polymerase chain reaction (PCR) testing. Thus, in the present paper, a larger sample size was used to reconfirm WSSV prevalence in pleopods, gills, hemplymph, stomach, abdominal muscle, heart, periopods and integument.

Kou GH, Peng SE, Chiu YL, Lo CF (1998) Tissue distribution of white spot syndrome virus (WSSV) in shrimp and crabs. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail:ghkou@ccms.ntu.edu.tw

Lo et al. (1996) and Lo and Kou (1998) have already used PCR, *in situ* hybridization, and transmission electron microscopy to show that WSSV positive specimens exist in captured populations of wild crabs (*Calappa philarigus*, *Charybdis feriatus*, *Charybdis natator*, *Helice tridens*, *Portunus pelagicus*, *Portunus sanguinolentus*, *Scylla serrata*) collected from the natural environment in coastal waters around southern Taiwan. In the present paper, we more fully document the WSSV prevalence in these same captured crab specimens and also investigate the virus tissue distribution in these crabs.

MATERIALS AND METHODS

Shrimp and crabs

Adult *Penaeus monodon* (black tiger shrimp) were either cultured or captured from their natural environment in the coastal waters around southern Taiwan in 1996-1997. Adult crabs *Calappa philarigus*, *Charybdis feriatus*, *Charybdis natator*, *Helice tridens*, *Portunus pelagicus*, *Portunus sanguinolentus*, *Scylla serrata* were all captured from their natural environment in the coastal waters around southern Taiwan on 18th July 1996. In these crabs, the prevalence of WSSV was first tested by WSSV diagnostic PCR using a piece of the last segment of the 5th pereiopod as the PCR template source.

WSSV tissue distribution in Shrimp and crabs

From the organs to be tested, PCR templates were prepared with proteinase K, N-cetyl N,N,N-trimethylammonium bromide (CTAB) treatments, phenol/chloroform extraction and ethanol precipitation (Lo et al. 1996a, Lo et al. 1997). For each sample, the quality of the extracted DNA was checked by PCR with a decapod 18S rRNA gene specific primer pair, before it was subjected to WSSV diagnostic PCR (Lo et al. 1996a). WSSV diagnostic PCR was performed as described previously (Lo et al. 1996b). Specimens were then divided into three groups based on WSSV PCR results. Specimens whose tissues were all two-step WSSV PCR negative were assigned to Group I; Group II comprised lightly infected specimens which had at least some tissues positive after re-amplification; Group III comprised heavily infected specimens whose tissues tested mostly one-step WSSV PCR positive. WSSV tissue distribution analysis in 5 crab specimens (three *C. feriatus* and two *P. sanguinolentus*) was also examined by *in situ* hybridization analysis (Lo et al. 1997).

RESULTS

WSSV tissue distribution in black tiger shrimp

In terms of clinical observation, all of the tested shrimp appeared to be healthy. However, of the 27 shrimp specimens, only five were 2-step PCR negative (Group I), 14 were lightly infected (Group II) and 8 were heavily infected (Group III). In lightly infected specimens (Group II), WSSV was particularly prevalent in the gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, stomach, pleopods, heart, integument, pereiopods, eyestalks and the hepatopancreas (Table 1). In Group III, WSSV prevalence in the hepatopancreas was relatively low (50%). Of the 8 specimens in Group III, only one specimen was 1-step WSSV PCR negative in abdominal muscle, integument and pereiopods (Table 2).

WSSV tissue distribution in crabs by PCR

The prevalence in several species as revealed by 2-step WSSV PCR was very high (Table 3). WSSV tissue distribution was investigated in three *Charybdis feriatus*, two *C. natator*, one *Portunus pelagicus*, and eight *P. sanguinolentus* specimens. Four of these crabs (two *P. sanguinolentus* and two *C. natator*) were 2-step WSSV PCR negative. The results for the other 10 crabs are shown in Tables 4 & 5. Almost all the tested tissues or organs of the heavily infected crabs (Group III) were one-step PCR positive (Table 5), while tissues or organs of the lightly infected crabs were positive only after re-amplification (Table 4). In lightly infected specimens, the prevalence of WSSV was particularly high in the gills, pereiopods and hemolymph, followed in order of decreasing prevalence by the stomach, eyestalk, maxillipeds, heart, integument, reproductive organs, midgut, abdominal muscle, nervous tissue and the hepatopancreas.

WSSV tissue distribution in crabs by *in situ* hybridization

Results of PCR testing of the pereiopods of the three *C. feriatus* and two *P. sanguinolentus* specimens are shown in Table 6. One of these crabs was 2-step PCR negative, two were positive only after reamplification and two were positive by 1-step PCR. After *in situ* hybridization, cells showing WSSV positive signals were observed in all of the tested organs. All sections from these crab specimens were also evaluated semiquantitatively. Signal prevalence scores of + (low), ++ (mild), +++ (moderate), ++++ (high) were defined as 1 to 10 positive cells, 11 to 100 positive cells, 101 to 1000 positive cells, and > 1000 positive cells, respectively, per 200 high power microscopic fields (´400 magnification). All the tested tissues of the two 1-step WSSV PCR positive crabs had moderate or high numbers of positive cells, while in the two crabs that were positive only after re-amplification, tissue signal prevalence scores were mostly nil, low or mild. In the infected crabs, the heart, gills and stomach tended to have the highest signal prevalence scores (Table 6).

DISCUSSION

While the results in Tables 2 and 5 are consistent, Tables 1 and 4 show both positive and negative results. In Tables 1 and 4, the shrimp and crabs were in the carrier state, while in Tables 2 and 5, they were in the transition state. It is important to note that they were not in the patent state (i.e., they had no gross signs of WSSV infection). Although the carrier state might persist for months, the transition state usually lasts for only a few hours and once a specimen becomes 1-step PCR positive, it will die within a few days at the most (Lo et al. 1998). So the transition interval is short-lived and during this time the disease/infection can progress rapidly (Lo et al. 1998).

Table 1. WSSV tissue tropism in lightly infected *Penaeus monodon* (Group II) as revealed by 2-step WSSV diagnostic PCR.

	2-step WSSV PCR									
Shrimp no.	Gill	Abdominal muscle	Hemolymph	Stomach	Pleopod	Heart	Integument	Pereiopod	Eyestalk	Hepatopancreas
1	+	-	-	-	+	-	-	-	nd	-
2	-	+	nd	-	-	-	-	+	nd	-
3	+	+	nd	+	+	+	-	-	nd	-
4	+	-	-	+	+	+	-	+	nd	-
5	+	+	+	-	+	-	-	+	nd	-
6	-	+	+	+	+	-	-	+	nd	+
7	+	-	+	+	+	+	+	-	nd	-
8	+	+	+	+	+	+	+	-	nd	-
9	+	+	+	+	+	-	+	-	nd	+
10	+	-	nd	+	-	-	+	-	+	-
11	-	+	nd	+	+	+	-	-	-	-
12	+	+	nd	-	-	+	+	+	-	-
13	+	+	nd	-	-	+	+	-	-	-
14	+	+	nd	+	-	+	+	+	+	+
Prevalence (%)	**78**	**71**	**71**	**64**	**64**	**57**	**50**	**42**	**40**	**21**

In the present study, pms146 F1/R1 and F2/R2 primer sets and the PCR reaction conditions described by Lo et al. (1996a) were utilized for WSSV diagnostic PCR. With this 2-step WSSV diagnostic PCR, it is in fact possible to detect 10-50 copies of target DNA in a PCR reaction, and the sensitivity of the 2-step amplification protocol is about 10^3 to 10^4 times greater than that of 1-step amplification alone. We conclude from the foregoing that to produce the consistent results seen in Table 2 and 5, the virus must have replicated very rapidly, by a factor greater than 10^3 (i.e., to well over the sensitivity threshold of 1-step WSSV diagnostic PCR). So with these pms146 primer sets, a 1-step positive diagnosis with clinically normal animals is a very clear indication of the transition state. It is important to note however, that

Table 2. WSSV tissue tropism in heavily infected *P. monodon* (Group III) as revealed by 1-step WSSV diagnostic PCR.

	1-step WSSV PCR								
Shrimp no.	Gill	Pleopod	Heart	Stomach	Abdominal muscle	Integument	Pereiopod	Hemolymph	Hepatopancreas
1	+	+	+	+	+	+	+	nd	-
2	+	+	+	+	+	+	+	+	-
3	+	+	+	+	+	+	+	+	-
4	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+
8	+	+	+	+	-	-	-	-	-
Prevalence (%)	**100**	**100**	**100**	**100**	**87**	**87**	**87**	**86**	**50**

Table 3. Results of two-step WSSV diagnostic PCR with *Calappa philarigus*, *Charybdis feriatus*, *Charybdis natator*, *Helice tridens*, *Portunus pelagicus*, *Portunu sanguinolentus* and *Scylla serrata* captured from their natural environment in the coastal waters around southern Taiwan.

	Prevalence of WSSV in crabs detected by PCR													
WSSV	*P. sanguinolentus*		*C. feriatus*		*P. pelagicus*		*S. serrata*		*H. tridens*		*C. natator*		*C. philarigus*	
PCR	No	%	No	%	No	%	No	%	No	%	No	%	No	%
1-step positive	28/48*	58	1/5	20	1/5	20	2/10	20	2/14	14	1/10	10	0/1	0
2-step positive	42/48	87	4/5	80	4/5	80	6/10	60	7/14	50	4/10	40	1/1	100
2-step negative	6/48	13	1/5	20	1/5	20	4/10	40	7/14	50	6/10	60	0/1	0

* Values represent the no. of crabs positive in the first and second step PCR per no. of crabs examined.

Table 4. WSSV tissue tropism in lightly infected crabs (Group II) as revealed by 2-step WSSV diagnostic PCR.

		2-step WSSV PCR														
Crab no.	Species	Pereiopod	Gill	Hemolymph	Stomach	Eyestalk	Maxilliped	Testis	Heart	Integument	Pleopod	Nerve	Midgut	Muscle	Ovary	Hepatopancreas
1	*Portunus sanguinolentus*	+	+	+	-	-	-	-	-	-	-	-	-	-		-
2	*Portunus sanguinolentus*	+	-	-	-	-	-		-	-	-	-	-	-	-	-
3	*Portunus sanguinolentus*	+	+	+	+	+	+	+	+	+	+	+	-	+		-
4	*Portunus sanguinolentus*	+	+	+	+	+	+	-	+	+	-	-	+	-		-
5	*Charybdis feriatus*	+	+	+	+	+	+	+	-	-	+	-	-	-		-
	Prevalence (%)	**100**	**80**	**80**	**60**	**60**	**60**	**50**	**40**	**40**	**40**	**20**	**20**	**20**	**0**	**0**

this is not necessarily true for other primer sets. For example, a 1-step PCR positive result with the more sensitive pms94 primer set (about 10x more sensitive and yielding a shorter amplicon) may sometimes be obtained with specimens that are still only in the carrier state (Lo et al in press, Lo & Kou unpublished data)

In very lightly infected shrimp (Group II), WSSV was particularly prevalent in gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, stomach, pleopods, heart, integument, periopods, eyestalks and the hepatopancreas (Table 1). This order, which is based on a

Table 5. WSSV tissue tropism in heavily infected crabs (Group III) as revealed by 1-step WSSV diagnostic PCR.

		1-step WSSV PCR														
Crab no.	Species	Heart	Gill	Stomach	Integument	Pleopod	Hemolymph	Nerve	Muscle	Pereiopod	Eyestalk	Maxilliped	Midgut	Hepatopancreas	Testis	Ovary
1	*Portunus sanguinolentus*	+	+	+	+	+	+	+	+	+	+	+	nd	+	+	
2	*Portunus sanguinolentus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
3	*Charybdis feriatus*	+	+	+	+	+	+	+	+	+	+	+	nd			-
4	*Charybdis feriatus*	+	+	+	+	+	-	-	-	-	-	-	-	-	-	
5	*Portunus pelagicus*	+	+	+	+	+	+	+	+	+	+	+	+			+
	Prevalence (%)	**100**	**100**	**100**	**100**	**100**	**80**	**80**	**80**	**80**	**80**	**80**	**75**	**75**	**66**	**50**

Table 6. WSSV tissue tropism in crabs *Charybdis feriatus* and *Portunus sanguinolentus* as revealed by signal prevalence scores of tissue sections after *in situ* hybridization.

Crab no.	Species	1-step WSSV PCR	2-step WSSV PCR	Heart	Signal prevalence scores: Gill	Stomach	Midgut	Hepatopancreas	Nerve	Integument	Muscle	Pereiopod	Eyestalk	Eyeball	Testis	Vas deferens
1	*Charybdis feriatus*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	*Charybdis feriatus*	-	+	+++	++++	+++	++	+	++	++	++	++	++	nd	+	+++
3	*Portunus sanguinolentus*	-	+	+	++	++	-	-	-	+	-	+	+	-	nd	nd
4	*Portunus sanguinolentus*	+	+	++++	++++	++++	++++	+++	+++	++++	+++	++++	nd	+++	+++	+++
5	*Charybdis feriatus*	+	+	++++	++++	++++	+++	+++	++++	++++	+++	+++	++++	+++	nd	nd

larger sample size, is slightly different to the order reported in our previous paper (Lo et al. 1997). However, pleopods, gills, hemolymph, stomach and abdominal muscle were still the 5 most prevalently infected organs in both studies. Thus, in the carrier state, it is in these organs that the virus most frequently appears and replicates. Also, because of a problem with false negatives (Lo et al. 1997), WSSV is likely to be more prevalent in eyestalks than its position in this list would imply. Therefore, for non-destructive screening of asymptomatic brooder carriers, we recommend that the best sources for PCR template preparation would be (a small piece of) gills or (a small aliquot of) the hemolymph. There is also some evidence to suggest that an ablated eyestalk would be a good alternative, provided that the compound eye is removed before use.

Acknowledgments. This work was supported by the Council of Agriculture under grant no. 87-BT-2.1-FID-01 (5-2). We thank Dr. I-Chiu Liao, Director General, Taiwan Fisheries Research Institute (TFRI), for his constructive suggestions. We are indebted to Mr. Paul Barlow for his helpful criticism of the manuscript.

LITERATURE CITED

Cai S, Huang J, Wang C, Song X, Sun X, Yu J, Zhang Y, Yang C (1995) Epidemiological studies on the explosive epidemic disease of prawn in 1993-1994. J. Fish. China 19: 112-117

Chou HY, Huang CY, Wang CH, Chiang HC, Lo CF (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 23:165-173

Flegel TW (1997) Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. In Y. Inui (ed) New approaches to viral diseases of aquatic animals. NRIA International Workshop proceedings. National Research Institute of Aquaculture, Nansei, Watarai, Mie 516-01, Japan. p. 167-187

Flegel TW (1997) Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World journal of microbiology & Biotechnology 13: 433-442

Inouye K, Miwa S, Oseko N, Nakano H, Kimura, T (1994) Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: Electron microscopic evidence of the causative virus. Fish Pathol. 29: 149-158 (in Japanese)

Lightner DV (ed) (1996) A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Soc, Baton Rouge, LA, Section 3.11

Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, Chen YT, Chou CM, Yeh PY, Huang CJ, Chou HY, Wang CH, Kou GH (1996 a) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25: 133-141

Lo CF, Ho CH, Peng SE, Chen CH, , Hsu HC, Chiu YL, Chang CF, Liu KF Su MS, Wang CH, Kou GH (1996 b) White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis Aquat Org 27: 215-225

Lo CF, Ho CH, Chen CH, Liu KF, Chiu YL, Yeh PY, Peng SE, Hsu HC, Liu HC, Chang CF, Su MS, Wang CH, Kou GH (1997) Detection and tissue distribution of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis Aquat Org 30: 53-72

Lo CF, Wang CH, Kou GH (1997) White spot syndrome (WSS): pathology, hosts and prevalence in captured shrimp and crabs in Taiwan. In Y. Inui (ed) New approaches to viral diseases of aquatic animals. NRIA International Workshop proceedings. National Research Institute of Aquaculture, Nansei, Watarai, Mie 516-01, Japan. p. 206-217

Lo CF, Hsu HC , Ho CH, Tsai MF , Kou GH, Lightner DV (1998) Specific genomic DNA fragment analysis of different geographical isolates of shrimp white spot syndrome associated viru Dis Aquat Org (in press)

Lotz JM (1997) Special review: Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. World journal of microbiology & Biotechnology 13: 405-413

Spann KM, Lester RJG (1997) Special topic review: Viral diseases of penaeid shrimp with particular reference to four viruses recently found in shrimp from Queensland. World journal of microbiology & Biotechnology 13: 419-426

Wang CH, Lo CF, Leu JH, Chou CM, Yeh PY, Chou HY, Tung MC, Chang CF, Su MS, Kou GH (1995) Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 23: 239-242

Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat Org 21: 69-77

Wongteerasupaya C, Wongwisansri, Boonsaeng V, Panyim S, Pratanpipat P, Nash GL, Withyachumnarnkul B, Flegel TW (1996) DNA fragment of *Penaeus monodon* baculovirus PmNOBII gives positive *in situ* hybridization with white-spot viral infections in six penaeid shrimp species. Aquaculture 143: 23-32

Primary Shrimp Cell Culture: Applications for Studying White Spot Syndrome Virus (WSSV)

[1]J. Kasornchandra, [2]S. Boonyaratpalin

[1]Marine Shrimp Research and Development Center, Pawong, Muang, Songkhla 90100, Thailand
[2]Department of Fisheries, Chatuchak, Bangkok 10900 Thailand

ABSTRACT: Primary shrimp cell culture from lymphoid organs of *Peneaus monodon* was successfully developed in our laboratory. Minced tissues of lymphiod organs were seeded and cultured in 2x Liebovitz-15 supplemented with 15% fetal bovine serum, 10% shrimp meat extract, and a salt mixture, with an osmolarity of 710-730 mmol/kg. Plates were then incubated at 28°C until 70-80% cell monolayers were formed. Even though these primary lymphoid cell cultures could not be subcultured, they proved to be useful for studying shrimp viruses which could induce unequivocal cytopathology in them. Cytopathic effect (CPE) induced by white spot syndrome virus (WSSV) showed characteristics of cell rounding, detachment and lysis. Quantitative analysis of WSSV infected *P. monodon* was determined using primary lymphoid cell systems. Among those tissues and organs observed, cuticular epidermis gave the highest titer of $10^{8.25}$ $TCID_{50}$/ml. Pathogenesis of shrimp viral infections and the relative virulence of WSSV infecting various species of crustaceans were also examined using this primary shrimp cell culture system.

KEY WORDS: primary shrimp cell culture, white spot syndrome virus (WSSV), *P. monodon*

INTRODUCTION

Disastrous failures have occurred in the shrimp farming industry in Thailand in the past decade mostly due to virus infection. One of the most serious viruses (known in Thailand as systemic ectodermal and mesodermal baculovirus or SEMBV) causes reddish body discoloration and white spots or patches on the inside surface of the carapace and shell of affected shrimp (Kasornchandra et al. 1995; Wongteerasupaya et al. 1995). Similar viruses with similar disease symptoms have now been reported elsewhere throughout Asia (Flegel 1997). This virus has been called white spot syndrome baculovirus (WSSV) by Lightner (1996) and that abbreviation is now widely used but to stand for white spot syndrome virus (i.e., the "baculo" prefix is usually dropped since the relationship of the virus to baculoviruses is now widely questioned). Therefore, in this paper we will use WSSV instead of SEMBV. This virus is a DNA virus of bacilliform to cylindrical morphology with an average size of 120x275±22 nm (Kasornchandra et al. 1995; Wongteerasupaya et al. 1995). Characterization of this virus is based on histological observations, electron microscopy and molecular studies. However, to study its infectivity, one has to rely on *in vivo* bioassay which requires large numbers of animals. Although much new information is available for this virus, many issues remain to be examined, and especially those relating to interaction between this virus and its host. These studies are difficult due to the lack of shrimp cell lines for production of the virus and for *in vitro* studies. Although numerous attempts have been made, no marine shrimp cell line has yet been established. Recently, the successful development of primary shrimp cell cultures has been reported from many laboratories (Chen et al. 1986, Hsu et al. 1995, Luedeman & Lightner 1992, Nadala et al. 1993, Toulled et al. 1996). Lu et al. (1995b) and Tapay et al. (1997) developed an *in vitro* quantal assay for YHV and China Baculo-like virus (CBV) using primary lymphoid cells of the white shrimp *Penaeus vannamei* and *P. stylirostris*.

In this present study, we prepared primary shrimp cell cultures derived from the lymphoid organ of *P. monodon* and we also demonstrated the use of these primary shrimp cell cultures for propagation of WSSV, for a viral titration assay, for determining the WSSV tissue and organ specificity in *P. monodon*, and for determining the relative virulence of WSSV to various species of crustaceans.

MATERIALS AND METHODS

Primary cell culture preparation

Primary culture of shrimp lymphoid cells are prepared according to the method of Kasornchandra et al. (1998) with slight modifications. Briefly, lymphoid organs were obtained from adult black tiger shrimp *P. monodon* (40-50 g), washed three times in cold antibiotic-buffered mixture consisting of 1,000 IU/ml penicillin, 1,000 µg/ml streptomycin, 250 µg/ml gentamicin, 250 µg/ml Fungizone, and 1M phosphate buffered saline (PBS). After a final wash and further incubation for 10 min in cold antibiotic-buffered solution, the lymphoid organs were then minced to small fragments of approximately 1 mm^3. Four or five tissue fragments were transferred to each well in 24-well plates and 1 ml of the culture medium containing 2x L-15 supplemented with 15% FCS, 10% shrimp meat extract (SME), 1% glucose, 5 g/L NaCl, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml

Kasornchandra J, Boonyaratpalin S (1998) Primary shrimp cell culture: Applications for studying white spot syndrome virus (WSSV). *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Fungizone, osmolarity 720±20 mmol/kg, was added to each well. Plate was then sealed and incubated at 28°C until 70-80% confluent monolayers were formed. These cells were then ready for propagation of the virus. Before propagation of the virus, 0.1 ml of medium in each well was replaced with fresh culture mediim containing 5% FCS. The plate was then ready for use.

Propagation of WSSV

Primary shrimp cell cultures were examined for thier ability to propagate WSSV. Viral suspension was prepared as described by Boonyaratpalin *et al*. (1993) with slight modifications. Briefly, 10% (w/v) gill tissue of *P. monodon* experimentally infected with WSSV was homogenized in 2xL-15 medium and filtered through a 0.2 µm sterile membrane. The virus suspension was then diluted 100 times in 2xL-15 medium and 10 µl of the diluted suspension was inoculated onto primary shrimp cell cultures. Control wells were inoculated with an extract of normal gill tissue prepared in the same manner. The inoculated plates were incubated at 28°C and observed daily for cytopathic effect (CPE). To confirm viral propagation in the shrimp cells, they were fixed with 6.25% glutaraldehyde in cacodylate buffer pH 7.2 and processed for examination with the electron microscopic.

Titration of WSSV

The viral titer was determined in primary lymphoid cell cultures by end-point dilution assay ($TCID_{50}$/ml) according to the method of Rovozzo and Burke (1973). Primary shrimp lymphiod cells were prepared in 96-well plates as described by Lu et al. (1996). Briefly, two to three tissue fragments were seeded in wells of a 96-well plate and a drop of culture medium was added to each well. The plate was then sealed and incubated at 28°C until 70-80% confluent monolayers were formed. The cells were then ready for virus titration. A day before performing the quantitative bioassay, 0.1 ml of medium in each well was exchanged with fresh culture medium containing 10% FCS and the plate was then incubated at 28°C until used. Viral suspension was prepared as previously mentioned. The 10-fold diluted WSSV suspension was diluted serially in 2x L-15 medium, and 0.1 ml of each dilution (10^{-2}-10^{-8}) was then inoculated into 3 wells of a confluent lymphoid cells monolayer. Control wells were inoculated with the same amount of normal gill tissue extract in inoculation medium. The plate was then sealed and incubated at 28°C for 7 days and examined daily for cytopathic effect (CPE). Dilution end-points were calculated by the method of Reed and Muench (1938).

Pathogenesis of WSSV infected shrimp

Specificity of tissues and organs of *P. monodon* for WSSV in experimentally infected shrimp was determined using primary lymphoid cells. WSSV suspension was prepared from gills, lymphoid organs, cuticular epidermis, hepatopancreas, heart, hemolymph, eyestalks, muscle and perieopods of infected animals as previously described. A bacterial-free suspension of these homogenated WSSV infected tissues and organs (0.1 ml) was added separately to sterile test tubes containing 0.9 ml 2xL-15 medium, and then serially diluted 10-fold. A portion (0.1 ml) of each dilution was added to tissue culture wells (3 wells each) followed by incubation for 10 days at 28°C to determine the viral titer.

Virulence of WSSV to various species of crustaceans

The infectivity of WSSV to various species of crustacean was conducted in aquaria at the Marine Shrimp Research and Development Center (MSRDC), Songkhla. Four species of penaeid and metapenaeid shrimp (*P. monodon, P. merguiensis, Metapenaeus ensis* and *M. brevicornis*) and three species of crabs (*Portunus pelagicus, Scylla serrata* and *Sesarma* spp.) collected from uninfected shrimp ponds and reservoirs inside the center and nearby were injected intramuscularly with $10^{6.0}$ $TCID_{50}$/ml at 0.1% body weight (ten shrimp or crabs of each species per dosage). Control shrimp and crabs were injected with L-15 basal medium. Both injected and control animals were kept separately in 30L aquaria equipped with aeration. Chlorinated sea water of 28-30 ppt salinitywas changed daily (75%). Experimental shrimp and crabs were fed 3 times daily. The animals were observed daily for clinical signs for 10 days. Dead and moribund shrimp and crabs were recorded and removed. In order to confirm WSSV infection, portions of the crustaceans were homogenated immediately after death in 2x L-15 medium. The homogenate was then filtered and inoculated onto primary shrimp cell cultures. Moribund shrimp and crabs were also fixed in Davidson's fixative and then subjected to histological preparation. At the termination of the experiment, all remaining crustaceans were subjected to histological analysis and shrimp cell culture assays.

RESULTS

Cells obtained from the lymphoid organ tissue fragments exhibited fibroblastic-like morphology by 18 h post-seeding. After 3-4 days, 70% confluent monolayers were achieved and these cells could be maintained for 8-10 days without changing medium.

Infectivity of WSSV in primary lymphoid cells showed unequivocal cytopathology in which the cells became rounded followed by detachment and lysis. (Fig. 1). The completion of cells lysis occurred within 4-5 days post-inoculation. No CPE was observed in control wells (Fig. 2). To confirm the cause of primary lymphoid cells lysis, infected cells were processed and subjected to electron microscopy. The results revealed the presence of a bacilliform virus having the same size as WSSV in nucleus of the infected cells.

Titration of the WSSV in primary lymphoid cells was $10^{8.0}$ $TCID_{50}$/ml. This titration was repeated twice and similar results were obtained. The recovery of infectious WSSV from selected tissues and organs of infected *P. monodon* is shown in Table 1. Among those tissues and organs examined, cuticular epidermis gave the highest titer of $10^{8.5}$ $TCID_{50}$/ml., followed by hemolymph and gills. Perieopods, eyestalks, muscle and heart produced moderate titers ranging from $10^{6.0}$-$10^{5.0}$ $TCID_{50}$/ml. Hepatopancreatic tissue gave the lowest titer of $10^{4.0}$ $TCID_{50}$/ml.

The infectivity of WSSV to various types of crustaceans was investigated and compared. *P. monodon* and *P. merguiensis* were the most susceptible to WSSV infection. They produced clinical symptoms after 4 days post-injec-

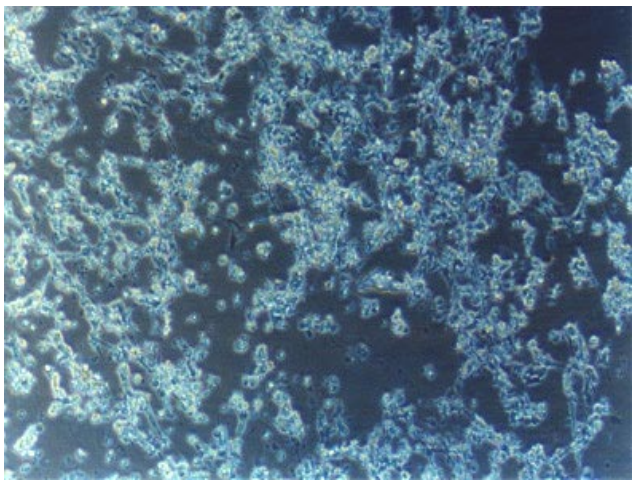


Figure 1. Primary lymphoid cell culture showing cytopathic effect (CPE) 5 days p.i. with WSSV extract from infected shrimp gill tissue.

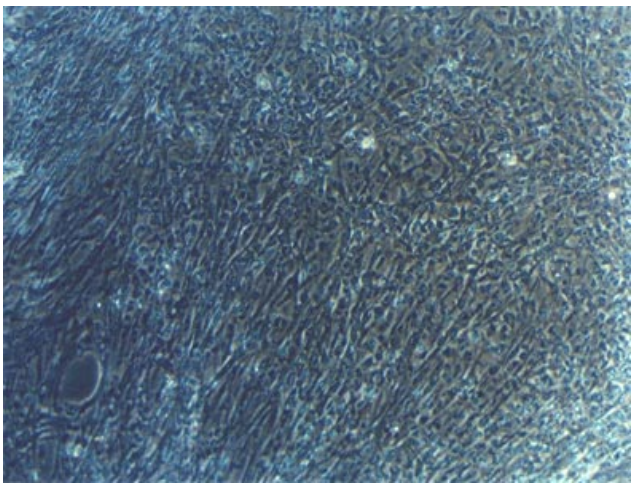


Figure 2. Primary lymphoid cell culture 5 days after inoculation with extract from normal shrimp gill tissue.

Table 1. Titer of WSSV recovered from selected tissues and organs of experimentally infected *P. monodon.*

Tissue/Organ	$TCID_{50}$/ml
Cuticular epidermis	$10^{8.25}$
Gills	$10^{7.75}$
Hemolymph	$10^{7.50}$
Lymphoid organ	$10^{7.00}$
Perieopods	$10^{6.00}$
Eye-stalk	$10^{5.50}$
Muscle	$10^{5.25}$
Heart	$10^{5.00}$
Hepatopancreas	$10^{4.00}$

tion and all shrimp died within 9 days post-injection (Table 2). *Metapenaeus ensis* and *M. brevicornis* were also susceptible to WSSV infection. However, some of these shrimp still survived 10 days post-injection (Table 2). *Portunus pelagicus, Scylla serrata* and *Sesarma* spp. were also infected by WSSV. Only a few crabs (*Portunus pelagicus* and *Scylla serrata*) died within 7 and 9 days post-injection. The others still survived 10 days post-injection (Table 2). Only one mangrove crab, *Sesarma* spp., died during this experiment. Histological examination of the moribund and surviving shrimp revealed the presence of hypertrophied nuclei in various tissues of ectodermal and mesodermal origin. The characteristics of CPE induced by their tissue extracts in primary shrimp cells were also similar to those induced by WSSV extracts as previously described. Interestingly, high numbers of hypertrophied nuclei were observed in the infected tissues of surviving crabs.

DISCUSSION

Primary shrimp cell cultures obtained from lymphoid organs of *P. monodon* were successfully developed in our laboratory. These cells can be maintained up to 10 days without changing medium. This is enough time to conduct viral titration assays. The study also demonstrated that our primary lymphoid cells supported growth of WSSV and that CPE induced by this virus exhibited cell rounding followed by detachment and lysis. Similar CPE was reported by Tapay et al. (1997) for Chinese Baculo-like virus or CBV (which produced white spot disease syndrome in China) in primary lymphoid cells derived from lymphoid organs of the white shrimp, *P. stylirostris*. Moreover the viral titer produced in their primary lymphoid cells was as high as that for our primary cells (i.e., giving a $TCID_{60}$ titer of $10^{8.0}TCID_{50}$/ml).

Table 2. Percent survival of seven species of crustaceans after experimental injection with WSSV. Number of animals in each group = 10.

Crustaceans	Days post injection									
	1	2	3	4	5	6	7	8	9	10
Penaeus monodon	100	100	100	100	80	60	20	10	0	0
P. merguiensis	100	100	100	100	100	70	50	20	0	0
Metapenaeus ensis	100	100	100	100	100	80	80	60	40	20
M. brevicornis	100	100	100	100	100	100	80	60	50	30
Portunus pelagicus	100	100	100	100	100	100	80	80	70	60
Scylla serrata	100	100	100	100	100	100	100	90	80	70
Sesarma spp.	100	100	100	100	100	100	100	100	100	90

Susceptibility of tissues and organs of *P. monodon* supporting growth of WSSV was determined in primary shrimp cells. The amount of infectious WSSV recovered was highest from cuticular epidermis followed by hemolymph and gills suggesting that those organs greatly support viral multiplication and would enhance the recovery of WSSV. A similar finding was reported by Tapay et. al. (1997) who demonstrated that cuticular epidermis of experimentally infected *P. stylirostris* was the most susceptible target for CBV multiplication, followed by gills and hemolymph. Thus, those tissues and organs seem to be the best choice for virus isolation.

Many scientists have reported that WSSV is capable of infecting various species of crustaceans including larval insects (Kasornchandra et al. 1997, Lo et al. 1997, Supamattaya et al. 1998). However, the relative virulence of this virus to the various species of crustaceans has not yet determined, due to the lack of cell lines for *in vitro* studies. We described here a method using primary shrimp cell cultures for viral quantitation to determine relative virulence of WSSV for four species of shrimp and three species of crabs. The results suggest that *P. monodon* and *P. merguiensis* are the most susceptible hosts for WSSV infection while the two species of metapenaeid shrimp, *M. brevicornis* and *M. ensis*, were moderately susceptible hosts. The shrimp survivors also showed typical characteristics of hypertrophied nuclei in infected tissues. For the three species of crabs tested (*Portunus pelagicus*, *Scylla serrata* and *Sesarma* spp.), most still survived 10 days post-injection. However, histological examination showed high levels of infection but little tissue damage in these survivors. Therefore, they may be implicated as carriers for white spot disease. Even though no continuous shrimp cell lines are available as yet, primary lymphoid cell cultures established not only in our laboratory but elsewhere have proven to be useful for studying shrimp viruses *in vitro* (Chen and Kou 1989, Lu et al. 1996a,b, Tapay et. al. 1997).

LITERATURE CITED

Boonyaratpalin S, Supamattaya K, Kasornchandra J, Direkbusaracom S, Ekpanithanpong U, Chantanachooklin C (1993) Non-occluded baculo-like virus, the causative agent of yellow head disease in the black tiger shrimp (*Penaeus monodon*). Fish Pathol 28:103-109

Chen SN, Kou GH (1989) Infection of cultured cells from the lymphoid organ of *Penaeus monodon* Fabricius by monodon-type baculovirus (MBV). J Fish Dis 12:73-76

Chen SN, Chi SC, Kou GH, Liao IC (1986) Cell culture from tissues of grass prawn, *Penaeus monodon*. Fish Pathol 21:161-166.

Flegel TW (1997) Special topic review:major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J Microbiol Biotech 13:433-442

Hsu Y-L, Yang Y-H, Chen C-Y, Tung M-C, Wu J-L, Engelking MH, Leong JC (1995) Development of an in vitro subculture system for the oka organ (Lymphoid tissue) of *Penaeus monodon*. Aquaculture 136:43-55

Kasornchandra J, Boonyaratpalin S, Khongpradit R, Ekpanithanpong U (1995) Mass mortality caused by systemic bacilliform virus in cultured penaeid shrimp, *Penaeus monodon*, in Thailand. Asian Shrimp News 5: 2-3

Kasornchandra J, Khongpradit R, Ekpanithanpong U, Boonyaratpalin S (1997) Progress in the development of shrimp cell culture in Thailand. In: the Proceeding of the Biotech Forum on Shrimp Biotechnology, BIOTECH/NSTDA, Bangkok, pp. 132-136

Lightner DV (1996) Handbook of pathology and diagnostic procedures for dieases of penaeid shrimp. World Aquaculture Soc, Baton Rouge

Lo CF, Wang CH, Kou GH (1997) White spot syndrome (WSS): pathology, hosts and prevalence in captured shrimp and craps in Taiwan. In: "NRIA International Workshop: new approaches to viral diseases of aquatic animals, Watarai, Mie, Japan pp. 206-217

Lu Y, Tapay LM, Loh PC, Brock JA, Gose RB (1995a) Distribution of yellow-head virus in selected tissues and organs of penaeid shrimp *Penaeus vannamei.* Dis Aquat Org 23:67-70

Lu Y, Tapay LM, Loh PC, Brock JA, Gose RB (1995b) Development of a quantal assay in primary shrimp cell culture for yellow head baculovirus (YBV) of penaeid shrimp. Virmet 52:231-236

Luedeman RA, Lightner DV (1992) Development of an in vitro primary cell culture system from the penaeid shrimp, *Penaeus stylirostris* and *Penaeus vannamei.* Aquaculture 101:205-211

Nadala ECB Jr, Lu Y, Loh PC (1993) Primary culture of lymphoid, nerve, and ovary cells from *Penaeus stylirostris* and *P. vannamei.* In Vitro Cell Dev Biol 29A:620-622

Reed LJ, Muench H (1938) A simple method of estimating fifty percent end points. Am J Hyg 27: 493-497

Rovozzo GC, Burke CN (1973) A manual of basic virological techniques. Prentice-Hall, Englewood Cliffs, New Jersey

Tapay LM, Lu Y, Gose RB, Nadala ECB Jr., Brock JA, LohPC 1997. Development of an in vitro quantal assay in primary cell cultures for a non-occluded baculo-like virus of penaeid shrimp. Virmet 64:37-41

Toulled JY, Crozat Y, Patrois J, Porcheron P (1996) Development of primary cell culture from the penaeid shrimps *Penaeus vannamei* and *P. indicus*. J Crustacean Biol 16:643-649

Supamattaya K, Hoffman RW, Boonyaratapalin S, Kanchanaphum P (1998) Transmission of white spot disease (bacilliform virus) from black tiger shrimp *Penaeus monodon* to portunid crab *Portunus pelagicus* and krill *Acetes* sp. Dis Aquat Org 5:79-85.

Wongteerasupaya C, Vickers JE, Sriurairatana S, Nash GL, Akarajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat Org 21:69-77

White Spot Virus Infective Properties Determined by a Single Tube Nested and Competitive PCR

Lee Kok Leong, Samson Soon, Tan Lee Tung, Mohd. Shariff Mohd. Din

Aquatic Animal Health Unit, Department of Clinical Veterinary Studies, Faculty of Veterinary Medicine Universiti Putra Malaysia, 43400 Serdang, Malaysia

ABSTRACT: Analysis of white spot syndrome virus (WSSV) infection in experimentally infected *Penaeus monodon* shrimp was conducted using a single tube nested PCR approach. Severity of infection was also evaluated by competitive quantitative PCR for relative viral burden determination. WSSV DNA was first detected after 20-24 hours post infection in muscle tissue and increased in signal intensity as assessed by semi-quantitative properties of single tube nested PCR after 32, 36, 40, 44 and 48 hours, reaching a plateau after 72 hours post infection. Significant shrimp mortalities with clinical signs of white spot disease were recorded at 72 h for all experimental tanks. Detection of single tube nested PCR signal was thus shown to correlate closely with WSSV load in the experimentally infected shrimp and to indicate the extent of viral infection in the diseased animals. Evaluation of the PCR data was further analysed using competitive PCR for relative quantitation and estimation of WSSV burden in infected shrimp muscle cells. Importance and significance of using this novel PCR approach in white spot disease research is discussed and its implication explored.

KEY WORDS: *Penaeus monodon*, PCR, white spot syndrome virus (WSSV), quantitation

Leong LK, Soon S, Tung TL, Shariff M (1998) White spot virus infective properties as determined by single tube nested and competitive PCR. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

One-step Nested PCR for Grading the Severity of White Spot Syndrome Virus Infections in *Penaeus monodon*

[1]Wansika Tongchuea, [2]Vichai Boonsaeng, [3]Chainarong Wongteerasupaya
[2]Karnyupha Jittivadhna, [4]Anchalee Tassanakajon, [2]Sakol Panyim

[1]National Center for Genetic Engineering and Biotechnology, Rama 6 Road, Bangkok 10400, Thailand
[2]Dept. of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
[3]Dept. of Biochemistry, Faculty of Medicine, Srinakharinwirot University
Sukhumvit 23 Road, Bangkok 10110, Thailand
[4]Dept. of Biochemistry, Faculty of Science, Chulalongkorn University
Phya Thai Road, Bangkok 10400, Thailand

ABSTRACT: We have developed a one-step, nested PCR technique for the detection of white-spot syndrome virus (WSSV) in *Penaeus monodon*. The new nested PCR protocol has been performed in a single tube containing 1 forward primer and 3 reverse primers. The primers produced either 3, 2 or 1 bands of nested PCR products (1100 bp, 526 bp and 250 bp) depending on the severity of WSSV infection in *P. monodon* samples. Severe infections gave 3 bands of PCR products (1100 bp, 526 bp and 250 bp) after agarose gel electrophoresis, while moderate infections gave 2 bands (1100 bp and 526 bp) and light infections 1 band (250 bp). In addition, internal control primers were included to yield a shrimp characteristic band for both infected and non-infected samples to assure integrity and reproducibility of the PCR assays. This new technique could detect as little as 0.1 fg WSSV DNA in crude postlarval samples. No PCR product was detected from the DNA of hepatopancreatic parvo like virus (HPV), nuclear polyhedrosis virus (NPV), salmonella or *E. coli*. This one-step, nested PCR assay is more simplified and more convenient than conventional two-step nested PCR assays and it gives additional information for grading the severity of WSSV infections.

KEY WORDS: *Penaeus monodon*, white spot syndrome virus, WSSV, one-step nested PCR

Tongchuea W, Boonsaeng V, Wongteerasupaya C, Jittivadhna K, Tassanakajon A, Panyim S (1998) One-step nested PCR for grading the severity of white spot syndrome virus infections in *Penaeus monodon*. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

PCR Monitoring of Cultured Shrimp for White Spot Syndrome Virus (WSSV) Infection in Growout Ponds

[1]Chu-Fang Lo, [1]Yun-Shiang Chang, [2]Chin-Te Cheng, *[1]Guang-Hsiung Kou

[1]Department of Zoology, National Taiwan University, Taipei, Taiwan, ROC
[2]Tainan City Animal Disease Diagnosis Control Center, Tainan, Taiwan, ROC

ABSTRACT: White spot syndrome (WSS) is a viral disease which affects most of the commercially cultivated marine shrimp species, not just in Asia but globally. WSS can cause up to 100% mortality, with a correspondingly devastating economic impact. So far no significant resistance to this disease has been reported for any species of shrimp. The causative agent of WSS, white spot syndrome virus (WSSV) is extremely virulent and has a wide host range. Based upon the sequence of the most conserved region of white spot syndrome virus (WSSV), a nested WSSV polymerase chain reaction (PCR) has been developed into one of the most powerful diagnostic tool available to date. It is very sensitive, being able to detect 20 copies of target DNA in a PCR reaction. In the present study, in addition to the 2-step nested WSSV PCR that we reported previously, we also used single-step nested WSSV PCR to monitor the cultured shrimp for WSSV infection during their growth period. Based on the single-step nested WSSV PCR results, WSSV infection can be divided into very severe infection (level 1), severe infection (level 2), light infection (level 3) and very light infection (level 4). In our previous paper, we classified WSSV infection into three stages, that is the asymptomatic carrier, transition and patent stages. In asymptomatic carriers, the infection was at levels 3-4, while that in transition and patent states, the infection was at levels 1-2. While the carrier stage may persist for months, under certain triggering conditions (e.g., stress), it may also progress to the transition and patent stages within a few hours to a few days. We found that shrimp at infection levels 3 and 4 were able to survive from the larval stages through till they were harvested. As expected, once the infection of the cultured shrimp became level 1-2, mortality inevitably occurred within a few days. PCR can thus predict mortality, but unfortunately by the time this prediction can be made (i.e., at the transition stage) mortality is unavoidable. The challenge at this point is to design a PCR test which will warn of an impending transition stage in time to take [stress reducing] action to prevent mass mortality.

KEY WORDS: WSSV infection in shrimp, monitoring by PCR.

INTRODUCTION

White spot syndrome (WSS) is a viral disease which affects most of the commercially cultivated marine shrimp species, not just in Asia but globally (Chou et al. 1995, Lightner 1996, Flegel 1997, Lotz 1997, Span & Lester 1997). In 1993, WSS was first observed in cultured Penaeid shrimp in Taiwan (Chou et al. 1995). Since then, WSS has become wide spread and the most economically damaging disease of cultured shrimp in Taiwan. White spots in the exoskeleton and epidermis are the most commonly observed clinical sign of WSS in diseased shrimp. However, the presence of white spots does not always mean that the condition is terminal. For instance, under non-stressful conditions, infected shrimp that have white spots may survive indefinitely. However, if the shrimp also appear: lethargic, if their color changes to pink or reddish-brown, if they gather around the edges of ponds at the surface during the day, or if there is a rapid reduction in food consumption, then a very high mortality rate in the shrimp population can be expected within a few hours to a few days of the onset of the signs. WSS can cause up to 100% mortality, with a correspondingly devastating economic impact. Lightner (1996) pointed out that no significant resistance to this disease had been reported for any species of shrimp, and this still remains true today. The causative agent of WSS, white spot syndrome virus (WSSV) is extremely virulent and has a wide host range (Lo et al. 1996b). Based upon the sequence of the most conserved region of this virus, a 2-step nested WSSV polymerase chain reaction (PCR) has been developed into one of the most powerful diagnostic tool available to date. With its high specificity and sensitivity, WSSV diagnostic PCR can be used for screening carriers in shrimp larvae, parental spawners and invertebrate populations that share the same habitat as well as in helping to ascertain the transmission and infection cycle of WSSV. WSSV diagnostic PCR is also suitable for monitoring cultured shrimp for WSSV infection during their growth period (Lo et al. 1998).

Our laboratory is presently carrying out a field survey of WSSV infection in cultured shrimp in more than 50 earthen

Lo CF, Chang YS, Cheng CT, Kou GH (1998) PCR monitoring of cultured shrimp for white spot syndrome virus (WSSV) infection in growout ponds. In Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: ghkou@ccms.ntu.edu.tw

ponds in southern Taiwan at two week intervals. With this investigation, we are attempting to discover the pattern of growth and survival of WSSV-infected and WSSV-free shrimp on typical shrimp farms, as well as the interaction between the cultured shrimp and endemic WSSV carriers (WSSV-infected copepods and crabs). Although, this work is still in progress, in the present paper, we report on some early PCR monitoring results in 4 of the study ponds in order to demonstrate how PCR monitoring might be used to predict the outcome for the shrimp in each of the culture ponds. We also introduce a new single-step (as opposed to 2-step) nested PCR protocol.

MATERIALS AND METHODS

Sensitivity of 2-step nested WSSV PCR and single-step nested WSSV PCR.

A plasmid (10^6 copies/mol) containing the WSSV DNA fragment pms146 (Lo et al. 1996a) was used as the WSSV DNA standard. This standard was serially diluted with 40 ng/µl yeast tRNA to 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 copies/µl and these diluted DNA solutions were used as WSSV positive samples to evaluate the sensitivity of 2-step nested and single-step nested PCR. Yeast tRNA (40 ng/µl) was used as the negative control.

2-step nested WSSV PCR

An aliquot (2 µl) of each of the WSSV positive samples was subjected to 2-step nested WSSV PCR (previously referred to as 2-step WSSV PCR) with the primer sets pms146 F1/R1 and F2/R2 as described previously (Lo et al. 1996b). Briefly, the nested primer set 146F1, 5'-ACT ACT AAC TTC AGC CTA TCT AG-3'; 146R1, 5'-TAA TGC GGG TGT AAT GTT CTT ACG A-3' and 146F2, 5'-GTA ACT GCC CCT TCC ATC TCC A-3'; 146R2, 5'-TAC GGC AGC TGC TGC ACC TTG T-3' was utilized for 2-step WSSV diagnostic PCR (Lo et al. 1996a). The amplification was performed in a 100-µl reaction mixture containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM of each dNTP, 100 pmol of each primer, 2 units of DynaZyme™II DNA Polymerase (Finnzymes Oy, Riihitontuntie 14 B, SF-02200 Espoo, Finland) in an AG-9600 Thermal Station (Biotronics Corp. Lowell, Mass. USA) for one cycle of 94°C for 4 min, 55°C for 1 min, and 72°C for 2 min; 39 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; plus a final 5 min extension at 72°C after 40 cycles. After completion of the first step, 10 µl of the reaction mixture was added to 90 µl of PCR cocktail containing the inner primer pair, 146F2 and 146R2, and this was then subjected to a second step of amplification over the same 40 cycles. For the analysis of PCR products, each of the completed PCR reactions (10 µl) of the first and second amplifications was mixed with 1 ml loading buffer and subjected to electrophoresis on 1% agarose gels containing ethidium bromide at a concentration of 0.5 µg/ml, and visualized by ultraviolet transillumination.

Single-step nested WSSV PCR

A commercialized WSSV detection kit (WSBV Specific Sequence Amplification Kit; Farming IntelliGene Tech. Co., Taipei, Taiwan) which includes First Run PCR Reagent PreMix, Nested PCR Reagent PreMix, Positive Control, Yeast tRNA, Thermostable DNA Polymerase, DNA Molecular Weight Marker (848, 630, 333 bp) and 6×Loading Dye was used for single-step nested WSSV PCR. The primers used in this detection kit are derived from the sequence of the WSSV pms146 fragment (Lo et al. 1996a). Each of the serially diluted WSSV positive samples (2 µl) was mixed with 7.5 µl First Run PCR Reagent PreMix (with primers yielding 910 bp WSSV-specific PCR products), 0.5 µl Thermostable DNA Polymerase (2 units/µl) in a PCR 0.2 ml microfuge tube. The amplification was performed in an AG-9600 Thermal Station (Biotronics Corp. Lowell, Mass. USA). The program for the first PCR run was five cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec; 20 cycles of 94°C for 15 sec, 62°C for 15 sec, and 72°C for 20 sec; plus a final cycle of 72°C for 30 sec and 20°C for 30 sec. After completion of the first run PCR, 14 µl of the Nested PCR Reagent PreMix (containing a pair of internal primers yielding a 296 bp WSSV DNA-specific PCR product, but which in combination with the Fist Run PCR primers also yields two other WSSV-specific PCR products both of approximately 550 bp, and also containing shrimp DNA-specific primers yielding an 848 bp shrimp DNA- specific PCR product) and 1 µl Thermostable DNA polymerase (2 units/µl) was added to each of the completed PCR reactions, which were then subjected to the nested PCR. The program for the nested PCR was 45 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 40 sec, plus a final cycle of 72°C for 30 sec and 20°C for 30 sec. To analyze the PCR products, 5 µl of each of the completed nested PCR reactions was mixed with 1 µl loading dye and subjected to electrophoresis on 2% agarose gels containing ethidium bromide at a concentration of 0.5 µg/ml, and visualized by ultraviolet transillumination.

Monitoring cultured shrimp for WSSV

Cultured *Penaeus monodon* (black tiger shrimp) in four earthen ponds located in the Tainan area of southern Taiwan were monitored for WSSV infection during their growth period by either 2-step nested PCR (before May 14, 1998) or single-step nested PCR (after May 14, 1998). A DNA extraction kit (AcuGen Asia CO., LTD Taipei, Taiwan) which comprised DTAB solution, CTAB solution and dissolving solution, was used to prepare PCR templates from the tested specimens. Briefly, 100 mg of abdominal muscle (juvenile through subadult), or postlarvae with the heads removed (after PL10) or the entire body of larvae or postlarvae (up to and including PL10) was ground with a disposable bamboo stick or the equivalent in a 2 ml microfuge tube with 600 µl of DTAB solution, and then incubated at 75°C for 5 min before cooling to room temperature. An aliquot (0.5 ml) of chloroform was added to the mixture, which was then vortexed briefly and centrifuged at 12000 g for 2 min. The upper aqueous phase was transferred to a fresh 2 ml tube with 100 µl of CTAB solution and 900 µl double distilled (dd) H_2O, vortexed briefly and then incubated at 75°C, for 5 min. After centrifugation at 12000 g for 10 min, the pellet was collected and resuspended with 150 µl dissolving solution and incubated at 75°C for 5 min. After centrifugation at 12000 g for

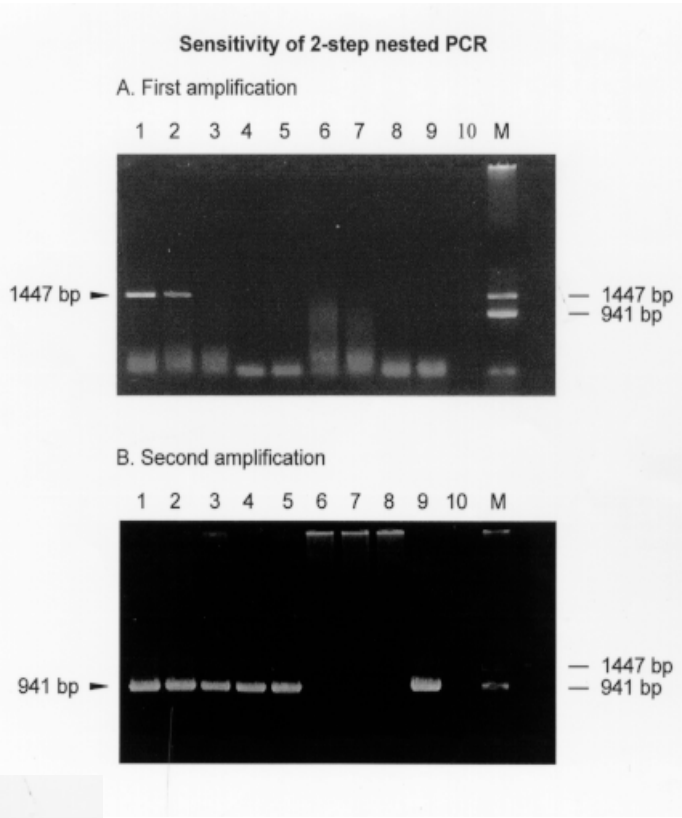


Figure 1. The sensitivity of 2-step nested PCR after (A) first amplification and (B) second amplification. In lanes 1 through 6 the number of copies of pms146 plasmid in each PCR reaction solution were successively reduced by a factor of 10 from 2×10^5 (lane1) to 2×10^0 (lane 6). Lane 7: tRNA (40 ng/µl); lane 8: dd water; lane 9: lightly infected shrimp; lane 10: blank; M: 1447 bp and 941 bp DNA markers.

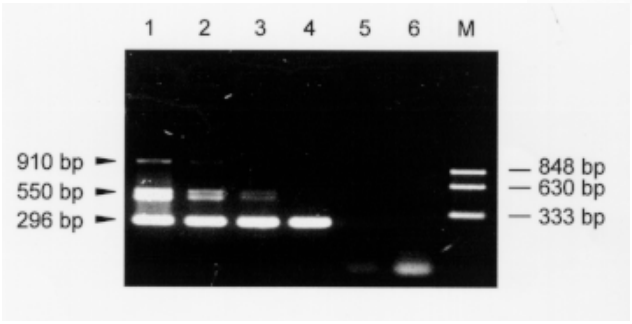


Figure 2. Single-step nested PCR products (910 bp, 550 bp and 296 bp) yielded by pms146 plasmid at concentrations of 2 x 10^4 (lane 1), 2 x 10^3 (lane 2), 2 x 10^2 (lane 3), 2 x 10^1 (lane 4), 2 x 10^0 (lane 5). Lane 6: tRNA (40 ng/µl). M: DNA markers of 848 bp, 630 bp and 333 bp.

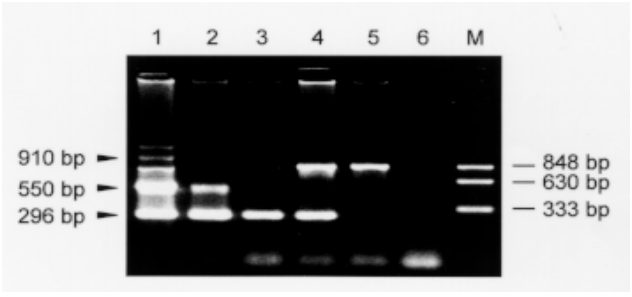


Figure 3. Four profiles of the PCR products (910 bp, 550 bp and 296 bp and their polymers) yielded by the shrimp specimens tested with the single-step nested WSSV PCR corresponding to WSSV infection at level 1 (lane 1), level 2 (lane 2), level 3 (lane 3) and level 4 (lane 4) and WSSV PCR negative shrimp (lane 5). Lane 6: tRNA (40 ng/µl). M: DNA markers of 848 bp, 630 bp and 333 bp.

5 min, the clear solution was transferred to a fresh 0.5 ml tube with 300 µl 95% ethanol, mixed well and pelleted at 12000 g for 5 min. After being washed with 200 µl of 70% ethanol, the pellet was resuspended in 200 µl dd H_2O and 2 µl of DNA solution was used for each PCR reaction.

RESULTS

Sensitivity of 2-step nested WSSV PCR

In lanes 1 through 6 (Fig.1) the number of copies of the pms146 plasmid in each PCR reaction solution was successively reduced by a factor of 10. The results revealed that the sensitivity of the 2-step nested PCR (Fig. 1B) was about a thousand times greater than that of 1-step PCR alone (Fig. 1A). With 2-step nested PCR, it was possible to detect 20 copies of the target plasmid in a PCR reaction (Fig. 1B lane 5).

Sensitivity of single-step nested WSSV PCR

Although the single-step nested WSSV PCR involves a 2-step sequential reaction, its PCR products are analyzed by electrophoresis only after completion of the second step of amplification. As shown in Figure 2, the single-step nested WSSV was also able to detect 20 copies of the target plasmid in a PCR reaction (Fig. 2 lane 4). Different concentrations of plasmid DNA generated different PCR product profiles (Fig. 2). The PCR products produced by the pms146 plasmid at different concentrations are shown in Table 1.

Monitoring cultured shrimp for WSSV

The PCR product profiles of the 83 specimens tested with the single-step nested WSSV PCR fell into 4 groups (Table 2; Fig. 3 lanes 1-4) corresponding to 4 levels of in-

Table 1. PCR products yielded by pms146 plasmid at different concentrations in single-step nested PCR.

Plasmid copies/PCR reaction	Single-step nested PCR products			
	296 bp	550 bp	910 bp	Other bands*
2×10^4	+	+	+	+
2×10^3	+	+	±**	+/-***
2×10^2	+	±	-	-
2×10^1	+	-	-	-
2×10^0	-	-	-	-

*Tentatively identified as dimers or other polymers of 296 bp, 550 bp and 910 bp; **Band with very low intensity; ***Not always observed

Table 2. Four distinct PCR product profiles yielded by the shrimp specimens tested with the single-step nested WSSV PCR.

Designated Infection Level	Single-step nested PCR products				
	296 bp	550 bp	910 bp	848 bp*	Other bands**
1 (very severe)	+	+	+	-	+
2 (severe)	+	+	-	-	-
3 (light)	+	-	-	-	-
4 (very light)	+	-	-	+	-

*Shrimp DNA-specific PCR product; **Tentatively identified as dimers or other polymers of 296 bp, 550 bp and 910 bp

fection: very severe infection (level 1), severe infection (level 2), light infection (level 3) and very light infection (level 4).

Monitoring data for the four ponds during the shrimp growth period are shown in Table 3. Once the specimens showed 1-step PCR positive in 2-step nested WSSV PCR or showed level 1-2 reaction patterns in the single-step nested PCR, mass mortality inevitably occurred within a few days (Table 3, ponds 1 & 2). None of the shrimp specimens collected from ponds which were successfully harvested (Table 3, ponds 3 & 4) were found to be 1-step PCR positive in 2-step nested WSSV PCR or showed the level 1 or level 2 reaction patterns in the single-step nested PCR.

DISCUSSION

Due to its rapid spread and broad host range, we anticipate WSSV will continue to inflict serious damage to the shrimp aquaculture industry worldwide. Effective prevention and control methods are urgently needed. For the moment, WSSV diagnostic PCR can be applied immediately to screen for carrier broodstock and shrimp larvae used for stocking ponds. This should allow for better control of WSSV in the culture system. In pilot studies that investigated the effect of culture conditions on the growth and survival of WSSV-infected and WSSV-free *P. monodon* on shrimp farms in southern Taiwan, our preliminary data suggest that mass mortality will predictably occur sometime during the growth period for any pond stocked with WSSV-infected postlarvae

Table 3. Data for four shrimp ponds monitored during the cultivation period.

Pond 1 (Stocked on Apr 5, 1998 at a density of 50/m^2)

Sampling Date	Length (cm)	2-step nested PCR					
		1-step PCR positive		2-step PCR positive		Negative	DF*
Apr 30	2.0-5.0	0		1/10		9/10	0
May 14	7.0-9.0	1/5		4/5		0	0
		Single-step nested PCR					
		Level 1	Level 2	Level 3	Level 4	Negative	DF
May 28	3.5-8.0	1/11	4/11	6/11	0	0	0

Culture was abandoned

Pond 2 (Stocked on May 10, 1998 at a density of 24/m^2)

Sampling Date	Length (cm)	2-step nested PCR					
		1-step PCR positive		2-step PCR positive		Negative	DF*
May 14	1.0-1.5	0		10/11		1/11	0
		Single-step nested PCR					
		Level 1	Level 2	Level 3	Level 4	Negative	DF
May 28	4.5-5.5	0	0	0	1/3	2/3	0
Jun 11	3.0-3.5	4/12	6/12	1/12	1/12	0	0

Culture was abandoned

Pond 3 (Stocked on Mar 10, 1998 at a density of 48/m^2**)

Sampling Date	Length (cm)	2-step nested PCR					
		1-step PCR positive		2-step PCR positive		Negative	DF*
Apr 30	3.0-9.0	0		1/10		9/10	0
May 14	3.5-9.0	0		1/3		2/3	0
		Single-step nested PCR					
		Level 1	Level 2	Level 3	Level 4	Negative	DF
May 28	3.5-11.0	0	0	0	1/7	2/7	4/7
Jun 11	6.0-15.0	0	0	0	4/5	1/5	0

Harvest on Jun 20, 1998 at density of 18/m^2

Pond 4 (Stocked on Apr 30, 1998 at a density of 23/m^2)

Sampling Date	Length (cm)	2-step nested PCR					
		1-step PCR positive		2-step PCR positive		Negative	DF*
Apr 30	2.0-4.0	0		2/10		8/10	0
May 14	3.0-8.0	0		20/25		5/25	0
		Single-step nested PCR					
		Level 1	Level 2	Level 3	Level 4	Negative	DF
May 28	3.5-9.0	0	0	0	0	10/10	0
Jun 11	4.5-12	0	0	0	8/20	12/20	0
Jun 26	9.5-14	0	0	0	0	15/15	0
Jul 9	10-14	0	0	0	3/10	7/10	0

Harvested on Jul 19, 1998 at density of 13/m^2 ; *DF: Detection failure (both WSSV and shrimp DNA-specific PCR products were not yielded); **This nominal stocking density was not consistent with field sampling and growout data and the value given is very likely too high (by about 2×).

at a relatively high culture density (e.g., 50-100 shrimp per m^2). This is true even if the WSSV is detected only by 2-step nested PCR or as level 3 or 4 by single-step nested PCR, which indicate light or very light infections. Pond 1 in the present study illustrated this point (Table 3). Furthermore, when many (> 50%) of the postlarvae used to stock a pond are infected at levels 3-4, mass mortality will predictably occur within a few days to a few weeks. This is true even for ponds stocked at relatively low culture densities. This is illustrated by Pond 2 in the present study. Pond 2 was located in a relatively isolated area. Prior to this study it had functioned as a reservoir and had not been used for culturing shrimp or any other aquatic animal for at least 3 years. Furthermore it was a closed system in the sense that all water came from an underground source or from rainfall. Consequently the water quality was good. Plankton (mostly copepods in the first five samples that were tested) collected with a sterilized plankton net (250 mesh) in this pond on 30 April, 1998 was 2-step PCR negative. Nonetheless on May 14, just 2 weeks later – and only 4 days after stocking – plankton samples were already 2-step PCR positive (data not shown). The postlarvae used to stock Pond 2 had been infected with WSSV at a high prevalence in the hatchery and this led to failure of cultivation.

The situation in Pond 2 contrasted with the situation in Pond 4, where the high prevalence (80%) of 2-step PCR positive shrimp at week 2 (Table 3) developed in the culture pond itself. Evidence that Pond 4 itself was the source of infection is provided both by the facts that all of the plankton specimens collected on 30 April were 2-step PCR positive (data not shown) and that the hatchery postlarvae used to stock the pond had only a low prevelance (20%) of 2-step PCR positive animals. The differences between Ponds 2 and 4 were crucial. Pond 4, stocked with postlarvae lightly infected with WSSV at low prevalence was successfully cultured through to harvest. This was true also for Pond 3.

None of the above should be interpreted as any kind of encouragement or endorsement for culturing WSSV-carrier postlarvae! On the contrary, the use of WSSV-free postlarvae for stocking plays a key role in successful culturing, even though the shrimp may subsequently be infected (usually lightly) via WSSV reservoir hosts. It is also important to point out that although these reservoir hosts are hard to eliminate completely from the culture system, the farmer should nonetheless make an effort to eliminate them as nearly as possible. The more their numbers are reduced, the less threat they pose to the shrimp population. We also strongly recommend that brooders be screened, and that larvae be monitored very carefully while in hatchery, after spawning and hatching. It should be emphasized that 1-step PCR negative tests or level 1 and level 2 negative tests by the single-step nested PCR technique (true for all our tested hatchery postlarvae) are insufficient criteria to pass postlarvae as safe for stocking. We maintain that the more sensitive nested PCR diagnosis is absolutely essential. Although stocking a pond with WSSV-free postlarvae would go a long way towards insuring a successful shrimp harvest, the shrimp should still be monitored during the growth period by random samples for WSSV detection by PCR. In this way any infection would be detected in its early stages, and farmers could forestall a serious outbreak by taking extra preventative action to reduce possible losses. Although monitoring the shrimp by PCR would increase a farmer's operating costs, it nonetheless would seem to be the best approach for increasing the probability of obtaining a profitable and successful harvest of market quality shrimp.

In our previous paper (Lo & Kou 1998), we classified WSSV infections into three stages, that is the asymptomatic carrier stage, the transition stage and the patent stage. The criteria that define these states include the presence/absence of white spots in the exoskeleton, the presence/absence of terminal clinical signs, and PCR results (1-step or 2-step nested PCR positive). Briefly, the 3 stages were defined as follows: shrimp at the asymptomatic carrier stage of infection are 2-step nested PCR positive only and they may or may not display any clinical signs of white spot syndrome; shrimp at the transition stage are 1-step PCR positive, and may have tiny spots in their exoskeleton, but they swim normally, are generally active and do not show any terminal clinical signs; shrimp at the patent stage of infection have white spots in their exoskeleton, display terminal clinical signs and are both 1-step PCR positive and 2-step nested PCR positive. It should also be emphasized that the asymptomatic carrier stage might persist for months, while the transition stage might last for only a few hours and is invariably followed by the patent stage of infection. Furthermore, once a specimen becomes 1-step PCR positive, it is certain to die within days, if not hours. In the present study, however, the results of the single-step nested WSSV PCR suggested an alternative classification: very severe infection (level 1), severe infection (level 2), light infection (level 3) and very light infection (level 4). Broadly, the correspondence between these 2 classification schemes is as follows: asymptomatic stage carriers would give level 3 or 4 results while transition and patent stage animals would give level 1 or 2 results. We found that ponds stocked with larvae at infection levels 3 and 4 were able to go through to harvest. As expected, however, once the infection stage of the cultured shrimp became level 1-2, mortality inevitably occurred within a few days. We should also mention here that both systems (i.e., single-step and 2-step nested PCR) have their specific advantages and disadvantages and the decision as to which test is appropriate will depend on specific circumstances.

The single-step nested PCR detection kit used here is based on the principle of competitive PCR. The primers in this kit are derived from the sequence of the WSSV pms 146 fragment described by Lo et al. (1996a). The entire sequence of this fragment (1461 bp) has been submitted to the EMBL/GenBank Data Libraries under Accession No. U50923 (Lo & Kou, 1996). Provided that the PCR protocol is properly followed as specified in the detection kit, a very lightly infected shrimp (level 4 infection) will yield both the shrimp-specific PCR product (848 bp fragment) and the shortest WSSV-specific amplicon (296 bp fragment). As the level of infection becomes more severe, the larger WSSV-specific amplicons (i.e., first the 550 bp and then the 910 bp fragments) will appear, while the shrimp specific PCR product will no longer appear. The general pattern is that larger

amplicons will result as the severity of the infection increases (L. Liu and C. Su of Farming IntelliGene Tech. Co. pers. comm.). Thus, infected shrimp can be graded in severity according to their PCR product profiles.

PCR can predict mortality, but unfortunately by the time this prediction can be made (i.e., at the transition stage) mortality is unavoidable. The challenge at this point is to design a PCR test which will warn of an impending transition stage in time to take [stress reducing] actions to prevent mass mortality. On the other hand, since stressful conditions frequently cause mass mortality not only in diseased shrimp but also in healthy shrimp populations, an alternative – or complementary – approach would be to identify a detectable common key response of shrimp to stressful conditions so that a rapid detection method could be developed to identify shrimp that are under stress.

Acknowledgments. This work was supported by the Council of Agriculture under grant no. 87-AST-1.4-FID-06 (12-3). We are indebted to Mr. Leo Liu and Mr. Chen Su, Farming IntelliGene Tech. Co. for numerous discussions concerning the Detection Kit for WSSV used in the present study and their constructive suggestions for PCR template preparation. We are indebted to Mr. Paul Barlow for his helpful criticism of the manuscript.

LITERATURE CITED

Chou HY, Huang CY, Wang CH, Chiang HC, Lo CF (1995) Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. Dis Aquat Org 23:165-173

Flegel TW (1997) Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World journal of microbiology & Biotechnology 13: 433-442

Lightner DV (ed) (1996) A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Soc, Baton Rouge, LA, Section 3.11

Lo CF, Kou GH (1996) WSBV 1461 bp *Sal* I fragment. EMBL/GenBank Data Libraries.

Lo CF, Leu JH, Ho CH, Chen CH, Peng SE, Chen YT, Chou CM, Yeh PY, Huang CJ, Chou HY, Wang CH, Kou GH (1996 a) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25: 133-141

Lo CF, Ho CH, Peng SE, Chen CH, , Hsu HC, Chiu YL, Chang CF, Liu KF Su MS, Wang CH, Kou GH (1996 b) White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis Aquat Org 27: 215-225

Lo CF, Ho CH, Chen CH, Liu KF, Chiu YL, Yeh PY, Peng SE, Hsu HC, Liu HC, Chang CF, Su MS, Wang CH, Kou GH (1997) Detection and tissue distribution of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis Aquat Org 30: 53-72

Lo CF, Hsu HC , Ho CH, Tsai MF, Kou GH, Lightner DV (1998) Specific genomic DNA fragment analysis of different geographical isolates of shrimp white spot syndrome associated virus. Dis Aquat Org (in press)

Lo CF, Kou GH (1998) Virus-associated white spot syndrome of shrimp in Taiwan: A review. Fish Pathol 30: (in press)

Lotz JM (1997) Special review: Viruses, biosecurity and specific pathogen-free stocks in shrimp aquaculture. World journal of microbiology & Biotechnology 13: 405-413

Spann KM, Lester RJG (1997) Special topic review: Viral diseases of penaeid shrimp with particular reference to four viruses recently found in shrimp from Queensland. World journal of microbiology & Biotechnology 13: 419-426

Application of PCR and Formalin Treatment to Prevent White Spot Disease in Shrimp

[1]Pornlerd Chanratchakool, [2]Chalor Limsuwan

[1]Aquatic Animal Health Research Institute, Department of Fisheries, Bangkaen, Bangkok, Thailand
[2]Faculty of Fisheries, Kasetsart University, Bangkaen, Bangkok, Thailand

ABSTRACT**:** Polymerase chain reaction (PCR) has proven to be a sensitive and accurate technique for detecting white spot syndrome virus (WSSV). However, in large populations where only a few individuals are infected, PCR is not very effective. In order to prevent outbreaks of disease, formalin is used to stress larvae prior to stocking. Animals responding poorly to the formalin test are eliminated. Studies were conducted to compare the occurrence of WSSV disease in ponds stocked with PCR-screened postlarvae and ponds stocked with non-screened postlarvae. The study also compared the health of shrimp submitted to a formalin test before stocking and those which were not formalin tested. The results revealed that only 1 pond out of six stocked with non-PCR-screened postlarvae survived to harvest, whereas all 5 ponds stocked with animals both PCR-screened and formalin tested survived. Among 108 ponds stocked with PCR-screened and formalin tested postlarvae, fifty-one were found to have shrimp with WSSV disease. Only 14 ponds with WSSV were harvested. The other 55 ponds were not adversely affected by the disease. Two other ponds suffered problems prior to harvest. Most of the ponds with WSSV disease developed patent infections during a heavy rain period in October and November.

KEY WORDS: white spot, virus, WSSV, treatment, PCR

INTRODUCTION

White spot syndrome virus (WSSV) outbreaks can be prevented by eliminating potential vectors and screening intake water. However, a serious outbreak can still occur in ponds stocked with infected postlarvae (PL). Using polymerase chain reaction (PCR) to screen for infected PL is one way to reduce this risk (Flegel et al. 1997; Kasornchandra et al. 1997). The technique is now used in many countries with varying results. PCR is very sensitive and can therefore detect small numbers of the virus in individual animals. However, the technique is not as effective where populations are large and only a small number of individuals are infected. This might be one reason why outbreaks still occur in ponds with postlarvae certified as PCR-negative.

Since outbreaks of white spot disease (WSD) often stem from infected PL, elimination of such animals from the population before stocking, can reduce the incidence of the disease. Formalin, at a concentration of 150 ppm, can be used to treat postlarvae for 30 minutes. After treatment, weak and unhealthy individuals can be separated from actively swimming shrimp and discarded. This technique successfully reduces the number of infected PL stocked into ponds (Limsuwan 1997a).

This study compared the occurrence of WSD in ponds stocked with PCR-screened and non-screened postlarvae. It also looked at the effect of the formalin test prior to stocking on PCR-screened PL. The results from the study provide a better understanding of WSD outbreaks.

MATERIALS AND METHODS

Study groups

Two studies were carried out. In Study 1, WSD occurrence was monitored in ponds stocked with PCR-screened and non-screened PL. The study was conducted at a commercial farm located in Trat province, eastern Thailand. It lasted from August 1996 until February 1997. The data was collected from the following two groups of ponds: Group I (6 ponds, stocked with non-screened postlarvae in August 1996) and Group II (5 ponds, stocked with PCR-screened and formalin-tested postlarvae in October 1996).

In Study 2, the occurrence of WSD was examined in ponds stocked with PCR-screened post-larvae given a formalin test prior to stocking. The study was conducted at a commercial farm located in Surathani province, southern Thailand. The following three groups of ponds were examined: Group I (32 ponds, stocked in June 1996), Group II (40 ponds, stocked in July 1996) and Group III (36 ponds, stocked in August 1996).

In both studies, samples for PCR analysis consisted of approximately 500 postlarvae fixed in 95% ethanol. PCR negative PLs were used for stocking in Group II in Study 1 and for all groups in Study 2. During the culture period, formalin was applied once at a concentration of 30 ppm between days 20-29, once between days 45-60 and at any time WSSV was detected.

Chanratchakool P, Chalor Limsuwan C (1998) Application of PCR and formalin treatment to prevent white spot disease in shrimp. *In* Flegel TW (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

Formalin treatment methods

Use to separate unhealthy postlarvae

Two hundred thousand PL were released into a 500 litre fibreglass tank containing 200 litres of pond water. Strong aeration was provided. The water volume was adjusted to 400 litres and left for 10-15 minutes. Next, 40 cc (100 ppm) of formalin was added. The aeration was stopped 30 minutes later. The tank water was then circulated by hand so that weak and dead postlarvae congregated in the centre of the tank bottom. The moribund shrimp were siphoned out of the tank and only the remaining, actively swimming PL were used for stocking the pond (Limsuwan 1997a).

Use during culture

Once between days 20-30 post-stocking, 30 ppm formalin was added to ponds in order to eliminate protozoa. The formalin was diluted with pond water before being applied. Thereafter, the pond water was aerated continuously for at least 48 hours to maintain oxygen levels. Once between days 45-60 post-stocking or when WSD was detected, a similar dosage of formalin was added. Moribund shrimp found along the pond side after formalin treatment were removed and discarded.

RESULTS AND DISCUSSION

Study 1 results

WSD occurrence from ponds stocked with PCR-screened and non-screened PL are given in Table 1. There was clear evidence that combined use of PCR and formalin was beneficial. Group I postlarvae probably carried the WSSV responsible for the outbreak which occurred on day 35. The disease might have been triggered by an adverse pond environment. Formalin treatment following positive detection of the virus was not always effective. This may be related to the strong PCR signals noted during testing. The shrimp died soon after detection of the virus. However, one pond in Group I survived until harvest and PCR assays from that pond gave a much weaker signal than the assays for other ponds in Group I. This probably indicated that fewer animals from that pond were infected. The results suggest that formalin treatment is more beneficial when WSSV prevalence is low or at an early stage of infection.

Moribund shrimp were usually observed along the sides of the ponds after formalin application. These animals tested positive for WSSV infection. We suggest that these infected shrimp be removed from the pond before they die to reduce the risk of a larger outbreak resulting from cannibalism (Limsuwan 1997b). In the case of an outbreak at a more advanced stage, formalin treatment could actually exacerbate the situation by seriously stressing the shrimp and leading to accelerated mortality. Dead, infected shrimp act as a disease vector via cannibalism and contribute to fast-spreading outbreaks.

Study 2 results

Results for the occurrence of WSD in ponds stocked with PCR-screened postlarvae given a formalin test prior to stocking are given in Table 2. Ponds stocked in June and July showed the most successful harvests with less than 20% of the ponds suffering from WSD. More than 70% of the ponds stocked in August were affected before harvest. Most of the ponds in Group III showed WSD during the heavy rain period of October and November. Stress arising from the rains may have been responsible for the higher mortalities. During initial PCR screening of the PL, the number of infected animals was probably so small that a negative PCR result was obtained because of sample size or low template concentration. However, the stressful conditions could have brought on an outbreak exacerbated by cannibalism. Alternatively, the virus could have entered the pond via a carrier during cultivation. In any case, formalin testing and removal of weak PL appears to effectively reduce the number of infected PL stocked into ponds (Limsuwan 1997a). However,

Table 1: WSSV detection in ponds stocked with PCR screened and non-screened postlarvae. In Group II, PL were screened by PCR before stocking. Subsequent PCR checks were performed at the time of formalin treatments (given in the table as day post stocking). Ponds harvested before 138 days constituted emergency harvests.

		PL pre-stocking	Juvenile PCR Results and Formalin Treatment Data						
			Treatment 1		Treatment 2		Treatment 3		Total
PCR Screening Group	Pond No.	PCR Result	Day	PCR results	Day	PCR results	Day	PCR results	No. Days Cultured
Not screened (I)	1	NA	30	++	35	+++			42
	2	NA	30	++	34	+++			44
	3	NA	30	++	35	++			48
	4	NA	30	++	35	++			39
	5	NA	30	++	34	++			41
	6	NA	30	++	35	+	43	+	145
Screened (II)	7	+	28	-	40	-	60	-	150
	8	-	28	-	40	-	60	-	148
	9	-	28	-	40	-	60	-	148
	10	-	28	-	40	-	60	-	145
	11	-	28	-	40	-	60	-	138

Notes: + = Weak positive PCR result; ++ = Moderately strong positive PCR result; +++ = Strong positive PCR result; - = Negative PCR result; NA = Not analysed

Table 2. Occurrence of WSSV and harvest results from ponds stocked with PCR-screened and formalin tested PL during different months.

Stocking Month	WSSV detection	No. of Ponds	Emergency Harvest	Successful Harvest
Jun 1996	WSSV detected during culture	13	6	7
	WSSV free during culture	19	-	19
	Total # ponds	32	6	26
	Percentage of ponds	100	19	81
Jul 1996	WSSV detected during culture	12	7	5
	WSSV free during culture	28	-	28
	Total # ponds	40	7	33
	Percentage of ponds	100	18	83
Aug 1996	WSSV detected during culture	26	24	2
	WSSV free during culture	10	2	8
	Total # ponds	36	26	10
	Percentage of ponds	100	72	28

if the incidence of infected postlarvae is high, the technique may not be practical and this could increase the risk of serious patent infections, especially when climatic conditions fluctuate.

CONCLUSIONS

To reduce the risk of WSD, postlarvae should be screened using PCR, especially when ponds are stocked during periods of climatic fluctuation (e.g., heavy rains, low temperatures). Sampling techniques used to select PL for PCR assays requires further study in order to ensure more reliable results. Formalin testing prior to stocking does seem to be effective in eliminating at least some infected animals from the population such that the risk of a serious WSSV outbreak is minimised.

Acknowledgements. This study was supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency. Special thanks to Phuket Kula Co., Ltd. and Siam Natural Resources Co., Ltd. for providing help and facilities during the study.

LITERATURE CITED

Flegel TW, Boonyaratpalin S, Withyachumnarnkul B (1997) Progress in research on yellow-head virus and white-spot virus in Thailand. *In* Flegel TW, MacRae IH (eds) Diseases in Asian aquaculture III. Fish Health Section, Asian Fisheries Society, Manila, pp. 285-295.

Kasornchandra J, Boonyaratpalin S, Itami T (1997) Detection of white spot syndrome in cultured penaeid shrimps in Asia : microscopic observation and polymerase chain reaction. Technical paper No. 8/1997. NICA, Department of Fisheries, Thailand. 16 p

Limsuwan, C (1997a) Reducing the effects of white-spot baculovirus using PCR screening and stressors. The AAHRI Newsletter, Vol. 6, No. 1. P. 1-2

Limsuwan, C (1997b) What kind of white spot kills shrimp? The AAHRI Newsletter,Vol. 6, No. 2. P. 4-5

Possible Prevention of White Spot Syndrome (WSS) in Kuruma Shrimp, *Penaeus japonicus*, in Japan

[*1]Toshiaki Itami, [1]Minoru Maeda, [1]Nobutaka Suzuki, [2]Kazuo Tokushige
[2]Atsushi Nakagawa, [1]Oscar Hennig, [1]Masakazu Kondo, [3]Jiraporn Kasornchandra
[4]Ikuo Hirono, [4]Takashi Aoki, [5]Riichi Kusuda, [1]Yukinori Takahashi

[1]National Fisheries University, Shimonoseki, Yamaguchi, Japan
[2]Kyowa Hakko Kogyo Co. Ltd., Ube, Yamaguchi, Japan
[3]Shrimp Research and Development Center, Songkla, Thailand
[4]Tokyo University of Fisheries, Tokyo, Japan
[5]Fukuyama University, Fukuyama, Hiroshima, Japan

ABSTRACT

Outbreaks of virus infection causing serious mortality to kuruma shrimp, *Penaeus japonicus*, have been occurring in Japan since 1993. Diseased shrimp show white spots in the carapace and reddish discoloration of the body. A non-occluded bacilliform virus, having a tail-like structure, was observed under the transmission electron microscope. We designed DNA primers for white spot syndrome virus (WSSV) and could detect the virus using the polymerase chain reaction (PCR). We successfully detected this viral DNA in captured shrimp by 2-step PCR amplification. Ninety-one out of 374 (24.3%) captured shrimp spawners collected in 1996 and 1997 were shown to be WSSV-positive by 2-step PCR. The filtrate of the heart homogenate of these virus-carrying shrimp was pathogenic to healthy kuruma shrimp by injection challenge. In order to confirm the prophylactic efficacy of oral administration of peptidoglycan (PG), an immunostimulant derived from *Bifidobacterium thermophilum*, we fed PG (0.2 mg /kg b.w./day) to kuruma shrimp for 30 days and exposed them to WSSV by a water-borne route during that period. The average survival rate of PG-fed shrimp was 97.6%, whereas it was only 19.0% in the control group. We then replace the PG feed with the control diet while the challenge continued. The shrimp that had been fed PG started dying within 20 days and final average survival rates were 7.2% 33 days after the termination of PG-feeding. These results confirmed that oral administration of PG was effective in preventing WSS in kuruma shrimp. WSSV was detected by 2-step PCR in PG-fed shrimp up to 60-95 days after challenge was discontinued. This result indicated that PG should be fed for at least 2-3 months after challenge because the shrimp still carry the virus even if they are fed PG and survive.

KEY WORDS: Penaeid acute viremia, Penaeid Rod-shaped DNA Virus, PCR, Prevention, *Bifidobacterium thermophilum*, singlet oxygen

INTRODUCTION

An outbreak of a virus infection in kuruma shrimp, *Penaeus japonicus*, referred to as penaeid acute viremia (PAV) and caused by penaeid rod-shaped DNA virus (PRDV) has been seriously damaging the culture of this species in Japan (Takahashi et al. 1994, 1996, Inouye et al. 1994, 1996). A similar virus infection of penaeid shrimp showing typical signs of white spots in the carapace has been reported in Thailand (Wongteerasupaya et al. 1995), Taiwan (Wang et al. 1995; Lo et al. 1996, 1997), Korea (Kim et al. 1998) and the USA, where it has been named white spot syndrome (WSS) (Durand et al. 1996). WSS has spread widely in, and caused much damage to, penaeid shrimp farms in Asia. These two viruses, PRDV and WSS virus (WSSV), are thought to be closely related, because the infections caused by them produce similar gross signs, affect similar target tissues and cause similar histopathological changes. Although PAV and PRDV are the standardized names for this infection in Japan, we use the names WSS and WSSV, respectively, in this paper since they are recognized worldwide.

In this paper, we emphasize the usefulness of 2-step PCR that is sensitive enough to detect WSSV in carrier shrimp and other crustaceans. In an attempt to develop a prophylaxis for WSS, the efficacy of orally administered peptidoglycan derived from *Bifidobacterium thermophilum* was examined and found to have promise as an immunostimulant.

The typical external signs of WSS are white spots in the carapace. Under the light microscope, the spots were observed to have a dark area in the center. The body color of the infected shrimp also becomes pale and reddish in color. Rod-shaped virions are found in the nucleus of infected lymphoid organ cells obtained from both experimentally challenged and naturally infected shrimp. The virions possess an

Itami I, Maeda M, Suzuki N, Tokushige K, Nakagawa, Hennig O, Kondo M, Kasornchandra J, Hirono I, Aoki T, Kusuda R, Takahashi T (1998) Possible Prevention of white spot syndrome (WSS) in Kuruma shrimp, *Penaeus japonicus*, in Japan. *In* T.W. Flegel (ed) Advances in shrimp biotechnology. National Center for Genetic Engineering and Biotechnology, Bangkok.

*e-mail: itamit@fish-u.ac.jp

envelope around a central nucleocapsid and a tail-like structure found in ultrathin sections. On average, the complete virions are 83 nm in diameter and 275 nm in length while the nucleocapsid is 54 nm in diameter and 216 nm in length. No occlusion bodies have been found in any of TEM observations. The virus has been purified by sucrose continuous density gradient ultracentrifugation. Negatively stained virus particles possess tail-like protrusions that were not mentioned by Kimura et al. (1995).

DETECTION OF THE VIRUS

PCR for diagnosis

We previously described a pair of primers designed from our original strain of WSSV (Takahashi et al. 1996). The DNA probe used in this paper did not react with baculoviral mid-gut gland necrosis virus (BMNV), yellow-head disease virus (YHV), *Baculovirus penaei* (BP) or uninfected shrimp. We therefore concluded that this DNA probe could specifically amplify DNA of WSSV.

Two-step PCR for the virus in carrier state crustaceans

In order to detect WSSV in carrier state shrimp and other crustaceans, we designed a nested primer for 2-step PCR. This was used for epidemiological and carrier studies of kuruma shrimp in the natural environment and other crustaceans in shrimp farms (Maeda et al. 1988). We collected 202 kuruma shrimp female spawners in 1996 and 172 in 1997 (body weight, 71.0 - 140.0 g) from 4 ports on the island of Kyushu from April through September in each year. The heart of each shrimp was used for extraction of template DNA for the 2-step PCR. The frequencies of virus positive samples by 2-step PCR were 25.2% and 23.3% in 1996 and 1997, respectively (Table 1). When we examined the hearts of immature shrimp (body weight, 13.3 - 48.0 g) that were collected in 2 ports from June through December, 16.5% and 16.9% were positive in 1996 and 1997, respectively (Table 1). These results suggest that the wild population of kuruma shrimp have been infected with WSSV. In this experiment, we found that the incidence of WSSV among captured females was significantly lower in immatures (14.5%) than in spawners (23.3 - 25.2%) ($p<0.05$). Interestingly, the frequency of the virus-positive shrimp increased in the mature shrimp. However, it is not clear whether this was due to sexual maturation or to multiple spawning behavior.

Table 1. Two-step PCR detection of WSSV in captured kuruma shrimp in Japan.

Shrimp group	1996	1997	Average
Female spawners	51/202* (25.2%)	40/172 (23.3%)	91/374 (24.3%)
Immature shrimp	45/272 (16.5%)	14/83 (16.9%)	59/355 (16.7%)

* No. of WSSV positive / no. of tested

Fourteen species of crustacean were collected in shrimp ponds and examined by 2-step PCR. We found that 5 species of shrimp and 6 species of crabs gave positive reactions. The shore crab *Helice tridens* showed an especially high frequency of positive reactions (66.7%, 26 out of 39 specimens).

PATHOGENICITY OF THE VIRUS DETECTED BY 2-STEP PCR

In order to clarify the pathogenicity of the virus detected by 2-step PCR in captured immature shrimp and spawners, we conducted stress tests and injection tests. In the stress tests, immature shrimp were placed in 500-*l* tanks without sand on the bottom to provide a stressful condition for them immediately after the they were transferred to our laboratory. Mortality was monitored every day for 15 days. Three groups of immature shrimp were examined. All the shrimp collected in July and November died within 14 days, and 16 out of 18 shrimp collected in December died within 15 days. All dead shrimp showed the typical symptoms of WSS (Table 2).

In the injection tests, immature shrimp hearts or spawners' hearts, both of which gave positive 2-step PCR results, were homogenized, filtered through a 0.45 µm membrane and injected into 6 or 10 healthy shrimp. Shrimp were kept in 55-*l* aquaria after the injection and observed for 10 days. All shrimp died within 10 days and showed the typical symptoms of WSS (Table 2). No mortality was found in the control shrimp injected with filtrate of 2-step PCR-negative shrimp hearts. These results indicated that a few virus particles detected in captured shrimp by 2-step PCR were virulent to shrimp and that the use of captured kuruma shrimp for seed production might cause vertical transmission of this virus.

Table 2. Pathogenicity of latent virus detected by 2-step PCR in captured immature shrimp and spawners.

Shrimp group	Date	No. of PCR positive / no. of tested (%)	Stress test[2]	Injection test[3]
Immature	Jul. 17, '96	–[1]	89/89[4]	–
	Nov. 7, '96	6/28 (22.4%)	22/22[4]	10/10[4]
	Dec. 4, '96	8/15 (53.3%)	16/18[4]	–
Spawners	Sep. 2. '96	8/30 (26.7%)	–	6/6[4]

[1]Not tested; [2]Stress test (i.e., shrimp kept in an aquarium without sand after arrival at laboratory from the landing port); [3]Injection test (i.e., shrimp injected with filtrates of heart homogenate from captured immature shrimp or spawners that showed positive 2-step PCR reactions for WSSV); [4]No. dead / no. challenged.

To determine whether the virus could be transmitted horizontally, a cohabitation trial was conducted using virus-carrying crabs and healthy shrimp. Healthy shrimp (n=10) were placed in a holding tank with other, untested shore crabs (n=10), a portion of which were proven to be infected with the virus by 2-step PCR. All shrimp died in 28 days and 60% of the crabs died in 30 days. This result indicated the possibility of horizontal transmission of this virus.

PROPHYLAXIS

Disinfectants and treatments

The sensitivity of WSSV to chemicals, temperature and drying were examined (Maeda et al 1998). The pathogenicity of the treated virus was determined by injecting it into shrimp followed by monitoring for 10 days. The virus was

inactivated by exposure to sodium hypochlorite at a concentration of 5 ppm for 10 min or 1 ppm for 30 min. A 12.5% sodium chloride solution inactivated the virus in 24 h at 25°C. Povidone-iodine inactivated the virus at a concentration of 10 ppm for 30 min. The virus lost its pathogenicity by heating at 50°C for 20 min in sterile seawater and by a drying treatment at 30°C for 1 h. It was sensitive to ethyl ether. When suspended at a high concentration in sterile seawater, it maintained pathogenicity for 120 days at 25°C and for more than 120 days at 4°C. However, when suspended at a low concentration (a 10^{-7} dilution of the above solution), it maintained pathogenicity for only 10 days at 25°C and for only 15 days at 4°C.

In an attempt to develop a new technique to inactivate WSSV, we exposed it to singlet oxygen ($^1O_2$) that was generated when the immobilized dye rose bengal was irradiated with visible light (Gollnick 1968, Suzuki et al. 1990). The mortality rate of shrimp that received the $^1O_2$-treated virus was significantly lower than that of the control shrimp (p<0.001) (Table 3), indicating that $^1O_2$ could inactivate

Table 3. Effect of singlet oxygen ($^1O_2$) on the infectivity of WSSV for kuruma shrimp.

Treatment[1]				
WSSV	Rose bengal	Visible light	$^1O_2$ generation[2]	No. dead /no. tested
+	+	+	Yes	4/21[a]
+	+	–	No	22/22[b]
+	–	+	No	22/22[b]
–	+	+	Yes	4/21[a]

[1]Treatment was done at room temperature; [2]Generated from rose bengal by irradiation with visible light; [a, b]Significant differences between different letters (P<0.001).

WSSV. Recently, application of UV irradiation and ozonation has been developed for disinfecting sea water for aquaculture (Brown and Russo 1979, Kobayashi et al. 1993). These techniques have been demonstrated to be effective for the inactivation of fish pathogens (Sugita et al. 1992). However, the equipment for both methods is expensive. Moreover, ozonation produces residual Br and Cl coupled oxidants that are toxic to animals and have a long half-life. Hence, these toxic residues in sea water must be removed by activated charcoal before the sea water is transferred to aquaria (Sugita et al. 1992). On the other hand, the $^1O_2$ used in this study was proven to be an effective disinfectant having a short half-life (Saito and Matsugo 1988) and the process was simple and inexpensive. Therefore, we believe that this $^1O_2$ generating system has a high potential for future applications, at least in shrimp and fish hatcheries where relatively small volumes of water are used. We need to collect more information on the quality of the dye, the intensity of the light and the $^1O_2$ itself in shrimp culture ponds with heterotrophic populations of microorganisms and high concentrations of organic materials.

Immunostimulant

We reported that the oral administration of PG derived from *Bifidobacterium thermophilum* was effective in preventing WSS in shrimp (Itami et al. 1998). In order to confirm the effectiveness of PG, we challenged the PG-fed shrimp with WSSV again and then examined the effect of termination of feeding PG during water-borne challenge.

Three groups of shrimp were studied: two were duplicated groups of PG-fed shrimp and one constituted the control group fed an identical diet without added PG. Each group contained 21 shrimp (average body weight: 2.5 g) kept in aerated, 100-*l* tanks with a flow-through water system. The concentration of PG for oral administration was 0.2 mg/kg body weight / day. PG was fed continuously for 7 days prior to the viral challenge and then at intervals in a 7-day cycle in which PG was fed for 4 days followed by 3 days of control diet for a total of 30 days (day 0 - 29 in Fig. 1) after the challenge started. After this 30-day period, the two groups of shrimp to which PG had been fed were switched to the control diet without PG for a further 33 days. Shrimp were challenged with WSSV by exposure to effluent seawater from an upstream holding tank (100 *l*) in which a constant number of diseased shrimp (n=15, body weight: 5-10 g) were maintained throughout the experiment period (Itami et al. 1988). Mortality in the test tanks was monitored daily and the heart of each dead shrimp was examined for the presence of WSSV by PCR.

The survival rates of shrimp challenged with WSSV are shown in Fig. 1. The survival rates of PG-fed shrimp on day 30 (95.2% and 100%) were significantly higher than that of the control shrimp (19.0 %) (p<0.05). The control shrimp were replaced with a new group of control shrimp (n=21, average body weight: 5.2 g) on day 30 and the water-borne challenge with WSSV was continued for a further 33 days. On day 62 the final survival rates of PG-fed groups (PG discontinued at day 30) were 14.3% and 0% and this did not differ (p>0.05) from the control group (4.8%). WSSV was detected in all the dead shrimp by PCR. These results demonstrated that resistance to WSSV was enhanced by PG because shrimp fed PG showed higher survival than control shrimp and because the PG-fed shrimp started dying once the PG-feeding was stopped.

To examine the fate of WSSV in PG-fed shrimp during the post-feeding interval, we checked the virus in surviving shrimp by 2-step PCR. We set up PG-fed groups in duplicate (n=150, average body weight: 5.2 g) and a control group (n=30, average body weight: 5.2 g). PG was fed for 7 days prior to the start of the water-borne challenge and then in a 7-day cycle as described above throughout 30 days of continuous challenge. The average survival rates for the PG-fed shrimp and control shrimp were 60.0% and 11.7%. On day 30, the challenge was stopped and 8 to 10 shrimp from the PG-fed groups were sampled and tested by 2-step PCR at various time intervals for a further 195 days. DNA extracts of individual shrimp hearts were used as PCR templates. Table 4 shows the incidence of WSSV detection in shrimp during this convalescent period in the absence of WSSV challenge. The virus could be detected by 2-step PCR up to 95 days after challenge termination. Previous work had shown that small numbers of virus particles detected only by 2-step PCR were pathogenic to shrimp (Maeda et al. 1998). Hence, these results suggested that PG-fed shrimp that survive WSSV challenge may still carry small numbers of virus particles and may be a source of infection. Therefore, shrimp

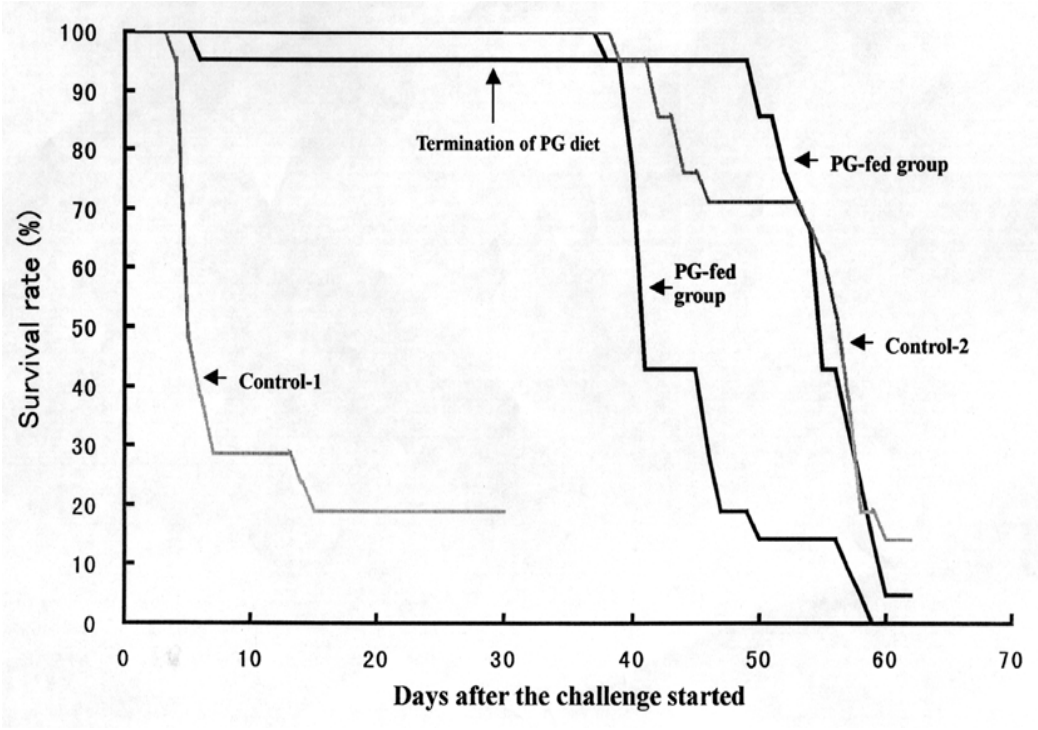


Figure 1. Survival rates of shrimp fed peptidoglycan (PG) derived from *Bifidobacterium thermophilum*. They were fed with PG for 30 days (days 0-29) followed by another 33 days (days 30-62) without PG. They were exposed to water-borne challenge throughout (days 0-62). The date of starting challenge for Controls 1 and 2 were days 0 and day 30, respectively.

that survive WSSV epizootics through PG-administration should receive PG for at least 2-3 months after exposure to infection in order for the virus to be cleared by shrimp defense factors that have been strengthened by PG.

Table 4. Detection of WSSV by 2-step PCR in convalescent shrimp after termination of WSSV challenge.

	Days after the termination of the challenge						
	10	35	60	95	125	165	195
Exp. 1	4/10*	4/10	6/8	0/10	0/10	NT	NT
Exp. 2	NT	9/10	4/10	4/10	0/10	0/10	0/10

*No. PCR positive/ no. examined; NT = not tested.

CONCLUSIONS

In 1988, annual shrimp culture production in Japan reached a maximum and since then has been decreasing. Three major diseases, *Vibrio penaeicida* infection, WSS (named PAV in Japan) and baculoviral mid-gut gland necrosis (BMN), are the primary causes of decreased production and they are of a great concern to the shrimp culture industry.

To control *V. penaeicida* infection, antibacterial agents are available in Japan and widely used. The proper use of these drugs is strongly recommended to avoid the appearance of strains that are resistant to these drugs.

In order to reduce the incidence of WSS and BMN, and other virus diseases, avoiding exposure to the virus is most important. Therefore, an early and accurate diagnosis is needed to detect the virus in latently infected shrimp and in virus-carrying crustaceans, before explosive infection occurs. In this respect, PCR and 2-step PCR are sensitive, simple and applicable methods for WSSV detection in hatchery-reared larvae, cultured shrimp and wild spawners.

The 2- step PCR method will be useful for screening brood stock and post larvae for WSSV, not only to protect cultured shrimp, but also to protect wild shrimp populations, which could be harmed by the release of infected shrimp. We also recommend that this technique be applied routinely to check the shrimp in hatcheries and growout ponds, so that virus-carrying shrimp can be found in early infection stages when effective countermeasures may still be devised (e.g., destroying all shrimp, eliminating the environmental stress factors, reducing the stocking density, strengthening shrimp immunodefense activities) before the infection becomes epizootic.

In order to prevent these bacterial and viral infections in shrimp, administration of immunostimulants is another approach. They could be used to enhance shrimp immunodefense activities whenever stress factors and/or diseases are expected to occur or whenever latent infections are detected by 2-step PCR.

Establishment of disease-resistant shrimp strains is one possible solution to the disease problems that are now threatening the world shrimp industry. Because artificial breeding techniques of kuruma shrimp have not been sufficiently developed, this will be an important area for future research. Recent developments in molecular biology such as identification of cDNA markers and microsatellite markers for growth performance and viral disease resistance, may lead to a higher rate of genetic improvement in shrimp (Acacia et

al. 1997).

LITERATURE CITED

Acacia A-WA, Dhar AK, Carr WH, Lotz JM (1997): Development of molecular markers in *Penaeus vannamei* for resistance to Taura syndrome virus (TSV) infection. Book of Abstracts, World Aquaculture'97, Seattle, WA, p8.

Brown C, Russo D J (1979) Ultraviolet light disinfection of shellfish hatchery sea water. I. Elimination of five pathogenic bacteria. Aquaculture 17: 17-23

Durand S, Lightner DV, Nunan LM, Redman RM, Mari J, Bonami J-R (1996) Application of gene probes as diagnostic tools for white spot baculovirus (WSBV) of penaeid shrimp. Dis Aquat Org 27: 59-66

Gollnick K (1968) Photooxygenation reactions in solution. In: Noyes Jr WA et al. (eds) Advances in photochemistry. John Wiley & Sons, New York, p 1-122

Inouye K, Miwa S, Oseko N, Nakano H, Kimura T, Momoyama K, Hiraoka M (1994) Mass mortality of cultured kuruma shrimp *Penaeus japonicus* in Japan in 1993: Electron microscopic evidence of the causative virus. Fish Pathol 29:149-158

Inouye K, Yamano K, Ikeda N, Kimura T, Nakano H, Momoyama K, Kobayashi J, Miyajima S (1996) The penaeid rod-shaped DNA virus (PRDV), which causes penaeid acute viremia (PAV). Fish Pathol 31: 39-45

Itami T, Kubono K, Asano M, Tokushige K, Takeno N, Nishimura H, Kondo M, Takahashi Y (1998) Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. Aquaculture 164: 277-288

Kim CK, Kim PK, Sohn SG, Sim DS, Park MA, Heo MS, Lee TH, Lee JD, Jun HK, Jang KL (1998) Development of a polymerase chain reaction (PCR) procedure for the detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimp. J Fish Dis 21:11-17

Kimura T, Nakano H, Momoyma K, Yamano K, Inouye K (1995) Purification of the rod-shaped nuclear virus (RV-PJ) from kuruma shrimp, *Penaeus japonicus*. Fish Pathol 30: 287-288

Kobayashi T, Yotsumoto H, Ozawa T, Kawahara H (1993) Closed circulatory system for mariculture using ozone. Ozone Sci Eng 15: 311-330

Lo C-F, Ho C-H, Peng S-E, Chen C-H, Hsu H-C, Chiu Y-L, Chang C-F, Liu K-F, Su M-S, Wang C-H, Kou G-H (1996) White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. Dis Aquat Org 27: 215-225

Lo C-F, Ho C-H, Chen C-H, Liu K-F, Chiu Y-L, Yeh P-Y, Peng S-E, Hsu H-C, Liu H-C, Chang C-F, Su M-S, Wang C-H, Kou G-H (1997) Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. Dis Aquat Org 30: 53-72

Maeda M, Kasornchandra J, Itami T, Suzuki N, Hennig O, Kondo M, Albaladejo JD, Takahashi Y (1998a) Effect of various treatments on white spot syndrome virus (WSSV) from *Penaeus japonicus* (Japan) and *P. monodon* (Thailand). Fish Pathol, in press

Maeda M, Itami T, Furumoto A, Hennig O, Imamura T, Kondo M, Hirono I, Aoki T, Takahashi Y (1998b) Detection of penaeid rod-shaped dna virus (PRDV) in captured shrimp and other crustaceans. Fish Pathol, in press

Saito I, Matsugo S (1988) Chemistry of active oxygen species. Protein, Nucleic Acid and Enzyme 33: 2665-2677

Sugita H, Asai T, Hayashi K, Mitsuya T, Amanuma K, Maruyama C, Deguchi Y (1992) Application of ozone disinfection to remove *Enterococcus seriolicida*, *Pasteurella piscicida* and *Vibrio anguillarum* from seawater. Appl. Environ. Microbiol 46: 1157-1162

Suzuki N, Nomoto T, Misumoto I, Kobayashi M, Wada M, Wada B, Inaba H. (1990) Chemiluminescence of luminol with singlet oxygen generated from a rose bengal system. Chem Express 5: 505-508

Takahashi Y, Itami T, Kondo M, Maeda M, Fujii R, Tomonaga S, Supamattaya K, Boonyaratpalin S (1994) Electron microscopic evidence of bacilliform virus infection in kuruma shrimp (*Penaeus japonicus*). Fish Pathol 29: 121-125

Takahashi Y, Itami T, Maeda M, Suzuki N, Kasornchandra J, Supamattaya K, Khongpradit R, Boonyaratpalin S, Kondo M, Kawai K, Kusuda R, Hirono I, T. Aoki (1996) Polymerase chain reaction (PCR) amplification of bacilliform virus (RV-PJ) DNA in *Penaeus japonicus* Bate and systemic ectodermal and mesodermal baculovirus (SEMBV) DNA in *Penaeus monodon* Fabricius. J Fish Dis 19: 399-403

Wang C-H, Lo C-F, Leu J-H, Chou C-M, Yeh P-Y, Chou H.-Y, Tung M-C, Chang C-F, Su M-S, Kou G.-H (1995) Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis Aquat Org 23: 239-242

Wongteerasupaya, C, Vickers JE, Sriurairatana S, Nash G.L, Akrajamorn A, Boonsaeng V, Panyim S, Tassanakajon A, Withyachumnarnkul B, Flegel TW (1995) A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. Dis Aquat.Org 21: 69-77