Characteristics of a phage effective for colibacillosis control in poultry

Article *in* Journal of the Science of Food and Agriculture · October 2012

DOI: 10.1002/jsfa.5683 · Source: PubMed

4 authors, including:

Sieo Chin Chin
Universiti Putra Malaysia
59 PUBLICATIONS **368** CITATIONS
SEE PROFILE

Wen Siang Tan
Universiti Putra Malaysia
162 PUBLICATIONS **1,545** CITATIONS
SEE PROFILE

Research Article

Received: 18 May 2011 Revised: 8 November 2011 Accepted: 4 March 2012 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/jsfa.5683

Characteristics of a phage effective for colibacillosis control in poultry

Gee Leng Lau,[a] Chin Chin Sieo,[a,b]* Wen Siang Tan[a,b] and Yin Wan Ho[b]

Abstract

BACKGROUND: Colibacillosis is one of the main causes of economic loss in the poultry industry worldwide. Although antibiotics have been used to control this infection, the emergence of antibiotic-resistant bacteria poses a threat to animal and human health. Phage therapy has been reported as one of the potential alternative methods to control bacterial infections. However, efficient phage therapy is highly dependent on the characteristics of the phage isolated. In the present study the characteristics of a lytic phage, ØEC1, which was found to be effective against the causative agent of colibacillosis in chickens in a previous *in vivo* study, are reported.

RESULTS: Examination by transmission electron microscopy revealed that ØEC1 is a DNA phage belonging to the Podoviridae family. ØEC1 showed an optimum multiplicity of infection of 0.1-1. The latent period of ØEC1 was 25 min, with a burst size of 200 particles per infected cell. Under the experimental conditions the maximum adsorption rate for ØEC1 was 99.9% within 8 min. ØEC1 demonstrated an optimum phage lytic activity at pH 6-9 and 25–41 °C.

CONCLUSION: These characteristics can serve as a guideline for selection of effective candidates for phage therapy, in this case for collibacillosis control in chickens.

Keywords: characteristics; colibacillosis; phage

INTRODUCTION

Colibacillosis, which is caused by avian pathogenic *Escherichia coli* (APEC), remains one of the major endemic diseases afflicting the poultry industry.[1,2] Outbreaks of the disease occur mostly in broiler and turkey flocks, either as primary or secondary disease,[3] resulting in airsacculitis, pericarditis, perihepatitis, peritonitis, septicaemia and sudden death. Losses result at all stages of age through high mortality, decreased reproductive performance, decreased hatching rate and the cost incurred in eradicating the disease from the flock.[4,5]

In order to protect animals and poultry from the adverse effects of various pathogenic bacteria, antibiotics have been employed. However, imprudent and prolonged medication with antibiotics has contributed to the emergence of antibiotic-resistant organisms.[6] This has created dramatic therapeutic problems, leading to the need to introduce alternative therapeutic methods for treatment of diseases.

Bacteriophage or phage therapy, which employs phages or bacterial viruses to lyse host bacteria, has proved to be an attractive candidate to cope with persistent pathogenic infections.[7] Although numerous studies have reported the presence of phages in various environments,[8,9] not all phages are suitable for phage therapy. Successful phage therapy is dependent on many factors, e.g. the specificity of phage adsorption, the degree of phage tolerance to gastric acid and body temperature of the infected organism and the stability and viability of phage preparations.[10] Phages that are not properly selected may lead to inappropriate phage choice, poor phage preparation, phage decay before application[11] and subsequent failure of the selected phage as either a prophylactic or therapeutic agent against the targeted bacteria. In many cases, extensive characterisation of a phage is conducted before its *in vivo* efficacy is tested.[12,13] However, phages that are well characterised and show superior characteristics *in vitro* may not work efficiently *in vivo*. Thus, in the present paper, we report the characteristics of a phage, ØEC1, that was found to be effective against colibacillosis control in chickens in a previous *in vivo* study.[14] These characteristics may serve as a reference for isolation of effective phages from the environment, especially for the purpose of applications in chickens.

EXPERIMENTAL

ØEC1 and APEC O78:K80

ØEC1 was isolated against APEC O78:K80 from a chicken's faecal sample using the soft agar overlay technique.[15] The host bacterium APEC O78:K80, which was kindly provided by Veterinary Research Ipoh (Veterinary Research Institute, Perak, Malaysia), is a virulent strain causing high mortality in infected chickens. A previous

* *Correspondence to: Chin Chin Sieo, Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia. E-mail: ccsieo@biotech.upm.edu.my*

a *Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

b *Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

in vivo study showed that ØEC1 could significantly improve the colibacillosis condition in infected chickens.[14]

Growth and maintenance of APEC O78:K80 and ØEC1

Escherichia coli O78:K80 was either maintained on MacConkey agar (Merck, Darmstadt, Germany) or grown in Luria–Bertani (LB) broth (BD Difco, Sparks, MD, USA) with constant agitation (180 rpm) at 37 °C. For long-term storage, bacteria were stored at −80 °C in 200 mL L^{-1} glycerol as stocks.

Routine maintenance of ØEC1 was carried out by infecting 0.1 mL of phage filtrate with 0.1 mL of log phase ($OD_{600nm} \approx 0.6$) host bacterial (*E. coli* O78:K80) culture. The mixture was added to 3 mL of melted LB top soft agarose (10 g L^{-1} tryptone, 10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 6 g L^{-1} agarose), vortexed and poured evenly onto the surface of an LB baseplate. After incubation for 4–5 h at 37 °C, plaque was picked and inoculated into 5 mL of LB broth containing 200 µL of fresh host inoculum. The mixture was then incubated at 37 °C with agitation at 180 rpm for the phage to lyse the host cells. The sample containing the lysed bacteria was centrifuged at 5600 × *g* for 10 min at 4 °C and the supernatant was filtered through a 0.2 µm membrane filter. The phage filtrate could be stored at 4 °C for at least 6 months.

Large-scale propagation, purification and dialysis of phage suspension

Large-scale propagation of the phage was carried out by infecting 500 mL of mid-log phase ($OD_{600nm} \approx 0.6$) *E. coli* O78:K80 with 30 mL of phage at a concentration of 10^8–10^9 plaque-forming units (PFU) mL^{-1}. After 3 h of incubation at 37 °C and 180 rpm, the lysate was treated with 0.2 µg mL^{-1} DNase I (Vivantis, Chino, CA, USA) and incubated for an additional 15 min under the same conditions. Then 12.5 g of NaCl was added, followed by incubation on ice for 1 h. After centrifugation of the lysate at 11 000 × *g* for 10 min at 4 °C, the phage in the supernatant was precipitated with 100 g L^{-1} polyethylene glycol 8000 (Sigma-Aldrich, Steinheim, Germany), in which overnight incubation at 4 °C was carried out. The phage was subsequently pelleted by centrifugation at 11 000 × *g* for 10 min at 4 °C and this partially purified phage was used for physiological characterisation. For electron microscopy and genomic analyses, further purification of the phage was required. The pellet of the partially purified phage was mixed with 2.5 mL of NaCl/TE buffer (1 mol L^{-1} NaCl, 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA), 10 mmol L^{-1} Tris-HCl, pH 8), vortexed and centrifuged again at 7525 × *g* for 10 min at 4 °C. The supernatant was used for purification of the phage by CsCl (Amresco, Solon, OH, USA) step gradient centrifugation in UltraClear tubes (Beckman Coulter, Brea, CA, USA). The tubes were centrifuged at 210 000 × *g* for 1 h at 10 °C using a Beckman Optima™Max ultracentrifuge (Beckman Instruments, Fullerton, CA, USA). The purified phage was dialysed against 100 volumes of dialysis buffer over 1 h using 10 000 MWCO SnakeSkin Pleated Dialysis Tubing (Thermo Scientific, Rockford, IL, USA).

Electron microscopy observation of phage morphology

A 10 µL aliquot of 10^{10}–10^{11} PFU mL^{-1} dialysed phage was applied to a carbon-coated copper grid. After 6 min of incubation at room temperature, excess liquid on the grid was removed by filter paper. The sample was then stained with 20 g L^{-1} uranyl acetate (pH 4) for an additional 10 min. Excess staining solution was removed by filter paper and the grid was air dried for 2 min. The grid was then examined using an HMG 400 electron microscope (Philips, Eindhoven, The Netherlands) to determine the morphology and size of the phage.

Genomic analysis of phage

For preparation of purified nucleic acid to determine the nature of the phage's nucleic acid, a high titre of 10^{10}–10^{11} PFU mL^{-1} dialysed phage was treated with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24:1 v/v/v), and the DNA extracted was precipitated with chilled alcohol. The extracted nucleic acid was stored at −20 °C or used to determine the type of nucleic acid. To achieve this, the purified nucleic acid was incubated with either 4 mg mL^{-1} DNase or 4 mg mL^{-1} RNase for 2 h at 37 °C. The resulting products were electrophoresed through 7 g L^{-1} agarose gel at 60 V for 60 min.

The phage genome size was determined by pulsed field gel electrophoresis (PFGE). For phage genome preparation, 10 µL of 10^{10}–10^{11} PFU mL^{-1} dialysed phage was diluted in 40 µL of TE buffer (10 mmol L^{-1} Tris, 1 mmol L^{-1} EDTA, pH 7.5). This was mixed with an equal volume of 14 g L^{-1} molten agarose (PFGE grade) in TE buffer and dispensed into plug moulds. The plugs were allowed to solidify at 4 °C for 10 min. They were then immersed in 5 mL of lysis buffer and incubated for 18 h at 55 °C for lysis of the phage capsid and digestion of the protein components. The plugs were rinsed with warm (55 °C) distilled water and washed six times for 30 min each with successive changes of washing buffer (20 mmol L^{-1} Tris, 50 mmol L^{-1} EDTA, pH 7.2). This was followed by cutting the plugs into four segments and inserting the segments into wells of 10 g L^{-1} agarose gel. The gel was then run using a CHEF DRII apparatus (Bio-Rad, Hercules, CA, USA) in 0.5× TBE (45 mmol L^{-1} Tris-HCl, 45 mmol L^{-1} borate, 1 mmol L^{-1} EDTA, pH 8.3) for 24 h at 6 V cm^{-1} with 2.2 and 63.8 s switch times at 14 °C buffer temperature. A lambda ladder PFG marker (New England Biolabs, Hitchin, UK) was used.

Physiological characterisation

Multiplicity of infection

To determine the optimum multiplicity of infection (MOI), the phage was mixed with log phase host bacteria at MOIs ranging from 0.01 to 100. The mixtures were incubated at 37 °C with agitation (180 rpm). Samples were taken every 20 min for 2.5 h to determine the titre of phage by the soft agar overlay method, and the number of viable bacteria was determined by the spread plate technique.[16–18]

Single-step growth curve

A single-step growth curve was constructed according to the method of Chow *et al.*[19] with slight modifications to determine the latent period and burst size of the phage. The phage was added to log phase host bacteria at an MOI of 0.01. After a short period of probable adsorption (∼10 min), free phage was removed by centrifugation at 5000 × *g* for 5 min at 4 °C. The supernatant was discarded and the infected pellet was resuspended in the original volume of prewarmed LB broth. The suspension was then incubated again at 37 °C with agitation. Samples were taken at various time intervals and titrated immediately to determine the titre of phage. Phage titres were then plotted against time intervals.

Adsorption rate of phage

Log phase host bacteria were infected with a known titre of phage at an MOI of 0.01 at 37 °C. After infection, 300 µL of sample was

removed every 2 min for the first 10 min and then every 5 min for the next 15 min. The samples were centrifuged at 5000 × *g* for 3 min at 4 °C to remove the bacteria and adsorbed phage. The supernatant was serially diluted immediately with SM buffer (0.1 mol L^{-1} NaCl, 8 mmol L^{-1} $MgSO_4 \cdot 7H_2O$, 50 mmol L^{-1} Tris-HCl, pH 5.5, 1 g L^{-1} gelatin), and the concentration of unabsorbed phage was determined by the soft agar overlay method. The adsorption rate of the phage was calculated as [(initial titre of phage – titre of unadsorbed phage)/initial titre of phage] × 100%.

Determination of optimum pH

To determine the optimum pH for phage lytic activity, 0.5 mL of phage was added to 0.5 mL of log phase host bacteria at an MOI of 0.1 and resuspended in 4 mL of LB broth with pHs ranging from 3 to 11. Control tubes containing either phage only or host bacteria only were set up for each tested pH. All tubes were incubated at 37 °C with agitation. The incubation period was as determined in the phage adsorption study, i.e. the time required for the highest amount of phage to adsorb to the host. After incubation, samples from the phage control and phage/bacteria mixture tubes were removed and centrifuged at 5000 × *g* for 3 min. The supernatants were used for determination of free phage at different pHs. Simultaneously, incubation proceeded until lysis of host bacteria was observed (the time required was estimated from the results of the MOI study). At this point, samples were removed from the host bacteria control and phage/bacteria mixture tubes to determine the reduction in host bacteria at different pHs using the spread plate technique.

Determination of optimum temperature

The assay was carried out following the method described for determination of optimum pH, with the modification that the phage and bacteria (MOI of 0.1) were resuspended in 4 mL of LB broth (pH 7) and incubated at different temperatures (25, 37, 39, 41 and 60 °C).

Statistical analysis

The experiments were repeated three times in duplicate. Analysis of variance and the paired *t* test were performed to analyse the data. In all analyses the confidence interval used was 95%. These tests were performed using SPSS for Windows Version 13 (SPSS Inc., Chicago, IL, USA).

RESULTS

Morphology of ØEC1

The electron micrograph of ØEC1 is shown in Fig. 1. ØEC1 was observed to have a pentagonal outline, indicating an icosahedral nature. It had a head diameter of 82.33 ± 0.03 nm and a tail length of 23.7 ± 2.6 nm. In accordance with the International Committee on Taxonomy of Viruses,[20] ØEC1 can be tentatively classified into the Podoviridae family.

Genomic analysis of ØEC1

The nucleic acid extracted from ØEC1 was treated with DNase and RNase to determine the type of phage genome. Figure 2(a) (lane 2) shows that the genome of ØEC1 disappeared after 2 h of incubation with DNase (4 mg mL^{-1}) at 37 °C, whereas no degrading effect was shown by RNase under the same conditions

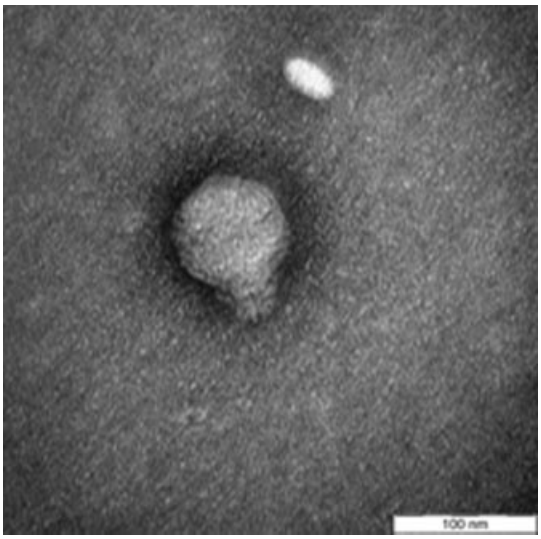

Figure 1. Electron micrograph of ØEC1 (magnification ×300 000).

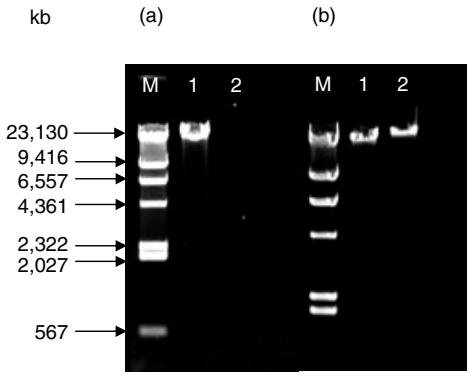

Figure 2. Genome nature of ØEC1 treated with (a) 4 mg mL^{-1} DNase and (b) 4 mg mL^{-1} RNase for 2 h at 37 °C. Lanes: M, λ DNA/HindIII markers; 1, untreated genome; 2, treated genome.

(Fig. 2(b), lane 2). This result indicated that ØEC1 is a DNA phage. The genome size of ØEC1 was estimated to be 59 kb as revealed by PFGE (Fig. 3).

Multiplicity of infection

The phage was added at different MOIs to early log phase host bacteria to determine the effect of phage titre on bacterial growth. As shown in Fig. 4(a), infection of host bacteria by ØEC1 at all tested MOIs led to a decrease in bacteria titre to a similar level (from ~8.5 to ~4.5 log colony-forming units (CFU) mL^{-1}) at the end of the experiment. However, the efficiency of reduction was dependent on the MOI. At an MOI of 0.01 a gradual decrease in bacteria titre was more obvious at 60 min post-infection. At this MOI the phage titre increased from 6.5 to 11.1 log PFU mL^{-1} as recorded at the end of the experiment (Fig. 4(b)). At MOIs of 0.1 and 1 a drastic decrease in bacteria titre occurred at 40 and 20 min post-infection respectively. At the end of the experiment the titre of ØEC1 increased by 3.5 log PFU mL^{-1} at an MOI of 0.1 and by 2.3 log PFU mL^{-1} at an MOI of 1. When the phage titre was higher than the host bacteria titre (MOIs of 10 and 100), rapid lysis of host bacteria (>5 log CFU mL^{-1}) was observed immediately after the two micro-organisms were mixed. However, in both cases the

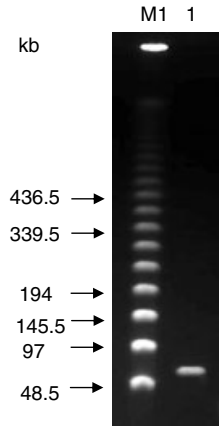

Figure 3. PFGE of ØEC1 genome. Lanes: M1, lambda ladder PFG marker; 1, genome of ØEC1.

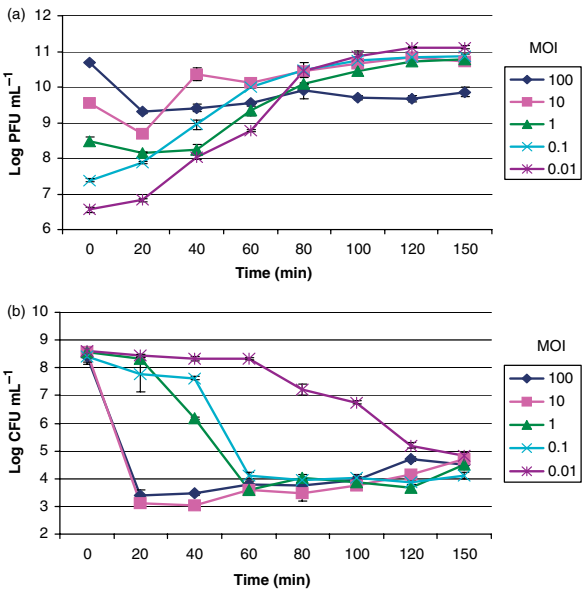

Figure 4. Populations of (a) ØEC1 and (b) bacteria (*Escherichia coli* O78:K80) at different MOIs. Values represent mean ± standard deviation of triplicate determinations.

bacteria showed a trend of regrowth at 40 min post-infection. In terms of phage titre, at an MOI of 10 the ØEC1 titre decreased significantly ($P<0.05$) from 9.5 to 8.7 log PFU mL^{-1} during the initial 20 min post-infection, followed by a slight increment to reach 10.7 log PFU mL^{-1} at the end of the experiment. In contrast, at an MOI of 100 the phage titre decreased significantly ($P<0.05$) from 10.7 to 9.9 log PFU mL^{-1} at the end of the experiment.

Single-step growth of phage

The growth pattern of ØEC1 is presented in Fig. 5. Based on the single-step growth curve, a latent period of approximately 25 min was recorded for ØEC1. The burst size was approximately 200 particles per infected cell. The infection cycle for ØEC1 was found to be approximately 45 min.

Adsorption rate of phage

As shown in Fig. 6, 98.9% of ØEC1 adsorbed to the host bacteria within 2 min of infection at 37 °C. The adsorption rate increased

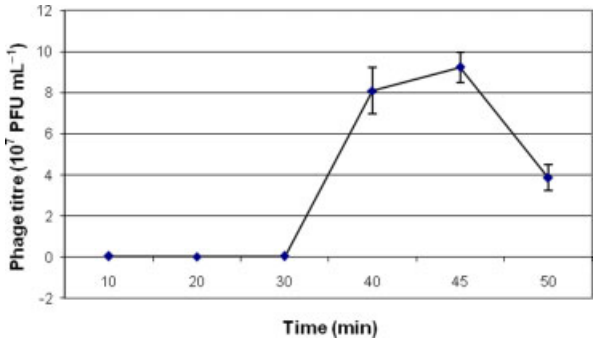

Figure 5. Single-step growth curve of ØEC1. Values represent mean ± standard deviation of triplicate determinations.

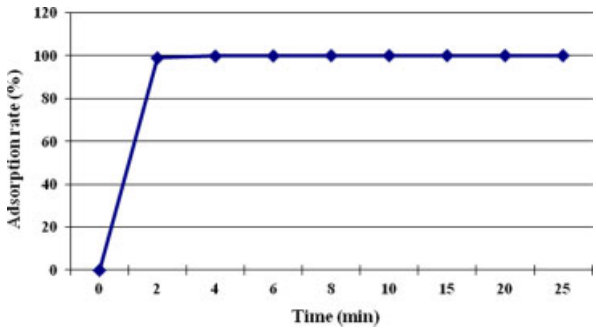

Figure 6. Adsorption ability of ØEC1.

to its maximum value (99.9%) at 8 min and remained at that level until the end of the experiment (25 min).

Determination of optimum pH

As shown in Fig. 7(a), ØEC1 was relatively stable at pH 5–11, where the titre of free phage (in the absence of bacteria) after incubation was not different from the initial phage titre. At pH 3 and 4, ØEC1 reduced by 3.3 and 0.7 log PFU mL^{-1} respectively. The adsorption ability of ØEC1 was also found to be affected by the pH. At pH 3, after 15 min of incubation, ØEC1 could not adsorb to the host bacteria. A significantly lower ($P<0.05$) titre of phage (0.9 log PFU mL^{-1}) adsorbed to the host bacteria at pH 4 in comparison with pH 5–11. At pH 5–11, more than 2.1 log PFU mL^{-1} of ØEC1 adsorbed to the host after incubation, with significantly higher ($P<0.05$) adsorption ability ($\geq$2.5 log PFU mL^{-1}) being observed at pH 6–9 (Fig. 7(a)). Owing to the higher adsorption ability, a higher bacteria reduction ability (5–6 log CFU mL^{-1} reduction) was observed at pH 6–9 (Fig. 7(b)). The host bacteria (*E. coli* O78:K80) did not survive incubation at pH 11.

Determination of optimum temperature

As shown in Fig. 8(a), ØEC1 was stable and adsorption of ØEC1 to the host bacteria was insignificantly different ($P>0.05$) at all tested temperatures (25, 37, 39, 41 and 60 °C). Under these experimental conditions, more than 1.6 log PFU mL^{-1} of phage adsorbed to the host bacteria, and the concentration of bacteria decreased by 5–6 log CFU mL^{-1} at 25, 37, 39 and 41 °C (Fig. 8(b)). The host bacteria (*E. coli* O78:K80) did not survive incubation at 60 °C.

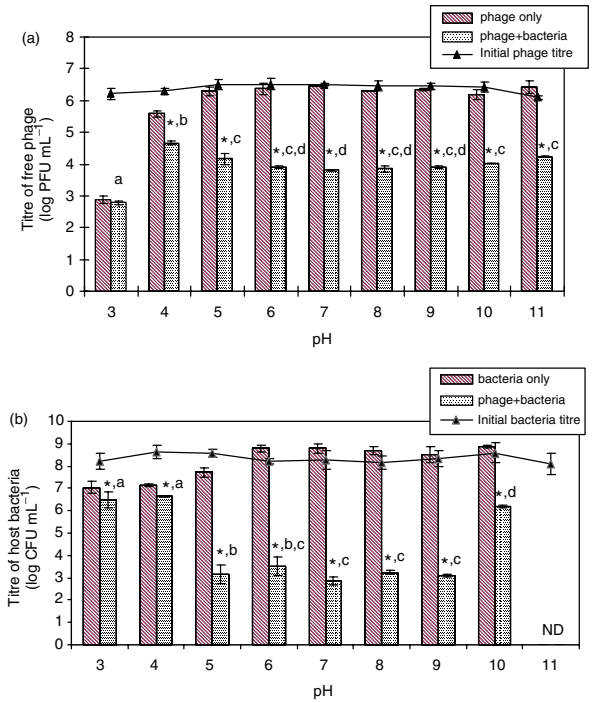

Figure 7. Determination of optimum pH for lytic activity of ØEC1: (a) titre of free phage before and after 15 min of incubation with host bacteria; (b) titre of host bacteria before and after 2 h of infection with ØEC1. *Asterisk indicates significant difference ($P<0.05$) from control group (phage or bacteria exposed to different pHs). [a–d]Different letters indicate significant difference ($P<0.05$) in population of phage titre or bacteria titre at different pHs. ND, population of host bacteria not detected. Values represent mean ± standard deviation of triplicate determinations.

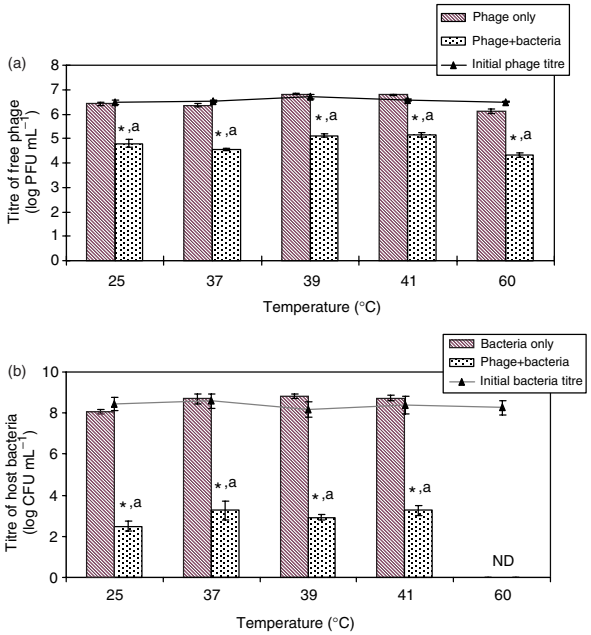

Figure 8. Determination of optimum temperature for lytic activity of ØEC1: (a) titre of free phage before and after 15 min of incubation with host bacteria; (b) titre of host bacteria before and after 2 h of infection with ØEC1. *Asterisk indicates significant difference ($P<0.05$) from control group (phage or bacteria exposed to different temperatures). [a]Same letter indicates insignificant difference ($P>0.05$) in population of phage titre or bacteria titre at different temperatures. ND, population of host bacteria not detected. Values represent mean ± standard deviation of triplicate determinations.

DISCUSSION

In this study the characteristics of ØEC1, which was found to be an effective biocontrol agent for colibacillocis when inoculated intratracheally into *E. coli*-infected chickens,[14] are presented. ØEC1 was preliminary identified by morphological characterisation using electron microscopy, one of the most common methods for virus identification. In previous reports, most lytic phages against *E. coli* isolated from different environments were from the Siphoviridae family.[21,22] However, in the present study, transmission electron microscopy examination and classification based on Ackermann[23] revealed that ØEC1 may belong to the Podoviridae family. According to statistics of phage distribution, the frequency of isolating phages from this family was considered low. Ackermann[24] reported that, among all phages isolated, only 14% were classified into the Podoviridae family.

Analysis of the genome showed that ØEC1 is a DNA phage. This observation is consistent with phages in the Podoviridae family.[24] However, slight differences in the genome size of ØEC1 compared with other members of the same family were detected. The genome size of ØEC1 was estimated to be 59 kb, slightly larger than the 42 kb demonstrated by a T7-like ØKMV phage.[25]

The virulency of a phage may depend on the phage–host interaction and environmental conditions such as pH and temperature. One of the important determining factors is the MOI, which refers to the ratio of phage to host bacteria during co-infection.[26] Efficient lysis of host bacteria will only occur at the right ratio of phage to host bacteria. In the present study the optimum MOI range for ØEC1 was determined as 0.1 – 1. At these MOIs, effective elimination of bacteria along with substantial mass production of phage progeny for subsequent cycles of phage lytic activity occurred. Although higher MOIs (10 and 100) resulted in an earlier and sometimes sharper decline in host bacteria number, a lower increment in phage titre was detected. Also, at higher MOIs a threshold for bacteria reduction and phage replication was observed. For instance, at MOI $\geq$ 10, bacteria lysis occurred rapidly initially. However, there was a rapid regrowth of the host bacteria, resulting in a subsequent increase in the level of bacteria. This could be due to lack of new progenies of the phage for continuous infection, as self-replication of the phage did not occur effectively (as indicated by the low increment in phage titre). Rabinovitch *et al.*[27] reported that such a phenomenon can occur during infection at high MOI, where the bacteria are attacked by a very large number of phages, resulting in premature lysis of host bacteria without free phage progeny being liberated. This phenomenon is also known as 'lysis from without'.[28]

The growth characteristics of ØEC1 were studied by carrying out a single-step growth experiment in which the time required for one complete phage infection cycle, the latent period and the burst size of the phage could be determined. Based on the results of this study, ØEC1 showed a latent period of approximately 25 min and an infection cycle of approximately 45 min. The burst size of ØEC1 was approximately 200 phage particles. Theoretically, bacteria can be lysed within a shorter time when a phage with a shorter latent period and infection cycle is used. However, a too short latent period may result in an insufficient burst size to sustain maximal phage population growth. Longer latent periods are normally associated with larger burst sizes, since a longer time period is provided for the mass synthesis of phage material and subsequent assembly of phage progeny before lysis of bacteria occurs.[29,30] It is important to have a large burst size so that the

progeny produced can continue to infect the remaining host bacteria for complete elimination of the targeted bacteria.

The adsorption ability and lytic efficiency of phages may be affected by environmental conditions such as pH and temperature. This may then determine the method of preparation and the route of administration of the phage. For ØEC1 the adsorption activity was affected at pH 3 and 4, an indication that ØEC1 may not be suitable for oral administration. This finding was supported by Muller-Merbach *et al.*,[31] who reported that phage adsorption was inhibited when it was added to host bacteria under low-pH conditions. In such an acidic environment, with increased H_3O^+ concentration, the receptor-binding proteins of the phage might have been protonated. Alteration of the phage receptors subsequently reduces the adsorption ability of phages and lowers their efficiency in elimination of host bacteria. In the present study the optimum lytic activity for ØEC1 was determined at pH 6–9. The optimum pH range for ØEC1 is considered wide compared with the results reported by Leverentz *et al.*,[32] who found that the highest reduction of *Listeria monocytogenes* (∼2.5 log CFU mL^{-1}) by its isolated phage occurred at pH 7–8. Ahmad and Morgan[33] also observed an optimum pH of 8 for a rhizobiophage. Besides pH, environmental temperature also plays an important role in determining the adsorption and lytic efficiency of phages. The effects are dependent on the physiology of individual phages. For instance, previous findings by Caso *et al.*[34] showed that phage *fri* against *Lactobacillus plantarum* demonstrated a normal lytic cycle at 30 °C. However, the process was completely unproductive at 37 °C even after incubation with the host bacteria for 240 min. Keogh[35] reported that the phage adsorption rate decreased when the incubation temperature was higher than the optimum growth temperature of the host. In another experiment, Yoon *et al.*[36] demonstrated that the proliferations of lytic *Pediococcus* bacteriophage at 25 and 30 °C were not significantly different. In the present study, no significant differences in adsorption ability and amount of bacteria reduction for ØEC1 at 25, 37, 39 and 41 °C were observed. Muller-Merbach *et al.*[31] also found that low temperature did not reduce the phage adsorption ability. However, as the replication of phages is highly dependent on the machinery system of the host bacteria, their lytic activity would also be slowed down together with the bacteria if the bacteria could not grow optimally at this temperature. Thus the bacteria reduction activity by the phage would be reduced.

In conclusion, ØEC1, which was previously found to be able to function *in vivo* to reduce infection by *E. coli* O78:K80,[14] demonstrated an optimum MOI of 0.1–1, with a latent period of 25 min, a burst size of 200 particles per infected cell, an adsorption rate of 99.9% within 8 min and an optimum lytic activity at pH 6–9 and 25–41 °C. These characteristics can serve as a guideline for selection of effective candidates for phage therapy, in this case for colibacillosis control in chickens.

ACKNOWLEDGEMENTS

This research was supported by the Ministry of Science, Technology and Innovation of Malaysia under the ScienceFund. We wish to thank Professor Dr Son Radu for providing the apparatus to carry out pulse field gel electrophoresis.

REFERENCES

1 Vandemaele F, Assadzadeh A, Derijcke J, Vereecken M and Goddeeris BM, Avian pathogenic *Escherichia coli* (APEC). *J Vet Med* **127**:582–588 (2002).
2 Dziva F and Stevens MP, Colibacillosis in poultry: unraveling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. *Avian Pathol* **37**:355–366 (2008).
3 Vandekerchove D, De Herdth P, Laevens H and Pasmans F, Colibacillosis in caged layer hens: characteristics of the disease and the etiological agent. *Avian Pathol* **33**:117–125 (2004).
4 Saleque MA, Rahman MH and Hossain MI, A retrospective analysis of chicken diseases diagnosed at the BRAC poultry disease diagnostic centre of Gazipur. *Bangladesh J Vet Med* **1**:29–31 (2003).
5 Vandekerchove D, De Herdth P, Laevens H, Butaye PP, Meulemans G and Pasmans F, Significance of interactions between *Escherichia coli* and respiratory pathogens in layer hen flocks suffering from colibacillosis-associated mortality. *Avian Pathol* **33**:298–302 (2004).
6 Patterson CA, Mackson JM and Weekes LM, Antibiotic prescribing for upper respiratory-tract infections in primary care. *Commun Dis Intell* **27**:S39–S41 (2003).
7 McVay C, Velasquez SM and Fralick JA, Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. *Antimicrob Agents Chemother* **51**:1934–1938 (2007).
8 Greer GG, Review: bacteriophage control of foodborne bacteria. *J Food Protect* **68**:1102–1111 (2005).
9 Bliss RM, Bacteriophages as novel antimicrobials for food safety. [Online]. (2008). Available: http://www.allbusiness.com/science-technology/life-forms-bacteria-coliform-e-coli/11467908-1.html [17 May 2010].
10 Sulakvelidze A, Alavidze Z and Morris JG, Bacteriophage therapy. *Antimicrob Agents Chemother* **45**:649–659 (2001).
11 Gill JJ and Hyman P, Phage choice, isolation, and preparation for phage therapy. *Curr Pharmaceut Biotechnol* **11**:2–4 (2009).
12 Shin H, Bandara N, Shin E, Kim RS and Kim KP, Prevalence of *Bacillus cereus* bacteriophages in fermented foods and characterization of phage JBP901. *Res Microbiol* **162**:791–797 (2011).
13 Yang H, Liang L, Lin S and Jia S, Isolation and characterization of a virulent bacteriophage AB1 of *Acinetobacter baumannii*. *BMC Microbiol* **10**:131–141 (2010).
14 Lau GL, Sieo CC, Tan WS, Hair-Bejo M, Jalila A and Ho YW, Efficacy of a bacteriophage isolated from chickens as a therapeutic agent for colibacillosis in broiler chickens. *Poultry Sci* **89**:2589–2596 (2010).
15 Adams MH, Appendix: methods of study of bacterial viruses, in *Bacteriophages*, Adams MH (ed.), by Interscience, New York, NY, pp. 450–451 (1959).
16 Kudva IT, Jelacic S, Tarr PI, Youderian P and Hodve CJ, Biocontrol of *Escherichia coli* O157 with O157-specific bacteriophages. *Appl Environ Microbiol* **65**:3767–3773 (1999).
17 Lu Z, Breidt Jr F, Fleming HP, Altermann E and Klaenhammer TR, Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, ΦJL-1, from a cucumber fermentation. *Int J Food Microbiol* **84**:225–235 (2003).
18 Pasharawipes T, Manopvisetcharean J and Flegel TW, Phage treatment of *Vibrio harveyi*: a general concept of protection against bacterial infection. *Res J Microbiol* **6**:560–567 (2011).
19 Chow JJ, Batt CA and Sinskey AJ, Characterization of *Lactobacillus bulgaricus* bacteriophage ch2. *Appl Environ Microbiol* **54**:1138–1142 (1988).
20 Fauquet CM, Mayo MA, Maniloff I, Desselberger U and Ball LA (eds), *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses*. Elsevier/Academic Press, San Diego, CA, USA (2005).
21 Chibani-Chennoufi S, Sidoti J, Bruttin A, Dillmann M, Kutter E, Qadri F, *et al.*, Isolation of *Escherichia coli* bacteriophages from the stool of pediatric diarrhea patients in Bangladesh. *J Bacteriol* **186**:8287–8294 (2004).
22 Yoichi M, Morita M, Mizoguchi K, Fischer CR, Unno H and Tanji Y, The criterion for selecting effective phage for *Escherichia coli* O157:H7 control. *Biochem Eng J* **19**:221–227 (2004).
23 Ackermann HW, Bacteriophage observations and evolution. *Res Microbiol* **154**:245–251 (2003).
24 Ackermann HW, Brief review: 5500 phages examined in the electron microscope. *Arch Virol* **152**:227–243 (2007).
25 Burkaltseva MV, Pleteneva EA, Shaburova OV, Kadykov VA and Krylov VN, Conserved genomes of KMV-like bacteriophages (T7 serogroup) active on *Pseudomonas aeruginosa*. *Russ J Genet* **42**:27–31 (2006).
26 Huff WE, Huff GR, Rath NC and Donoghue AM, Evaluation of the influence of bacteriophage titer on the treatment of colibacillosis in broiler chickens. *Poultry Sci* **85**:1373–1377 (2006).

27 Rabinovitch A, Aviram I and Zaritsky A, Bacterial debris – an ecological mechanism for coexistence of bacteria and their viruses. *J Theor Biol* **224**:377–383 (2003).
28 Abedon ST, Lysis from without. *Bacteriophage* **1**:1–4 (2011).
29 Mudgal P, Breidt Jr F, Lubkin SR and Sandeep KP, Quantifying the significance of phage attack on starter cultures: a mechanistic model for population dynamics of phages and their hosts isolated from fermenting sauerkraut. *Appl Environ Microbiol* **72**:3908–3915 (2006).
30 Shao Y and Wang I, Bacteriophage adsorption rate and optimal lysis time. *Genetics* **180**:471–482 (2008).
31 Muller-Merbach M, Kohler K and Hinrichs J, Environmental factors for phage-induced fermentation problems: replication and adsorption of the *Lactococcus lactis* phage P008 as induced by temperature and pH. *Food Microbiol* **24**:695–702 (2007).
32 Leverentz B, Conway WS, Camp MJ, Janisiewicz WJ and Abuladze T, Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Appl Environ Microbiol* **69**:4519–4526 (2003).
33 Ahmad MH and Morgan V, Characterization of a cowpea (*Vigna unguiculata*) rhizobiophage and its effect on cowpea nodulation and growth. *Biol Fertil Soils* **18**:297–301 (1994).
34 Caso JL, Reyes-Gavilan CGDL, Herrero M, Montilla A, Rodriguez A and Suarez JE, Isolation and characterization of temperate and virulent bacteriophages of *Lactobacillus plantarum*. *J Dairy Sci* **78**:741–750 (1995).
35 Keogh BP, Adsorption, latent period, and burst size of phages of some strains of lactic streptococci. *J Dairy Res* **40**:303–309 (1973).
36 Yoon S, Barrangou-Poueys R, Breidt F and Fleming HP, Detection and characterization of a lytic *Pediococcus* bacteriophage from the fermenting cucumber brine. *J Microbiol Biotechnol* **17**:262–270 (2007).

