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Retention of virulence in viable but non-culturable halophilic *Vibrio* spp.

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Abstract

The viable but non-culturable (VBNC) forms of two environmental strains of *Vibrio alginolyticus* 1 and *Vibrio parahaemolyticus* 66 and one strain of *V. parahaemolyticus* ATCC 43996 showing virulence characteristics (hemolysin production, adhesive and/or cytotoxic ability, in vivo enteropathogenicity) were obtained by culturing bacteria in a microcosm consisting of artificial sea water (ASW) and incubating at 5 °C with shaking. Every 2 days, culturability of the cells in the microcosm was monitored by spread plates on BHI agar and total count and the percentage of viable cells were determined by double staining with DAPI and CTC. When cell growth was not detectable (< 0.1 CFU/ml), the population was considered non-culturable and, then, the VBNC forms were resuscitated in a murine model. For each strain, eight male Balb/C mice were intragastrically inoculated with 0.1 ml of concentrated ASW bacterial culture. Two mice from each group were sacrificed at 2, 4, 8, and 12 days after challenge for autopsy and re-isolation of the microorganisms from the intestinal tissue cultures. Isolation was obtained in 25% of the animals challenged with the VBNC *V. alginolyticus* strain, in 37.5% of those challenged with the VBNC *V. parahaemolyticus* strain of environmental origin and in 50% of the animals infected with VBNC *V. parahaemolyticus* ATCC 43996. The strains thus isolated were again subjected to biological assays to determine the retention of pathogenicity. The virulence characteristics that seemed to disappear after resuscitation in the mouse were subsequently reactivated by means of two consecutive passages of the strains in the rat ileal loop model. The results obtained indicate that VBNC forms of the strains examined can be resuscitated and retain their virulence properties.

Keywords: VBNC *Vibrio* spp.; Virulence; Retention

1. Introduction

In recent years, the incidence of gastroenteritis involving bacteria of the genus *Vibrio* has increased. The cases reported have most frequently been attributed to the consumption of raw or undercooked seafood, or the ingestion of contaminated water (Piersimoni et al., 1991; Bravo Farinas et al., 1992).

Alongside the classic pathogen *Vibrio cholerae*, other *Vibrio* species including *V. cholerae* non-O1, *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*, which normally produce less

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severe symptoms have occasionally been reported to cause gastroenteritis, septicemia and wound infections (Blake et al., 1986; Bockemuhl et al., 1986; Chowdhury et al., 1987; Hansen et al., 1990; Mouzin et al., 1997)). These bacteria are widely found in different aquatic environments, including fresh and marine waters (Chowdhury et al., 1990; Chakraborty et al., 1997).

Although the mechanisms of pathogenicity by which *V. cholerae* 01 is able to induce gastrointestinal disorders are well known, relevant questions regarding the mode of the pathogenic action exerted by other halophilic *Vibrios* still remain unanswered. In fact, most of the virulence factors identified for the *Vibrio* species mentioned above have not been unequivocally associated with their pathogenicity, which instead appears to be strain-specific and due to the production of cytotoxic substances such as enterotoxins and hemolysins (Chowdhury et al., 1987; Stelma et al., 1992; Okuda et al., 1997).

A number of recent reports (Lavine et al., 1993; Matsiota and Nauciel, 1993; Uh et al., 2001) have associated contaminated aquatic environments with *Vibrio* infections in humans, suggesting the importance of systematic monitoring of these environmental strains and the need to define their possible pathogenic potential and clinical significance. *Vibrio*, like other bacterial species including *Aeromonas*, *Campylobacter*, *Salmonella* and *Shigella* under unfavourable environmental conditions, carry out a survival strategy based on the ‘‘viable but not culturable’’ (VBNC) state (Roszak et al., 1984; Colwell et al., 1985; Rollins and Colwell, 1986; Wolf and Oliver, 1992; Islam et al., 1993; Morgan et al., 1993).

Bacteria in the so-called VBNC state still show metabolic activities, but cannot be cultured under standard laboratory procedures. In the last decade, it has been frequently reported that under appropriate conditions these microorganisms can nevertheless be able to recover from this dormant state, became metabolically active and fully culturable (Roth et al., 1988; Nilsson et al., 1991; Whitesides and Oliver, 1997). This problem is even more complex for bacterial pathogens, since these microorganisms in VBNC state may remain pathogenic (Rahman et al., 1994; Stern et al., 1994; Ravel et al., 1995; Colwell et al., 1996; Pommepuy et al., 1996).

The aim of the present study was to verify the retention of virulence factors in experimentally induced VBNC forms of halophilic *Vibrio* strains isolated from coastal waters.

2. Materials and methods

2.1. Bacterial strains

Environmental strains of *V. parahaemolyticus* 66 and *V. alginolyticus* 1, isolated from Italian coastal waters of the central Adriatic Sea, were examined for their ability to enter the VBNC state by lowering the incubation temperature from 37 to 5 °C. These strains had been previously demonstrated (Baffone et al., 2000, 2001) to be able to colonize the gut of mice and to express virulence characteristics including haemolysin production, adhesiveness and cytotoxicity.

V. parahaemolyticus ATCC 43996 and *Vibrio harvey* ATCC 33868 were used as positive and negative controls strains, respectively.

2.2. Microcosm features and spread plate counts

Stationary-phase cells were obtained by overnight growth of the microorganisms in brain–heart infusion (BHI) broth (Oxoid, code CM0225R, Garbagnate Milanese, Milan, Italy) at room temperature (ca. 22 °C), with shaking. The bacterial cultures were then washed twice with artificial seawater (ASW) (Wolf and Oliver, 1992) to remove any nutrients, then resuspended in ASW in order to obtain 10^8 CFU/ml, as measured by plate counts. Five milliliters of this suspension were diluted in 495 ml of ASW in order to obtain a starting cell number in the microcosm of approximately 10^6 CFU/ml. The microcosms were then stored at 5 °C in ambient atmosphere, with shaking, and were monitored every 2 days to determine the colony-forming ability following the procedure of Whitesides and Oliver (1997) with slight modification. Each microcosm was serially diluted in cold sterile phosphate-buffered saline to have appropriate dilutions, and a volume of 100 μl was plated in triplicate onto cold (5 °C) brain heart infusion (BHI) agar (Oxoid, code CM 0375B, Garbagnate Milanese). The plates were incubated at room

temperature for 24–72 h and CFU were counted and related to the initial sample at each appropriate dilution. At the same time, 10 ml of the microcosm were filtered through a 0.22-μm-pore-size membrane filter (Millipore, code GSWP04700, Vimodrone, Milan, Italy), which was placed on the agar medium (BHI agar) and incubated as described above. The bacterial cells were considered to be in the non-culturable state when <0.1 CFU/ml could be detected in the population.

2.3. Total and viable cell counts

Double staining (CTC-DAPI) for the counting of viable *Vibrio* cells (i.e., respiring bacteria) was performed as described by Rodriguez et al. (1992). A portion (0.5 ml) of the BHI broth was added to 0.5 ml microcosm suspension for analysis. 5-Cyano-2.3 ditolyl tetrazolium chloride (CTC, Polysciences, Trimital, code 07919292, Magenta, Milan, Italy) was diluted and added to obtain a final concentration of 8.0 mM and the mixture was incubated for 4 h at 37 °C. Then, bacteria were captured by filtration through a 0.2-μm-pore-size black polycarbonate membrane filter (25 mm in diameter, Millipore, code GTBP0255500, Vimodrone) and were counterstained for 5 min with DAPI solution (4,6-diamidino-2-phenylindoldihydro chloride; Merck, Sigma, code D9564, Milan, Italy). Filters were air-dried and mounted on glass microscope slides (Zeiss, Axiolab, Carl Zeiss, Milan, Italy) for fluorescence observation. Microscopical observations were performed with a fluorescent light fitted with a 0.45-mm dichroic mirror, which allows the simultaneous visualisation of both dyes. Counting was carried out randomly on the basis of 20 microscope fields per filter. For each sample, two filters were counted. Active cells showing CTC crystals and total cell counts (i.e., viable and non-viable) were determined using DAPI. Results are expressed as the number of bacteria per milliliter of the initial sample.

2.4. Resuscitation of VBNC cells in animal experiments

Balb/C mice (weight: 20–25 g, Charles River, Calco, Lecco, Milan, Italy) were used as animal model in our experiments to resuscitate VBNC cells. The VBNC forms were obtained from the microcosm after ca. 30 days of incubation at 5 °C, while the bacillary forms were obtained after incubation of each strain for 24 h at 37 °C in BHI broth. Before inoculation in mice, all of the broth cultures were centrifuged at 3000 × *g* for 20 min and resuspended in BHI broth to obtain a 5 McFarland standard; 0 CFU/ml was the value for VBNC forms, whereas 10^8 CFU/ml was the final value for the bacillary culture (Cellini et al., 1994). Broth culture (0.1 ml) was inoculated intragastrically into Balb/C mice. In this study, 64 mice were used and subdivided into eight groups, each comprising of eight mice. Four groups were inoculated with the bacillary culture; the first and the second groups (1a and 2a) were inoculated with *V. parahaemolyticus* 66 and *V. alginolyticus* 1 of environmental origin; whereas the third group (3a) was inoculated with the positive reference strain (*V. parahaemolyticus ATCC* 43996). The same experimental scheme was repeated for the strains in VBNC form (1b, 2b, 3b). The inactive cultures (those unable to colonise the intestine of experimental animal model and without the above-mentioned virulence characteristics) of *V. harvey* 33868 in the bacillary and VBNC forms were administered to the remaining two groups (4a and 4b) as negative controls.

At 2, 4, 8, and 12 days after challenge, two mice from each group were sacrificed via CO_2 asphyxia before autoptic examination of the stomach and gut. The stomach and small and large intestines were aseptically removed and suspended in BHI broth. Each suspension was homogenized with a stomacher and 1 ml of this suspension was diluted with into 9 ml of alkaline peptone water and incubated at room temperature (ca. 22 °C) for 18 h. Subcultures were carried out on TCBS (Oxoid, code CM0333B, Garbagnate Milanese) or BHI agar and incubated at 37 °C for 24–48 h. The test was considered positive when the organisms were isolated on culture media. Colonies were identified as *Vibrio* spp. using the scheme described in our previous work (Baffone et al., 2001).

2.5. Passage in rat ileal loops (RIL)

Ligated intestinal loops were prepared in albino rats (strain CD SPF/VAP M, average weight 150–250 g, Charles River, Calco, Lecco) using the method suggested by Saha et al. (1998). In duplicate experiments,

the rat intestines were exposed by abdominal incision under light ether anaesthesia and 5–6 loops of 5 cm in length were tied, separated by 2-cm control loop intervals, starting at the ileo–caecal junction.

The inoculum, consisting of 0.5 ml of suspension containing 10^8 cells/ml of the *Vibrio* strains re-isolated from murine model and grown in BHI broth for 24 h at 37 °C, was injected into these loops. The rats were sacrificed via CO_2 asphyxia after 48 h, the abdomen was reopened, and the gut was externalised and examined for fluid accumulation (weight/length of the intestine). The bacterial suspension that caused fluid accumulation in the range of 0.4–0.5 ml/cm of gut was considered positive. A sample of fluid was collected from each loop and plated directly on TCBS or BHI agar in order to re-isolate the bacterial strains. The re-isolated strains were then confirmed by their known biotype patterns, as referred to in our previous work (Baffone et al., 2001). Re-isolated strains that caused little or no accumulation of fluid or which did not show the initial virulence characteristics were subject to two consecutive passages through rat ileal loops in order to favour the reactivation of their virulence expression.

2.6. Biological assays

Assays of haemolysin production, adhesiveness and cytotoxicity of the supernatant obtained by centrifugation of the bacterial suspensions were carried out as described in our previous works (Barbieri et al., 1999; Baffone et al., 2001): hemolytic activity was assayed using Wagatsuma agar, while adhesiveness was assayed using Hep-2 cells and cytotoxicity was determined using CHO cells. The assays of these biological activities were repeated for each strain re-isolated from the experimental mice and from the rat ileal loops.

2.7. Statistical analyses

Statistical analyses were performed using the chi-square test and the Fisher's exact *P*-test.

3. Results

VBNC forms were induced in two strains of *V. parahaemolyticus* and in one strain of *V. alginolyticus* showing virulence characteristics. Their ability to colonise the gut of mice and, subsequently, to maintain their virulence properties was evaluated after resuscitation in the murine model.

3.1. Studies on entry into the VBNC state

As shown in Fig. 1, both strains of *V. parahaemolyticus* 66 and *V. parahaemolyticus* ATCC

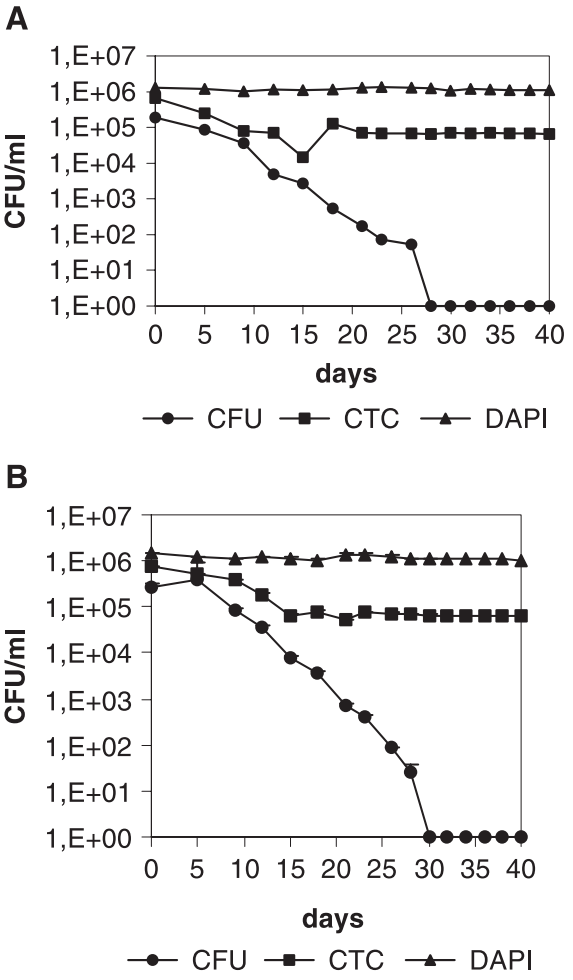

Fig. 1. Evidence for different physiological states of *V. alginolyticus* 1 (A) and *V. parahaemolyticus* 66 (B) strains incubated in an ASW microcosm stored at 5 °C in ambient atmosphere, with shaking, for 40 days. Culturable cell counts were obtained by spread plate counts on BHI agar. Active cells were counted after addition of 5 $mmol^{-1}$ CTC and total cell counts were determined using DAPI as counterstain and epifluorescence microscopy. Culturable cell counts (–●–); active cell counts (–■–); total cell counts (–▲–).

43996 and the strain of *V. alginolyticus* 1, in the stationary phase, entered a VBNC state within 28–30 days of incubation in the ASW microcosm, as evaluated by spread plate counts on BHI agar. Throughout this period, all total direct counts in the studies remained near the original inoculum level of ca. 10^6 CFU/ml and viable cells fluctuated between 5×10^4 and 7.4×10^5 CFU/ml for *V. parahaemolyticus* 66 and between 1.4×10^4 and 6.6×10^5 cell/ml for *V. alginolyticus* 1, as demonstrated by CTC staining. Similar values were obtained for *V. parahaemolyticus* ATCC 43996.

3.2. Resuscitation studies from the VBNC state

A murine model was used to resuscitate VBNC strains of *V. parahaemolyticus* and *V. alginolyticus*. As regards the bacillary forms, the microorganisms were found 2, 4, 8 and 12 days after challenge, and 67% of the mice were found to have been colonised (Table 1). In some cases, autopsy revealed hepatosplenomegaly and an increased intestinal volume, while a few animals died spontaneously (Table 1). The same strains in the VBNC state were isolated from a smaller number (37.5%) of mice at 2, 4 and 8 days after inoculation (Table 1). Therefore, considering the overall results, the VBNC forms showed a lesser ability to colonize the mouse gut ($P = 0.04$). When inoculated in bacillary form, cells were later re-isolated in 75% of the animals, regardless of the strain (66 or ATCC) of *V. paraginolyticus* used; in contrast, cells with the VBNC morphology were re-isolated in 37% of the animals inoculated with *V. parahaemolyticus* 66 and in 50% of those challenged with *V. parahaemolyticus* ATCC. The difference, in this case, was not statistically significant. Similar results were obtained for *V. alginolyticus* 1 in the bacillary form (50%) and in the corresponding VBNC form (25%); no statistically significant differences were verified. *V. harvey* ATCC 33868, which was used as negative control, was never isolated.

3.3. Reactivation of virulence in rat ileal loop assays

Data regarding fluid accumulation in the rat ileal loops are given in Table 2. Although the strains were enterotoxigenic prior to incubation in the microcosm and caused the accumulation of approximately 0.45–0.50 ml of fluid per cm of rat gut, they caused little or no fluid accumulation at the time of resuscitation in the murine model. However, with two consecutive passages in the rat gut, the fluid accumulation caused by the strains increased, reaching approximately the original levels.

Table 1
Number of mice found to have been colonised by the *Vibrio* strains vs. the total number of mice inoculated with the bacillary and VBNC forms of the same strains

Vibrio strain		Days after challenge				Total no. of mice colonised
Bacillary form	Mouse group	2	4	8	12	
		No. of mice colonised				
V. parahaemolyticus 66[a]	1a	1/2	2/2[b]	2/2[b]	1/2	6/8
V. alginolyticus 1[a]	2a	0/2	1/2	2/2	1/2	4/8
V. parahaemolyticus ATTC 43996[c]	3a	2/2	2/2[b]	2/2	0/2	6/8
VBNC form	Mouse group	No. of mice colonised				Total no. of mice colonised
V. parahaemolyticus 66[a]	1b	1/2	1/2	1/2	0/2	3/8[d]
V. alginolyticus. 1[a]	2b	0/2	1/2	1/2	0/2	2/8[d]
V. parahaemolyticus ATTC 43996[c]	3b	0/2	2/2	2/2	0/2	4/8[d]

The mice were sacrificed at 2, 4, 8, and 12 days after intragastric challenge.

[a] Environmental strains.

[b] Animals which died spontaneously.

[c] Reference strains.

[d] All strains isolated from each colonised animal were examined in biological assays to determine whether they had retained virulence characteristics.

Table 2
Reactivation of virulence by two consecutive passages in the rat ileal loop (RIL) model of *Vibrio* strains

Strains	Before incubation in ASW at 5 °C				Strains (S) re-isolated from the murine model (see Table 1)	After incubation in ASW at 5 °C							
						After recovery in murine model				After two consecutive passages in RIL model			
	Haemolysin	Adhesivity	Cytotoxicity	Accumulation of fluid (ml/cm)		Haemolysin	Adhesivity	Cytotoxicity	Accumulation of fluid (ml/cm)	Haemolysin	Adhesivity	Cytotoxicity	Accumulation of fluid (ml/cm)
V. parahaemolyticus 66	+	+	+	0.50	S1	+/−	+/−	+/−	0.15	+	+	+	0.40–0.45
					S2	−	+/−	−	0.15	+	+	+	0.35–0.45
					S3	−	+/−	−	0.10	+	+	+	0.35–0.40
V. alginolyticus 1	+	+	+	0.40	S1	−	+/−	−	0.15	+	+	+	0.30–0.40
					S2	−	+/−	−	0.10	+	+	+	0.30–0.40
V. parahaemolyticus ATCC 43996	+	+	+/−	0.55	S1	+/−	+	+/−	0.15	+	+	+/−	0.40–0.55
					S2	+/−	+/−	−	0.10	+	+	+/−	0.40–0.55
					S3	+/−	+/−	−	0.15	+	+	+/−	0.35–0.45
					S4	−	+	−	0.10	+	+	+/−	0.30–0.40

These strains had been shown to have lost their virulence characteristics when resuscitated in a murine model.

Biological analyses (hemolysin production, adhesiveness and cytotoxicity) were also carried out on *Vibrio* strains re-isolated from each challenged animal (Table 1) to determine if it retained its virulence characteristics after incubation in the ASW microcosm. Before passage in the RIL model, the strains resuscitated from the VBNC state in the murine model showed a reduction in their virulence expression. Moreover, the virulence properties were restored when the strains were re-isolated on BHI agar after two consecutive passages in the ileal loop of the rat (Table 2).

4. Discussion

Like other microorganisms, *Vibrio* species enter a viable but non-culturable state under adverse conditions (Colwell et al., 1985; Rollins and Colwell, 1986; Islam et al., 1993; Oliver et al., 1995).

The environmental strains of *V. parahaemolyticus* 66 and *V. alginolyticus* 1 used in this study were inoculated at 5 °C into an ASW microcosm and became non-culturable after 28–30 days of incubation (<0.1 CFU/ml). CTC-DAPI staining was used to monitor the transition of *Vibrio* cells to the viable but non-culturable state in ASW. CTC was reduced by *Vibrio* cells and counterstaining with DAPI allowed the enumeration of both the total number of cells and the number of metabolically active bacteria. The double-staining technique used herein is a rapid single-step method suitable for use in studying the VBNC forms of the same *Vibrio* species.

Another important characteristic of the VBNC forms is their ability to be resuscitated in vitro (Nilsson et al., 1991; Weichert et al., 1992) and in natural estuarine environments (Hite et al., 1994) as a consequence of environmental changes, i.e., an increase in temperature. In agreement with the results reported by other authors for *V. vulnificus* (Oliver and Bochian, 1995), our experience suggests that resuscitation of the *V. parahaemolyticus* and *V. alginolyticus* cells can also occur in the murine model, without necessarily leading to serious damage to the internal organs or death of the experimental animals. In our opinion, the fact that resuscitation from VBNC to the bacillary form in the murine model did not cause the death of the animals or serious damage to their internal organs could be explained as a temporary inability of these forms to

express their virulence characteristics. In fact, hemolysin production, adhesiveness and cytotoxin production were not observed when using bacterial cells which had been stored at 5 °C in the VBNC state and then resuscitated in mice. This observation initially led us to suspect that the VBNC bacteria might have lost their pathogenic potential.

Studies on the virulence of other bacterial species present in the VBNC state were reported by other authors. VBNC *Escherichia coli* and *V. cholerae* cell, for example, were recovered from rabbit ileal loops in which enterotoxigenicity was exhibited (Colwell et al., 1985; Grimes and Colwell, 1986). In other studies, chlorine-stressed *Yersinia enterocolitica* displayed virulence characteristics similar to those of control cultures (Singh and McFeters, 1987) and chick embryos died when injected with non-culturable cells of *L. pneumophyla* (Hussong et al., 1987).

Because our initial results did not seem to support those reported in the literature (Colwell et al., 1985; Singh and McFeters, 1987), we performed further studies using the method of consecutive passages in rat ileal loops, in an attempt to obtain comparable results. In the rat intestinal loops, the strains examined after the first passage showed a decreased ability to cause fluid accumulation and, when tested for hemolysin production, adhesiveness and cytotoxicity, showed a decreased (at times virtually absent) expression of virulence factors. However, a return to values similar to the initial ones was obtained after another two consecutive passages in the RIL model. This technique had been used by Saha et al. (1991, 1998) for freeze–thaw injured strains of *Campylobacter*, which were subsequently resuscitated by passing them through rat gut and re-isolating on them specific cultural medium.

As hypothesised by Saha et al. (1998), the complex microenvironment in the intestine of the rat may facilitate the multiplication of *Vibrio* cells and, in particular, the phenotypic expression of virulence characteristics revealed by fluid accumulation. This accumulation of fluid in rat ileal loops inoculated with both *V. parahaemolyticus* strains (*V. parahaemolyticus* 66 and *V. parhaemolyticus* ATCC 43996) could be related to hemolysin production (thermostable direct hemolysin—TDH) by this species which, as suggested by other authors (Nishibuchi et al., 1992), could take part in an enterotoxin-like mechanism.

Because the environmental strain of *V. parahaemolyticus* 66 used in our study was able to produce cytotoxin, as demonstrated in vitro by their ability to cause rounding of CHO cells, we can hypothesize that—as recently suggested by some authors (Fabbri et al., 1999; Raimondi et al., 2000)—the mechanism of action of TDH may be cytotoxic in nature.

Epidemiological evidence has revealed that most cases of infections caused by *V. vulnificus* occur in the summer months, when water temperatures are at their highest levels (Oliver, 1989). Nevertheless, cases have been reported during periods in which water temperatures are low, as demonstrated by the epidemics of *V. vulnificus* infection that occurred in Florida between 1981 and 1993, when the microorganism was repeatedly isolated from patients but never found in the waters of the five states of the Gulf Coast (Oliver and Bochian, 1995). These episodes, alongside others in which *Vibrio* species were implicated, lead us to believe that when these microorganisms, even if in the VBNC form, penetrate the human body through the occasional ingestion of contaminated recreational waters or, even more likely, through ingestion of contaminated raw sea food; they find the intestine an ideal habitat in which they can be resuscitated. Upon returning to the vegetative state, they are able to reactivate their pathogenic potential and presumably to cause diseases.

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