Comparative study of bacterial infections responsible for mass mortality in penaeid shrimp hatcheries of the Pacific zone

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Abstract

Mass mortality associated with luminescence has been observed in some Thai and Ecuadorian penaeid shrimp hatcheries in recent years. Samples collected from hatcheries in these two different areas revealed luminescent sucrose-negative *Vibrio* strains in larval rearing tanks where mortality occurred. Pathogenicity trials were carried out to identify the causative agents of mortality and to study the course of infection. Results showed that strains isolated from Thai hatcheries were more virulent than Ecuadorian strains when tested on different larval stages of *Penaeus monodon*. The virulence of these strains was also estimated. Trials on other species of penaeid shrimp demonstrated the specific action of Thai strains. Drug sensitivity patterns revealed a high resistance level of these strains to chemotherapeutants used in shrimp hatcheries, such as chloramphenicol and oxytetracycline. In order to characterize the virulence factors, *in vitro* tests were performed. Virulence activities of extracellular products (ECPs) and adhesion on cell lines were studied using primary cultures of different tissues of penaeids. No detectable differences were noticed between these strains using these *in vitro* tests.

Introduction

Vibriosis in shrimp hatcheries is a severe infectious disease with a worldwide occurrence. Samples of infected larvae were collected from hatcheries in Ecuador and Thailand in 1992 and 1993 during epizootics. The gross symptom observed by technicians was the same everywhere: green luminescence accompanied by mass mortality. Previous studies of *Vibrio-*

infected larvae using transmission electron microscopy suggested that pathogenicity in Asia and in Ecuador was different (Lavilla-Pitogo *et al.*, 1990; Morales, 1993). Lavilla-Pitogo *et al.* (1990) reported luminous bacteria associated with larval mortality in the Philippines. They isolated *Vibrio harveyi* and *V. splendidus* from infected larvae, proved the pathogenicity of *V. harveyi* in tests conducted on *P. monodon* larvae and postlarvae, and found bacteria adhering to the feeding apparatus and the oral cavity of infected individuals. Mass mortality of larval *P. vannamei* in Ecuador was reported by Morales (1993), who studied the ultrastructural stages of "bolitas negras". The intestinal cells of shrimp first begin to lose their intercellular junctions and their attachment to the basal membrane. This is followed by the total loss of intestinal cells. Bacterial ecological disorder in rearing tanks was suspected, but no causative agent was isolated from the digestive tract.

This paper discusses comparative experiments using luminescent bacterial strains isolated from *P. monodon* larvae in Thailand and water from larval rearing tanks in Ecuador. The results of studies on the identification and virulence of the isolated strains are presented.

Materials and Methods

Origin of samples

Shrimp hatcheries used the same protocol in Thailand and in Ecuador; shrimp larvae were reared in 10-12 m^3 tanks at densities of 100 larvae per litre and at a temperature of 28 to 30°C. Shrimp species bred in Thailand and Ecuador are different, *P. monodon* being the main species in the former country, and *P. vannamei* in the latter. Food for larva consisted of algae, *Artemia* and micro-particulated diets. Bacterial strains from Ecuador were collected in the course of ecological bacterial surveys. Bacterial strains from Thailand were isolated during mass mortality events in one hatchery. Bacterial samples were collected daily for comparison from both rearing water and larvae in tanks with mass mortality, as well as in tanks with no mortality.

Bacteriological analyses

Larvae were washed and homogenized and the homogenized tissue pipetted up and down in a sterile test tube to break up clumps and aggregates. It was then diluted ten-fold and plated on to marine agar 2216 E (Difco) and thiosulphate-citrate-bile-sucrose agar (TCBS, Difco) to determine bacterial concentration. The colonies were counted after 1 and 2 d of incubation at 25°C, respectively, and recorded as "colony forming units" (CFU).

Pure cultures of the dominant heterotrophic flora were identified using morphological and biochemical tests according to the procedures of Kelly *et al.* (1991): the identification of the isolates was performed by using Gram's stain, oxidase test, morphology and motility, fermentation of glucose and sensitivity to the vibriostatic agent O/129. The complete taxonomic identification of these strains was obtained by additional biochemical tests according to Dodin and Fournier (1991).

Sensitivity of these isolates to various drugs was assayed by the disc diffusion method on Mueller-Hinton agar (Sanofi Diagnostic Pasteur). The minimum inhibitory concentrations were specified for the drugs commonly used in aquaculture i.e., chloramphenicol, thiamphenicol, quinolones and tetracyclines.

Experimental infections and statistical analyses

Virulence assays on larvae were performed according to Lavilla-Pitogo *et al.* (1990) with slight modifications. Pathogenicity experiments were conducted using 100 zoea III/L, and 100 mysis III/L, and 60 pl 8/L of *P. monodon*. Each assay was done in five replicates in 500-mL jars. For bacterial screenings, 1 mL of bacterial suspension at 10^7 CFU/mL was added to 500 mL of seawater containing larvae. For comparison of pathogenic effects of strains from Thailand and Ecuador, we performed ten-fold dilutions of Thai strain suspensions, and assays were then performed as previously described. For negative control, larvae were reared in the same conditions, but no bacterial suspension was added.

Survival was examined after 24 h or 48 h at 28°C. Survival percentages were arc-sine transformed prior to comparison using a one-way analysis of variance followed by a multiple range test. Statistical analyses were performed using the Statgraphics (MS-DOS) software.

In vitro *assays*

In vitro assays were developed to identify the specific virulence factor(s) involved in the pathogenic effects of the strains. Primary cell cultures from a variety of adult shrimp organs from *Penaeus stylirostris, P. vannamei* and *P. monodon* were established in minimum essential medium eagle (MEM) and Grace's medium supplemented with fetal bovine serum (FBS) and antibiotics. Embryonic cells from *P. indicus* were also used. Subcultures of these cell cultures have been obtained for more than six months from five primary cell cultures (Le Groumellec *et al.*, 1995).

Bacterial adhesion to shrimp cell monolayers

The adhesion of bacteria to target cells was assayed on cell monolayers from ovary, hepatopancreas and embryo cultured on glass slides through Giemsa staining as previously described by Amaro *et al.* (1992).

The second method used a new fluorescing product, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Coger) as described in Rodriguez *et al.* (1992). This method distinguished attached from non-attached bacteria.

Cytotoxicity assays

Filtered supernatants were obtained from bacterial cultures in brain-heart broth (Sanofi-Diagnostic Pasteur) using a 0.22 μm filter (millipore), thereby obtaining extracellular products (ECPs). These ECPs were assayed for cytotoxicity on shrimp ovarian primary cell cultures. Half of the medium was removed from the different monolayers in culture wells, replaced with 1 mL of fresh MEM complete medium and 1 mL of each *Vibrio* culture filtrate. Plates were then incubated at 25°C and examined every 2 d for at least 1 mo. Wells showing totally or partially destroyed monolayers were recorded as positive for cytotoxic response.

Results

Bacterial numeration

Bacterial numerations were performed on samples from Thailand. In seawater samples collected daily during 2 wk from a rearing tank of mysis II showing mass mortality, bacterial numerations in marine agar 2216 E were 10^6 CFU/mL, and $5X10^5$ CFU/mL in TCBS. Sixty-four percent of the flora on TCBS consisted of luminescent strains. The negative control showed only 2% of luminescent strains on TCBS, despite the fact that the same levels of bacteria were found on marine agar 2216 E and TCBS. In larvae, although there was no luminescent strain in negative control, nearly 100% of the TCBS flora was composed of luminescent strains in samples collected during mass mortality events.

Screenings of strains

In order to find out the causative agents of mass mortality, screenings of strains were performed on larvae of penaeid shrimp. Data presented in Figures 1 and 2 showed that four strains from Thailand (BL 1, BL 2, BL 3, and BL 11), induce high mortality rates at 10^4 CFU/mL, statistically different ($p<0.05$) from the negative control and from the other strains tested. B8-2 at 10^4 CFU/mL appears to induce no mortality significantly higher than the negative control for *P. monodon* larvae, despite the fact that this strain is biochemically equivalent to *V. harveyi* from Thailand (BL 1, 2, 3, 11). On the contrary, B8-2 has formerly been recognised for inducing mortality significantly higher than the negative control in *P. vannamei* larvae, using the same protocol, whereas BL 1 was shown to induce no significant mortality for *P. vannamei* in the same test. We concluded that these strains have a specific virulence against their natural host.

Bacterial identification and drug sensitivity patterns

The identification of the pathogenic strains showed that the dominant strains belong to the genus *Vibrio*. Two strains were resistant to the vibriostatic agent O/129, but this character appears to vary among *Vibrio* strains (Dodin and Fournier 1991) according to recent taxonomic studies.

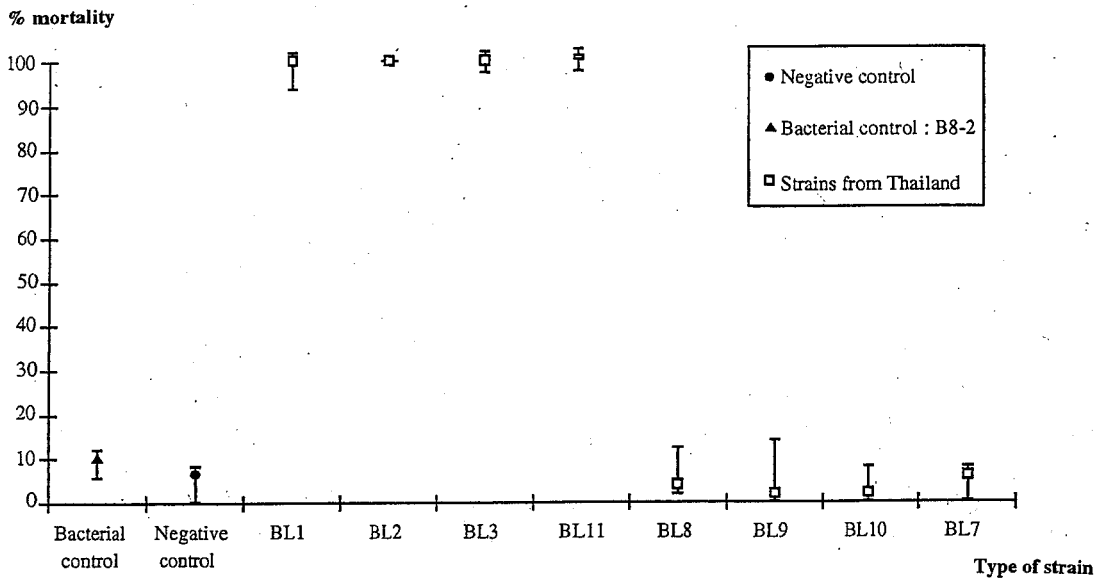

Fig. 1. Screening of *Vibrio* strains from Thailand: comparison of mortality observed in experimentally infected *Penaeus monodon* larvae (zoea III) after 24 h.

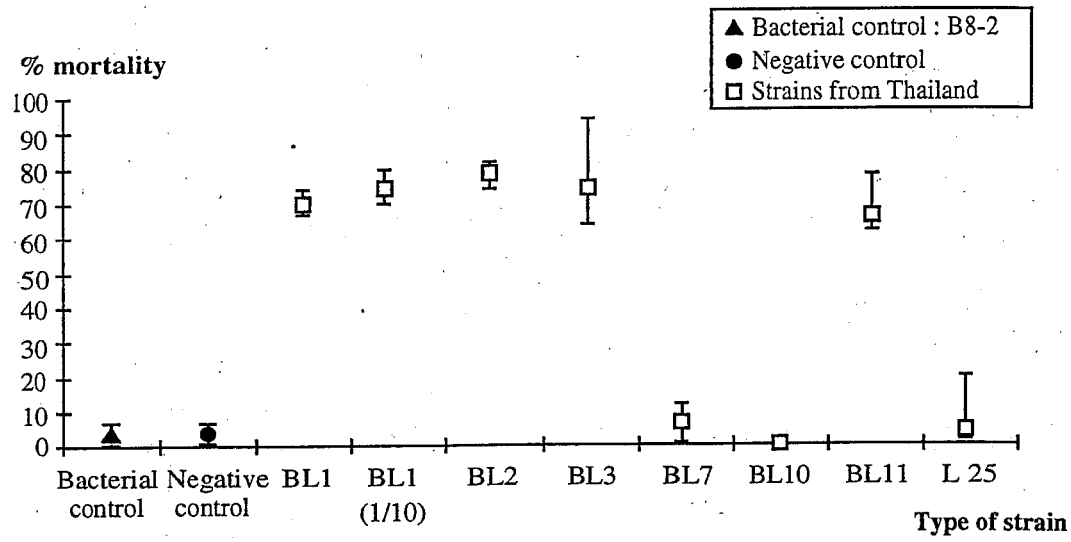

Fig. 2. Screening of *Vibrio* strains from Thailand: comparison of mortality observed in experimentally infected *Penaeus monodon* larvae (mysis III) after 48 h.

No difference was found between the biochemical characteristics of BL 1, BL 2, BL 3, and BL 11. One of these, BL 1, was arbitrarily chosen and further compared to B8-2. The pathogenic strains from Thailand (BL 1) and Ecuador (B8-2) have been both identified as *V. harveyi* using additional biochemical tests (Table 1).

Table 1. Main morphological and biochemical identification tests performed on BL 1 and B8-2. Literature data are reported for comparison.

	Strains		Literature data	
Characteristics	BL 1	B8-2	*V. harveyi*[1]	*V. harveyi*[2]
Gram	-	-	-	-
Motile	+	+	+	+
O/129 sensitivity	-	-	+	+
Oxidase	+	+	+	+
Swarming	-	-	-	-
Luminescence	+	+	+	d
Metabolism				
Resp.	+	+	+	+
Ferm.	+	+	+	+
ADH	-	-	-	-
LDC	+	+	+	+
ODC	+	+	+	+
ONPG	+	+		d
NO_3 to NO_2	+	+	+	+
Growth in % NaCl				
0	-	-	-	-
3	+	+	+	+
6	+	+	+	+
8	+	+	+	d
10	-	-	-	d

[1] from Lavilla-Pitogo *et al.* (1990).
[2] from Dodin and Fournier (1991).
d positive or negative depending on the strain

Drug sensitivity patterns of isolates were different between BL 1 and B8-2 (Table 2). Moreover, the minimum inhibitory concentrations for chloramphenicol, thiamphenicol, quinolones and tetracyclines exhibited difference between BL 1 and B8-2. The latter appeared to be more resistant to antibiotics commonly used in aquaculture, especially chloramphenicol and tetracyclines (Table 3).

In our bacteriological analysis the two pathogenic strains BL 1 and B8-2 were identified as *V. harveyi*, but as clinical signs described in Thailand and Ecuador were not exactly the same, we further investigated the virulence of BL 1 and B8-2 in comparative assays.

Table 2. Antibiotic sensitivity testing by diffusion tests (Discs Sanofi-Diagnostic Pasteur).

		Strain	
Category	Antibiotic	BL1	B8-2
BETA-LACTAMINES	Penicillin (10 IU, 6 µg)	R[1]	R
	Ampicillin (10 µg)	R	
	Cefalotin (30 µg)	R	
AMINOSIDES	Streptomycin (10 IU)	R	R
	Kanamycin (30 IU)	R	
	Gentamicin (10 IU, 15 µg)	R	S
PHENICOLS	Chloramphenicol (30 µg)	R	R
	Thiamphenicol (30 µg)	R	
TETRACYCLINES	Tetracycline (30 IU)	R	R
	Oxytetracycline (30 IU)	R	
MACROLIDES	Erythromycin (30 IU)	R	
POLYPEPTIDES	Polymixin B (300 IU)	R	R
	Colistin (50 µg)	R	R
SULFONAMIDES and associated antibiotics	Sulfonamides (200 µg)	R	
	Trimethoprim (5 µg)	R	
	T + S (1.25 µg + 23.75 µg)	R	R
NITRO-FURANES	Furanes (300 µg)	S	S
QUINOLONES	Nalidixic acid (30 µg)	S	S
	Flumequine (30 µg)	S	S
OTHERS	Novobiocine (5 µg)	R	

[1] R = Resistant, S = susceptable

Table 3. Minimum Inhibitory Concentrations (MIC) of common antibiotics for pathogenic strains BL 1 and B8-2.

	Thiamphenicol		Chloramphenicol		Quinolone		Tetracycline	
Conc. Tested	Strains		Strains		Strains		Strains	
(ppm)	BL 1	B8-2	BL 1	B8-2	BL 1	B8-2	BL 1	B8-2
10	+[1]	+	+	+	-[2]	-	+	+
20	+	+	+	+	-	-	+	+
30	+	+	+	+	-	-	+	+
40	+	+	+	+	-	-	+	+
50	+	+	-	+	-	-	-	+
60	+	+	-	+	-	-	-	+
70	+	+	-	+	-	-	-	+
80	+	+	-	+	-	-	-	-
90	+	+	-	+	-	-	-	-
100	+	+	-	-	-	-	-	-

[1] + = growth, [2] - = no growth

Virulence assays

For comparative studies, 1 mL of BL 1 bacterial suspension at various bacterial levels (10^7 to 10^2 CFU/mL) was added to larval rearing jars in order to obtain a dose-effect curve that permits comparison it with the effect of B8-2 bacterial suspension. Results are presented in Figure 3. Statistically significant difference was observed at 10^6 CFU/mL between BL1 and B8-2. The latter appears to be less pathogenic in experimental conditions. The mortality of *Penaeus monodon* zoea I larvae induced by B8-2 at 10^6 CFU/mL was not significantly different from the mortality induced by BL1 suspensions at 10^3 CFU/mL and 10^2 CFU/mL, according to the results of Duncan's multiple range test. All bacterial treatments were statistically different ($p<0.05$) from the negative control.

In vitro *assays*

Bacterial adhesion was assayed on various shrimp cell monolayers. Using Giemsa staining, pathogenic *Vibrio* BL 1 and B8-2 seemed to attach more to penaeid cells than the "bacterial control", consisting of cell monolayers treated with the non-pathogenic strain BL 10. Nevertheless, no differences were detected on *P. vannamei* ovarian cells in binding ability between pathogenic and non-pathogenic strains labelled by CTC were detected

In cytotoxicity assays, gaps were noticed in the monolayers treated with *Vibrio* (BL 1 and B8-2) culture supernatants (ECPs). Their size and number were not different.

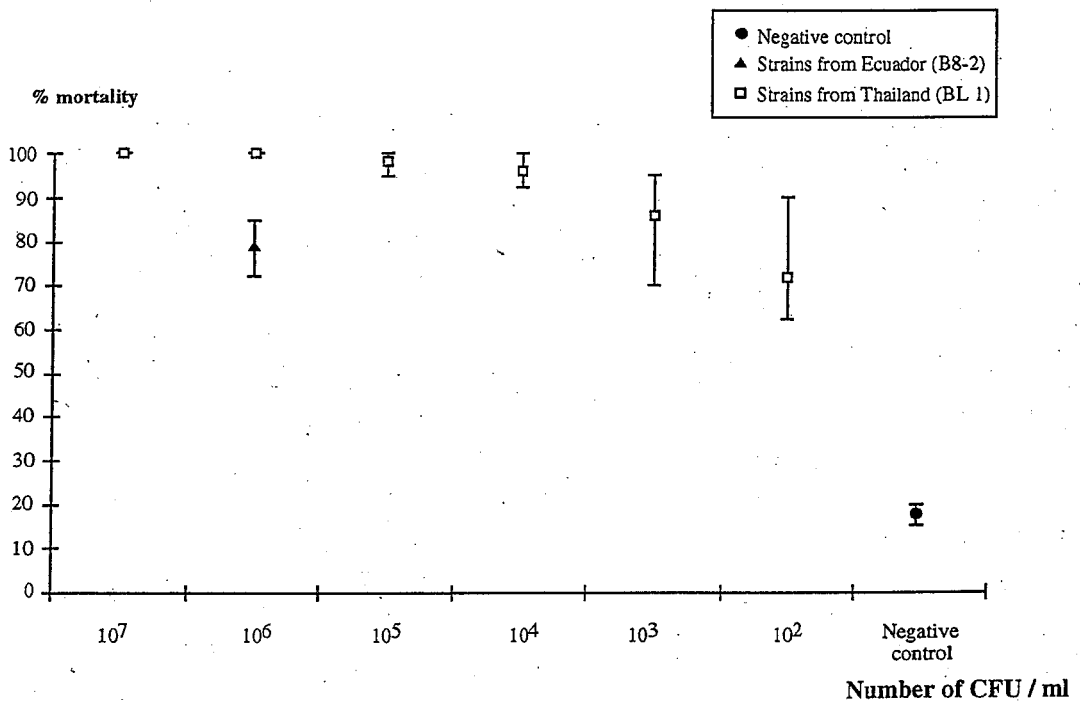

Fig. 3. Comparison of the pathogenic effects of *Vibrio* strains from Ecuador and Thailand in experimental infections of *Penaeus monodon* larvae (zoea I). Mortality was observed after 24 h.

In vitro assays did not show any difference between BL 1 and B8-2. Moreover, the strains had no extracellular products with strong cytotoxic effect on the monolayers tested. These *Vibrio* did not show any particular attachment to the monolayers tested.

Discussion

During mass mortality events in shrimp hatcheries in Thailand and Ecuador, bacterial samples were collected from water and larvae in rearing tanks. Comparison of pathogenic strains from Thailand and Ecuador was substantiated by similarities between diseases previously described. In this study, bacteriological analysis demonstrated that the main samples belonged to the same bacterial species (*V. harveyi*) and constituted the dominant flora on TCBS medium during mass mortality events, in both Ecuador and Thailand. Bacterial samples were screened by experimental infections in larvae of *P. monodon* pointing to five strains inducing high mortality rates. Pathogenic strains of *V. harveyi* have already been involved in larval mortalities with luminescence (Lavilla-Pitogo *et al.*, 1990). Experimental infections performed by Lavilla-Pitogo *et al.* (1990) also showed high pathogenicity of the isolated strains. Scanning electron microscopy demonstrated that the digestive tract was the site where pathogenic effects occurred. In our study, we found two strains of *V. harveyi* with different virulence levels in *P. monodon* larvae.

Experimental infections emphasized differences between the two strains. BL 1 was more pathogenic than B8-2 in assays performed on *P. monodon* larvae. According to Karlsson *et al.* (1992) and Neu (1992), bacterial adhesion to target cells is an important virulence factor. Moreover, for *Vibrio* strains, virulence is often related to the presence of toxins, especially cytotoxins, in fish pathogens. Our current hypothesis is that part of this virulence relies on species and tissue specificity. In order to test this hypothesis, investigations on bacterial adhesion and cytotoxicity in *in vitro* assays were begun on shrimp primary cell cultures. Drug sensitivity patterns of BL 1 and B8-2 showed differences. B8-2 appeared to be resistant to more antibiotics than BL 1, and the levels of resistance to the same antibiotics were higher in B8-2. This could be related to differences in the use of antibiotics in aquaculture in the two countries. Nevertheless, the pathogenic strain BL 1 from Thailand had higher minimum inhibitory concentrations for the antibiotics tested than strains of *V. harveyi* previously isolated from Southeast Asia (Baticados *et al.*, 1990).

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