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Experimental infection with different bacterial strains in larvae and juvenile *Litopenaeus vannamei* reared in Santa Catarina State, Brazil

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ABSTRACT. This study evaluated the pathogenic characteristics of bacteria isolated from *Litopenaeus vannamei* during an outbreak at the Laboratory of Marine Shrimp, UFSC, Santa Catarina State, Brazil. Their virulence potential in larvae and juvenile shrimp and the effects on the total haemocyte count, phenoloxidase activity and serum agglutinate titre were examined after experimental infection. Bacterial strains were isolated from larvae and adult shrimps, identified by the AP120E biochemical system as: two strains of *Vibrio alginolyticus*, three of *Aeromonas salmonicida* and one of *Pasteurella multocida* sp. and *Pasteurella* sp. All the bacterial strains isolated in this study caused mortality in shrimp. One strain of *V. alginolyticus* was responsible for 97.3 and 88.7% mortality in larvae and juvenile shrimps, respectively. The shrimp immunological system was influenced by experimental infection with *V. alginolyticus*. Decrease in the total haemocyte count and increase in the phenoloxidase activity and the serum agglutinate titre ($p < 0.05$) were observed. The results showed the high pathogenicity of *V. alginolyticus* isolated from larvae and juvenile reared marine shrimp.

Key words: shrimp, experimental infection, *Vibrio*, virulence, immunology.

RESUMO. Infecção experimental em larvas e juvenis de *Litopenaeus vannamei* cultivados no Estado de Santa Catarina, Brasil. Este estudo avaliou as características patogênicas de cepas de bactérias isoladas de *Litopenaeus vannamei* durante surto de mortalidade no Laboratório de Camarões Marinhos, UFSC, Estado de Santa Catarina, Brasil. Seu potencial de virulência em larvas e juvenis de camarão marinho e os efeitos sobre a contagem total de hemócito, atividade de fenoloxidase e título aglutinante do soro foram avaliados após infecção experimental. As cepas bacterianas foram isoladas de larvas e de camarões adultos e identificadas bioquimicamente pelo sistema API20E como: duas cepas de *Vibrio alginolyticus*, três de *Aeromonas salmonicida* e uma de *Pasteurella* sp. e *P. multocida*. Todas as cepas isoladas provocaram mortalidade em *L. vannamei*, e uma de *V. alginolyticus* resultou em mortalidade de 97,3 e 88,7% para larvas e juvenis de camarões, respectivamente. O sistema imunológico dos camarões juvenis sofreu influência da infecção experimental com *V. alginolyticus*, representado por redução significativa no número total de hemócitos circulantes e aumento na atividade da fenoloxidase e do título aglutinante do soro ($p < 0,05$). Os resultados deste ensaio demonstraram que a cepa de *V. alginolyticus* isolada apresentou alto poder de virulência para larvas e juvenis de camarão marinho cultivado.

Palavras-chave: camarão, infecção experimental, *Vibrio*, virulência, imunologia.

Introduction

Shrimp production in the State of Santa Catarina has experienced great losses due to disease outbreaks. In 2004 the presence of white spot syndrome virus (WSSV) was diagnosed for the first time in the shrimp farms (MADRID, 2005) which was responsible for huge economical losses in the shrimp industry.

With the appearance of WSSV, the Marine Shrimp Laboratory of the Federal University of Santa Catarina has followed strategies in

reproducer maintenance in the super intensive system, named Zero Exchange, Autotrophic and Heterotrophic System (ZEAH). According to Hargreaves (2006) this culture is characterized by a suspended-growth system, in which low water flow, dissolved organic carbon, ammonia and nitrite stimulate the formation of suspended microorganisms in rearing units. The low water flow increases the biosecurity in this system that prevents the entrance of pathogenic microorganisms. According to Montgomery-

Brock et al. (2007) the water temperature is maintained at 30°C, in which some important viral strains do not reproduce decreasing their virulence. On the other hand, the disadvantage of the ZEAH system is the low control of water quality, phytoplankton density, metabolism and community composition compared to other systems (HARGREAVES, 2006).

In the aquatic environment the microorganism population normally consists of non-pathogenic bacteria, as well as obligatory and opportunist organisms (SCHULZE et al., 2006). This equilibrium can be broken due to inadequate handling that favors pathogenic bacteria reproduction (KARUNASAGAR et al., 1994). Because of the high water temperature, high nutrient concentration and high stocking density in the ZEAH system, the lack of adequate handling may be responsible for rapid reproduction of *Vibrio* (WASIELESKY et al., 2006).

Vibriosis is one of the most important diseases in marine aquaculture and on several occasions has caused massive mortalities (KARUNASAGAR et al., 1994; VANDENBERGHE et al., 1998). Diseases caused by *Vibrio* are considered as a secondary infection that proliferates when shrimp is immunologically weakened (MORIARTY, 1998).

Vibrio proliferation has been controlled by prophylaxis and chemical products in commercial shrimp farms (GATESOUBE et al., 1989). This fact might provoke serious problems in the resistance to antibiotics (HAMEED et al., 2003; KARUNASAGAR et al., 1994). As a consequence, new alternatives to control bacterial diseases have been explored.

The objectives of this assay were to isolate and characterize bacterial strains in marine shrimp, *Litopenaeus vannamei*, during an outbreak at the Laboratory of Marine Shrimp, Santa Catarina State, Brazil. The pathogenicity potential of the isolates was analyzed by larval quality, total haemocyte count, phenoloxidase activity and serum agglutinate titre after experimental infection.

Material and methods

Bacterial isolation and identification

Bacteria were isolated during an outbreak in larval rearing and reproducers of *L. vannamei*. Shrimp were disinfected with 70% alcohol, macerated in 3% sterile saline solution (1 g sample to 1 mL water), diluted in solutions (1:10), in order to be scattered on Petri dishes with Agar TCBS (Tiosulfate Citrate Salt of Bilis Sucrose, Difco™) and incubated at 30°C for 24h.

In the ZEAH system, shrimps were collected with clinical signs such as melanized necrosis in the first

segment of the abdomen. Samples of haemolymph were collected from the ventral sinus, scattered on Petri dishes with agar TCBS and incubated at 30°C for 24h. After incubation green and yellow colored colonies were pricked again to drain to the last drop and complete isolation. The isolated strains were then identified biochemically in an API 20E (Biomerieux™) kit (LIGHTNER, 1996).

Experimental infection in larvae

The 1 L aquaria were filled with sterile marine water, aerated, maintained at 28°C and stocked with 100 larvae at the mysis 2 stage to test seven strains (treatments) and four replications.

Bacteria were inoculated directly in the water at a concentration of 1×10^6 colony forming units (CFU mL⁻¹) to raise the mortality rate and larval quality 48h after inoculation. The larval quality was observed by swimming activity homogeneous stage, lipid contents and hepatopancreas color relative to diet, cromatophorus color, the presence of food in the intestines, necrosis, cannibalism, deformities, epibionts, fouling, luminous appearance (suggesting bacterial contamination) determined by a protocol for larval quality FM-03/PO-4/LCM (FAO, 2004) and slightly modified by Mouriño et al. (2008a).

Experimental infection in juveniles

A total of 420 shrimps (6.86 ± 0.86 g body weight) were randomly distributed in twelve 100 L aquaria, 35 shrimps in each aquarium. In six of these aquaria the animals were injected with 25 µL sterile saline solution containing 1×10^7 CFU *Vibrio alginolyticus* 1 mL⁻¹ (the strain that caused the highest mortality in larvae) in the first segment of the abdomen with the aid of a 1 mL syringe (MOURIÑO et al., 2008b). In the other six aquaria, the shrimps received 25 µL saline solution (control group). Ten hours after injection the survival rate, haemolymph samples from five shrimps per aquarium for total count of haemocytes, phenoloxidase activity and serum agglutinate titre were collected, as well as samples of hepatopancreas and haemolymph for microbiological analysis (*Vibrio* ssp. count).

Collection of haemolymph and hepatopancreas

A sample of 10 µL of haemolymph was collected with 4°C refrigerated syringes, fixed in 4% formaldehyde-MAS (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) for total haemocyte count (THC). Another 10 µL sample was left to coagulate in ice, to freeze and defrost three times, centrifuged at 2000 rpm for 10 minutes to remove the serum and stored at 20°C. After removing the haemolymph, a hepatopancreas sample was carefully collected.

Hepatopancreas and haemolymph for microbiological analysis

The haemolymph and a sample of macerated hepatopancreas were scattered in sterile conditions on a Petri dish containing Agar TCBS to incubate at 30°C for 24 hours (to *Vibrio* spp. count).

Immunological analysis

The total haemocyte count was determined directly using a Neubauer chamber. The serum protein concentration was determined according to the Bradford (1976) method using bovine serum albumin (BSA) as standard.

Phenoloxidase activity (PO) activity was detected spectrophotometrically (490 nm) by the formation of the DOPA-chrome pigment, after oxidation of the L-dihydroxyphenylalanine substrate (L-DOPA, Sigma). Briefly, serum samples (8 pools of 3 shrimp) were diluted (1:9) in TBS-1 (1 mM Tris, 336 mM NaCl, 5 mM CaCl₂, 10 mM MgCl₂, pH 7.4) and 50 µL of the solution was incubated in triplicate with 50 µL trypsin enzyme inducer (Sigma, 1 mg L⁻¹) for 5 minutes in 96-microwell plates. After incubation, 50 µL L-DOPA (3 mg mL⁻¹) was added to each well. Control was done with 100 µL TBS added to 50 µL of the 1:9 diluted serum in TBS. DOPA-chrome formation was monitored after 0, 5 and 10 minutes. One unit of enzymatic activity is equivalent to a variation of 0.001 in the absorbance min.⁻¹ milligram⁻¹ of protein (SÖDERHÄLL; HALL, 1984). Each test was performed in triplicates.

To determine agglutinating activity, 50 µL samples of shrimp serum were added to a U-shaped 96-well microplate plate and a twofold serial

dilution was prepared using TBS-2 (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM CaCl₂) as the diluent. The same volume of a 2% suspension of rat red blood cells in TBS was added to each well and incubated for 2–3h at room temperature in a humid chamber. In controls, the shrimp serum was replaced by TBS. The titer of the natural agglutinating activity of the shrimp serum was expressed as the reciprocal of the highest dilution showing a positive agglutination pattern. Each test was performed in duplicate.

Statistical analyses

The results obtained were first submitted to the Bartlett test, to evaluate variance homogeneity. Microbiological counts and THC were log(x+1)-transformed to homogenize variances before analysis. Agglutinating titre were log₂(x) transformed. Data from experimental infection in larvae were analyzed by one-way ANOVA (p < 0.05). When analysis of variance indicated difference, the Tukey test was used at the 0.05 level of significance. Data from experimental infection in juveniles were analyzed by the t-test analysis (p < 0.05) (ZAR, 1984).

Results and discussion

Five strains of bacteria from larvae were isolated and identified as one strain of *Vibrio alginolyticus*, two of *Aeromonas salmonicida*, one of *Pasteurella* sp. and one of *P. multocida*. Two strains were isolated from the adult shrimp haemolymph: one of *Vibrio alginolyticus* and one of *Aeromonas salmonicida*. All bacteria presented different biochemical features as shown in Table 1.

Table 1. Mortality rate, larval quality index (QI), source of isolated strains evaluated after the virulence test and biochemical characteristics.

Strains	Mortality (%)	QI	Source	ONPG	ADH	LDC	ODC	CIT	H ₂ S	URE	TDA	VP
<i>V. alginolyticus</i> 1	97.3 ± 4.6 ^a	30.0 ^b	Larval rearing	-	-	+	-	-	-	-	-	-
<i>V. alginolyticus</i> 2	88.7 ± 19.6 ^{ab}	36.3 ^b	Reproduction	-	+	+	+	-	-	-	-	-
<i>A. salmonicida</i> 1	82.0 ± 8.0 ^b	93.8 ^a	Larval rearing	-	-	-	-	-	-	-	-	+
<i>A. salmonicida</i> 2	79.3 ± 4.6 ^b	73.8 ^a	Larval rearing	-	-	-	-	-	-	-	-	+
<i>A. salmonicida</i> 3	75.3 ± 12.8 ^b	77.5 ^a	Reproduction	-	-	-	-	-	-	-	-	+
<i>Pasteurella</i> sp.	72.0 ± 8.0 ^c	65.0 ^a	Larval rearing	-	-	-	-	-	-	-	-	-
<i>P. multocida</i>	67.0 ± 6.8 ^c	90.0 ^a	Larval rearing	-	-	-	-	-	-	-	-	-
Control	32.0 ± 10.4 ^c	105.0 ^a										
Strains	SAC	MEL	IND	RHA	GEL	GLU	MAN	INO	SOR	AMY	ARA	OX
<i>V. alginolyticus</i> 1	+	-	+	-	+	-	+	-	-	-	-	-
<i>V. alginolyticus</i> 2	+	-	+	-	-	+	+	-	-	+	-	+
<i>A. salmonicida</i> 1	-	-	-	-	-	+	-	-	-	-	-	+
<i>A. salmonicida</i> 2	-	-	-	-	-	+	-	+	-	-	-	+
<i>A. salmonicida</i> 3	-	-	-	-	-	+	-	-	-	-	-	+
<i>Pasteurella</i> sp.	-	-	+	+	-	+	-	+	-	+	-	+
<i>P. multocida</i>	+	-	+	-	-	+	-	-	-	-	-	+
Control												

*Different letters indicate significant difference among the strains by the Tukey test (p < 0.05). Biochemical characteristics: β-galactosidase (ONPG), arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC), citrate utilization (CIT), H₂S production (H₂S), urease (URE), tryptophane deaminase (TDA), Voges Proskauer (VP), sucrose (SAC), melibiose (MEL), indole production (IND), Rhamnose (RHA), gelatinase (GEL), Glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), amygdalin (AMY), arabinose (ARA), oxidase (OX).

After experimental infection the strains isolated showed pathogenicity to larvae with mortalities higher than the control larvae ($p < 0.05$). Two strains of *V. alginolyticus* caused the highest mortality in larvae and the worst larval quality index (Table 1).

The larvae infected by *A. salmonicida* showed the lowest mortality and higher larval quality than ($p < 0.05$) those infected by *V. alginolyticus* (Table 1). On the other hand, *Pausterella* sp. and *P. multocida* provoked the lowest mortalities but higher than ($p < 0.05$) observed in the control (Table 1).

Juvenile shrimp infected by *V. alginolyticus* showed high mortality and high counts ($p < 0.05$) of *Vibrio* spp. in the haemolymph and hepatopancreas when compared to the control (Table 2).

In normal conditions the haemolymph and hepatopancreas do not contain bacteria. The crustacean immune system is formed by strong physical barriers (thick cuticle) further to cellular and humoral defenses mediated by the haemolymph (JIRAVANICHPAISAL et al., 2006). When the physical barrier is broken by an infectious agent, the immune system is responsible for reducing the circulating microorganisms (VAN DE BRAAK et al., 2002). These microorganisms attach to other organs such as the hepatopancreas (SUNG et al., 1999) and lymphoid organ (VAN DE BRAAK et al., 2002). This fact was proved in this trial when 10 hours after infection the concentration of bacteria in the hepatopancreas was higher than that observed in the haemolymph. In spite of the antiseptics at the injection site the bacteria found in the haemolymph and hepatopancreas in saline injected shrimp may be related to the wound caused by injection that allowed the opportunistic microorganisms in from the water.

The reduction in the total haemocyte count ($p < 0.05$) in the *V. alginolyticus* injected shrimp (Table 2) is possibly related to haemocytes migration to the infected area as assumed by Lorenzon et al. (2002). In addition, the haemocytes can agglutinate in several layers to capture microorganisms (ALVARENGA et al.,

1990) to be removed from the circulation of the gills (MARTIN et al., 2000).

Phenoloxidase activity in the infected animals was higher than that reported in non-infected animals ($p < 0.05$) (Table 2). The prophenoloxidase system named proPO is one of the most important immune responses in crustaceans and is activated by microorganism surface compounds such as lipopolysaccharide (LPS) from Gram negative bacterium, peptidoglycan from Gram positive bacterium and B-1,3-glycan from fungus (SÖDERHÄLL; CERENIUS, 1998). On the other hand, with the activation of compounds proPO system, toxic molecules such as quinone, hemiquinone and free radicals (reactive oxygen species) are responsible for destruction of the invader pathogens.

In crustaceans the agglutination is mediated by lectin, plasmatic proteins normally synthesized in the hepatopancreas (GROSS et al., 2001; LUO et al., 2006) that recognize sugar *N*-acetylate, especially those derived from sialic acid and sialoglycoconjugate (MARQUES; BARRACCO, 2000). A group of agglutinins capable of binding to bacterial LPS was isolated and characterized in crawfish, *Pacifastacus leniusculus* (KOPÁČEK et al., 1993), in shrimps, *Farfantepenaeus californiensis* (VARGAS-ALBORES et al., 1993), *Litopenaeus schmitti* (COMINETTI et al., 2002) and *Penaeus monodon* (LUO et al., 2006). All of them bind to a LPS that suggests an important role to control the infection caused by Gram negative bacteria such as *Vibrio*, a microorganism pathogenic to reared shrimp. This explains the high agglutinate titre ($p < 0.05$) in infected shrimp (Table 2).

The increased phenoloxidase and agglutinate titre in the haemolymph of infected shrimp was possibly stimulated by recognition of LPS in the *V. alginolyticus* membrane by activating the humoral defense system.

The larval quality indexes confirmed their relation with survival rates that was directly proportional to high mortality. Nevertheless, the reduced larval quality index occurred after mortalities in larviculture.

Table 2. Mortality rate (MOR), total count of *Vibrio* spp. in the hemolymph and hepatopancreas, total count of haemocytes (THC), phenoloxidase activity (PO) and agglutinate titer (AGT) in serum of shrimp injected with saline solution or *Vibrio alginolyticus* after 10 hours.

Treatments	PO (U mg ⁻¹)	THC (cells mL ⁻¹ x 10 ⁶)	AGT (x 10 ³)	MOR (%)	<i>Vibrio</i> spp.	
					Haemolymph (UFC mL ⁻¹ x 10 ³)	Hepatopancreas (UFC g ⁻¹ x 10 ³)
Shrimp injected with saline solution	24.32 ± 10.84 ^a	36.4 ± 7.00 ^a	28.67 ± 10.03 ^a	0	0 ^a	1.50 ± 2.00 ^a
Shrimp injected with <i>V. alginolyticus</i>	49.67 ± 7.03 ^b	6.58 ± 1.72 ^b	80.00 ± 48.23 ^b	52.74 ± 5.21 ^b	2.00 ± 2.40 ^b	59.0 ± 67.0 ^b

*Different letters indicate significant difference among the treatments by the t-test ($p < 0.05$).

Indeed, the bacterial strains isolated in this assay were pathogenic to cultivated marine shrimp larvae and *V. alginolyticus* was the most virulent. Consequently, the immunological system of juvenile shrimp was damaged by the experimental infection with *V. alginolyticus*.

Conclusion

Of the isolated strains, *V. alginolyticus* showed the highest virulence to shrimp larvae.

Vibrio alginolyticus was virulent to juveniles of shrimp and did modify their immune parameters such as total haemocytes count, phenoloxidase activity and agglutinate titer after infection.

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