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Susceptibility and immune response of Catarina scallop (*Argopecten ventricosus*) against *Vibrio parahaemolyticus*, the causative agent of AHPND in shrimp

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ABSTRACT

Susceptibility and immune response of *Argopecten ventricosus* challenged with *Vibrio parahaemolyticus* were determined. Mortality and median lethal concentration of *V. parahaemolyticus* were assessed in scallop seeds and adults challenged by immersion. Histopathological analysis was done in adults challenged by immersion. The immune response was analyzed in adults by injecting heat-inactivated bacteria in the adductor muscle. Hemolymph samples were taken at 0, 8, 24, 48, and 72 h. Time zero corresponded to samples of scallop hemolymph before challenging with vibrio. In bioassays, scallops were fed with microalgae concentrate Shellfish Diet 1800®. Seeds and adults of *A. ventricosus* were susceptible to *V. parahaemolyticus* showing a LC_{50} of 623 CFU mL^{-1} and 62,853 CFU mL^{-1}, respectively. *V. parahaemolyticus* toxins caused lesions in digestive glands of adults analyzed. Significant variations were observed in hemocyte number, phenoloxidase activity, and lysosomal hydrolytic enzymes, mainly at 8 and 24 h, post infection. Results confirm that seeds and adults of *A. ventricosus* are susceptible to *V. parahaemolyticus* infection, but seeds are more susceptible than adults. In adults, the immune response of *A. ventricosus* was modulated by *V. parahaemolyticus*.

1. Introduction

The Catarina scallop, *Argopecten ventricosus* (Sowerby II, 1842), is an important fishery resource in Nortwest Mexico, mainly in Baja California Sur (Ruiz-Verdugo et al., 2016). Because of this, efforts have been made to develop mariculture of this species with the final purpose of recovering populations in fishery areas (Medina et al., 2007). However, as in other bivalve mollusc species, diseases are the main issues that the pectinid culture is facing (Barbosa et al., 2001). The incidence of *Vibrio* genus, which causes massive mortalities in bivalve molluscs, is considered the most common sanitary problem afecting larvae, juveniles, and adults (Beaz- Garnier et al., 2007; Gómez-León et al., 2008; Beaz-Hidalgo et al., 2010). Studies on the diversity of *Vibrio* species found that the predominating species associated with bivalve molluscs from different geographical locations were *V. splendidus*, *V. alginolyticus*, *V. harveyi* (Beaz-Hidalgo et al., 2010). However, other vibrios like *V. parahaemolyticus* are virulent to small abalones (*Haliotis diversicolor supertexta*) (strain 880915) (Lee et al., 2003). In cultured molluscs, the severity of *Vibrio* attack is related to suboptimal culture conditions (high density, contaminated microalgae, residual gametes, high temperatures), which increase bacterial proliferation and compromise the immune response of infected organisms (Elston, 1984). In the case of crustaceans, *Litopenaeus vannamei* is severely affected by *Vibrio parahaemolyticus*, causing acute hepatopancreatic necrosis disease (AHPND). This bacterium becomes virulent by acquiring a plasmid that expresses toxins (PirA and PirB), which induce cell death by membrane pore forming (Lee et al., 2015). In addition, *V. parahaemolyticus* has type III (T3SS1) and VI (T6SS1 and T6SS2) secretion systems that are important virulence factors (Cascales, 2008; Kongrueng et al., 2014). Nowadays, there are no reports about pathogenicity of *V. parahaemolyticus* in bivalve molluscs.

Compared to larvae, diseases caused by bacteria in juveniles and

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adults exist but are less frequent (Sindermann, 1990; Lane and Birkbeck, 2000), which indicates that a process of maturation of the immune system occurs during the ontogeny of these organisms (Bachère et al., 1995; Luna-González et al., 2003).

The internal immune defense is fundamental to protect animals against pathogens (Matozzo et al., 2016). In bivalves, internal defense relies solely on innate immune mechanisms, including cellular and molecular effectors. Hemocytes play a key role in the immune response, mainly through the phagocytosis of foreign particles, and the production of reactive oxygen intermediates, hydrolytic enzymes, and antimicrobial peptides (Carballal et al., 1997; Donaghy et al., 2012; Hine, 1999; Mitta et al., 2000). Because of the fact that hemocytes are a key element of internal defense, their concentration or size can provide information about cell division processes or pathologic states (Lambert et al., 2007; Ramírez-Castillo et al., 2011).

In this paper, we describe, for the first time, the effect of *Vibrio parahaemolyticus* (at different concentrations), pathogenic for penaeid shrimp, on the susceptibility and immune response of seeds and adults of *A. ventricosus*.

2. Materials and methods

2.1. Experimental animals

A. ventricosus seeds were obtained from Acuacultura Robles, S.P.R. de R.I. (La Paz, B.C.S., Mexico) and transported by plane in a wet sponge to the Immunology Laboratory of the Aquaculture Department at Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional-Instituto Politécnico Nacional (CIIDIR-IPN) in Guasave, Sinaloa, Mexico. Although animals had a pathogen-free certificate, they were quarantined during 10 d and discharged water was chlorinated. Adult clams were collected by diving in El Caracol (Guasave, Sinaloa, Mexico) (108°74′W 25°49′N) and transported in 250-L plastic tanks containing seawater and constant aeration to the laboratory facilities. In the laboratory, the organisms were acclimated for 3 d in 2 plastic tanks (1000*L*) with filtered (20 μm) seawater at 30 psu and under constant aeration. The scallops were fed thrice a day with the microalgae concentrate Shellfish Diet 1800® (Reed Mariculture Inc., Campbell, CA, USA) composed of *Isochrysis*, *Pavlova*, *Tetraselmis*, and *Thalassiosira pseudonana*, according to Reed Mariculture's instructions. The tanks were cleaned daily by siphoning and the discarded water was recovered immediately.

2.2. Bioassays 1 and 2 (*A. ventricosus* susceptibility to *V. parahaemolyticus* and LC_{50})

Two bioassays, one for seeds (height: 5.3 ± 1.1 mm) and one for adults (height: 4.3 ± 0.57 cm) were conducted for 5 and 10 d, respectively, in an open culture system, using glass tanks (4 L) for seeds and plastic tanks (60 L) for adults with filtered (20 μm) seawater and constant aeration. Ten seeds or adults were placed per tank and fed with the microalgae concentrate, Shellfish Diet 1800®. Seeds were fed twice a day with 4×10^7 cells L^{-1}, 2×10^7 cells L^{-1} at 08 h and 2×10^7 cells L^{-1} at 17 h. Adults were fed fed twice a day with 5×10^7 cells L^{-1}, 2.5×10^7 cells L^{-1} at 08 h and 2.5×10^7 cells L^{-1} at 17 h. *V. parahaemolyticus* strain was isolated, characterized, and tested in shrimp by López-León et al. (2016). Treatments in triplicate were as follows: I) Control, without *Vibrio*; II) *Vibrio* (1×10^2 CFU mL^{-1}); III) *Vibrio* (1×10^3 CFU mL^{-1}); IV) *Vibrio* (1×10^4 CFU mL^{-1}); V) *Vibrio* (1×10^5 CFU mL^{-1}). Debris were removed by siphoning before feeding and water recovered. Survival was determined daily and dead animals were removed.

2.2.1. Determination of median lethal concentration

Cumulative mortality from bioassays were used to calculate the median lethal concentration (LC_{50}) by using Probit analysis (Finney, 1952) with StatPlus® 2009 professional 5.8.4.

2.3. Bioassay 3 (histological analysis in scallops infected with *V. parahaemolyticus*)

Two groups of scallops (height: 4.3 ± 0.57 cm) were used, one as control (4 scallops) and one for challenging (4 scallops) with *V. parahaemolyticus* (1×10^6 CFU mL^{-1}) by immersion. Animals were fed fed twice a day with 1.2×10^9 cells, 6×10^8 cells at 08 h and 6×10^8 cells at 17 h. All challenged scallops were moribund at 24 h and were removed for histological analysis. Tissue samples (digestive glands) were previously fixed in 10% formaldehyde, neutralized with dibasic sodium phosphate ($2\ g\ L^{-1}$) and monobasic sodium phosphate ($4\ g\ L^{-1}$), and processed according to the histological techniques (Kim et al., 2006). Tissues were dehydrated in alcohols of ascending concentration (50 to 100%) and embedded in Paraplast-TX®. Samples were sectioned at 4 μm in a rotating microtome (Leica RM 2155). The histological sections were stained with hematoxylin and eosin (Humason, 1979). The slides were observed with a composite microscope (Olympus BX50) and the visual fields (10 ×, 20 ×, and 40 × magnifications) were digitized with a digital camera (Nikon DS-Ri1) and the images processed with the Image Pro Premier® (version 9.0, Media Cybernetics, USA) software.

2.4. Bioassay 4 (immune system of *A. ventricosus*)

A 72-h bioassay was performed in which the effect of *V. parahaemolyticus* infection was evaluated in adult scallops. The culture system consisted of a 130-L plastic tank with 120 L of filtered (20 μm) seawater at 30 psu and constant aeration. Twenty-eight animals (height: 4.4 ± 0.51 cm) were placed in the tank and cultured under natural photoperiod. The feeding was done as in previous bioassays. Animals were challenged by injection into the adductor muscle with 1.5×10^6 dead bacterial cells in 100 μL^{-1} saline solution (2.5% NaCl). Bacterial cells were killed by heat at 60 °C for 10 min. The tank was cleaned daily by siphoning and the discarded water was recovered immediately. Physicochemical parameters, such as temperature, pH, salinity and dissolved oxygen, were determined daily. Hemolymph was withdrawn from 5 scallops randomly chosen per time (0, 8, 24, 48, and 72 h) for analysis of the immune system. Time zero corresponded to samples of scallop hemolymph before challenging with vibrio. Hemocytes were counted and measured (diameter) and the activity of phenoloxidase (PO) and lysosomal hydrolytic enzymes was determined. Total protein was determined by the method of Bradford (1976), using bovine serum albumin for the standard curve.

2.4.1. Hemolymph extraction

One milliliter hemolymph was withdrawn from each adult scallop sampled from bioassay 4 by puncturing the hemolymphatic sinus of the adductor muscle with a sterile 1-mL syringe (27 G × 13 mm needle). The hemolymph samples were individually stored in pre-cooled Eppendorf tubes to be used immediately.

2.4.2. Hemocyte quantification and measurement

Hemolymph sample was diluted 1:3 (50 μL:150 μL) in a 6% formalin solution (Luna-González et al., 2004) to prevent hemocyte agglutination. Hemocytes were counted in a Neubauer's chamber and measured from digital photographs. Fifty hemocytes from each specimen were measured using the Sigma Scan Pro (V 5.0) software. The outline of each hemocyte was manually drawn and the lengths of the larger and smaller axes were measured. Hemocyte diameter was calculated as the average length of both axes (Ramírez-Castillo et al., 2011), then, the diameter measurement was used to calculate the hemocyte area.

2.4.3. Hemolymph lysate samples

Withdrawn hemolymph samples were subjected to two freezing-