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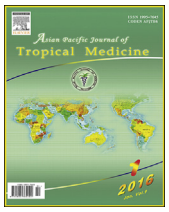

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Enterobacteria and *Vibrio* from *Macrobrachium amazonicum* prawn farming in Fortaleza, Ceará, Brazil

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ABSTRACT

Objective: To investigate the isolation of enterobacteria associated with *Macrobrachium amazonicum* (*M. amazonicum*) farming and evaluate the *in vitro* antimicrobial susceptibility of *Vibrio* strains.

Methods: Strains were isolated from female *M. amazonicum* prawns and environmental and hatchery water. Biochemical assays were used to identify bacterial genera and those belonging to the genus *Vibrio* were submitted to further analyses for species identification, through Vitek 2 automated system and serotyping. Susceptibility test was performed according to Clinical Laboratory Standards Institute.

Results: The following genera of enterobacteria were recovered: *Enterobacter* (n = 11), *Citrobacter* (n = 10), *Proteus* (n = 2), *Serratia* (n = 2), *Kluyvera* (n = 2), *Providencia* (n = 2), *Cedecea* (n = 1), *Escherichia* (n = 1), *Edwardsiella* (n = 1) and *Buttiauxella* (n = 1). As for *Vibrio*, three species were identified: *Vibrio cholerae* non-O1/non-O139 (n = 4), *Vibrio vulnificus (V. vulnificus)* (n = 1) and *Vibrio mimicus* (n = 1). *Vibrio* spp. showed minimum inhibitory concentrations values within the susceptibility range established by Clinical Laboratory Standards Institute for almost all antibiotics, except for *V. vulnificus*, which presented intermediate profile to ampicillin.

Conclusions: Enterobacteria do not seem to be the most important pathogens associated with *M. amazonicum* farming, whereas the recovery of *Vibrio* spp. from larviculture, with emphasis on *Vibrio cholerae* and *V. vulnificus*, deserves special attention due to their role as potentially zoonotic aquaculture-associated pathogens. Furthermore, the intermediate susceptibility of *V. vulnificus* to ampicillin reflects the importance of monitoring drug use in prawn farming.

1. Introduction

The favorable climate and the technological development for prawn/shrimp production make Brazil one of the main producers in the Americas. In 2014, Brazil exported 216 metric tons of prawn, standing out in the international export market, and the state of Ceará is a leader in production [1]. *Macrobrachium amazonicum* (*M. amazonicum*) has a particularly high potential for aquaculture in South America, because it is present in the most important South American river basins, including the Amazon [2]. In Northern and Northeastern Brazil, *M. amazonicum* is important for artisanal and subsistence fishing and it has been gaining attention for commercial purposes [2,3].

Infectious diseases in aquatic organisms are one of the main risks for economical losses in the aquaculture industry and many

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of these diseases are caused by bacteria that are potentially pathogenic to humans [4]. The risk of zoonotic infections with these microorganisms, by either handling or ingesting aquaculture products, rises with the increase in aquaculture production and consumption of its products [5]. Bacteria belonging to the family Enterobacteriaceae are not only one of the main indicators of poor sanitary conditions for farmed shrimp, but also one of the main bacterial families causing seafood associated infections [6,7]. In addition, bacteria of the genus *Vibrio* are important pathogens for farmed crustaceans and also have been reported as primary agents of bacterium-associated illness due to seafood consumption and handling, with emphasis on the species *Vibrio cholerae* (*V. cholerae*), *Vibrio vulnificus* (*V. vulnificus*) and *Vibrio parahaemolyticus* [8,9].

Thus, this study initially sought to isolate enterobacteria associated with *M. amazonicum* farming. Then, due to the incidental recovery of *Vibrio* spp. from hatchery water, the pursuit for this bacterial genus in prawn farming and in the natural environment and the evaluation of the *in vitro* antimicrobial susceptibility of the recovered *Vibrio* strains were included as goals.

2. Materials and methods

2.1. Research licensing

This study was previously approved by the Chico Mendes Institute for Conservation of Biodiversity/Biodiversity Authorization and Information System – SISBIO, under the number 28175-1.

2.2. Collection of hatchery water

Duplicate 5-mL-aliquots of water from *M. amazonicum* hatchery were collected with sterile syringes, from different areas of the larviculture tanks (bottom, substrate, surface and near the walls of the tank), according to Brilhante *et al.* [10]. Each cultivation tank had a capacity of 70 L, density of 20 larvae/L and water salinity of 4 mg/L salinity. The samples were weekly collected, for two consecutive hatchery cycles of *M. amazonicum* prawns at the Laboratory of Shrimp Farming of the State University of Ceará. A total of 18 samples of hatchery water were obtained and these samples were taken to the Laboratory of Emerging and Reemerging Pathogens for microbiological processing and recovery of bacterial strains.

2.3. Collection of M. amazonicum and water from the natural environment

After the incidental recovery of *Vibrio* sp. from hatchery water, it was decided to investigate the presence of this bacterial genus in the environment where the ovigerous females were harvested, in order to obtain *M. amazonicum* larvae for hatchery in captivity. Thus, ovigerous females were collected in Sapiranga Lake (3°48′3.46″ S and 38°27′30.83″ W), Fortaleza, Ceará, Brazil and sent to the Laboratory of Shrimp Farming of the State University of Ceará. The digestive tracts of 10 females were removed by making a dorsal transverse incision, they were placed in sterile slants containing sterile saline (0.9% NaCl), and were treated as one single sample [10]. Overall, 20 *M. amazonicum* females were used, yielding two digestive tract samples.

In addition, water samples from shallow areas of the Sapiranga Lake were collected, according to Medeiros *et al.* [11], with some modifications, for two consecutive weeks, obtaining a total of two samples. The water samples were obtained with a 1-L Van Dorn bottle, which was rinsed three times with water from the lake, before collection. All collected samples were transported to Laboratory of Emerging and Reemerging Pathogens for microbiological processing and bacterial isolation.

2.4. Sample processing and bacterial isolation and identification

Initially, for the primary recovery of Enterobacteriaceae the specimens were seeded on BHI agar (HiMedia; India), MacConkey agar (Sigma–Aldrich; USA), and Salmonella-Shigella agar (HiMedia; India) [12]. Then, after the incidental recovery of Vibrio sp. from hatchery water, TCBS agar (BD Difco; USA) was used for bacterial primary recovery, in order to monitor the production system and the natural environment for the presence of this bacterial genus.

Hatchery and natural water samples were similarly processed. The samples were divided into two 2.5 mL-aliquots in hemolysis tubes. The tubes were then centrifuged at 3 000 rpm for 20 min. After centrifugation, the supernatant was discarded and the remaining material was transferred to a sterile test tube with sterile saline, reaching a total volume of 1 000 μL. After this procedure, 1 000 μL of sterile saline were added and each suspension was homogenized in a vortex for 3 min and left to settle for 30 min at 25 °C [11]. Subsequently, 10 μL-aliquots of the supernatant of each sample were seeded onto the agar plates and incubated at 35 °C, for 24 h–48 h.

The digestive tracts were opened and mixed in a sterile porcelain mortar, and a suspension was prepared with approximately 1 g of the material and sterile saline. Then the suspension was homogenized in a vortex for 3 min and left to settle for 30 min at 25 °C [10]. Aliquots of 10 μL of the supernatant of each sample were seeded onto the agar plates and incubated at 35 °C for 24 h.

The recovered colonies were individually subcultured on MacConkey agar and TCBS agar. Then, they were Gram stained, for the selection of Gram-negative microorganisms, and tested for the production of cytochrome-oxidase to differentiate between oxidase-negative microorganisms, which include enterobacteria, and oxidase-positive microorganisms, which include the genus *Vibrio* [13].

The genera of Enterobacteriaceae were identified through the following tests: carbohydrate utilization, with Triple Sugar Iron medium, citrate assimilation, phenylalanine desaminase and urease production, decarboxylation of amino acids (lysine, arginine and ornithine), Voges–Proskauer reaction, hydrogen sulfide and indole production and motility. The test results were read after 20 h and interpreted following the identification keys [12].

Vibrio species were initially identified through glucose fermentation, urease and indole production, and motility