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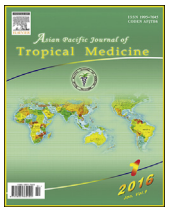

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Enterobacteria and *Vibrio* from *Macrobrachium amazonicum* prawn farming in Fortaleza, Ceará, Brazil

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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

tests [12]. Reading was performed after 20 h and interpreted following the identification keys [14]. Subsequently, the strains were identified through Vitek 2 automated system (bioMérieux; USA).

The recovered *V. cholerae* strains were also serotyped with antisera specific for serogroup O1 and O139 (PROBAC; Brazil). The strains that showed no agglutination with these antisera were described as non-O1/non-O139 *V. cholerae* [15].

2.5. In vitro susceptibility test of Vibrio spp.

Antimicrobial minimum inhibitory concentrations (MIC) were determined through the broth microdilution method, as described by the Clinical Laboratory Standards Institute, document M07-A9 [16]. The tested drugs were ampicillin, azithromycin, doxycycline, trimethoprim–sulfamethoxazole and chloramphenicol, against all *Vibrio* species, and ceftazidime and ciprofloxacin (Sigma Chemical Corporation; USA) against *V. vulnificus* and *Vibrio mimicus (V. mimicus),* document M45-A2 [17]. *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC29213 were included as quality control, according to document M100-S22 [18]. Susceptibility tests were performed in 96-well plates, which were incubated at 35 °C for 20 h [17]. All assays were performed in duplicate, and for each strain drug-free growth control and inoculum-free sterility control were included. The antimicrobial MIC were defined as the lowest concentration able to inhibit 100% bacterial growth, except for trimethoprim–sulfamethoxazole, for which MIC was defined as the minimum concentration capable of inhibiting 80% of bacterial growth, when compared to the growth control [16]. The strains were classified as susceptible, intermediate or resistant [17].

2.6. Statistical analysis

Analysis of variance with *post hoc* Fisher's LSD test were used to compare the recovery rate of Enterobacteriaceae and *Vibrio* spp. from each site. $P \leq 0.05$ indicated significant difference.

3. Results

In this study, 33 strains of Enterobacteriaceae were isolated, 16 from the hatchery water, 12 from the digestive tract of *M. amazonicum* and 5 from lake water. The recovery of enterobacteria was statistically more common ($P = 0.0002$) from the digestive tract of *M. amazonicum* and water from the natural environment, when compared to hatchery water. No other statistically significant conclusions were observed. In addition, six strains of *Vibrio* were isolated from hatchery water ($n = 5$) and the digestive tract of *M. amazonicum* females ($n = 1$) (Table 1).

The following genera of enterobacteria were obtained from hatchery water: *Citrobacter*, *Serratia*, *Proteus*, *Escherichia*, *Kluyvera*, and *Buttiauxella*. Two genera were found in lake water, *Enterobacter* and *Cedecea*, while five were found in the digestive tracts, *Enterobacter*, *Providencia*, *Citrobacter*, *Kluyvera* and *Edwardsiella* (Table 1). Among the identified species of *Vibrio*, *V. cholerae* serogroups non-O1/non-O139 ($n = 4$) and *V. vulnificus* ($n = 1$) were isolated from hatchery water and *V. mimicus* ($n = 1$) was isolated from the digestive tract of prawns (Table 1).

The antimicrobial MIC values obtained against *Vibrio* spp. are described in Table 2. Non-O1/non-O139 *V. cholerae* and *V. mimicus* were susceptible to all tested antibiotics. The strain of *V. vulnificus*, on the other hand, presented intermediate profile to ampicillin, with an MIC of 16 μg/mL. Briefly, the antibiotics azithromycin, doxycycline and trimethoprim–sulfamethoxazole presented MIC values against the strains of *Vibrio* spp. ranging from 0.25 to 1 μg/mL, from 0.031 to 0.062 μg/mL and from 0.0156/0.297 to 0.125/2.37 μg/mL, respectively. Chloramphenicol presented an MIC of 0.5 μg/mL against all tested strains. In addition, ceftazidime presented MIC values of 1 μg/mL against *V. mimicus* and 0.5 μg/mL against *V. vulnificus*, and ciprofloxacin showed MIC of 0.001 μg/mL against these two species. All tested drugs presented MIC values within the expected range against the quality control strains *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC29213.

Table 1
Bacteria isolated from *M. amazonicum* and water from lake and hatchery tank.

Microorganisms	Collection site							
	Prawn		Water				Total	
			Hatchery		Lake			
	n	%	n	%	*n*	%	*n*	%
Buttiauxella sp.	–	–	1	2.56	–	–	1	2.56
Cedecea sp.	–	–	–	–	1	2.56	1	2.56
Citrobacter spp.	1	2.56	9	23.08	–	–	10	25.64
Edwardsiella sp.	1	2.56	–	–	–	–	1	2.56
Enterobacter spp.	7	17.95	–	–	4	10.26	11	28.21
Escherichia sp.	–	–	1	2.56	–	–	1	2.56
Kluyvera spp.	1	2.56	1	2.56	–	–	2	5.13
Proteus spp.	–	–	2	5.13	–	–	2	5.13
Providencia spp.	2	5.13	–	–	–	–	2	5.13
Serratia spp.	–	–	2	5.13	–	–	2	5.13
V. cholerae non-O1/non-O139	–	–	4	10.26	–	–	4	10.26
V. mimicus	1	2.56	–	–	–	–	1	2.56
V. vulnificus	–	–	1	2.56	–	–	1	2.56
Total	13	33.33	21	53.85	5	12.82	39	100

Table 2
MIC of antibiotics against *Vibrio* spp. strains isolated from hatchery water and the digestive tract of *M. amazonicum*.

Vibrio spp.	MIC (μg/mL)						
	Ampicillin	Azithromycin	Doxycycline	Trimethoprim/ Sulfamethoxazole	Chloramphenicol	Ceftazidime	Ciprofloxacin
V. cholerae	4.000	0.50	0.062	0.0156/0.297	0.5	–	–
V. cholerae	4.000	0.25	0.031	0.031/0.590	0.5	–	–
V. cholerae	4.000	0.50	0.031	0.125/2.370	0.5	–	–
V. cholerae	4.000	1.00	0.062	0.062/1.187	0.5	–	–
V. vulnificus	16.000	0.25	0.031	0.031/0.590	0.5	0.500	0.001
V. mimicus	0.125	1.00	0.031	0.125/2.370	0.5	1.000	0.001
Escherichia coli (ATCC25922)	2.00	–	0.500	0.500/9.500	4.0	0.125	0.004
Staphylococcus aureus (ATCC29213)	–	2.00	–	–	–	–	–

4. Discussion

The recovery of potentially zoonotic bacteria is a frequent concern associated with crustacean farming [19–21]. The recovery of bacteria from *M. amazonicum* from the natural environment has already been reported [22], however data on the isolation of enterobacteria from *M. amazonicum* farming is limited. Therefore, the idea of this research emerged based on the potential use of this prawn for commercial cultivation and the scarcity of data on the bacterial microbiota and the zoonotic risk associated with *M. amazonicum* farming. In addition, during the analyses of the first water samples obtained from larviculture tanks, we recovered non-O1/non-O139 *V. cholerae*. This finding led us to include the pursuit for *Vibrio* spp. and the analysis of their antimicrobial susceptibility as goals of this research. Parallely, the natural environment from which ovigerous females were harvested to obtain *M. amazonicum* larvae for larviculture was investigated. Microbiological analyses of lake water and ovigerous females were performed, as an attempt to track the origin of the *Vibrio* isolates obtained from larviculture.

Microorganisms of the Enterobacteriaceae family are widely distributed in nature, water and intestinal tracts of humans and animals [12]. In this study, we recovered ten genera of this family, of which *Citrobacter* spp. was the most common genus in hatchery water and *Enterobacter* spp. was predominant in the digestive tract of prawns and environmental water. Even though these genera are potentially pathogenic to humans, especially immunocompromised individuals [23], they are not listed as important zoonotic agents [6,7]. Among the Enterobacteriaceae, the genera *Salmonella*, *Escherichia* and *Edwardsiella* have been reported as the main aquaculture-associated zoonotic agents of this bacterial family [6,7]. In the present study, only one *Escherichia* sp. and one *Edwardsiella* sp. isolate from hatchery water and prawn, respectively, were recovered. These findings demonstrate that enterobacteria are indeed widely distributed as commensal microorganisms of aquatic animals, as previously stated [7], but they do not seem to be the most relevant human pathogens when handling and consuming *M. amazonicum* prawns.

The genus *Vibrio* comprises bacteria that inhabit surface waters and estuarine ecosystems with a wide range of temperatures and salinities throughout the world [9,24]. The incidence of human *Vibrio*-associated diseases has increased worldwide over the last decade due to infection with *V. cholerae*, *V. vulnificus* and *Vibrio parahaemolyticus* [25]. These species are reported as the primary bacterial agents of aquaculture-associated infections, as a consequence of seafood consumption and handling, causing gastroenteritis, skin and soft tissue infections and sepsis [6,7,9,24]. In the present study, four non-O1/non-O139 *V. cholerae* and one *V. vulnificus* were recovered from hatchery water, but not from environmental water or wild-harvested *M. amazonicum* females. In addition, one strain of *V. mimicus* was recovered from the digestive tract of *M. amazonicum*. Even though this species is not commonly associated with human diseases, it is a mesophilic species that eventually causes food-borne and wound infections [7,24].

This is the first report of the recovery of non-O1/non-O139 *V. cholerae* and *V. vulnificus* from *M. amazonicum* farming, which is noteworthy considering that the analyses were carried out for a short period, since only hatchery water was assessed. Interestingly, these *Vibrio* species were not recovered from the natural environment, thus, the source of these isolates remains unknown. However, it seems that the larviculture system offers proper conditions for the viability of these potentially zoonotic bacteria. Among these conditions, the constantly high water temperatures (near 30 °C) of the production systems may enhance the growth of the mesophilic human pathogenic *Vibrio* species, as well as stimulate the expression of virulence genes, favoring the occurrence of human infections [26].

The prophylactic use of antimicrobial agents in aquaculture favors the emergence of resistant pathogens [27]. However, in this study we found that *Vibrio* strains were mainly susceptible to the tested antibiotics, corroborating the results of Yano *et al*. [28]. Only the strain of *V. vulnificus* presented an intermediate profile to ampicillin, which has been reported as the least effective antibacterial drug against *Vibrio* strains recovered from farmed shrimps [19,29].

In conclusion, enterobacteria do not seem to be the most important aquaculture pathogens associated with *M. amazonicum* farming. On the other hand, the recovery of *Vibrio* spp., with emphasis on *V. cholerae* and *V. vulnificus*, from larviculture of *M. amazonicum* prawns deserves special attention because they are important aquaculture pathogens with zoonotic potential. These findings highlight the importance of monitoring aquaculture systems for the presence of *Vibrio* species, in order to prevent not only production losses, but also the occurrence of aquaculture-associated human infections [24]. Furthermore, the intermediate susceptibility of *V. vulnificus* to ampicillin reflects the importance of monitoring drug use in prawn farming.

Conflict of interest statement

We declare that we have no conflict of interest.

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