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Full Length Research Paper

Detection of virulence and antibiotic resistance genes in environmental strains of *Vibrio* spp. from mussels along the coast of Rio de Janeiro State, Brazil

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Mussels have a filter system enabling them to take up nutrients from the water, so a microbiological analysis of these bivalve mollusks can show the contamination levels of their surrounding aquatic environment. The present work aimed to isolate *Vibrio* species from two hundred samples of mussels (*Perna perna*) incrusted on rocks of the Santana Archipelago and from longline mariculture in Ilha Grande Bay in Angra dos Reis and from Arraial do Cabo, all of which are in Rio de Janeiro state, Brazil. A total of 209 *Vibrio* were isolated. The most prevalent species was *Vibrio parahaemolyticus* (44.66%) followed by *Vibrio alginolyticus* (19.62%) and *Vibrio vulnificus* (12.44%). All 209 *Vibrio* isolates tested positive for the RNA polymerase alpha gene (*rpoA*). The *tlh* gene (thermolabile hemolysin), a genetic marker for *V. parahaemolyticus*, and *vvhA* (cytolysin hemolysin) of *V. vulnificus* were detected in 85 and 26 isolates, respectively. The MALDI-TOF MS proteomic technique was used to confirm the identification of the 41 *V. alginolyticus* isolates. Our most important finding was the detection of the *tdh* virulence gene in 68.20% (58/85) of *V. parahaemolyticus* environmental strains. Besides the circulation of the virulence gene, the spread of antimicrobial resistance was evaluated and 91.3% (191/209) of the isolates showed resistance to ampicillin, 23.9% (50/209) to ciprofloxacin, 18.6% (39/209) to nitrofurantoin, 5.7% (12/209) to tetracycline, 4.3% (9/209) to pefloxacin and 3.3% (7/209) to chloramphenicol. These findings indicate that environmental isolates can act as reservoirs of virulence and antibiotic resistance genes.

Key words: *vvh*A gene, mussels, public health, *rpoA* gene, *tlh* gene, *Vibrio.*

INTRODUCTION

Mussels are filter-feeding bivalve organisms that pump seawater through their digestive systems to obtain oxygen and food. They thus accumulate and concentrate both harmless as well as pathogenic microorganisms,

such as human enteric viruses and the pathogenic *Vibrio* species. So, the microbiological quality of bivalve mollusks is directly related to the environmental quality of their aquatic surroundings. Furthermore, due to their distribution along coastlines and in estuaries, these mussels are often exposed to sewage pollution, mainly in areas near big cities (Asplund, 2013).

The consumption of raw or undercooked marine bivalves, which is a common practice in Brazil, poses a potential health hazard for humans due to the possible ingestion of pathogens. Besides this potential of food-borne diseases, the sessile characteristic of mussels enables the encrustation of various sites, such as coastal rocks and cables. This represents a health risk to fishermen and handlers of underwater cables because they can be injured by the hard shells and become infected with any of the pathogenic bacteria found in these animals or from the water (Sousa et al., 2004; Pereira et al., 2007).

The marine bacteria genus *Vibrio* is widespread in tropical coastal waters worldwide. Pathogenic and nonpathogenic species are widely distributed, particularly in estuarine waters, where they accumulate in edible animals (Yeng and Boor, 2004; Pereira et al., 2007). Some *Vibrio* isolates are clinically significant in humans, and others are known to cause diseases in fish (Vieira et al., 2010). The main pathogenic species to humans are *V. cholera*, *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* (Thompson et al., 2004). In the present study, *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* were isolated from mussels incrusted on rocks of the Santana Archipelago and from longline mariculture in Ilha Grande Bay located in Angra dos Reis and Arraial do Cabo, all of which are important sites of marine activity in Rio de Janeiro state, Brazil. So due to their importance as food-borne pathogens and infections of tissue injuries we decided to investigate them as potential reservoirs of virulence and antibiotic resistance genes.

Vibrio parahaemolyticus is an autochthonous bacterium prevalent in marine and estuarine environments worldwide (Joseph et al., 1982). While the majority of environmental strains are innocuous members of the marine microbiota, small subpopulations are opportunistic pathogens for humans, associated with gastroenteritis (Johnson et al., 2008) and extra-intestinal infections such as secondary septicemia, ocular infections and otitis, besides contaminating open wounds after exposure (Drake et al., 2007). Potentially virulent strains are commonly differentiated from likely avirulent strains by the presence of the thermostable direct (*tdh*) and/or *tdh*-related (*trh*) hemolysin genes (Bej et al., 1999). Virulent strains are also able to produce thermolabile hemolysin (TLH), encoded by the *tlh* gene. The *Tlh* gene is not associated with pathogenicity, since it has been observed in all clinical and environmental *V. parahaemolyticus* strains, and is considered a species-specific marker which is employed in *V. parahaemolyticus* characterization studies (Ward and Bej, 2006; Rojas et al., 2011). Although there is widespread belief that environmental isolates lack the virulence genes usually found in clinical strains, some studies indicate that virulence genes, or their homologues, can also be present in strains from environmental sources and that acquisition of such genes might take place in the aquatic environment (DePaola et al., 2003; Nordstrom et al., 2007).

Vibrio vulnificus is a virulent pathogen that causes two distinct major conditions: Primary septicemia, caused by the consumption of raw or undercooked seafood, and necrotizing wound infections, acquired through the exposure of an open wound to warm seawater with high concentrations of *V. vulnificus* (Bross et al., 2007). In addition, *V. vulnificus* has also been associated with other clinical disorders, including pneumonia, osteomyelitis, spontaneous bacterial peritonitis, eye infections, and meningitis (Pfeffer et al., 2003). Hemolytic/cytolytic exotoxin has been the most studied virulence marker. This toxin, encoded by a gene known as *vvhA* or *cth*, is a heat-labile enzyme that lyses mammalian erythrocytes and is cytotoxic to a variety of mammalian tissue culture cell lines (Drake et al., 2007).

Vibrio alginolyticus is commonly found in marine environments. It is recognized as an opportunistic pathogen to both humans and marine animals (Campanelli et al., 2008). Rising ocean temperatures have led to reports of out-of-season *V. alginolyticus* outbreaks in the United States, Spain, Mexico, Japan and China (Sganga et al., 2009; Yoder et al., 2008; Cavallo and Stabili, 2002). The pathogenesis and epidemiology of *V. alginolyticus* infections are still unclear (Wang et al., 2008).

The great variability of the biochemical characteristics of *Vibrio* species makes it difficult to standardize an accurate phenotypic identification (Thompson et al., 2004; Tarr et al., 2007). Consequently, more specific, rapid and sensitive molecular methods for the identification of *Vibrio* species must be developed. A PCR based on the RNA polymerase alpha subunit (*rpo*A) gene has been used for the detection of the *Vibrio* genus (Dalmasso et al., 2009). Also, virulence genes related to some *Vibrio* species have been used for species identification (Panicker et al., 2004; Rojas et al., 2011). Additionally, as antimicrobial resistance has been a growing public health challenge (Chai et al., 2008), the antimicrobial resistance profile of the *Vibrio* isolates was

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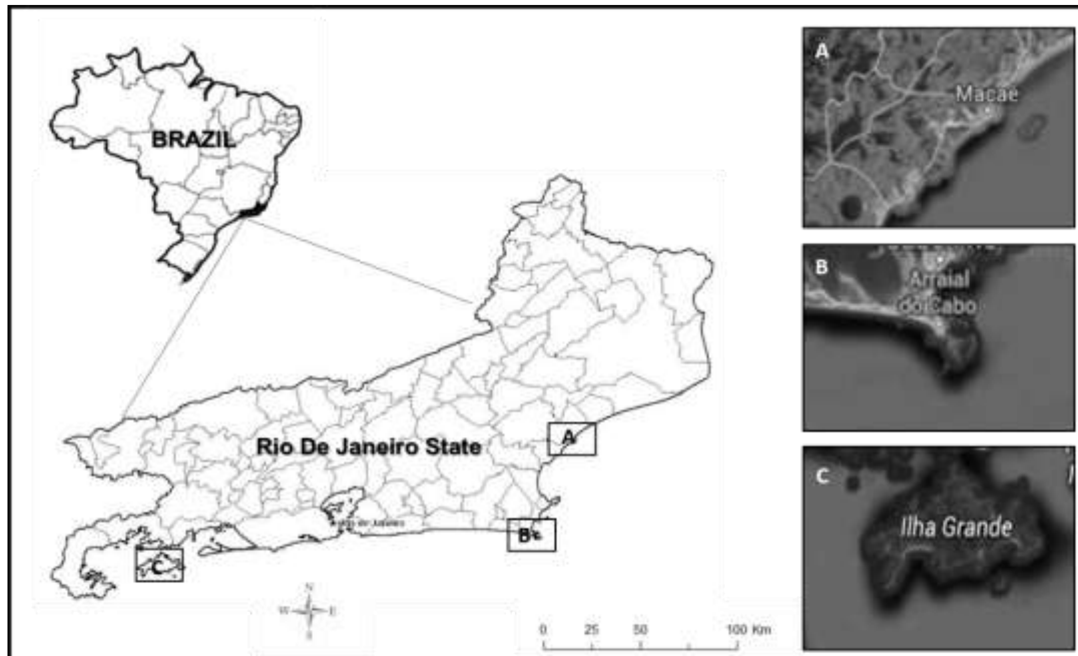

Figure 1. Map showing the locations where the mussels were collected; (A) Santana Archipelago, Macaé, (B) Arraial do Cabo Beach and (C) Ilha Grande Bay located in Angra dos Reis, Rio de Janeiro State, Brazil.

monitored to understand their possible role as resistance gene reservoirs. Zulkifli et al. (2009) stated that food contamination with antibiotic-resistant bacteria is a threat to public health, since the resistance determinants can be transferred to other bacteria of clinical significance, and that *Vibrio* isolates are candidate vehicles for such transfer because of their diversity and because they can survive in the gastrointestinal tracts of both humans and animals.

The inappropriate use of antibiotics in aquaculture is one of the causes for the high incidence of antimicrobial resistant bacteria isolated from aquatic environments that represent a danger for aquatic organisms and human health (Rebouças et al., 2011). Also, the discharge of untreated industrial and domestic waste in the aquatic environment contributes to spread of resistance (Sotomayor and Balcázar, 2003). Although the Brazilian seacoast is very extensive, there is little information available on the level of antimicrobial resistance in the pathogenic bacteria from this aquatic environment.

Accordingly to the aforementioned issues, the present work aimed to characterize the virulence and resistance profiles of the most prevalent species of *Vibrio* isolates isolated from mussels along the seacoast of Rio de Janeiro state, Brazil.

MATERIALS AND METHODS

Sampling

Mussels of the *Perna* species were collected at three different times and places in the state of Rio de Janeiro. In total, seven samples were taken: Three were from the rocks of the Santana Archipelago, offshore from Macae, from June 2007 to May 2008 where the animals were taken directly from the rocks, at depths ranging from 3 to 4 m by divers. The geographic coordinates of the collection points were: 22º 23' 45.43'' S - 41º 43' 42.52'' W. Two other samples were taken from mariculture longline in the Bay of Ilha Grande facing the town of Angra dos Reis in October 2010 and February 2011 between latitudes 22° 55’ S and 23° 15' S and longitudes 44° 43' W and two collections were made on the beach in Arraial do Cabo in May and July 2011 at 22° 56' 31" S and 42° 8' 19" W (Figure 1). At each sampling, 2 lots of 25 adults mussels were collected, with closed valves and the same size as used commercially (greater than 6 cm). The mussels were whashed individually with brush in running drinking water to remove the dirt. During this process were rejected the animals with open valves, totalizing two hundred samples of mussels (*Perna perna*). The samples were placed in polyethylene boxes and transported at 6 to 10°C to the Veterinary Bacteriology Laboratory of Federal Rural University of Rio de Janeiro.

Microbiological analysis

The body mass and intravalvular liquid was collected in beaker, where he was held crushing of the solid parts in order to promote homogenization of the material. In order to detect Vibrio spp., 25-g of the sample was added to 225 ml of alkaline peptone water (APW) containing 1% NaCl. Serial dilution of each sample was performed until 10^{-2} dilution in APW and was incubated at 37°C for 18 h. After that, the samples were inoculated in dishes containing Thiosulfate Citrate Bile Sucrose agar (TCBS) with the addition of 1, 2 and 3% NaCl and incubated at 37°C for 18 to 24 h (Konemam et al., 2008). Five to ten colonies from each dish were inoculated into tubes containing nutrient agar, LIA (lysine iron agar) and KIA (Kligler iron agar), along with 1% NaCl and incubated at 37°C for 24

h in order to carry out the differential diagnosis between *Vibrio* isolates and members of the Enterobacteriaceae family. The isolates were also submitted to the cytochrome-oxidase test to distinguish between the *Vibrio* isolates and members of the Enterobacteriaceae family (Koneman et al., 2008). *Vibrio* species identification was performed through biochemical tests based on the vibriostatic agent O/129 resistance (2,4 diamine-6,7 diisopropyl pteridine); production of ortho-nitrophenyl-β-galactoside (ONPG); production of acetoin by using the Voges-Proskauer test; glucose, sucrose, arabinose and mannose fermentation; lysine and ornithine decarboxylase; arginine dehydrogenase; halophilia production in different NaCl APW concentrations (0, 3, 6, 8 and 10%); motility observation, indole production; and nitrate reduction (Sewell, 2002).

Detection of *rpoA, tlh, tdh, trh* and *vvhA* genes

Bacterial DNA extraction was performed by thermal shock according to the method proposed by Dalmasso et al. (2009). A pair of primers that targets *rpo*A (Dalmasso et al., 2009), *tlh (*Bej et al., 1999), *tdh* (Honda and Iida, 1993; Frakuddin et al., 2012), *trh* (Honda and Iida, 1993) and *vvhA* (Brauns et al., 1991) genes was used to amplify DNA from *Vibrio* isolates and PCR conditions were performed as described previously by the authors mentioned above. Reference strains were kindly provided by Oswaldo Cruz Foundation - Fiocruz, Brazil: *V. parahaemolyticus* (ATCC 17802) was used as positive control in detection of genes *rpo*A, *tlh* and *tdh* (Frakuddin et al., 2012), *V. alginolyticus* (ATCC 17749) to detection of gene *rpo*A, and *V. vulnificus* (ATCC 27562) to detection of genes *rpo*A and *vvh*A.

Vibrio alginolyticus identification by Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI TOF MS)

To perform the MALDI-TOF MS, the samples were inoculated in BHI agar at 37°C for 24 h. Each culture was transferred to a microplate (96 MSP, Bruker - Billerica, USA). Each bacterial sediment was covered by a lysis solution (70% formic acid, Sigma-Aldrich). A 1-µL aliquot of matrix solution (alpha-ciano-4-hidroxi-cinamic acid diluted in 50% acetonitrile and 2.5% trifluoroacetic acid, Sigma-Aldrich). The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT Microflex, Bruker) equipped with a 337-nm nitrogen laser in a linear path, controlled by the FlexControl 3.3 (Bruker) program. The spectra were collected in a mass range between 2,000 and 20,000 m/s, and then were analyzed by the MALDI Biotyper 2.0 (Bruker) program, using the standard configuration for bacteria identification, by which the spectrum of the sample was compared with the references in the database. The results are given on a 0-3 scale, where the highest value means a more precise match and reliable identification. In this study, we accepted values for matching greater than or equal to two (2) as proposed by the manufacturer.

Antimicrobial susceptibility tests

The isolates were submitted to susceptibility tests by the disk diffusion technique, as standardized by CLSI (2011). The following antimicrobial disks were used: (SENSIFAR-Cefar): tetracycline (30 µg), chloramphenicol (30 µg), nitrofurantoin (300 µg), pefloxacin (5 µg), ampicillin (10 UI) and ciprofloxacin (5 µg). After 24-h incubation at 35°C, the diameters of the inhibition zones were measured in millimeters and analyzed by the interpretation criteria established in the CLSI standards (2011).

RESULTS AND DISCUSSION

A total of 209 *Vibrio* isolates were phenotypically characterized. The prevalent species were *V. parahaemolyticus* (n=85), *V. alginolyticus* (n=41) and *V. vulnificus* (n=26). We also detected *V. harveyi* (n= 13), *V. fischerii* (n=2), *V. anguillarum* (n=2) and *V. carchariae* (n=3) (Table 1).

All 209 isolates tested positive for *rpo*A gene yielding a 242-bp fragment (Figure 2). The *rpo*A gene detection is considered a specific, sensitive and fast method for *Vibrio* identification (Thompson et al., 2005; Dalmasso et al., 2009; Jeyasekaran et al., 2011).

The great variability of biochemical characteristics of *Vibrio* species makes it difficult to standardize an accurate phenotypic identification (Thompson et al., 2004; Tarr et al., 2007). So the prevalent species *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* were submitted to genotypic characterization in order to confirm the previous phenotypic identification.

The *tlh* (thermolabile hemolysin) is a species marker gene for *V. parahaemolyticus* (Rojas et al., 2011). A total of 85 isolates was classified as *V. parahaemolyticus* since they tested positive for *tlh* amplification yielding a 450-bp specific fragment (Figure 3). The *rpo*A gene (242-bp) was used as endogenous control.

All the 124 isolates (59.3%) that tested negative for the *tlh* gene were submitted to a PCR for the *vvh*A (cytolysin-hemolysin) gene, a species marker gene for *V. vulnificus* (Drake et al., 2007). Twenty-six isolates (20.9%) tested positive for *vvhA* gene yielding a 386-bp fragment (Figure 4). Although widely used and highly specific to *V. vulnificus*, this gene is not able to predicate the virulence potential of *V. vulnificus* strains, since there are other virulence mechanisms associated with *V. vulnificus*, such as the presence of the polysaccharide capsule, protease, elastase and phospholipase, which may be found in almost all clinical and environmental strains (Who, 2005). The close relation of *V. alginolyticus* to other species such as *V. parahaemolyticus* and the absence of a reliable specific genetic marker require additional techniques for its differentiation. Therefore, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for *V. alginolyticus* identification. This technique is based on proteomic technology and is considered a more specific, rapid and sensitive method. MALDI-TOF MS was a useful tool for reliable identification of *V. alginolyticus,* confirming the phenotypic identification of all 41 isolates. Several authors concluded that the whole-cell MALDI-TOF mass spectrometry analysis is a highly reproducible method for rapid discrimination of *Vibrio* isolates and related species including *V. alginolyticus* (Dieckmann et al., 2010; Hazen et al., 2009).

The spatial distribution of *V. parahaemolyticus* agrees with the important epidemiologic role due to the

Table 1. Phenotypic characterization percentage of *Vibrio* isolates from different harvesting sites according to number of isolates

Vibrio species	Phenotypic characterization % (number of isolates)			
	Macaé	Arraial do Cabo	Angra dos Reis	Total
V. harveyi	8(8)	3.4 (2)	5.8 (3)	6.2 (13)
V. vulnificus	20 (20)	5.1 (3)	5.8 (3)	12.4 (26)
V. carchariae	1 (1)	1.7 (1)	1.9 (1)	1.4 (3)
V. parahaemolyticus	44 (44)	31.0 (18)	45.0 (23)	40.6 (85)
V. alginolyticus	15 (15)	18.9 (11)	29.4 (15)	19.6 (41)
V. damsela	2 (2)	-	1.9 (1)	1.4 (3)
V. aestuarianus	-	1.7 (1)	-	0.4 (1)
V. costicola	-	1.7 (1)	1.9 (1)	0.9 (2)
V. cincinnatiensis	-	1.7 (1)	-	0.4 (1)
V. fischeri	-	1.7 (1)	1.9 (1)	0.9 (2)
V. anguillarum	2	-	-	0.9 (2)
V. metschnikovii	-	1.7 (1)	-	0.4 (1)
V. mimicus	-	1.7 (1)	-	0.4 (1)
Vibrio spp.	8 (8)	29.3 (17)	5.8 (3)	13.3 (28)
Total of isolated	100	58	51	290

-, detected.

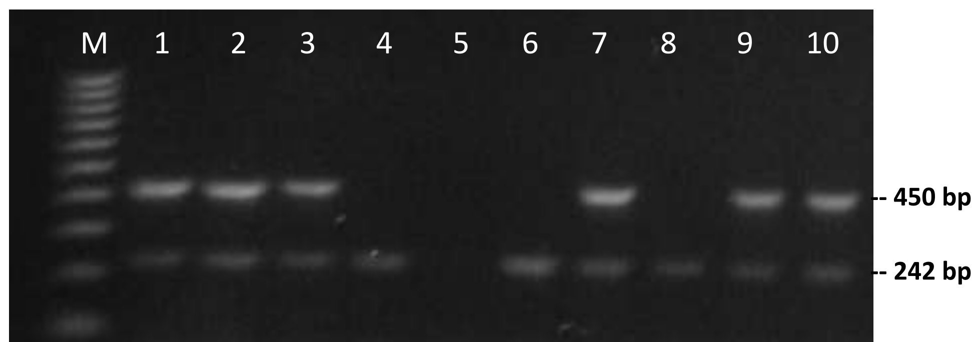

Figure 2. Amplification of *rpoA* and *tlh* genes in *Vibrio* spp. M: 100-bp ladder, 1: ATCC 17802 *Vibrio parahaemolyticus*; 2, 3, 7, 9, 10: *V. parahaemolyticus*; 6: *V. vulnificus*; 5: negative control.

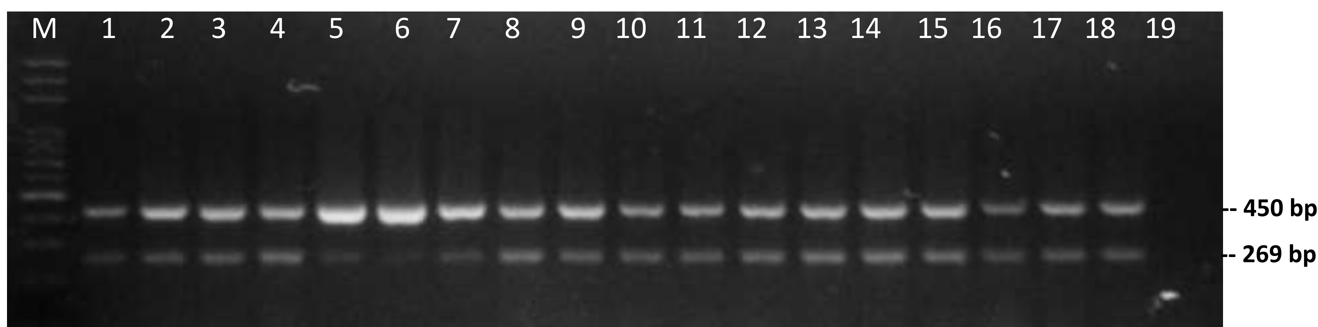

Figure 3. Amplification of *tdh* gene in *V. parahaemolyticus*. Internal control: *tlh* gene. M: 100 bp DNA Ladder, 1-18: *V. parahaemolyticus strains*; 19: negative control.

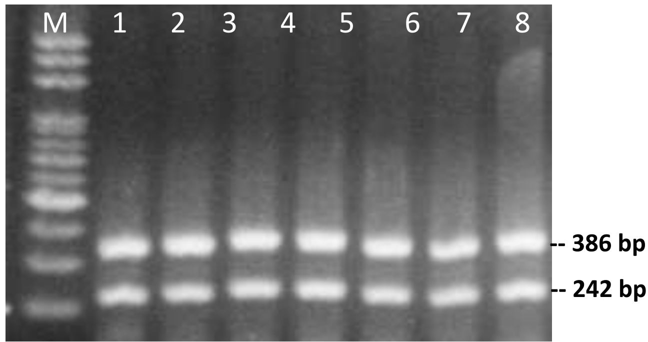

Figure 4. Amplification of *rpoA* and *vvhA* genes in *V. vulnificus.* M: 100-bp ladder; 2: ATCC 27562 *V. vulnificus*, 3, 4, 5, 6, 7; 8: *V. vulnificus*.

occurrence of epidemic outbreaks of this agent after the ingestion of either raw or undercooked seafood (Cabrera-Garcia et al., 2004). Similarly, *V. alginolyticus* was the second most isolated species, which reinforces its epidemiological importance, associated with the occurrence of diarrhea and wound infections, particularly among mussel handlers (Rodrigues et al., 2001). The detection of 26 *V. vulnificus* isolates represented an important microbiological finding, since this species is characterized as highly pathogenic to humans (Nascimento et al., 2001).

Although some authors have considered that environmental *V. parahaemolyticus* strains lack the thermostable direct (*tdh*) and *tdh*-related (*trh*) hemolysin virulence genes (Su and Liu, 2007; Rojas et al., 2011), we decided to investigate their presence in order to establish the circulation of these genes in the aquatic environment. Surprisingly, 58 *V. parahaemolyticus* strains (68.2%) tested positive for *tdh* gene (58/85) yielding a 386-bp fragment (Figure 4). The *tlh* gene was used as the endogenous control. However, the *trh* gene was not detected. Virulent strains can present both *tdh* and *trh* genes or just one of them (Su and Liu, 2007; Rojas et al., 2011).

According to Bhoopong et al. (2007), only 1 to 2% of environmental strains present these virulence genes. These data show the prospective occurrence of virulence genes circulating in aquatic environments, since most of the reported data in the literature have been obtained from clinical specimens. Our findings are similar to those of DePaola et al. (2003) and Nordstrom et al. (2007), who considered the recent detection of virulence genes in environmental *V. parahaemolyticus* strains is a consequence of the employment of more sensitivity molecular methods. Caburlotto et al. (2008) reported for the first time the pandemic potential of a *V. parahaemolyticus* strain presenting both *tdh* and *trh* genes from marine water in Caleri, Italy. These authors emphasized the marine environment as a reservoir of pandemic strains and virulence genes (Caburlotto et al., 2010). As far as we know, this is the first time that the *tdh* gene has been detected in *V. parahaemolyticus* environmental strains along the coast of Rio de Janeiro State. The observed *tdh*+ *V. parahaemolyticus* strains in our study can be associated with different sources of human and animal fecal contamination of seawater. In Arraial do Cabo, there is a floating restaurant nearby the collection sites. Similarly, in Ilha Grande Bay, in Angra dos Reis, domestic effluent is discharged without previous treatment into the bay. And although the Santana Archipelago is well offshore, there are oil platforms and plenty of seabirds that can also contribute to fecal contamination. Cabrera-Garcia et al. (2004) suggested that fecal contamination can be the origin of the *tdh*+ environmental strains by genetic material exchange between pathogenic and environmental strains. Besides virulence gene circulation, the spread of antimicrobial resistance is a challenge of utmost importance. The evaluation of the susceptibility profile of the 209 *Vibrio* strains showed 91.3% (191/209) resistance to ampicillin, 23.9% (50/209) to ciprofloxacin, 18.6% (39/209) to nitrofurantoin, 5.7% (12/209) to tetracycline, 4.3% (9/209) to pefloxacin and 3.3% (7/209) to chloramphenicol (Table 2). These data are of significant concern since *Vibrio* is usually considered highly susceptible to virtually all antimicrobials. *Vibrio* isolates live in coastal and estuarine waters, open areas particularly subject to environmental contamination by agricultural runoff or wastewater, which may contain significant levels of antimicrobials and heavy metals, exerting selective pressure for the development of antimicrobial-resistant aquatic bacteria (Han et al., 2007). One of the main findings in this study was the high ampicillin resistance among *Vibrio* isolates from all collection sites. Recently, Jun et al. (2012) reported the detection of beta-lactamase genes carried by the ampicillin-resistant *V. alginolyticus*, *V. cholerae* and *Photobacterium damselae* subsp. *damselae* strains isolated from marine environments with the primers designed from a novel beta-lactamase gene cloned from *V. alginolyticus* KV3, isolated from aquaculture water of Geoje Island, Korea. This information points to the possibility of developing new strategies for ampicillin resistance that need to be investigated further to confirm or refute the idea of the emergence of resistant *Vibrio* isolates in aquaculture (Han et al., 2007).

The resistance to ciprofloxacin was detected in both the Arraial do Cabo and Angra dos Reis samples. As in other gram-negative bacteria, resistance to fluoroquinolones in *Vibrio* isolates is mostly mediated by mutations within the quinolone resistance-determining regions (QRDRs) of the *gyrA* gene. This mutational change was present in the majority of fluoroquinolone-resistant *Vibrio* isolates and probably can explain the resistance detected in the present study.

The emergence of multi-drug resistant bacteria in recent years is worrying and is eroding the antibiotic

armamentarium and thus limiting the therapeutic options available to clinicians (Zulkifli et al., 2009). Considering this, frequent and careful monitoring of antibiotic use in aquaculture, for the purpose of preventing and treating microbial infections or as animal growth promoters, is important to ensure aquatic environmental safety (Noorlis et al., 2011).

The results of this study show the detection of the *tdh* virulence gene in 68.2% (58/85) of *V. parahaemolyticus* environmental strains and the high rate of ampicillin resistance among *Vibrio* isolates from all the collection sites. These findings indicate that environmental strains can act as reservoirs of virulence and antibiotic resistance genes. The detection of *rpoA* and *tlh* genes yielded reliable genus confirmation and *V. parahaemolyticus* identification, respectively. Also, the MALDI-TOF MS technique was very efficient in distinguishing *V. alginolyticus* from the closely related species *V. parahaemolyticus*.

Conflict of Interests

The author(s) declare there are no conflicts of interests.

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