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

Marcelo Santos de Oliva\(^1\), Greiciane França Bronzato\(^1\), Lidiane de Castro Soares\(^2\), Ingrid Annes Pereira\(^3\), Bruno Rocha Pribul\(^4\), Marco Antônio Soares de Souza\(^2\), Shana Mattos de Oliveira Coelho\(^1\), Irene da Silva Coelho\(^1\), Dália dos Prazeres Rodrigues\(^4\) and Miliane Moreira Soares de Souza\(^1\)*

\(^1\)Veterinary Institute, Veterinary Microbiology and Immunology Department, Federal Rural University of Rio de Janeiro, Seropédica, RJ, Brazil.

\(^2\)Severino Sombra University, Vassouras, RJ, Brazil.

\(^3\)Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil.

\(^4\)Oswaldo Cruz Institute – FIOCRUZ, Rio de Janeiro, 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.


**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 non-pathogenic 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 autchthonous 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 (*tht*) hemolysin genes (Bej et al., 1999). Virulent strains are also able to produce thermostable 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 (*rpoA*) 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

*Corresponding author. E-mail: miliane@ufrj.br, Tel/Fax: +55 21 26821711.

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Monitoring 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: 22° 23' 45.43'' S - 41° 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, totaling 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 10⁻⁵ 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 hours.
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. fischeri (n=2), V. anguillarum (n=2) and V. carchariae (n=3) (Table 1).

All 209 isolates tested positive for rpoA gene yielding a 242-bp fragment (Figure 2). The rpoA 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 thl (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 thl amplification yielding a 450-bp specific fragment (Figure 3). The rpoA gene (242-bp) was used as endogenous control.

All the 124 isolates (59.3%) that tested negative for the thl gene were submitted to a PCR for the vvhA (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

<table>
<thead>
<tr>
<th><em>Vibrio</em> species</th>
<th>Phenotypic characterization % (number of isolates)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Macaé</td>
</tr>
<tr>
<td><em>V. harveyi</em></td>
<td>8 (8)</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>20 (20)</td>
</tr>
<tr>
<td><em>V. carchariae</em></td>
<td>1 (1)</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>44 (44)</td>
</tr>
<tr>
<td><em>V. alginolyticus</em></td>
<td>15 (15)</td>
</tr>
<tr>
<td><em>V. damsela</em></td>
<td>2 (2)</td>
</tr>
<tr>
<td><em>V. aestuarianus</em></td>
<td>-</td>
</tr>
<tr>
<td><em>V. costicola</em></td>
<td>-</td>
</tr>
<tr>
<td><em>V. cincinnatiensis</em></td>
<td>-</td>
</tr>
<tr>
<td><em>V. fischeri</em></td>
<td>-</td>
</tr>
<tr>
<td><em>V. anguillarum</em></td>
<td>2</td>
</tr>
<tr>
<td><em>V. metschnikovii</em></td>
<td>-</td>
</tr>
<tr>
<td><em>V. mimicus</em></td>
<td>-</td>
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<tr>
<td><em>Vibrio</em> spp.</td>
<td>8 (8)</td>
</tr>
<tr>
<td>Total of isolated</td>
<td>100</td>
</tr>
</tbody>
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-, 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.
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 trh 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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