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ORIGINAL ARTICLE

PATHOGENIC POTENTIAL OF *Vibrio parahaemolyticus* ISOLATED FROM TROPICAL ESTUARINE ENVIRONMENTS IN CEARÁ, BRAZIL

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ABSTRACT

Vibrio parahaemolyticus is a potentially pathogenic bacterium that occurs naturally in estuarine environments worldwide. This research aimed to investigate the occurrence of *V. parahaemolyticus* in estuarine environments and determine the virulence profile in an aquaculture environment by molecular techniques and conventional microbiological methods. Sampling was conducted in four estuaries in the State of Ceará (Pacoti, Choró, Pirangi and Jaguaribe), Brazil, between January and April 2009. The analysis included 64 samples of water (n=32) and sediment (n=32) collected from the estuaries. The samples yielded 64 isolates suspected to be *V. parahaemolyticus*. The isolates were submitted to biochemical identification using a dichotomous key and PCR for the detection of the species-specific *tlh* gene. Virulence was assessed by testing for urea hydrolysis and β-hemolysis in erythrocytes (Kanagawa phenomenon) and simultaneous detection of the *tdh* and *trh* genes. All but one of the isolates (63/64) were confirmed to be *V. parahaemolyticus* by genotypic detection of *tlh* gene. The *tdh* and *trh* genes were detected in 57 and 19 isolates, respectively. The Kanagawa test was positive for 51 isolates. Only one isolate was positive for urease. The incidence of *tdh/trh*-positivity was very high in isolates recovered from the environment. The present study demonstrates the need to increase knowledge of the ecology and pathogeny of *V. parahaemolyticus*.

KEY WORDS: Estuary; public health; *Vibrio parahaemolyticus*; virulence; tdh; trh genes.

INTRODUCTION

The genus *Vibrio* includes opportunistic pathogenic species, capable of causing diseases in host organisms under stress or compromised immune defense systems (Defoirdt et al., 2008). Of particular concern to public health,

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the enteric pathogen *Vibrio parahaemolyticus* can cause conditions such as acute gastroenteritis and septicemia in humans exposed to raw or undercooked seafood (Zhao et al., 2011). The species is Gram-negative and halophilic and is widely distributed in estuarine and marine environments (Griffit et al., 2011; Hassan et al., 2012).

The earliest reports of diseases caused by seafood associated with *V. parahaemolyticus* date from the 1950s in Japan. The incidence has since been increasing, especially in the US, Southeast Asia, Canada and Mexico (Rahman et al., 2006; Tunung et al., 2011). The first Brazilian report of *V. parahaemolyticus* dates from mid-1975, associated with an outbreak of gastroenteritis in a small town in the Northeast (Cascavel, Ceará) (Hofer, 1983).

Despite the increasing incidence of *V. parahaemolyticus* in clinical samples, it is not as frequently detected in food and environment samples as might be expected, possibly due to limitations found in conventional microbiology techniques (Martinez-Urtaza et al., 2008).

Circumventing these limitations, several molecular biology techniques have been developed to identify and determine the pathogenicity of *V. parahaemolyticus* isolates, especially PCR-based techniques capable of targeting specific genes (Blanco-Abad et al., 2009). The thermolabile hemolysin (TL) is encoded by the *thl* gene, used for identification of clinical and environmental *V. parahaemolyticus* and *V. alginolyticus* isolates (Cariani et al., 2012).

Epidemiological studies have revealed a strong association between the Kanagawa phenomenon (characterized by β-hemolysis) and clinical isolates from outbreaks of gastroenteritis, although the phenotype is observed in only 1-2% of environmental isolates. β-hemolysis is therefore considered an important marker of virulence in *V. parahaemolyticus* isolates (Nishibuchi & Kaper, 1995; Rizvi & Bej, 2010). The main factors which enable *V. parahaemolyticus* isolates to induce β-hemolysis in human erythrocytes (hence markers of virulence) are the hemolysins TDH (thermostable direct hemolysin) and TRH (thermostable direct hemolysin-related hemolysin) encoded by the *tdh* and *trh* genes, respectively (Sobrinho et al., 2011; Zhao et al., 2011). The ability of certain clinical isolates to hydrolyze urea has also been identified as an indicator of virulence (Magalhães et al., 1991).

Considering the high incidence of *V. parahaemolyticus* in seafood and the potential threat it poses to human consumers, the purpose of the present study was to determine the virulence profile of isolates recovered from the environment using conventional (culture-dependent) microbiology techniques and molecular biology (PCR) techniques.

MATERIAL AND METHODS

Thirty-two water samples and thirty-two sediment samples were collected from the estuaries of 4 rivers in Ceará State, Brazil: Pacotí, Choró, Pirangi and Jaguaribe. Two points in each river were analyzed: one near and one far from the mouth of each river. Sample collections were performed monthly (Figure) (Menezes et al., 2017).

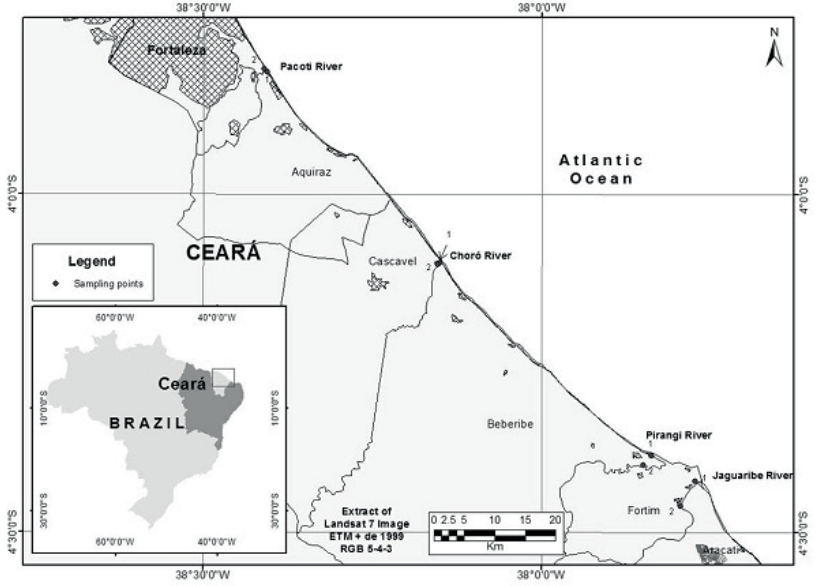

Figure. Map showing the sampling locations in the Pacoti, Choró, Pirangi and Jaguaribe estuaries (Ceará) investigated in the present study.

Water samples were collected at a depth of 50 cm. The water temperature was recorded at the time of collection and the salinity and pH determinations were performed in the Laboratory of Environmental and Fish Microbiology (LAMAP/LABOMAR/UFC) for rapid analysis. Important to note that amber 1 L sterilized bottles were used for sample collection. On the other hand, sediment samples were collected and transported to the LAMAP using isothermal boxes (Menezes et al., 2017).

Serial dilutions (from 10^{-1} to 10^{-4}) in alkaline peptone water (APW) at pH 7.5-8.5 were used for water and sediment analysis. Twenty-five grams of sediment were homogenized in 225 mL of APW for 30 minutes (10^{-1}) (Menezes et al., 2014).

For isolate selection Thiosulfate Citrate Bile salts Sucrose (TCBS) selective medium was used. Aliquots (100 µL) of each dilution were inoculated and incubated for 18h at 37ºC (Menezes et al., 2014). Green and blue colonies with morphological characteristics of *V. parahaemolyticus* (round, opaque, 2-3 mm in diameter) were selected for additional testing (Kaysner & Depaola, 2012).

Biochemical testing was performed using the Noguerola & Blanch (2008) dichotomous key, an update of the key proposed by Alsina & Blanch (1994a, 1994b). To detect virulence phenotypes among *V. parahaemolyticus*, two tests were conducted: the Kanagawa test, using Wagatsuma agar (Wagatsuma, 1968) (containing a freshly collected 20% suspension of washed human blood group O erythrocytes) and the urease test, using urea broth (DIFCO™) (ICMSF, 1978). A reference *V. parahaemolyticus* isolate (IOC 17082), supplied by the Oswaldo Cruz Institute (Rio de Janeiro, Brazil), was used as positive control.

The Spearman correlation coefficient (rs) was used to determine the correlation between environmental parameters and the abundance of *V. parahaemolyticus*. The SPSS software package (SPSS Inc., version 17.0 for Windows) was used for statistical analysis (Spearman, 1904).

The DNA was obtained using a commercially available kit (DNeasy Blood & Tissue Kit®, Qiagen, Brazil). *V. parahaemolyticus* identified by biochemical testing were further confirmed by PCR, using primers and conditions for detection of the *tlh* gene (450bp), while the presence of virulence-associated genetic determinants was evaluated by using primers for the *tdh* (269bp) and *trh* (500bp) genes (Croma BioTechnologies®, Brazil) (Bej et al., 1999). *V. parahaemolyticus* IOC 18950 was used as a positive control for the presence of all the genes under investigation. Agarose gels at 1% were used for electrophoresis for 60 minutes. Molecular size was estimated by using 1000-bp DNA ladder (Sigma, Brazil). The gels were photo-documented with a Kodak EDAS290 digital camera, Brazil (Menezes et al., 2016).

RESULTS

In relation to environmental studied parameters, the water temperature varied from 28 to 36.5°C, the salinity ranged from 2.0 to 48.0, and the pH between 6.96 and 8.32 in all four estuaries investigated. The abundance of *V. parahemolyticus* isolates in the samples collected was positively correlated with the water temperature ($r_s = 0.50$; $p < 0.05$) and negatively correlated with pH ($r_s = -0,43$; $p < 0.005$) and salinity ($r_s = 0.65$; $p < 0.005$).

Sixty-four *V. parahaemolyticus* isolates were recovered from 32 samples of water (n=27) and 32 samples of sediment (n=37) and identified phenotypically. Of these, 63 were confirmed genetically (Table 1). Using specific primers, 57 (89%) isolates were positive for tdh while 19 (29%) isolates were positive for *trh*. Both genes were present in 19 (29%) isolates. In other words, 45 (70%) isolates were negative for *trh* while only 7 (10%) were negative for *tdh*. Moreover, five isolates identified as *V. parahaemolyticus* were positive for *tdh* but did not express the corresponding phenotype in the Kanagawa test.

Table 1. Results of tests for detecting ß-hemolysis (Kanagawa), urease, species-specific gene (*tlh*) and virulence genes (*tdh* and *trh*) among *Vibrio parahaemolyticus* isolates recovered from water and sediment samples collected in four estuaries in Northeastern Brazil.

Origin (estuary)	Sample	Potential virulence profile					N
		Genotype			Phenotype		
		tlh [a]	*tdh* [b]	*trh* [c]	Kanagawa	Urease	
Choró	Water	+	+	+	-	-	1
		+	+	-	+	-	7
	Sediment	+	+	+	+	-	1
		+	+	+	+	-	2
		+	+	-	+	-	7
		+	-	-	-	-	2
Jaguaribe	Water	+	+	+	-	-	1
		+	+	+	+	-	2
		+	+	-	-	-	1
		+	+	-	+	-	6
	Sediment	+	+	+	+	-	2
		+	-	-	-	-	2
		+	+	+	-	-	1
Pacoti	Water	+	+	-	+	-	7
		+	+	-	+	-	1
		+	+	+	+	-	3
	Sediment	+	-	-	-	-	2
		+	+	-	+	-	6
		+	+	+	+	-	2
Pirangi	Water	+	+	+	-	-	1
		+	-	-	-	-	1
	Sediment	+	+	-	+	-	1
		+	+	+	+	-	3
		+	+	-	+	-	1

N = number of isolates analyzed; a) thermolabile hemolysin gene; b) thermostable direct hemolysin gene; c) thermostable direct hemolysin-related gene.

Isolates carrying virulence genes were more abundant in sediment than in water. Thirty-seven isolates were identified in sediment samples, with positivity for *tlh* (n=36), *tdh* (n=33), *trh* (n=11) and *tdh*+*trh* (n=11), while 27 isolates were identified in water samples, with positivity for *tlh* (n=27), *tdh* (n=24), *trh* (n=8) and *tdh*+*trh* (n=8). The Kanagawa test was positive for 30 (81%) isolates obtained from sediment and for 21 (77%) isolates recovered from water (Table 2).

Table 2. Number of *Vibrio parahaemolyticus* isolates according to sample type, presence of species-specific gene (*tlh*) and virulence genes (*tdh* and *trh*), and ability to induce hemolysis in erythrocytes (Kanagawa phenomenon), based on 64 water and sediment samples collected in four estuaries in Northeastern Brazil

Sample type	Genes				Kanagawa	
	tlh [a] +	*tdh* [b] +	*trh* [c] +	*tdh* [b] +/*trh* [c] +	+	-
Water	27	24	8	8	21	6
Sediment	37	33	11	11	30	7
Total	64	57	19	19	51	13

a) thermolabile hemolysin gene; b) thermostable direct hemolysin gene; c) thermostable direct hemolysin-related gene.

DISCUSSION

One of the factors that most affect the *Vibrio* distribution in the temperate environment is salinity (Noriega-Orozco et al., 2007). The highest population densities of *Vibrio* are noted during the warm seasons, however this is not valid for tropical waters since the temperature undergoes slight variations throughout the year.

The results of this study are in agreement with those by Tan et al. (2010) showing that the density of *Vibrio* species can be affected and highlight the relationship between virulence (presence of the *tdh* and *trh* genes) and variable responses to salinity alterations. Also, Randal et al. (2004) and Johnson et al. (2010) have found variable responses among isolates of two species, *Vibrio vulnificus* and *V. parahaemolyticus*, against different salinities tested: 5 to 10 ppt and 3 to 35 ppt, respectively.

The water temperature was the only parameter that showed a positive correlation with the amount of *V. parahaemolyticus* isolates. Although considered one of the determinant factors for proliferation of *Vibrio* spp. in tropical environments (Johnson et al., 2010; Johnson et al., 2012), a consistent correlation between the salinity levels and the amount of *V. parahaemolyticus* was not detected in the estuaries investigated. The effect of environmental factors on the *Vibrio* population are diversified.

Salinity is fundamental for the occurrence of marine *Vibrio*, as for example, *V. parahaemolyticus* (Takemura et al., 2014). In relation to pH, this parameter was stable within the favorable limits for *Vibrio* development, namely 7.5 to 8.5 (Sousa, 2004; Han et al., 2018).

The high level of positivity for the *tlh* gene observed in this study confirms the efficiency of the key proposed by Noguerola & Blanch (2008) for the identification of *Vibrio* species. Likewise, Croci et al. (2007) found the Alsina & Blanch (1994a, 1994b) *Vibrio* identification key - a forerunner to the Noguerola & Blanch (2008) key - to be more efficient for identifying environmental isolates than the commercial kits API 20E and API 20NE.

Vibrio parahaemolyticus is known to cause gastroenteritis and even septicemia. It is the *Vibrio* species most frequently implicated in outbreaks of food-borne infections due to its wide distribution in estuarine and marine environments and frequent association with seafood (Klein et al., 2014).

The virulence of *V. parahaemolyticus* depends on the presence of two toxins, TDH and TRH, encoded by the *tdh* and *trh* genes, respectively (Chao et al., 2009; Silva et al., 2018). Therefore, the presence of *tdh* gene characterized by a β-hemolysis on Wagatsuma agar (Kanagawa phenomenon), the *trh* gene correlated to a positive urease test, or both serve as markers for pathogenic isolates (Chao et al., 2009). According to Rodrigues et al. (1993) and Canizalez-Roman et al. (2011), the pathogenicity of *V. parahaemolyticus* isolates is closely correlated with their ability to induce β-hemolysis in human erythrocytes on Wagatsuma agar.

In the present study, isolates from environmental samples were positive for the *tdh* and *trh* genes, also presenting high values of positivity in the Kanagawa test. These results disagree with the conclusion reached by Pumipuntu & Indrawattana (2017) and Hiyoshi et al. (2010) that only clinical isolates of *V. parahaemolyticus* are capable of expressing β-hemolysis in that test. However, Hongping et al. (2011) reported high levels of Kanagawa positivity both in clinical isolates from patients with gastroenteritis (88%) and in isolates from water (76%) and fish (46%). Although an increased number of Kanagawa-positive isolates was detected among environmental isolates of *V. parahaemolyticus*, the correlation with outbreaks of *V. parahaemolyticus* gastroenteritis has not been established so far. Even so, this is a concerning public health issue since the Kanagawa test is correlated with the presence of the *tdh* gene and the expression of TDH toxin in *V. parahaemolyticus* (Klein et al., 2014).

The high incidence of virulence genes observed (tdh=84% and trh=29%) counters the claims of Ottaviani et al. (2010) and Hongping et al. (2011) that such genes are rare in environmental isolates and that only 0.3-3% of non-pathogenic environmental isolates are Kanagawa-positive. In fact, the expression of *tdh* and *trh* is regulated by the toxR gene which is present in Kanagawa-positive isolates (Gopal et al., 2005; He et al., 2014).

The virulence-associated tdh and trh genes were also observed in a study of environmental *V. parahaemolyticus* isolates recovered from water, sediment and shrimps from the coast of India (Gopal et al., 2005). However, only 2 out of 70 isolates were *tdh/trh*-positive, one (1%) from a shrimp sample and one (1%) from a sediment sample. On the other hand, Chang et al. (2011) detected the *tdh* and *trh* genes in 11% of *V. parahaemolyticus* isolates from sediment and 1% of isolates from water collected on oyster farms in Southwestern Taiwan.

The results reached by Nakaguchi (2013) and Johnson et al. (2010) support our finding of a high prevalence of tdh-positive *V. parahaemolyticus* isolates in environmental samples. In addition, according to Vezzulli et al. (2009), virulent isolates are more abundant in sediment than in water because the former serves as a reservoir for the microbiota (pathogenic and non-pathogenic) of pelagic environments.

Despite the recognized specificity of the PCR method for the identification of bacteria, our results show that the Noguerola & Blanch (2008) dichotomous key is a reasonably reliable technique to identify *V. parahaemolyticus* isolates from aquaculture environments. The high percentage of detection of phenotypes and genotypes markers of virulence among *V. parahamolyticus* isolated from aquatic environments is relevant to epidemiological studies on these pathogenic bacteria. The ability of *V. parahaemolyticus* to survive and maintain the virulence factors in aquaculture environments presents a risk to human health and causes significant economic issues in the aquaculture industry worldwide.

The present study demonstrates that the need to better understand the ecology and pathogeny of *V. parahaemolyticus* in aquatic environments is critical to identify and understand the risk for human and animal health related to the environment and activities in coastal areas.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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