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CHARACTERIZATION OF *Vibrio parahaemolyticus* ISOLATED FROM OYSTERS AND MUSSELS IN SÃO PAULO, BRAZIL

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SUMMARY

Vibrio parahaemolyticus is a marine bacterium, responsible for gastroenteritis in humans. Most of the clinical isolates produce thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) encoded by *tdh* and *trh* genes respectively. In this study, twenty-three *V. parahaemolyticus*, previously isolated from oysters and mussels were analyzed by PCR using specific primers for the 16S rRNA and virulence genes (*tdh*, *trh* and *tlh*) and for resistance to different classes of antibiotics and PFGE. Nineteen isolates were confirmed by PCR as *V. parahaemolyticus*. The *tlh* gene was present in 100% of isolates, the *tdh* gene was identified in two (10.5%) isolates, whereas the gene *trh* was not detected. Each isolate was resistant to at least one of the nine antimicrobials tested. Additionally, all isolates possessed the $bla_{\text{TEM-116}}$ gene. The presence of this gene in *V. parahaemolyticus* indicates the possibility of spreading this gene in the environment. Atypical strains of *V. parahaemolyticus* were also detected in this study.

KEYWORDS: *Vibrio parahaemolyticus*; *tlh*, *tdh* and *trh* Genes; $bla_{\text{TEM-116}}$

INTRODUCTION

Vibrio parahaemolyticus is a human pathogenic Gram-negative halophilic bacterium, a natural inhabitant of the marine environment and can be found in crabs, shrimps, fish, oysters, mussels and other seafoods[13,34,41]. *V. parahaemolyticus* infections are associated to the ingestion of contaminated raw or undercooked shellfish, especially bivalve molluscs[10,13,18].

It was first isolated in 1950, during a large outbreak of gastroenteritis that occurred in Japan[13]. Since then this bacterium has been recognized as one of the main agents causing foodborne diseases, in many countries including Asian countries, the United States, France, Mexico, Peru and Chile[5,14,15,17,20,22,28,41,45]. Moreover, *V. parahaemolyticus* infections have increased globally[28,33]. Studies in Brazil demonstrated the presence of *V. parahaemolyticus* in environmental samples from a variety of sources as well as in clinical samples[1,25,26,27,31,34,35,38]. In 2002, according to data of the National Health Foundation (FUNASA), in Ceará, Brazil, an outbreak of gastroenteritis occurred, and Kanagawa-positive strains of *V. parahaemolyticus* serovar O3:K6 were isolated[31].

The pathogenicity of this bacterium in humans is associated to the production of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH)[30]. Many studies have demonstrated that virulent strains of *V. parahaemolyticus* possess either the gene *tdh* or *trh*, or both[21]. The evidence of transmission of these genes through plasmids or insertion elements has already been demonstrated. Another thermolabile haemolysin (TLH) which is encoded by *tlh* gene is considered as a species-specific marker. This gene has been detected in all clinical and environmental *V. parahaemolyticus* strains[4].

Coastal waters and estuarine environments are subjected to waste contamination that could selectively contribute to antimicrobial resistance in bacteria from these environments[44]. Pathogenic strains of *V. parahaemolyticus* have been reported to be resistant to different classes of antimicrobials such as aminoglycosides, beta-lactams and quinolones[36,46]. Thus in this study, antimicrobial susceptibility of *V. parahaemolyticus* isolated from the environment was evaluated.

V. parahaemolyticus is an important foodborne pathogen, although in São Paulo outbreaks caused by *V. parahaemolyticus* have not been reported by the Center for Epidemiologic Surveillance (CVE). In addition, consumption of raw fish in Brazilian cities is popular[39]. Therefore, in this study, *V. parahaemolyticus* isolated from shellfish were characterized by PCR targeted to 16S rRNA and virulence genes, antibiotic resistance patterns and DNA profiles by PFGE.

MATERIAL AND METHODS

Strains. Twenty-three *V. parahaemolyticus* strains isolated from oysters and mussels, collected from aquatic environments, fish markets and restaurants in São Paulo, Brazil, between February 1989 and January 1990, were used in this study. Isolates were kept in Luria Bertani broth containing 60% glycerol at -80 °C, in the Public Health Laboratory

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Culture Collection, School of Public Health /University of São Paulo. The isolates were identified as *V. parahaemolyticus* by standard biochemical methods as described previously[26]. To determine the viability, the strains were cultured in thiosulphate citrate bile salts sucrose (TCBS) agar (Difco, France) at 35 °C for 24 hours and screened in Triple Sugar Iron (TSI) agar (Merck, USA) and citochrome oxidase production. A *V. parahaemolyticus* positive control was kindly provided by Fundação Instituto Oswaldo Cruz (FIOCRUZ - Rio de Janeiro, Brazil). *V. harveyi* was used as a negative control. DNA extraction was carried out for all samples using the heat-shock technique[7].

Kanagawa Test. Wagatsuma agar containing 5% human erythrocytes was used to screen all *V. parahaemolyticus* strains for β-hemolysis by incubation at 35 °C for 24 hours[43].

Antimicrobial Susceptibility Test. **A**ntimicrobial susceptibility test was performed using disc-diffusion, Kirby-Bauer method[29]. The antibiotics used were streptomycin (10 µg), gentamicin (10 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), cotrimoxazole (25 µg), tetracycline (30 µg), ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefpodoxime (10 µg) and imipenem (10 µg) (OXOID®, England).

Polymerase Chain Reaction (PCR). PCR was performed to identify *V. parahaemolyticus* using 16S rRNA as a targeted gene. Multiplex PCR was established for detection of virulence genes *tdh*, *trh* and *tlh*. In the present study, the majority of *V. parahaemolyticus* strains were resistant to ampicilin. In addition, bla_{TEM} genes have recently been detected in many Brazilian environmental bacteria[2]. Therefore, we also investigated the presence of bla_{TEM} genes in *V. parahaemolyticus* strains by PCR. All primers used are indicated in Table 1.

Pulsed Field Gel Electrophoresis (PFGE). PFGE technique was performed as previously described[40]. Briefly, DNA was digested with 40U of *Sfi*I restriction enzyme (GE Healthcare, UK). Restriction fragments were separated by pulsed field gel electrophoresis in a Chef Mapper apparatus (Bio-Rad Laboratories, Hercules, CA) in a two-step procedure as follows: Block 1: 6V/cm, 14 °C, for 13h, pulse ramp of 2s to 10s, 120° angle. Block 2: 6V/cm, 14 °C, for six hours, pulse ramp of 20s to 25s, 120° angle. Gels were stained with ethidium bromide. DNA profiles obtained by PFGE were visualized in an Epi Chemi II Darkroom (UVP Bioimaging Systems). PFGE profiles were analyzed using Gel Works 1D Advanced 4.01 and Gel Works 1D Database 1.12 (UVP Bioimaging Systems, Upland, CA).

RESULTS

Identification of V. parahaemolyticus. Twenty-three strains presumptively identified as *V. parahaemolyticus* were submitted to biochemical and molecular tests. Four strains (17.4%) were negative for PCR targeted to 16S rRNA. Nineteen strains (82.6%) including five strains with atypical biochemical tests were confirmed as *V. parahaemolyticus* by PCR. The presence of *tlh* gene was observed in all 19 *V. parahaemolyticus* strains. Biochemical tests revealed atypical characteristics in two (8.7%) strains which were sucrose positive. Three strains (13.0%), were negative for ethanol as sole carbon source. Figure 1 summarizes the results of biochemical tests, presence of virulence genes, Kanagawa phenomenon, antimicrobial susceptibility and PFGE profiles of *V. parahaemolyticus* strains evaluated in this study.

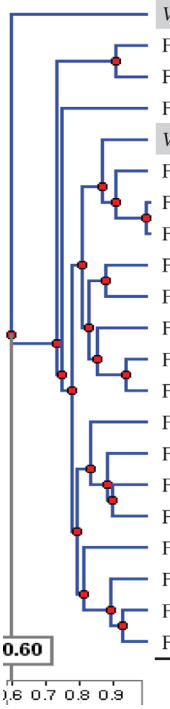
Virulence. The β-hemolysis in Wagatsuma agar (Kanagawa phenomenon) was observed in three strains of *V. parahaemolyticus* (15.8%). The presence of virulence gene *tdh* was observed in two (10.5%), but none of the strains were positive for *trh* gene.

Antibiotics resistance. From 19 strains tested, six were resistant to at least two antimicrobial agents (Fig. 1). All strains were susceptible to imipenem, nalidixic acid and ceftazidime. It is important to highlight that all strains had intermediate results for streptomycin. For bla_{TEM} genes evaluation, all *V. parahaemolyticus* isolates presented the expected 972pb fragment, and sequencing of these amplicons demonstrated 100% similarity to $bla_{TEM\text{-}116}$ (GenBank accession numbers of JF327790 to JF327797).

Table 1
Sequences of primers used to confirm the taxonomic position, detect virulence genes and detect bla_{TEM} genes

Gene target	Primer sequence	Band size (bp)	PCR cycle*	Reference
tlh	L-*tl*: (5'-AAAGCGGATTATGCAGAAGCACTG-3') R-*tl*: (5'-GCTACTTTCTAGCATTTTCTCTGC-3')	450	30 cycles: 94 °C - 1min. 58 °C - 1 min. 72 °C - 1 min	4
tdh	L-*tdh*: (5'-GTAAAGGTCTCTGACTTTTGGAC-3') R-*tdh*: (5'-TGGAATAGAACCTTCATCTTCACC-3')	269		
trh	L-*trh*: (5'-TTGGCTTCGATATTTTCAGTATCT-3') R-*trh*: (5'-CATAACAAACATATGCCCATTTCCG-3')	500		
16S rRNA	VparaF (5'-GCTGACAAAACAACAATTTATTGTT-3') VparaR (5'-GGAGTTTCGAGTTGATGAAC-3')	170	35 cycles: 94 °C - 1min. 55 °C - 1 min. 72 °C - 1 min	19
bla_{TEM}	TEM CR F (5'-CGWGTCGCCCTTATTCCCT-3') TEM R (5'-CCAAWGCTTAATCAGTGAG-3')	840	30 cycles: 94 °C - 45s 52 °C - 1 min. 72 °C - 1 min	6

*All amplifications had one initial cycle of 94 °C for 5 min., final extension of 72 °C for 10 min. and were maintained at 4 °C after PCR.



Isolates[a]	Sample/Origin[b]	Collection Site[c]	Kanagawa	PCR				Resistance patterns	Phenotypic Characteristics			
				16S	*tlh*	*tdh*	*trh*		Ethanol	Putrescine	Sucrose	
											TSI	TCBS
V. harveyi (outgroup)												
FSP358	3/O	MF-1	-	+	+	-	-	AMP	+	+	-	-
FSP402	6/O	R-1	-	+	+	-	-	AMP, CPD, TE*, CIP*	+	-	-	+
FSP367	12/O	R-2	+	+	+	+	-	AMP	+	+	+	+
Vibrio parahaemolyticus (positive control)												
FSP1017	5/M	E-1	-	+	+	-	-	AMP, CTX*, GEN*	+	+	-	-
FSP1020	5/M	E-1	-	+	+	-	-	AMP	+	+	-	-
FSP1016	5/M	E-1	-	+	+	-	-	AMP, CTX*	+	+	-	-
FSP365	7/O	R-3	-	+	+	-	-	AMP	+	+	-	-
FSP1010	9/O	R-4	-	+	+	-	-	AMP, CPD, TE*	+	+	-	-
FSP1028	6/M	E-2	-	+	+	-	-	AMP*	+	+	-	-
FSP1011	9/O	R-4	-	+	+	-	-	AMP	+	+	-	-
FSP366	10/O	MF-2	-	+	+	-	-	AMP	+	+	-	-
FSP1021	3/M	E-2	-	+	+	-	-	AMP	+	+	-	-
FSP1014	6/O	R-1	+	+	+	+	-	AMP	-	+	-	-
FSP364	6/O	R-1	+	+	+	-	-	AMP	-	+	-	-
FSP1022	3/M	E-2	-	+	+	-	-	AMP	+	+	-	-
FSP1006	15/O	R-5	-	+	+	-	-	AMP	+	+	-	-
FSP1013	6/O	R-1	-	+	+	-	-	AMP	-	+	-	-
FSP1018	4/M	E-3	-	+	+	-	-	AMP, CIP, GEN*	+	+	-	-
FSP1019	4/M	E-3	-	+	+	-	-	CLO, SXT*	+	+	-	-

[a]FSP, Faculdade de Saúde Pública; [b]O, oysters; M, mussels; [c]MF, market fish; R, restaurant; E, environment; PCR, polymerase chain reaction; 16S, 16S rRNA gene sequence; *tlh*, thermolabile haemolysin gene; *tdh*, thermostable direct haemolysin gene; *trh*, thermostable-related haemolysin gene; TSI, triple-sugar-iron agar; TCBS, thiosulphate-citrate-bile salts-sucrose agar; AMP, ampicillim; CTX, cefotaxime; CPD, cefpodoxime; CLO, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; TE, tetracycline; SXT, cotrimoxazole; *Isolate exhibiting intermediate resistance against indicated antibiotic; +, positive; –, negative.

Fig. 1 - Phenotypic and genotypic characteristics of *V. parahaemolyticus* according to Pulsed Field Gel Electrophoresis profile.

PFGE. The PFGE analysis demonstrated heterogeneity among the *V. parahaemolyticus* isolates except for isolates FSP1020 and FSP1016 that were very close related, with only one band difference suggesting that they might have originated from the same clone. However, these strains showed differences in antimicrobial susceptibility. Overall, the graphic representation of the Dice's similarity coefficient matrix showed similarity above 80% (Fig. 1).

DISCUSSION

Four strains with typical biochemical reactions were negative for PCR identification of *V. parahaemolyticus*. On the other hand, FSP367, FSP402, FSP364, FSP1013 and FSP1014, which were strains with atypical phenotypic characteristics (positive reaction for sucrose and negative for ethanol as sole carbon source) were confirmed as *Vibrio parahaemolyticus* by PCR. It has been demonstrated that phenotypic tests are often unable to discriminate species of vibrios[9]. In this study, *tlh* gene was a useful marker for confirmation of *V. parahaemolyticus* and the results of detection of this gene corroborates the results obtained for 16S rRNA gene[12]. Therefore, molecular techniques are a useful tool for identification of environmental *V. parahaemolyticus* strains if atypical *V. parahaemolyticus* is detected[13,18,42].

In regard to the presence of virulence genes in *V. parahaemolyticus*, *tdh* was observed in two (10.5%) strains which corroborates the results of other authors that this gene was absent in the majority of environmental strains of *V. parahaemolyticus*[3,30]. The *trh* gene was not detected in any tested strains. In the present study it was observed that one strain showed beta-hemolysis on Wagatsuma agar (Kanagawa positive) but it was negative for *tdh* gene. This phenomenon has been reported in Brazil[1,4,16,24,32] and might be due to deletion or mutation in the *tdh* gene[33].

PFGE has been used to investigate diversity of *V. parahaemolyticus* and has been applied to characterize bacterial strains from environment and foodborne outbreaks[37,45]. In the present study, genotypic diversity of *V. parahaemolyticus* strains was achieved by PFGE using *Sfi*I. This methodology is widely used in epidemiological studies for many genus of bacteria[8] and this is the first application to access the diversity of *V. parahaemolyticus* strains isolated from Brazilian samples. These

results may be kept as a database of environmental strains to be used in investigations in case of outbreaks.

In the present study it was demonstrated that environmental isolates showed low susceptibility to penicillins and aminoglycosides, thus, they may have a low effectiveness in clinical treatment of *V. parahaemolyticus*.

It has been demonstrated that clinical isolates of *V. parahaemolyticus* show intermediate susceptibility to gentamicin, resistance to streptomycin and ampicillin, and susceptibility to trimethoprim-sulphamethoxazole[23]. In addition, clinical and environmental *V. parahaemolyticus* strains also show resistance to chloramphenicol, tetracycline and cefotaxime[46]. Results obtained in this study, demonstrated the same resistance profile to streptomycin and ampicillin that was observed in clinical strains, but most of our *V. parahaemolyticus* environmental isolates were susceptible to chloramphenicol, tetracycline and cefotaxime. Resistance to cotrimoxazole (combination of trimethoprim and sulfamethoxazole) and chloramphenicol was detected in FSP1019 strain.

The acquisition of resistance genes can occur by mobile elements such as plasmids and integrons, which have already been described in *V. parahaemolyticus* and other *Vibrio* species[42].

In the present study, bla_{TEM} genes were detected in all tested *V. parahaemolyticus* and sequencing of this gene revealed 100% similarity with $bla_{TEM-116}$. The presence of this gene in *V. parahaemolyticus* isolates from several sites in São Paulo State indicates that $bla_{TEM-116}$ genes have been disseminated in the marine environment. In fact, studies demonstrated the presence of this gene in *Aeromonas* spp. and *Klebsiella pneumoniae* isolated from environment and clinical samples in the same regions in São Paulo[2,11].

To contribute to a better characterization of the pathogen, further studies on *V. parahaemolyticus* from its natural environment, seafood or clinical samples are essential. Thus, the molecular methods used in this study may be useful in the monitoring of this microorganism. However, in order for this application to be viable, it is necessary to improve the clinical diagnosis for *V. parahaemolyticus* infections. Therefore, it is recommended that routine searching for halophilic vibrios and a systematic notification of clinical cases be undertaken in hospital areas located near coastal regions and in places where consumption of seafood is a regular practice in order to identify the prevalence of vibriosis in the population. Consequently, health professionals should be directed, in cases of gastroenteritis associated with seafood consumption, to request specific culture for the isolation and characterization of vibrios, including *V. parahaemolyticus*. Such measures could provide relevant information to Health Surveillance services.

RESUMO

Caracterização de *Vibrio parahaemolyticus* isolados de ostras e mexilhões em São Paulo, Brasil

Vibrio parahaemolyticus é uma bactéria marinha, responsável por gastroenterite em humanos. A maioria dos isolados clínicos produzem hemolisina termoestável direta (TDH) e hemolisina TDH-relacionada (TRH) codificadas por genes *tdh* e *trh*, respectivamente. Neste estudo, vinte e três *V. parahaemolyticus*, previamente isolados de ostras e mexilhões foram analisados por PCR utilizando indicadores específicos para o gene 16S rRNA, genes de virulência (*tdh*, *trh* e *tlh*), resistência a diferentes classes de antibióticos, e PFGE. Dezenove isolados foram confirmados por PCR, como *V. parahaemolyticus*. O gene *tlh* estava presente em 100% dos isolados, o gene *tdh* foi identificado em dois (10,5%) dos isolados, enquanto que o gene *trh* não foi detectado. Cada isolado foi resistente a pelo menos um dos nove antibióticos testados. Além disso, todos os isolados apresentaram resultado positivo para o gene $bla_{TEM-116}$. A presença deste gene em *V. parahaemolyticus* indica a possibilidade de propagação desse gene no ambiente. Cepas atípicas de *V. parahaemolyticus* foram também detectadas neste estudo.

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