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Kanagawa-Negative, *tdh*- and *trh*-Positive *Vibrio parahaemolyticus* Isolated from Fresh Oysters Marketed in Fortaleza, Brazil

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Abstract Between October 2008 and June 2009, 15 samples of 10 live oysters each (*Crassostrea rhizophorae*) measuring 8.31–10.71 cm were purchased from a restaurant on the seashore of Fortaleza, Brazil. The *Vibrio* count ranged from 75 (estimated) to 43,500 CFU/g. Fourteen species were identified among the 56 isolated *Vibrio* strains, with *V. parahaemolyticus* as the most prevalent. Two of the 17 *V. parahaemolyticus* strains were urease-positive and *tdh*- and *trh*-positive on multiplex PCR, but neither produced β-hemolysis halos in Wagatsuma agar. Thus, fresh oysters served in natura in Fortaleza, Brazil, were found to contain *Vibrio* strains known to cause gastroenteritis in humans.

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Introduction

The increasing consumption of shellfish in natura, especially oysters, collected from natural nurseries and marketed without proper depuration or processing, is drawing the attention of public health authorities worldwide, particularly in the tropics [20]. Exposure to high temperatures, poor handling hygiene, and unclean ice increase the risk of contamination by potentially harmful microorganisms.

Vibrios are part of the autochthonous microbiota of marine and estuarine environments. Some vibrios are known to cause infection in seafood consumers. Oysters filter the surrounding water, retaining and accumulating microorganisms and other substances. The ability of oysters to infect seafood consumers with pathogenic vibrios depends on the microbiological quality of their habitat as well as on handling and processing practices.

Vibrios are generally classified into sucrose-positive (Suc+) and sucrose-negative (Suc−), according to their ability to ferment sucrose [15]. The relative abundance of Suc+ and Suc− vibrios depends on the environment, but according to some authors, including Chan et al. [3] and Matté et al. [11], Suc+ vibrios are prevalent in seafoods. However, despite variations in prevalence, the two species most commonly associated with infectious outbreaks following oyster consumption in natura (*V. parahaemolyticus* and *V. vulnificus*) [12] are both sucrose-negative.

Vibrio parahaemolyticus is widely distributed in marine environments around the world and is often isolated from raw seafoods, especially mollusks. The consumption in natura of seafoods contaminated with *V. parahaemolyticus* can induce gastroenteritis accompanied by diarrhea, vomiting, abdominal pain, nausea, and headache [21]. *V. parahaemolyticus* strains capable of causing infectious outbreaks may present the genes *tdh* and/or *trh* [13], in

addition to be capable of hydrolyzing urea and inducing β-hemolysis in blood agar [9, 16].

The objective of this study was to identify and quantify Sac+ and Sac− vibrios in fresh oysters (*Crassostrea rhizophorae*) purchased from a restaurant on the seashore of Fortaleza, Brazil, and to test identified *Vibrio parahaemolyticus* strains for virulence factors such as urease and the genes *tdh* and *trh*.

Materials and Methods

Sampling

Between October 2008 and June 2009, 15 samples of live oysters (*Crassostrea rhizophorae*) were purchased from a single restaurant in Fortaleza, Brazil. Each sample consisted of 10 oysters, totaling 150 specimens. The oysters were transported live to the laboratory in an isothermal box and analyzed within 1 h of sampling.

Sample Preparation

The oysters were washed in tap water and vigorously brushed. Following measurement with a caliper, the shells were opened aseptically for removal of the soft tissues and intervalvular fluid. A 10-g aliquot of each sample was added to 90 ml alkaline peptone water containing 1% NaCl (pH 8.5) [17]. The mixture was homogenized in a magnetic stirrer for 15 min. The homogenate (dilution of 10^{-1}) was used to make serial decimal dilutions from 10^{-2} to 10^{-4}.

Vibrio Count

Vibrios were quantified by standard plate count, as described by Downes and Ito [5]. A 0.1-ml aliquot was taken from each dilution (10^{-1}–10^{-4}), spread plated in duplicate in thiosulfate citrate bile sucrose (TCBS) agar and incubated for 18 h at 35°C. Results were expressed in colony-forming units per gram of oyster sample (CFU/g).

Isolation and Identification of Vibrios

Fifty-six strains were isolated from the 14 samples (four from each). The isolates were shipped in trypticase soy agar (TSA) and 1% NaCl to the enterobacteriology laboratory at the Institute Oswaldo Cruz (IOC) for biochemical identification, as described by Noguerola and Blanch [15].

Virulence Factors in *Vibrio parahaemolyticus*

Strains identified as *V. parahaemolyticus* were submitted to two phenotypical virulence tests: urease testing with Difco™ urea broth, and hemolysis of human erythrocytes in Wagatsuma agar for detection of the Kanagawa phenomenon [9, 25]. A standard *V. parahaemolyticus* IOC 18950 strain from an outbreak in Cascavel (a town in Northeastern Brazil) was used as positive control [6].

DNA Extraction

The *V. parahaemolyticus* strains testing positive for virulence ($n = 2$) were inoculated in 1% alkaline peptone water and cultured for 24 h at 35°C. Then, 1.0 ml of culture was retrieved for DNA extraction with a commercially available extraction kit (DNeasy Blood & Tissue Kit, Qiagen). A 1.0-µl aliquot of each sample of extracted DNA was submitted to spectrophotometry (260 and 280 nm wavelength) for quantification and determination of extraction efficiency.

Detection of Virulent Genes

The detection of the genes *tdh* (thermostable direct hemolysin) and *trh* (thermostable direct hemolysin-related hemolysin) was done with the *tdh* primers L-*tdh*: 5′-gta aag gtc tct gac ttt tgg ac-3′ and R-tdh: 5′-tgg aat aga acc ttc atc ttc acc-3′ and the *trh* primers L-trh: 5′-ttg gct tcg ata ttt tca gta tct-3′ and R-trh: 5′-cat aac aaa cat atg ccc att tcc g-3′ [2]. The total extracted DNA was amplified by multiplex PCR in a Techne thermocycler set to 30 cycles starting with DNA template degradation for 1 min at 94°C followed by primer annealing in the target region of the template for 1 min at 58°C and strand extension for 1 min at 72°C. This was followed by 5 min final strand extension at 72°C. Reaction solutions of 25 µl were prepared containing 1 µl extracted DNA, 2 µl dNTP from each nucleotide (Amersham Pharmacia Biotech), 0.5 µl of each primer, 0.25 µl Taq DNA polymerase (Biotools), 2.5 µl buffer, and 17.25 µl ultrapure distilled water (Gibco, Invitrogen). DNA from the *V. parahaemolyticus* reference strain IOC 18950 (*tdh*+ and *trh*+) was used as positive control and included in each amplification run. With the exception of the DNA template, all reaction solution components were used as negative controls. A 6-µl aliquot was drawn from the final amplification product and submitted to 2% agarose gel electrophoresis in TBE buffer for 60 min at 60 V. Following staining with ethidium bromide gel, the DNA was viewed under UV light with a Spectroline transilluminator and photographed with a Kodak EDAS 290.

Detection of the *tl* Gene

The detection of the gene *tl* (thermolabile hemolysin, species-specific marker for *Vp*) was performed with the

primers L-*tl*: 5′-aaa gcg gat tat gca gaa gca ctg-3′ and R-*tl*: 5′-gct act ttc tag cat cat ttt ctc tgc-3′ [2]. Reaction solutions of 25 µl were prepared containing 1 µl extracted DNA, 2 µl dNTP from each nucleotide (Amersham Pharmacia Biotech), 0.5 µl of each primer, 0.25 µl Taq DNA polymerase (Biotools), 2.5 µl buffer, and 18.25 µl ultrapure distilled water (Gibco, Invitrogen). DNA from the *V. parahaemolyticus* reference strain IOC 18950 (*tl*+) was used as positive control. DNA amplification and final electrophoresis were performed as described in the previous section.

Results

The sampled oysters measured 8.31–10.71 cm. The *Vibrio* count ranged from 75 (estimated) to 43,500 CFU/g. Variations in pH were slight (6.22–7.11) (Table 1).

In the present study, the correlation between *Vibrio* counts and pH values was weak, with the values of 0.029963, 0.02573 e−0.03257 for total *Vibrio*, Sac+, and Sac−, respectively. The correlation between *Vibrio* counts and size was weak inverse for Sac− (−0.0346) and moderate inverse for total *Vibrio* (−0.30636) and Sac+ (−0.34349).

The 56 strains characterized could be affiliated to 14 *Vibrio* species (Table 2), with *V. parahaemolyticus* as the most prevalent (30.3%), followed by *Vibrio cholerae* non-O1 non-O139 (14.3%), *V. fisheri* (12.5%), *V. harveyi* (7.14%), *V. alginolyticus* (5.35%), *V. vulnificus* (5.35%), *V. mytili* (5.35%), *V. litoralis* (3.6%), *V. mimicus* (3.6%), *V. rumoiensis* (3.6%), *V. campbelli* (3.6%), *V. diazotrophicus* (1.8%), *V. calviensis* (1.8%), and *V. coralliilyticus* (1.8%).

DNA extraction yielded the following ratio: A(260/280) = 1.8 (#31) and A(260/280) = 1.7 (#32 and *V. parahaemolyticus* IOC 18950).

Two (#31 and #32) of the 17 strains confirmed by *tl* detection to be *V. parahaemolyticus* were urease-positive, but neither produced *β*-hemolysis halos in Wagatsuma agar. The genes *tdh* and *trh* were detected in both urease-positive strains (Fig. 1).

Table 1 *Vibrio* count, size, pH, and counted colonies (standard: sucrose fermentation) in samples of oysters (*Crassostrea rhizophorae*) purchased at a restaurant on the seashore of Fortaleza, Brazil

Sample	Length of oyster (cm)	pH	Total *Vibrio* count	Sac+	Sac−
1	8.31	6.61	4,800	1,950	2,850
2	9.46	6.30	200[a]	–	200[a]
3	8.53	6.70	43,500	32,000	11,500
4	9.38	6.22	200[a]	200[a]	–
5	9.58	6.25	1,750[a]	950[a]	800[a]
6	9.72	6.24	1,450[a]	50[a]	1,400[a]
7	10.71	6.96	50[a]	–	50[a]
8	9.83	6.72	100[a]	–	100[a]
9	10.39	6.64	12,000	125	11,875[a]
10	10.52	6.71	100[a]	–	100[a]
11	9.53	6.69	200[a]	–	200[a]
12	9.7	6.88	400[a]	50[a]	350[a]
13	8.64	6.87	–	–	–
14	8.47	7.11	425[a]	50[a]	375[a]
15	8.6	6.95	75[a]	50[a]	25[a]

[a] Estimated

Table 2 Distribution of *Vibrio* species in samples of oysters (*Crassostrea rhizophorae*) purchased at a restaurant on the seashore of Fortaleza, Brazil

Species	*n*	Sample (number of strains)
V. parahaemolyticus	17	1 (2), 2 (1), 3 (1), 4 (2), 5 (4), 6 (4), 9 (3)
V. cholerae non-O1 non-O139	8	14 (5), 15 (3)
V. fisheri	7	2 (2), 4 (3), 5(1), 11(1)
V. harveyi	4	1 (2), 3 (2)
V. alginolyticus	3	3 (1), 6 (1), 9 (1)
V. vulnificus	3	1 (2), 2 (1)
V. mytili	3	12 (3)
V. litoralis	2	3 (1), 5 (1)
V. mimicus	2	11 (1), 12 (1)
V. rumoiensis	2	8 (1), 9 (1)
V. campbelli	2	3 (2)
V. diazotrophicus	1	1 (1)
V. calviensis	1	4 (1)
V. coralliilyticus	1	10 (1)

Discussion

Vieira et al. [23] reported smaller *Vibrio* counts (330–16,000 CFU/g) in *C. rhizophorae* collected in the estuary of the Jaguaribe river (150 km east of Fortaleza) an environment contaminated by the discharge of organic matter. The origin of the oysters sampled for the present study could not be ascertained since the restaurant from which they were purchased is supplied by various shellfish distributors.

Many factors can influence the number of vibrios in oysters, such as the microbiota of the local ecosystem and favored storage practices (restaurant from which our samples were obtained stores shellfish at room temperature). In fact, in a study on pathogenic *Vibrio* strains in *C. rhizophorae*, Pereira et al. [18] found that approximately 40% of

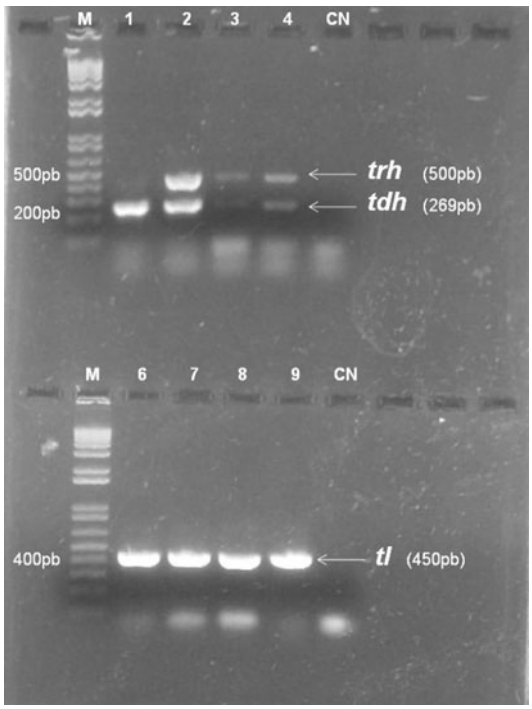

Fig. 1 1% agarose gel electrophoresis. *Lines 1 to 4* PCR products of the genes *tdh* and *trh* (1 to 4) and the gene *tl* (6 to 9), based on total DNA extracted from strains of *Vibrio parahaemolyticus*, *lines 1 and 6* control *V. parahaemolyticus* strain (Cascavel, 1983), *Lines 2 and 7 tdh+*, *trh+ V. parahaemolyticus* (IOC 18950), *lines 3, 4, 8 and 9 V. parahaemolyticus* isolated from oysters (strains #31 and #32). *M* Molecular weight marker (Ladder 1 Kb), *NC* negative control

the restaurants in Rio de Janeiro stored shellfish at room temperature. According to the authors, this practice represents a risk factor to public health by favoring the proliferation of potential pathogens, accelerating deterioration, and reducing effective shelf life.

In the present study, the variations in pH were slight (6.22–7.11) and the highest value registered (7.11; sample #14) did not coincide with the highest *Vibrio* count (sample #3). It should also be pointed out that, potentially, vibrio counts based on phenotypical characteristics may be underestimated. Vibrios may present as viable but non-culturable cells, as shown by Dileep et al. [4] in conventional biological and molecular analyses of 86 seafood samples (shrimp, crab, mollusk, and fish) obtained from retailers and from estuaries in Mangalore, India. Thus, toxR-targeted PCR detected almost twice as many samples with *V. parahaemolyticus* as conventional biological testing (53 vs. 28).

The same prevalence of *V. parahaemolyticus* was reported by Vieira et al. [24] for *Vibrio* populations in farmed oysters. *V. parahaemolyticus* was not only the most prevalent species, but it could be isolated from samples collected almost anytime during the 12-month culture cycle. The species is commonly found in seafoods [4], but some of the more virulent strains, identifiable by β-hemolysis in Wagatsuma agar [24], are known to cause gastroenteritis.

The fact that no strain displayed β-hemolysis halos in Wagatsuma agar does not rule out pathogenicity. Kanagawa-negative *V. parahaemolyticus* strains have previously been implicated in gastroenteritis, suggesting the involvement of other factors of virulence [7, 10].

The coincidence of urease-positivity and the presence of *trh* were also reported in 1995 by Suthienkul et al. [22]. According to these authors, urease-positivity is per se an indication of virulence. Kanagawa-negative strains carrying the *tdh* gene produce TDH and are therefore potentially toxigenic. In our study, Kanagawa-negativity was probably due to low levels of detectable hemolysin making positive strains unable to hemolyze blood. In a study by Nishibuchi et al. [14], 16% of Kanagawa-negative strains carried the *tdh* gene. Okuda and Nishibuchi [16] demonstrated that of the two genes *tdh*-1 and *tdh*-2, only the latter was responsible for hemolytic activity in Kanagawa-positive strains. The two genes are usually, though not exclusively, found in the chromosome.

The presence of both the *tdh* gene and the *trh* gene in two strains isolated from fresh oysters is a disquieting finding, since these genes are rarely observed in the environment. *V. parahaemolyticus* strains are only pathogenic and capable of inducing acute gastroenteritis if they produce the virulence factors TDH or TRH [13]. Robert-Pillot et al. [19] also isolated *trh*-, *tdh*-, and urease-positive strains of *V. parahaemolyticus* from two samples (a clinical sample and a sample of fresh oysters collected on the shores of France).

No less important, eight non-O1, non-O139 *V. cholerae* strains and three *V. vulnificus* strains were isolated from our oyster samples. Although the identified *V. cholerae* strains were not the ones known to cause cholera (O1 and O139), their presence in fresh oysters sold in restaurants represents a potential health risk. Bagchi et al. [1] described an outbreak of watery diarrhea among Khmers in a camp in Thailand caused by non-O1 *V. cholerae*. The other species (*V. vulnificus*) was also identified in samples collected during an oyster culture cycle in Eusébio, in the vicinity of Fortaleza, Brazil [24].

In conclusion, fresh oysters served in natura at restaurants on the seashore of Fortaleza, Brazil, were found to contain *Vibrio* strains known to cause gastroenteritis in humans. Oysters should, therefore, be transported and stored on ice and consumption in natura is not recommended.

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