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Short Communication

Hemolytic and urease activities in vibrios isolated from fresh and frozen oysters

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ABSTRACT

Introduction: The present study aimed to survey the *Vibrio* microbiota of oysters (*Crassostrea rhizophorae*) obtained from restaurants in Fortaleza, State of Ceará, Brazil, and to identify virulence factors. **Methods:** The isolated vibrios were submitted to biochemical identification and were tested for hemolytic and urease activities. **Results:** The isolated strains belonged to 13 species, with predominance of *Vibrio mimicus*. Of the strain isolates only from fresh samples, 20.5% and 2.8% showed hemolytic and urease activities, respectively. **Conclusions:** The findings support the little-publicized claim that *Vibrio* species other than *V. parahaemolyticus* and *V. vulnificus* can represent a health risk to public health.

Keywords: *Vibrio*. *Crassostrea rhizophorae*. Virulence factors.

Vibrios are part of the native microbiota of marine environments and organisms. Up to 40% of water-column bacteria are associated with zooplankton. Among these, vibrios have a competitive advantage in the colonization of chitinous exoskeletons[1]. Due to their abundance in the water column, vibrios may be up to a hundred times more concentrated in filter-feeding mollusks than in the immediate environment[2].

Since vibrios are part of the autochthonous bacterial community colonizing bivalves, it comes as no surprise that they have been implicated in many outbreaks related to oyster consumption. The vibrio species most often associated with disease from consumption of oysters *in natura* are *Vibrio parahaemolyticus*, *V. vulnificus*, and *V. cholerae*[3]. However, virulence factors similar to those displayed by *V. parahaemolyticus* are detected in other vibrio species with increasing frequency. Thus, Nishibuchi et al.[4] found that *V. hollisae* strains from clinical samples can carry the hemolysin-encoding gene *tdh*, a marker of virulence. Other vibrio species isolated from marine organisms have also been shown to be capable of β-hemolysis[5].

The objective of the present study was to survey the *Vibrio* microbiota of fresh and frozen oysters (*Crassostrea rhizophorae*) obtained from restaurants in Fortaleza, and to identify phenotypical virulence factors by testing strains for β-hemolysis and urease activity.

The study was based on 15 samples of fresh oysters and 15 samples of frozen oysters (*C. rhizophorae*) obtained from two restaurants in Fortaleza City (Northeastern Brazil) between August 2009 and August 2010. Each sample consisted of 10 specimens, for a total of 300 specimens examined. Oysters with closed valves were placed in labeled polyethylene bags and transported in isothermal boxes to the Laboratory of Seafood and Environmental Microbiology (LABOMAR/UFC) for processing. Transportation did not exceed two hours.

The oysters were washed in tap water and vigorously scrubbed, then opened under aseptic conditions for retrieval of the intervalvular tissues and fluid. A 50-gram portion was taken from each sample of 10 specimens and added to 450mL alkaline peptone water (pH 8.5) containing 1% NaCl. The sample was homogenized in a magnetic stirrer (Cole Parmer, Model 51450) for 15min. The homogenate (which corresponded to a 10^{-1} dilution) was used to make serial decimal dilutions from 10^{-2} to 10^{-4}.

Subsequently, 0.2mL aliquots of each dilution were spread-plated on thiosulfate-citrate-bile salt-sucrose agar (TCBS-Difco) and incubated at 35°C for 18h. Three yellow or blue-green colonies for each sample were randomly select and cultured in tryptone soy agar (TSA-Difco) supplemented with 1% NaCl. All colonies were submitted to biochemical identification using the key developed by Noguerola and Blanch[6]. The following metabolic parameters were measured: lysine and ornithine decarboxylation; arginine hydrolysis; indole and H_2S production; gelatinase; oxidase; sucrose consumption; mannitol; melibiose; arabinose and D-glucosamine; growth at 4°C, 35ºC, and 40°C; growth at 0, 6, 8, and 10% NaCl; ONGP (o-nitrophenyl-β-D-galactopyranoside); resistance to 10µg ampicillin and 10µg O/129; Voges-Proskauer test (acetoin); citrate; and nitrate reduction. Urease activity was tested in the medium Stuart, van Stratum & Rustigian. All the media used for identification contained 1% NaCl.

The isolates were grown on Wagatsuma agar supplemented with 20% defibrinated sheep blood (modification), incubated at 35°C for 24h. A positive control strain (Kanagawa-positive *V. parahaemolyticus* IOC 18950) was used.

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Eleven *Vibrio* species were identified in the microbiota of fresh samples, compared with five species in frozen samples **(Table 1)**. Only three species (*V. rumoiensis*, *V. coralliilyticus*, and *V. litoralis*) were isolated from fresh and frozen oysters concomitantly. The species *V. mimicus*, *V. ponticus*, *V. hepatarius*, *V. proteolyticus*, *V. lentus*, *V. natriegens*, *V. pelagius* I, and *V. scophthalmi* were isolated from fresh samples only. On the other hand, *V. vulnificus* and *V. diazotrophicus* were detected only in the microbiota of frozen samples.

Seven strains (20.5% of total) belonging to five species tested were hemolytic-positive in Wagatsuma agar. Only one (2.8%) of our 35 *Vibrio* strains was capable of ureolysis **(Table 2)**. None of our strains were both Kanagawa-positive and ureolytic.

TABLE 1 - Vibrios isolated from 15 fresh and 15 frozen samples of oyster obtained from restaurants in Fortaleza, State of Ceará, Brazil.

Isolated *Vibrio* species	Strains (n)	Origin of strains	
		Fresh oyster	Frozen oyster
Vibrio mimicus	5	5	-
Vibrio hepatarius	4	4	-
Vibrio ponticus	4	4	-
Vibrio proteolyticus	4	4	-
Vibrio vulnificus B3	4	-	4
Vibrio rumoiensis	3	1	2
Vibrio coralliilyticus	2	1	1
Vibrio diazotrophicus	2	-	2
Vibrio litoralis	2	1	1
Vibrio lentus	1	1	-
Vibrio natriegens	1	1	-
Vibrio pelagius I	1	1	-
Vibrio scophthalmi	1	1	-

TABLE 2* - Vibrio *species isolated from fresh oyster, with phenotypical profiles compatible with urease and hemolytic activities

Test	Strains (n)	Species (n of strains)
β-hemolysis	7	*Vibrio proteolyticus* (3)
		Vibrio pelagius I (1)
		Vibrio ponticus (1)
		Vibrio rumoiensis (1)
		Vibrio coralliilyticus (1)
Urease	1	*Vibrio litoralis* (1)

The *Vibrio* diversity was greater in fresh than in frozen samples, suggesting that handling and storage practices have an impact on bacterial diversity and confirming the claim that vibrio proliferation is greater at room temperature than under cold storage. This is supported by Pereira et al.[7], according to whom prolonged storage of oysters at room temperature favors bacterial multiplication.

The most frequently isolated species were *V. mimicus* (fresh samples) and *V. vulnificus* B3 (frozen samples). The former has been reported abundantly in the literature since the early 1980s. Thus, Shandera et al.[8] described the clinical and epidemiological characteristics of infection caused by *V. mimicus* and concluded that the species should be considered in the differential diagnosis of acute gastroenteritis following ingestion of seafood, especially oysters *in natura*.

The detection of *V. vulnificus* in frozen oyster samples does not match the findings of Prapaiwong et al.[9], who observed the species in fresh samples only. However, Bryan et al.[10] suggested that the effectiveness of rapid freezing of oysters to reduce *V. vulnificus* levels may be compromised by product handling procedures that permit cold adaptation. The authors emphasize than this adaptive nature to cold temperatures could be important for shellfish industry efforts to reduce the risk of *V. vulnificus* infection from consuming raw oysters.

The index of isolation of *V. vulnificus* in our study (11.4%) is disquieting and high enough to make positive samples a potential source of infection. According to Daniels[11], oysters are the most common vehicle of transmission of *V. vulnificus* infection, which manifests in the form of gastroenteritis, primary septicemia, or wound infection, accompanied by fever, chills, diarrhea, nausea, vomiting, septic shock, and characteristic skin lesions.

Seven (53.8%) of the 13 vibrio species identified in this study were recently described for the first time: *V. scophthalmi*, *V. rumoiensis*, *V. lentus*, *V. hepatarius*, *V. coralliilyticus*, *V. ponticus*, and *V. litoralis*. The type strains used to characterize the vibrio species above were isolated from aquatic organisms sampled in coastal areas, marine environments, and/or the fishing industry. Of particular interest is *V. lentus*, which was first characterized based on 12 strains isolated from Mediterranean oysters[12].

In view of the importance of determining the level of virulence in vibrio strains isolated from oysters destined for human consumption, all strains (n=35) were tested for their ability to hydrolyze urea and produce hemolysin – two well-established indicators of pathogenicity in vibrios. In the present study only fresh oyster samples showed hemolytic activity in Wagatsuma agar and strong positive urease production **(Table 2)**.

Detected phenotypically by the observation of β-hemolysis, the phenomenon Kanagawa is triggered by the expression of the gene *tdh*, which encodes thermostable direct hemolysin (TDH), a determining factor of virulence in *V. parahaemolyticus* strains. However, functional TDH production depends on two genes, *tdh*1 and *tdh*2, of which only the latter is capable of hemolytic activity[13].

Although most studies on Kanagawa positivity focus on the species *V. parahaemolyticus*, other vibrio species may occasionally carry *tdh* highly homologous to the genes known from *V. parahaemolyticus*[4]. This was borne out in our study by the finding of β-hemolysis in species other than *V. parahaemolyticus*.

The species *V. proteolyticus*, *V. pelagius* I, *V. ponticus*, *V. rumoiensis*, and *V. coralliilyticus* showed hemolytic activity **(Table 2)**. Likewise, strains of *V. coralliilyticus* isolated from scallops (*Nodipecten nodosus*) and coral (*Pocillopora damicornis*) were shown by Austin et al.[5] to produce hemolysin in sheep blood.

Isolation of urease-positive *V. litoralis* **(Table 2)** may be indicative of health risk to consumers of fresh oyster. According to Mobley[14], ureolysis is observed in a wide range of taxonomically different bacteria, the pathogenicity of which appears to be related to their ability to colonize the gastric mucosa and protect themselves from the acid environment of the stomach. In vibrios, especially *V. parahaemolyticus*, the phenotypical detection of urease may be considered a marker of virulence for TDH-related hemolysin (TRH) production and, consequently, indicates the presence of the gene *trh*[15].

The virulence factors were not expressed simultaneously in any strain. Similarly, Pereira et al.[7] only observed ureolysis in Kanagawa-negative strains of *V. parahaemolyticus*.

The findings of this study support the little-publicized claim that vibrio species other than *V. parahaemolyticus* and *V. vulnificus* can represent a health risk to consumers of oysters. In addition, frozen storage was shown to reduce vibrio diversity and, consequently, the risk of bacterial infection associated with the consumption of fresh oysters.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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