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DETECTION OF *Vibrio parahaemolyticus* AND *Vibrio cholerae* IN OYSTER, *Crassostrea rhizophorae*, COLLECTED FROM A NATURAL NURSERY IN THE COCÓ RIVER ESTUARY, FORTALEZA, CEARÁ, BRAZIL

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SUMMARY

Oysters are edible organisms that are often ingested partially cooked or even raw, presenting therefore a very high risk to the consumers' health, especially in tropical regions. The presence of *Vibrio cholerae* and *Vibrio parahaemolyticus* in oysters sampled at an estuary in the Brazilian northeastern region was studied, with 300 oysters tested through an 8-months period. The salinity of the water at the sampling point varied between 3‰ and 27‰. *V. cholerae* was the most frequently detected species (33.3% of the samples), and of the 22 *V. cholerae* isolates, 20 were identified as non-O1/non-O139, with two of the colonies presenting a rough surface and most of remaining ones belonging to the Heiberg II fermentation group. *V. parahaemolyticus* was isolated from just one of the samples. Other bacteria such as *Providencia* spp., *Klebsiella* spp. and *Morganella morganii* were also isolated.

KEYWORDS: Public health; *Vibrio parahaemolyticus*; *Vibrio cholerae*; Oyster; Northeastern Brazil.

INTRODUCTION

Due to their peculiar feeding procedure – filtering the surrounding water, retaining and accumulating microorganisms and other substances – bivalves are commonly associated with the transmission of diarrhea-related microbiological agents²².

Enteropathogenic bacteria may be present in the tissues of bivalves in concentrations higher than in the source water, and since oysters are usually eaten raw they are highly correlated with gastrointestinal disorders⁸.

The concentration of vibrios depends on geographical and hydrographical conditions. In filtering organisms the determining factors are species, location in the water column, and time of permanence in the water¹⁷. Vibrios are part of the natural microbiota of marine and estuarine environments and some may cause infections in humans which were exposed to seafood or sea water. However, organisms commonly employed to indicate contamination give no hint of the presence of these autochthonous bacteria. *Vibrio parahaemolyticus* and *Vibrio cholerae* are pathogenic to humans. They are found in the marine and estuarine environments and may cause gastrointestinal illnesses, either in isolated cases or in the form of outbreaks^{19,24}.

The increasing consumption of mollusks *in natura*, especially oysters, collected from natural nurseries and neither subjected to decontamination

procedures nor adequately packed, is a matter of great concern to public health authorities, mainly in tropical countries.

The objective of this study was to investigate *V. parahaemolyticus* and *V. cholerae* colonizing oysters collected from the estuary of Rio Cocó, a river in Northeastern Brazil, in view of the popularity of these mollusks as a source of raw food.

MATERIAL AND METHODS

Sampling: Twelve batches of the oyster species *Crassostrea rhizophorae* were collected from the Cocó river estuary (Fortaleza, Brazil) at fortnightly intervals between October 2000 and April 2001. The batches consisted of 20-25 pooled specimens each and were transported to the laboratory in isothermal boxes within 2-3 hours after collection. All dirt was removed from the valves by thorough washing and brushing in running water prior to aseptic opening.

Enrichment and isolation: Initially a 1:9 dilution was prepared with 25 g of meat and liquid (depending on size, 20-25 oysters of the same batch) in 225 mL alkaline peptone water (APW) at pH 8.6 with the addition of 3% sodium chloride. The homogenized sample was incubated at 42 °C for 18 h for enrichment and growth. Ten fold dilutions (from 10⁻² to 10⁻⁵) in APW were inoculated onto Petri dishes containing thiosulfate-citrate-bile-saccharose agar (TCBS, Difco). After incubation at 35 °C for 18 h, saccharose-positive and negative colonies (three on

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average) suspected of belonging to the *Vibrio* genus were transferred and inoculated into tubes containing triptone soy agar (TSA, Difco) with further incubation at 35 °C for 24 h.

Identification: Preliminarily, all cultures were submitted to an oxidase test. Strains testing oxidase-positive were identified phenotypically according to the procedures presented in the Laboratory Diagnosis Handbook⁶, including classification into the Heiberg groups for *V. cholerae* and the Kanagawa test for *V. parahaemolyticus*.

Results and discussion: Four out of a total of twelve samplings (with repeats), resulting from four different pools, tested positive for the presence of *V. cholerae* while one was positive for *V. parahaemolyticus* (Table 1).

Table 1

Presence of *V. cholerae* and *V. parahaemolyticus* in twelve oyster samples (*Crassostrea rhizophorae*) and the registered salinities at the natural nursery in the estuary of Rio Cocó (Fortaleza, Brazil)

Samplings	<i>V. cholerae</i>	<i>V. parahaemolyticus</i>	Salinity (‰)
1	–	–	27
2	–	–	27
3	–	–	27
4	–	–	27
5	–	–	27
6	–	–	10
7	–	–	8
8	–	–	8
9	+	–	17
10	+	–	3
11	+	–	3
12	+	+	3

Out of the 57 saccharose-positive strains isolated from plates with TCBS agar, 22 (39%) were confirmed as non-O1, non-O139 *V. cholerae*; two strains with wrinkled surfaces were classified as *V. cholerae*.

XU et al.²⁵ reported greater resistance in estuarine environments for *V. cholerae* than for *E. coli*, an important observation for public health authorities in charge of monitoring areas in which oysters are farmed and collected. The presence of longer-lived vibrios may compromise water quality evaluations based on coliform bacilli standards alone.

The occurrence of strains of *V. cholerae* coincided with the beginning of the local rainy season and with reduced levels of salinity in the source water (Table 1). Other authors have described the relation between salinity and isolation of *V. cholerae* in oysters. In fact, COLWELL & HUQ⁷ observed a higher frequency of *V. cholerae* in areas with salinities in the range 0.2–2.0‰.

V. cholerae is found in aquatic environments either as a free-swimming organism or in association with zooplankton. Strains of non-O1, non-O139 *V. cholerae* are often isolated from seafood or the marine environment, but – though they may be invasive and transmitted to man by way of raw seafood – are not considered as important as serotypes O1 and O139. The medical literature registers cases of non-O1, non-

O139 *V. cholerae* as the causal agent of bacteremia and septicemia in patients with chronic degenerative disorders, along with a considerable number of references to gastroenteric disease²⁰.

According to ALBERT *et al.*¹, cholera outbreaks have been increasingly associated with the consumption of contaminated food products. Among cholera-associated seafoods, bivalves and crustaceans are the most commonly implicated.

Of the 22 strains identified as *V. cholerae*, two strains pertaining to batch #10 had wrinkled surfaces. One of these was classified as belonging to the Heiberg I group. Of the remaining 20 strains classified as non-O1 *Vibrio cholerae*, five (four pertaining to batch #10 and one to batch #9) were found to belong to the Heiberg I group, while fourteen other strains (nine pertaining to batch #11 and five to batch #12) were Heiberg II and one (from batch #12) was Heiberg IV. Our findings match those reported by HOFER & ERNANDEZ¹⁵ working on strains of *V. cholerae* isolated from the outlet of sewage treatment plants in Rio de Janeiro. In their paper, *Vibrio* strains of type II were observed to prevail during the low-temperature season, a finding confirmed by the present study. As in HOFER & ERNANDEZ, our second-last sampling, performed during the month of April, displayed the highest number of non-O1 *V. cholerae* strains of Heiberg type II.

In accordance with the Bacteriological Analytical Manual¹¹, the detection of *V. parahaemolyticus* and *V. cholerae* in oysters requires a dilution in APW to reduce competition with other vibrios. As filtering organisms, oysters ingest water and plankton which may contain different vibrios or other potential competitors of the target bacteria. On the other hand, DePAOLA & HWANG⁹ associated the reduction of pH in the APW enrichment broth with failure to detect *V. cholerae*. Oyster meat contains plenty of glycogen, a substance producing lactic acid during fermentation and thereby reducing the pH during enrichment of the homogenized oyster samples³. RODRIGUES & HOFER²³ found the TCBS agar plating procedure after culture in APW at pH 8.6 and 3% NaCl to be an efficient means of detecting the majority of *Vibrio* spp., especially in oyster samples. They studied the presence of *Vibrio* in oysters and water from Bafa de Sepetiba (Rio de Janeiro) and isolated 576 cultures belonging to this genus. Out of an assemblage of 390 cultures seven species were identified: 65% were non-O1 *V. cholerae* and 14% were *V. alginolyticus*, while *V. parahaemolyticus* accounted for 11%, *V. fluvialis* for 6.7%, *V. harveyi* for 1.5% and *V. vulnificus* for 0.5%.

Plating onto a selective medium such as TCBS should be followed by a simple and reliable detection procedure for the isolation of suspected *Vibrio* colonies. Such conditions may be attained in samples of cooked food, but in highly contaminated foods *in natura* such as oysters vibrios will often be underestimated because of the large number of competing microorganisms. The most common competitors of *V. parahaemolyticus* and *V. cholerae* are non-fermenting gram-negative bacteria, other *Vibrio* subspecies, enterobacteriaceae and, to a lesser degree, *Enterococcus*, *Staphylococcus* and *Micrococcus* spp¹⁰.

In our study *V. parahaemolyticus* was detected in a single sampling and, as expected, was Kanagawa-negative.

The salinity of the water in the estuary ranged from 3 to 27‰ (Table 1). Such oscillation may in part account for the reduced number of isolated

strains. Thus, one would expect to detect a larger number of *Vibrio parahaemolyticus* strains in water with a salinity between 2 and 4%, considered to be ideal for the species¹⁸.

In a study involving seafood, HARA-KUDO *et al.*¹³ observed that colonies of *V. parahaemolyticus* in TCBS medium are difficult to distinguish from other colonies. The acids produced by saccharose-fermenting bacteria become diffused over the agar, changing the color of the medium from green to yellow in the vicinity of the colonies. The *V. parahaemolyticus* colonies near these bacteria may therefore become camouflaged making isolation a difficult task. The use of solid media containing substrates for the enzyme beta-galactosidase has been proposed as a means of minimizing this problem. In the case of mollusks, one must also take into account the organisms' own defense mechanisms such as the presence in the serum of phagocytes capable of eliminating vibrios as well as the presence of antibacterial and antiviral substances in the homogenized oyster samples^{5,12,14,16}.

Through its Resolution 12/01, the Department of Sanitary Vigilance has determined the maximum acceptable level of *Vibrio parahaemolyticus* in raw seafood-based dishes to be 10³ per gram⁴. Though oysters are not mentioned explicitly in this paragraph, they may be considered a raw seafood since they are traditionally consumed *in natura*.

The fact that some of the isolated strains presented sour gas, making the isolation of *Vibrio* more difficult, suggests an association between the gas-producing bacteria and bacteria of this genus. This inference was further supported by the identification, among the strains isolated from the TCBS plates, of *Proteus* spp. and *Morganella morganii*, which are known to produce the gas H₂S²¹. The bacteria belonging to these genera are found naturally in the environment and colonize the intestines of animals and healthy humans².

Furthermore, *Providencia* spp., *Klebsiella* spp., *Proteus* spp. and *Morganella morganii* were identified in 41%, 8.3%, 16.6%, and 8.3% of the oyster samples, respectively.

The presence of non-O1 *Vibrio cholerae* in the oysters analyzed in this study is disquieting, considering that the animals were collected in Fortaleza's largest mangrove swamp and that oysters from this area are marketed and consumed raw by the local population. With an appropriate thermal treatment such food represents a significant risk for enteric disease.

RESUMO

Isolamento de *Vibrio parahaemolyticus* e *Vibrio cholerae* em ostras, *Crassostrea rhizophorae*, coletadas em um criadouro natural no estuário do rio Cocó, Fortaleza, Ceará, Brasil

As ostras são alimentos marinhos frequentemente ingeridos crus ou parcialmente cozidos. Por esta razão, o risco para a saúde dos consumidores desses produtos é muito elevado, principalmente, quando são de regiões tropicais. Foi estudada a presença de *Vibrio cholerae* e *Vibrio parahaemolyticus* em ostras de um estuário na região Nordeste do Brasil. Trezentas ostras foram analisadas, em um período de 8 meses. A salinidade da água, no local de coleta, variou de 3 a 27‰. *V. cholerae* foi o vibrio mais frequentemente detectado (33,3% das amostras). Dos 22 isolados, 20 foram identificados como *V. cholerae* não-O1/não-O139,

duas delas apresentando forma rugosa sendo a maioria das demais pertencente ao tipo fermentativo Heiberg II. *V. parahaemolyticus* foi isolado em apenas umas das coletas. Foram, também, identificadas nas amostras isolados de *Providencia* spp., *Klebsiella* spp., *Proteus* spp. e *Morganella morganii*.

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