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RAW TROPICAL OYSTERS AS VEHICLES FOR MULTIDRUG-RESISTANT *Vibrio parahaemolyticus*

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SUMMARY

The following study aimed to determine the antimicrobial susceptibility profile of *Vibrio parahaemolyticus* strains from fresh and frozen oysters *Crassostrea rhizophorae* sold in Fortaleza-Brazil. An antibiogram was performed on 87 isolates using nine antibiotics: gentamicin (Gen 10 µg), ampicillin (Amp 10 µg), penicillin G (Pen 10U), ciprofloxacin (Cip 5 µg), chloramphenicol (Chl 30 µg), nalidixic acid (Nal 30 µg), tetracycline (Tet 30 µg), vancomycin (Van 30 µg) and erythromycin (Ery 15 µg). All strains were resistant to at least one antibiotic, and 85 (97.7%) were multi-resistant, with predominance of the Van+ Pen+Amp resistance profile (n = 46). Plasmid resistance to Pen, Amp and Ery was detected. Thus, the risk that raw oyster consumption poses to the health of consumers is highlighted, due to the fact that these bivalves may host antibacterial-resistant microorganisms.

KEYWORDS: *Vibrio parahaemolyticus; Crassostrea rhizophorae;* Antimicrobial resistance.

INTRODUCTION

The consumption of raw oysters has been constantly associated with bacterial etiology outbreaks, and *Vibrio parahaemolyticus* has been highlighted as one of the main species responsible for this phenomenon[5]. This species, frequently present in marine and estuarine environments, is part of the indigenous microbiota of aquatic organisms[16,17] and its ability to cause diseases seems to be related to virulence factors, such as the presence of *tdh* and *trh* genes[18].

Oyster-associated outbreaks caused by *V. parahaemolyticus* are well documented[9,15,7,10], and represent a worldwide problem. In the United States, McLAUGHLIN *et al.*[11] reported a large outbreak of gastroenteritis - involving episodes of watery diarrhea - associated with *V. parahaemolyticus* serotype O6:K18.

According to DANIELS & SHAFAIE[3], *V. parahaemolyticus* strains responsible for cases of gastroenteritis are usually sensitive to antibiotics commonly used in the treatment of enteric infections. However, for patients with *V. parahaemolyticus* wound infections and septicemia, the treatment - intravenous antimicrobial agents - is similar to that for patients with *V. vulnificus* infection. Thus, besides virulence, the threat of antimicrobial-resistant vibrios is also worth mentioning[6].

Considering the risk that the consumption of oysters may pose to human health, the following study aimed to determine the antimicrobial susceptibility profile of *Vibrio parahaemolyticus* strains from fresh and frozen oysters *Crassostrea rhyzophorae* sold in Fortaleza-Brazil.

MATERIAL AND METHODS

Strains origin: 87 *V. parahaemolyticus* strains - isolated from soft tissues with the intervalvar liquids of *C. rhizophorae* oysters - were taken from the bacterial collection of the Environmental and Fish Microbiology Laboratory at the Institute of Marine Sciences (LABOMAR-UFC). The study was based on 15 samples of fresh (sold at room temperature) and 15 samples of frozen (sold at -4 °C) oysters obtained from two restaurants in Fortaleza-Brazil in 2010. Each sample consisted of 10 specimens, for a total of 300 specimens examined. For isolation and purification of the strains, 50 g of the intervalvar tissues and fluid was taken from each sample of 10 specimens and added to 450 mL alkaline peptone water (1% NaCl). The homogenate (which corresponded to a 10^{-1} dilution) was used to make serial decimal dilutions from 10^{-2} to 10^{-4}. Thus, 0.2 mL aliquots of each dilution were spread plated on thiosulfate-citrate-bile salt-sucrose agar (TCBS-Difco) and incubated at 35 °C for 18h. Three blue-green colonies for each sample were randomly selected and cultured in tryptone soy agar (TSA-Difco) (1% NaCl).

Biochemical characterization of the strains: All colonies (n = 37 from fresh oysters, and n = 48 from frozen oysters) were submitted to biochemical identification using the key developed by NOGUEROLA & BLANCH[13]. The strains presented the following phenotypic profile: (1) Gram-negative curved rods, (2) oxidase (+) in oxidase strips (Laborclin), (3) sucrose (-) in Basal Media for Carbohydrate containing 0.5% (w/v) of sucrose (35 ºC for five days), (4) indol (+) in Sulfide-Indole-Motility Agar (35 ºC for 48 h), (5) ortho-Nitrophenyl-β-galactoside-ONPG (-) in saline solution with a drop of toluene and buffered solution

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of ONPG 13.3 mM (37 ºC for 24 h), (6) mannitol acid (+) in Basal Media for Carbohydrate containing 0.5% (w/v) of mannitol (35ºC for 5 days), (7) Voges-Proskauer (-) in MRVP broth (35ºC for 96 h), (8) D-glucosamine cs (+) in Basal Media for Carbohydrate containing 0.5% (w/v) of D-glucosamine (35 ºC for five days), (9) growth at 0% (-) and 8% (+) NaCl in Alkaline Peptone Water (35 ºC for 24 h), and (10) arginine dihydrolase (-), lysine decarboxylase (+), ornithine decarboxylase (+) in basal media (0.02 g of bromocresol purple, 5 g of peptone, 3 g of extract yeast, 10 g of sodium chloride and 1 g of glucose in one liter of distilled water, pH 8,5) containing 0.125**%** (w/v) of arginine, lysine and ornithine, respectively, with incubation at 35 ºC for seven days.

Antibiogram: The antimicrobial susceptibility pattern was carried out by disk diffusion method[1], with Muller-Hinton Agar (MH) containing 1% NaCl. Nine antibiotics were tested for each strain: gentamicin (Gen 10 µg), ampicillin (Amp 10 µg), penicillin G (Pen 10U), ciprofloxacin (Cip 5 µg), Chloramphenicol (Chl 30 µg), nalidixic acid (Nal 30 µg), tetracycline (Tet 30 µg), vancomycin (Van 30 µg) and Erythromycin (Ery 15 µg). Zones of inhibition were measured using a digital caliper (Digimess) and each strain behavior was classified as sensitive, intermediate or resistant, according to CLSI[1] recommendations.

Plasmid curing: Strains that showed resistance to at least one antimicrobial underwent plasmid curing in broth Luria Bertani supplemented with acridine orange (SIGMA A-6014) at 0.1 mg mL^{-1} [12]. After the curing procedure, the strains were again subjected to antibiotic susceptibility testing (described above). Thus, the resistance was considered chromosomal when observed after the curing procedure; otherwise it was characterized as plasmid.

RESULTS

From the 87 *V. parahaemolyticus* isolates tested, more than 96.5% were resistant to vancomycin and penicillin, and 74.7% showed resistance to ampicillin. Resistance to erythromycin was observed in 74.7% of the isolates. In contrast, all strains were sensitive to chloramphenicol, and more than 95.4% were sensitive to gentamicin, ciprofloxacin, tetracycline, nalidixic acid and gentamicin.

Isolates from fresh oysters showed resistance rates to the following antibiotics: Van (n = 38; 97.4%), Pen (n = 36; 92.3%), Amp (n = 19; 48.6%), Ery (n = 6, 15.4%). Resistance rates for the frozen oysters isolates were: Van (n = 48; 100%), Pen (n = 48; 100%), Amp (n = 46; 95.8%), Ery (n = 16, 33.3%).

A high rate of multiple resistance was observed in strains isolated from fresh (94.9%) and frozen (100%) oysters. The most recurrent multi-resistant profile in both fresh and frozen sources was Van+Pen+Amp (Table 1). *V. parahaemolyticus* strains isolated from both types presented a MAR oscillating from 0.2 to 0.4.

Plasmid curing indicated a chromosomal resistance profile in 100% of Van-resistant strains. Isolates with a plasmid resistance profile were more frequent in strains extracted from fresh oysters (Table 2).

DISCUSSION

The occurrence of antimicrobial-resistant vibrios in oysters poses a threat to their consumers. HAN *et al.*[6] investigated the susceptibility of vibrios isolated from oysters and reported a high rate of penicillin-resistant *V. parahaemolyticus*. This finding is similar to the results obtained in the present study, since the resistance to Amp was found in isolates from both types of oysters (Table 1).

DARAMOLA *et al.*[2] determined the antimicrobial resistance profiles of *V. parahaemolyticus* strains isolated from water samples, sediments and mussels from the Humber River estuary in the U.K. - an area where shellfish harvest and mussel culture occurs. The authors reported that all isolates were sensitive to chloramphenicol, presented a low level resistance to vancomycin (3.9%), ampicillin (1.3%), and high rates (73.7%) of resistance to gentamicin. In the present research, a large number of Van and Amp-resistant strains was detected; in contrast, sensitivity to Gen and Chlo were observed. Comparing the results to those of DARAMOLA *et al.*[2], it is possible to suggest that the mechanisms of antimicrobial resistance in the same bacterial species undergo a differentiation process according to the region.

Table 1
Multiple antimicrobial resistance in *Vibrio parahaemolyticus* strains isolated from samples of fresh and frozen oysters

Profile	Fresh	Frozen	MAR
Van+Pen+Amp+Ery	2	16	0.4
Van+Pen+Amp	16	30	0.3
Van+Pen+Ery	4	1	0.3
Van+Pen	13	1	0.2
Van+Amp	1	-	0.2
Van+Ery	1	-	0.2
Total	37 (94.9%)	48 (100%)	

*VAN: vancomycin 30 µg; PEN: penicillin 10U; AMP: ampicillin 10 µg; ERY: erythromycin 15 µg; MAR: multiple antibiotic resistance.

Table 2
Profile of chromosomal and plasmid resistance to antibiotics in *Vibrio parahaemolyticus* strains isolated from samples of fresh and frozen oysters

	Antibiotics			
	Van	Pen	Amp	Ery
	Fresh oysters			
Number of resistant strains	38	36	19	6
Chromosomal resistance	38	33	9	-
Plasmid resistance	-	3	10	6
	Frozen oysters			
Number of resistant strains	48	48	46	16
Chromosomal resistance	48	47	45	11
Plasmid resistance	-	1	1	5

*VAN: vancomycin 30 µg; PEN: penicillin 10U; AMP: ampicillin 10 µg; ERY: erythromycin 15 µg.

OTTAVIANI *et al.*[14], in a study on the susceptibility of *Vibrio* (including *V. parahaemolyticus*) isolated from fresh and frozen sold seafood (shellfish, shrimp, squid and cod), found *Vibrio* strains without resistance mechanisms to ciprofloxacin and nalidixic acid, as well as isolates with multiple resistance profiles to different combinations of antimicrobials, including ampicillin and penicillin, as in the present study. The authors suggest that for the plasmid role in *Vibrio,* multiple resistance to antibiotics must be investigated, even though most of the studies until that moment indicated that this characteristic is inherent to that genus.

The high rate of multiple resistance observed in this study raises questions as to the effectiveness of antimicrobial agents commonly used in the treatment of gastroenteritis caused by *Vibrio*. It is possible to consider that Chl, Nal, Cip, Tet and Gen should be selected to treat diseases caused by *V. parahaemolyticus*, as has been reported in the literature. KHAN *et al.*[8] determined the susceptibility of 27 strains of the same species isolated from cultured shrimp in Khulna (Bangladesh), and suggested that the tetracycline and gentamicin were the best choice for controlling diseases caused by enteric bacteria, including *V. parahaemolyticus.* Thus, it is necessary to establish therapy with appropriate antimicrobials for a more effective treatment of infections caused by *V. parahaemolyticus, V. vulnificus,* and others[19]. The authors above suggest that the antimicrobial ciprofloxacin is effective in these cases, in accordance with the findings of this study.

In accordance with the findings in this study, ZULKIFLI *et al.*[20] investigated the resistance of *V. parahaemolyticus* strains isolated from cockles in Indonesia, and reported rates of resistance to penicillin and ampicillin higher than 50%, as well as a 100% sensitivity to gentamicin.

LOZANO-LEÓN *et al.*[10] investigated an outbreak of gastroenteritis involving 64 people in Spain and revealed the presence of *V. parahaemolyticus* in fecal samples of all patients involved. Symptoms appeared within 12 to 24 hours after the consumption of raw oysters at a street market in the city of Vigo (Galicia, Spain). The study also revealed that 100% of the isolates were resistant to ampicillin, erythromycin and vancomycin, antibiotics commonly used in the treatment of gastroenteritis. The strains used in this study showed a similar resistance profile to those responsible for the aforementioned outbreak, a fact which confirms that the consumption of raw oysters poses a potential risk to human health.

The results of the susceptibility test after the plasmid curing suggest that the antimicrobial resistant profile from *V. parahaemolyticus* strains isolated from oysters are linked to chromosomal genes, in accordance with the literature. DEVI *et al.*[4], in a study on the antimicrobial susceptibility in strains from the same species isolated from shrimps cultivated in the southeast of India, also found low rates of plasmid resistance, reporting that there were no modifications in the resistance to chloramphenicol, oxytetracycline and trimethoprim before and after plasmid curing.

Considering the high rates of resistance, especially multiple resistance, the findings of this study support the assertion that oysters may serve as hosts to vibrios which are resistant to drugs used in the gastroenteritis treatment in human beings. Thus, the ingestion of those bivalve mollusks without prior cooking is strongly unadvisable.

RESUMO

Ostras tropicais cruas como fonte de *Vibrio parahaemolyticus* multirresistentes

O presente estudo objetivou determinar o perfil de suscetibilidade a antimicrobianos de cepas de *Vibrio parahaemolyticus* oriundas de ostras "in natura" e congeladas comercializadas em Fortaleza-Brasil. Oitenta e sete (87) cepas foram submetidas ao antibiograma com emprego de nove antibióticos: gentamicina (Gen 10 µg), ampicilina (Amp 10 µg), penicilina G (Pen 10U), ciprofloxacin (Cip 5 µg), cloranfenicol (Clo 30 µg), ácido nalidíxico (Nal 30 µg), tetraciclina (Tet 30 µg), vancomicina (Van 30 µg) e eritromicina (Eri 15 µg). Todas as cepas mostram-se resistentes a pelo menos um antibiótico, e 85 (97,7%) apresentaram multirresistência, com predomínio do perfil Van+ Pen+Amp (n = 46). Foi detectada resistência plasmidial a Pen, Amp e Eri. Dessa forma, o risco que o consumo de ostras cruas representa para a saúde dos consumidores merece ser destacado, uma vez que esses bivalves podem ser veículos de transmissão de micro organismos multirresistentes a fármacos antibacterianos.

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