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Aeromonas spp. ISOLATED FROM OYSTERS (*Crassostrea rhizophorea*) FROM A NATURAL OYSTER BED, CEARÁ, BRAZIL

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SUMMARY

Between April and October 2002, thirty fortnightly collections of oysters (*Crassostrea rhizophorea*) from a natural oyster bed at the Cocó River estuary in the Sabiaguaba region (Fortaleza, Ceará, Brazil) were carried out, aiming to isolate *Aeromonas* spp. strains. Oyster samples were submitted to the direct plating (DP) and the presence/absence (P/A) methods. *Aeromonas* were identified in 15 (50%) samples analyzed by the DP method and in 13 (43%) analyzed by the P/A method. *A. caviae*, *A. eucrenophila*, *A. media*, *A. sobria*, *A. trota*, *A. veronii* bv. *sobria*, *A. veronii* bv. *veronii* and *Aeromonas* sp. were isolated. The predominant species was *A. veronii* (both biovars), which was identified in 13 (43%) samples, followed by *A. media* in 11 (37%) and *A. caviae* in seven (23%). From the 59 strains identified, 28 (48%) presented resistance to at least one of the eight antibiotics tested.

KEYWORDS: *Aeromonas*; Oysters; Antibiotics.

INTRODUCTION

Shellfish consumption has been showing a sustainable growth in recent years, mainly in developing countries. The consumption of marine products is worthy due to its nutritious value and low cholesterol levels. However, marine products may be a vehicle for most known pathogenic bacteria[8].

Bivalve mollusks, due to their filtering characteristic, are used as bioindicators. Oysters' meat microbiota is directly related to the environment from which they come from, although in higher concentrations, due to their bioaccumulation mechanism.

The *Aeromonas* genus is primary autochthonous from the aquatic environment[25]. Its taxonomy has been constantly reevaluated based initially on its phenotype characteristics, including the metabolic and antigenic ones, this last being the one used solely as an epidemiological tool. The use of those methods capable of performing a genotype characterization indicated spatial genetic complexity among mesophilic species being characterized in phenospecies and genospecies, although its toxicity is not already well defined[11].

Some species of *Aeromonas* are responsible for a significant number of intestinal and extra-intestinal infections in humans and also in animals (fishes)[9,26]. Even though *Aeromonas* are isolated from patients with diarrhea its etiological role in gastroenteritis is still not clear[1]. It is believed that the difficulty in assigning an unequivocal role to the causation of diarrhea is because aeromonads are heterogeneous and because maybe only some subgroups are pathogenic[15]. Despite the identification of a variety of virulence factors in *Aeromonas* spp., including enterotoxins, cytotoxins, hemolysins, aerolysins, proteases, hemagglutinins, and the ability to adhere to and invade tissue culture cell lines[26], the linkage of these factors to the diarrheagenic ability of the isolates has not been clearly demonstrated. Besides, a high prevalence of mixed infections of *Aeromonas* sp. with other pathogens has been observed; it is also possible that multiple pathogens act synergistically to produce diarrhea[1].

Aeromonads are currently divided into 14 DNA HGs, genomospecies, or genospecies and 14 phenospecies. The HGs are identified by a variety of methods, including DNA-DNA hybridization, PCR amplification, rDNA restriction, restriction fragment length polymorphism analysis, and pulsed-field gel electrophoresis[9]. However, these methods are relatively complex and not amenable to use in many laboratories. Fortunately, it has been reported that 98% of aeromonads can be accurately identified to the genospecies level by a battery of biochemical tests[1,9].

Among the 14 *Aeromonas* species described, only five (*A. hydrophila, A. caviae, A. jandaei, A. schubertii* and *A. veronii* [both biovars]) are currently recognized as human pathogens. *A. hydrophila, A. caviae* and *A. veronii* bv. *sobria* are regularly isolated from laboratory

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materials and related to extra-intestinal and systemic infections, including septicemia, wound infections, meningitis, peritonitis and hepatobiliary disease[9].

Mesophilic, motile *Aeromonas* are found in freshwater, brackish water and estuaries[25]. They are also common contaminants of fresh foods, including fish and other seafoods[6]. These bacteria are plentiful in tropical regions, mainly on summer months, and have been described as the probable cause of bacterial intestinal bacterial enteritis which affects mostly children and travelers who present a moderate form of diarrhea and, sometimes, a severe form similar to cholera[17].

There are a few published cases in which *Aeromonas* spp. have been strongly suspected as a cause of food-borne gastroenteritis. Among suspected foods, prefrozen or inadequately cooked seafood and oysters predominate[12].

Studying oyster samples, TSAI & CHEN[27] isolated *A. hydrophila* strains in 50% of them, indicating the high potential of infection which could come from eating raw oysters.

A. caviae and *A. hydrophila* of particular relevance have been frequently mentioned in the last ten years as being related to intestinal infections in men, relatively to the consumption of a variety of foods, although all species of this genus are presently known as emergent pathogens[22]. Such designation involves also the increase of its incidence, particularly on skin and soft tissues, especially after traumas exposed to aquatic or terrestrial environment. It is a very harsh condition to immunocompromised patients, where it is usually more severe and eventually fatal. These aspects make it difficult to recognize the infecting dose pointed out as being 10^5 to 10^8, even though studies in some countries have effectively determined it at around 10^8 cells/g in food related to foodborne diseases[14].

The present study aimed to quantify and identify *Aeromonas* spp. from oysters (*Crassostrea rhizophorea*) collected from a natural oyster bed at the Cocó River estuary in the Sabiaguaba region (Fortaleza, Ceará, Brazil); as well as to verify the susceptibility of the isolated strains to stand some antimicrobial agents.

MATERIAL AND METHODS

Sample collection: From April to October/2002 thirty oyster (*Crassostrea rhizophorea*) collections were made fortnightly, in a natural oyster bed, at the Cocó river estuary, region of Sabiaguaba (Fortaleza, Ceará, Brazil), during low tide. The site chosen is an area of oysters (*Crassostrea rhizophorea*) exploitation, located 200 m from the river's mouth between latitudes 03°46'S and 03°47'S. In each collection 35 to 40 units were examined, totalizing about 1200 units.

Samples were transported in refrigerated isothermal boxes to the microbiology laboratory at the *Instituto de Ciências do Mar/LABOMAR/UFC* and analyzed immediately.

Bacterial isolation: The oysters were washed under running water and opened aseptically. Twenty five grams of the intervalvar liquid and soft meat were homogenized in a sterile blender with 225 mL of alkaline peptone water (APW), other dilutions following until 10^{-4}.

The strains were isolated by the direct plating (DP) and the presence/absence methods (P/A) on *Pseudomonas Aeromonas* selective agar (GSP, Merck), with 20 µg/mL of ampicillin (GSPA) added.

For *Aeromonas* enumeration the DP method was used by spreading 0.1 mL of each dilution onto the surface of two GSPA agar plates and incubating at 28 ºC for 24 h. After 24 h presumptive colonies (yellow colonies of 2-3 mm, surrounded by a yellow zone) were counted.

For A*eromonas* P/A test the trypticase soy broth (TSB, Difco) added with 20 µg/mL of ampicillin (TSBA) was used. Aliquots of 10 mL from the initial dilution (10^{-1}) were inoculated, in duplicate, in 10 mL of TSBA and incubated at 28 ºC for 24 h. After incubation, portions from each tube were streaked onto the surface of two GSPA agar plates and re-incubated.

Bacterial identification: From each plate typical colonies (2 to 5) were transferred to trypticase soy agar (*Difco*) for biochemical identification tests according to PALUMBO *et al*.[21]. The positive control was *Aeromonas* ATCC 7966.

Antimicrobial susceptibility test: Susceptibility to antibiotics was tested using the agar disk diffusion method advocated by the National Committee for Clinical Laboratory Standards[18]. Antimicrobial disks (ceftriaxone 30 µg; cephalothin 30 µg; chloramphenicol 30 µg; ciprofloxacin 5 µg; nalidixic acid 30 µg; nitrofurantoin 300 µg; sulfamethoxazole-trimethoprim 23.75/1.25 µg and tetracycline 30 µg) were obtained from Oxoid.

RESULTS AND DISCUSSION

From thirty collections of oysters, 20 (67%) presented positive results for *Aeromonas*. Its presence in the aquatic environment where mollusks may be recognized as indicators of fecal contamination present significant correlation[2]; ORMEM & OSTENOVIK[20] referred to an increase in human infections risk levels through direct exposure of wounds to the contaminated environment or the consumption of food, particularly those consumed raw. Meanwhile, cross contamination and asymptomatic food handlers are also relevant sources in such products as milk and milk products, poultry, pork, vegetables and eggs[16,19].

In general *Aeromonas* spp. are common contaminants of fish and seafood, since they are ubiquitous in the water environment[6]. It is important to point out that the stream that drains into the estuary mentioned in this study bathes several shantytowns which have no basic sewage systems, thus increasing environmental contamination.

In Table 1 results from the oysters analysis are presented with species identified and the number of strains isolated from each method.

With direct plating (DP) method 15 (50%) collections were positive for *Aeromonas* and by P/A method 13 (43%) collections were found. However, considering analysis from both methods, total positive results increase to 20 (67%) collections. These results are similar to those found by RALL *et al*.[23] who isolated 24 (48%) samples of *Aeromonas* from commercial fish in São Paulo, Brazil with direct plating (DP) method and 21 (42%) with P/A method.

Using both methods HÄNNINEN *et al*.[6] isolated *Aeromonas* from fish samples (93%), fish eggs (100%) and shrimp (16%) in Helsinki, Finland.

In 30 collections carried out eight species were identified, most frequent ones being *A. veronii* bv. *sobria* and *A. media* (11 collections), followed by *A. caviae* (7), *A. trota* (3), *A. sobria* and *Aeromonas* sp. (2) and *A. eucrenophila* (1) (Table 1).

Even though no virulence tests were performed it is known that *A. veronii* bv. *sobria* (HG8) and *A. caviae* (HG4) are associated with human diarrhea[6]. According to CHOPRA & HOUSTON[3] *A. caviae* and *A. veronii* have been frequently isolated from human infections and their capacity to produce a variety of biologically active extra cellular products, including hemolysins, cytotoxins, enterotoxins and endotoxins have been demonstrated. GRANUM *et al*.[5] analyzed nine *A. caviae* strains from food and water in Norway and observed cytotoxins production in four of them. ALBERT *et al*.[1] analyzed 115 samples from children with diarrhea and isolated *A. caviae* and *A. veronii* bv. *sobria* in 33.9% and 18.3% of them respectively.

Bacteremia from *Aeromonas* has been more frequent in male adults with classic signs of septicemia (fever in 90% or more patients and shivers in 70% of them) *A. hydrophila* and *A. veronii* (both biovars) being responsible for more than 90% of the reported episodes[10]. Although it has not been implicated in any kind of infection, *Aeromonas media* is frequently isolated from fecal samples[9].

A. trota has been mentioned in the literature as the responsible for a diharrea episode in a 3-year old child[24], while GRANUM *et al*.[5] found cytotoxin production in three strains of this same species isolated from crab, water and powdered eggs.

Aeromonas sobria and *A. caviae* may cause enteritis in anyone or septicemia in immunocompromised persons[4]. The first one is commonly isolated from recreational lakes and rivers, while *A. caviae* prevails in marine water and is also found in sewage contaminated water[25].

Sabiguaba's mangrove is known to bear contamination both from the river that bathes it as from the presence of countless beach shacks near its margins with meager hygiene conditions.

Table 1
Aeromonas species isolated from mangrove oysters (*Crassostrea rhizophorea*), by presence/absence (P/A) and direct plating (CFU/g) methods

Collect	P/A	*Species (No. of strains)	CFU/g	*Species (No. of strains)
1º	A	-	$< 10^2$	-
2º	A	-	1.2×10^3	Avs (1)
3º	A	-	$< 10^2$	-
4º	A	-	3.1×10^4	Avs (1)
5º	A	-	$< 10^2$	-
6º	P	Ac (1), Am (1), Avv (1)	1.2×10^3	Ac (1)
7º	A	-	4.0×10^4	Am (1)
8º	A	-	1.5×10^3	Avs (1)
9º	P	Am (1), At (3)	$< 10^2$	-
10º	P	Am (1), Avv (1)	1.4×10^4	Am (1)
11º	A	-	$< 10^2$	-
12º	P	Ac (1), Am (1), Avs (1)	2.8×10^3	Ac (1)
13º	P	Am (2)	6.9×10^3	Ac (2), Am (1), Avs (1)
14º	A	-	9.0×10^3	Ac (2)
15º	P	At (1)	9.0×10^2	Ac (1)
16º	A	-	7.3×10^3	Asp (2), Avs (1)
17º	P	Asp (1), At (1), Avs (4)	1.4×10^4	Asp (2), As (1), Avs (1)
18º	P	Am (1)	2.0×10^3	Ac (1), Ae (1), Am (2), As (1), Avs (1)
19º	A	-	$< 10^2$	-
20º	P	Ac (1), Am (2)	$< 10^2$	-
21º	A	-	$< 10^2$	-
22º	A	-	$< 10^2$	-
23º	P	Am (1)	$< 10^2$	-
24º	A	-	$< 10^2$	-
25º	P	Am (1)	$< 10^2$	-
26º	A	-	9.4×10^3	Avs (1)
27º	A	-	$< 10^2$	-
28º	A	-	$< 10^2$	-
29º	P	Am (1)	1.5×10^3	Avs (1)
30º	P	Avs (2)	$< 10^2$	-

*Ac (*Aeromonas caviae*), Ae (*A. eucrenophila*), Am (*A. media*), As (*A. sobria*), Asp (*Aeromonas* sp.), At (*A. trota*), Avs (*A. veronii* bv. *sobria*) and Avv (*A. veronii* bv. *veronii*)

Table 2
Antibiotics susceptibility of *Aeromonas* strains isolated from mangrove oysters (*Crassostrea rhizophorea*)

Antibiotics* / Species	No. (%) susceptible to TE	F	KF	CIP	CRO	SXT	C	NA
Aeromonas sp. (n = 5)	2(40)	4(80)	4(80)	5(100)	4(80)	1(20)	5(100)	5(100)
A. caviae (n = 11)	9(82)	11(100)	10(91)	11(100)	9(82)	6(55)	11(100)	11(100)
A. media (n = 17)	15(88)	17(100)	16(94)	17(100)	17(100)	14(82)	17(100)	17(100)
A. sobria (n = 2)	2(100)	2(100)	2(100)	2(100)	2(100)	0(0)	2(100)	2(100)
A. veronii bv. sobria (n = 16)	16(100)	16(100)	14(88)	16(100)	16(100)	5(31)	16(100)	14(88)
A. veronii bv. *veronii* (n = 2)	2(100)	2(100)	2(100)	2(100)	2(100)	1(50)	2(100)	2(100)
A. trota (n = 5)	5(100)	5(100)	5(100)	5(100)	5(100)	4(80)	5(100)	5(100)
A. eucrenophila (n = 1)	1(100)	1(100)	1(100)	1(100)	0(0)	1(100)	1(100)	1(100)
Total (n = 59)	52(88)	58(98)	54(92)	59(100)	55(93)	32 (54)	59(100)	57(97)

*Tetracycline (TE), nitrofurantoin (F), cephalothin (KF), ciprofloxacin (CIP), ceftriaxone (CRO), sulfamethoxazole-trimethoprim (STX), chloramphenicol (C) and nalidixic acid (NA). n = number of strains tested

In Table 2 results of the susceptibility tests of *Aeromonas* to eight antimicrobial agents are shown.

From the 59 strains tested, 28 (48%) showed resistance to at least one of the antimicrobial agents. Increasing resistance of microbes to medication has been heightening worries of sanitary authorities.

According to literature most *Aeromonas* species are susceptible to tetracyclines, trimethoprim-sulfamethoxazole, third-generation cephalosporins and the quinolones[9], even though a 1996 study from Taiwan found increasing resistance[13].

All *Aeromonas* strains were sensitive to ciprofloxacin and chloramphenicol. The majority of them were sensitive to nalidixic acid (97%), nitrofurantoin (98%), ceftriaxone (93%) and cephalothin (92%).

The susceptibility profile facing tetracycline varied. All *A. sobria*, *A. veronii* (both biovars), *A. trota* and *A. eucrenophila* strains were sensitive, while *A. media*, *A. caviae* and *Aeromonas* sp. showed 88%, 82% and 40% susceptibility, respectively. Similar susceptibility results were found by RALL *et al*.[23] for *A. sobria* (100%) and *A. caviae* (93%), but different results were found for *Aeromonas* sp. (93%). Meanwhile KO *et al*.[13] found 59% suceptible strains to tetracycline for *A. caviae* and 42% for *A. sobria*. HEDGES *et al*.[7] suggested that low resistance could be due to a plasmid.

Strains were resistant to Sulfamethoxazole-trimethoprim (46%).

The results obtained in this study show the food examined may represent an increase in human infections risk levels, mainly due to the habit of raw oyster consumption. Although no *A. hydrophila*, has been isolated, the presence of *A. caviae* and *A. veronii* (both biovars) should be pointed out, as these species have been involved in several gastroenteritis cases[6,23]. Available data indicate that further epidemiological studies together with new taxonomic data on genospecies and on aeromonad pathogenicity are needed to elucidate the public health significance of these pathogens in food and drinking water.

RESUMO

Aeromonas spp. isoladas de ostras (*Crassostrea rhizophorea*) coletadas em um criadouro natural, Ceará, Brazil

Foram realizadas 30 coletas quinzenais, entre abril e outubro de 2002, de ostras (*Crassostrea rhizophorea*) de um criadouro natural, no estuário do rio Cocó (Fortaleza/Ceará/Brasil), objetivando-se isolar cepas de *Aeromonas* spp. As amostras de ostras foram submetidas aos métodos de plaqueamento direto (PD) e presença/ausência (P/A). Foram identificadas *Aeromonas* em 15 (50%) amostras analisadas pelo método PD e em 13 (43%) pelo método P/A. Foram isoladas: *A. caviae*, *A. eucrenophila*, *A. media*, *A. sobria*, *A. trota*, *A. veronii* bv. *sobria*, *A. veronii* bv. *veronii* e *Aeromonas* sp. A espécie predominate foi *A. veronii* (ambos biovars), identificada em 13 (43%) amostras, seguida de *A. media* em 11 (37%) e *A. caviae* em 7 (23%). Das 59 cepas identificadas, 28 (48%) apresentaram resistência a pelo menos um, dos oitos antibióticos testados.

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