

CHARACTERIZATION OF *Aeromonas* SPECIES ISOLATED FROM AN ESTUARINE ENVIRONMENT

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ABSTRACT

Thirty water samples were collected, at two week intervals, from the estuary of the River Cocó. The aim was to characterize the presence, distribution and types of *Aeromonas* spp, in the estuary of the River Cocó, Ceara, Brazil (03°46'28.83''S e 38°26'36.52''S). *Aeromonas* were identified in 19 (63%) samples analyzed by plating and CFU counts. Presence/absence tests were positive for 11 (37%) of the samples resulting in the detection of *Aeromonas* in a total of 23 (77%) of samples. CFU counts varied from < 10 to 1.4×10^4 CFU mL⁻¹. From the isolated strains seven species of *Aeromonas* were identified: *A. caviae* (29/69), *A. veronii* bv. *sobria* (13/69), *A. veronii* bv. *veronii* (8/69), *A. trota* (6/69), *A. media* (5/69), *A. sobria* (4/69) and *A. hydrophila* and *Aeromonas* sp. (2/69). Of the 38 strains tested, 23 (60%) showed resistance to at least one of the eight antimicrobials. Multiple resistance to antibiotics was observed in *A. caviae*, *A. media*, *A. sóbria* and *A. veronii* bv. *sobria*. *Aeromonas caviae* showed the highest multiple resistance, being resistant to four antibiotics. The presence of those microorganisms may contribute to the occurrence of gastroenteritis, mainly in children, since they are considered opportunists.

Key words: *Aeromonas*, Estuary, Antibiotics

INTRODUCTION

Aquatic environments are used worldwide for water supply, energy production, irrigation, navigation, aquaculture, and primary and secondary contact activities. Nevertheless, in the last decades these environments have been threatened by human misuse with detrimental consequences for mankind as a whole (25). Today, many countries are suffering from serious

environmental impacts caused for example by domestic and industrial sewage discharged into rivers (37). Therefore, environmental pollution has become a public issue around the world.

According to the IBGE census (2000), the city of Fortaleza has an annual population growth of 2.8% due to immigration from rural areas or from other Brazilian states. These immigrants contribute to the settlements in river shore

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areas, thus increasing waterborne disease outbreaks (5). The River Cocó, like most of Brazilian rivers, has been affected by human activities which altered its characteristics and development. Hence, it is important to develop water quality monitoring programs in the affected areas to collect information which may help institutions make the right decisions.

Members of the genus *Aeromonas* currently belong to the family *Aeromonadaceae*, and are characterized as short, Gram negative, oxidase-positive, rod-shaped bacteria, which metabolise glucose by both the respiratory and fermentative pathways and show resistance to the vibriostatic agent O/129. It can be divided into two groups: the first includes the psychrotrophic *Aeromonas*, represented by *Aeromonas salmonicida* and the second, by the mesophyllic *Aeromonas* (9).

In Brazil, several studies appoint the occurrence of these pathogens isolated from aquatic ecosystem, food origin (fish, oysters, mussels and crabs) and coetaneous lesions in humans caused by fisheries (8, 31). These studies are important to Public Health due to the association of some of these microorganisms on foodborne disease and extraintestinal infections (14, 30). Previous studies have demonstrated that the genus *Aeromonas* is the second cause of gastroenteritis in children and the fifth in adult patients (15).

The genus *Aeromonas* is composed of a large number of different taxa. Currently this group is included in the family *Aeromonadaceae*, at least 17 genome-species are recognized in the genus (23). However, *Aeromonas hydrophila*, *A. veronii*, *A. caviae*, *A. jandaei* and *A. schubertii* are recognized as human and animal (fish) pathogens (16). Despite the high incidence of infections of *Aeromonas* combined with other pathogens isolated from patients with diarrhea, the virulence factors that cause gastroenteritis are still not totally defined (1). Among the species above *A. hydrophila* has attracted attention due to its frequent association with infections in humans (22). Infections are principally found in patients with a history of accidents, where there has been exposure of wounds to aquatic

environments, in immunodeficient individuals and commonly are associated with meningitis, endocarditis, peritonitis, hemolytic uremic syndrome or septicemia (24).

The increasing bacterial resistance to antibiotics has become a public health problem due to the fact that bacteria can be found in different niches. Among those niches, the aquatic environment is considered the most efficient for the selection of resistant populations, as well as for the exchange of resistance genes by means of mobile genetic elements (38), such as plasmids and transposons (33) which encode resistance to antibiotics. Evangelista-Barreto (8) analysed isolated oysters from the Coco River and observed antibiotic resistance in 48% of the aeromonas studied.

The Food and Agriculture Organization/World Organization Health (FAO/WHO) commission recommends that to prevent waterborne disease in developing countries, aquatic environments directly impacting human populations should be characterized physically, chemically and microbiologically. In light of this recommendation, the present study aimed to quantify and identify *Aeromonas* spp in water samples collected from the Cocó River estuary in the Sabiaguaba region (Fortaleza, Ceará, Brazil), and to verify the isolated strains susceptibility to antibiotics.

MATERIALS AND METHODS

Sample collection

Thirty water samples were collected on a weekly basis from april to october from one point in the Cocó river estuary (Brazil), located 0.2 Km from the river's mouth (03°46'28.83''S and 38°26'36.52''W).

Water samples were collected at margins and in the middle of the river, about 30 cm depth in 1000 mL sterilized bottles. Samples were transported in refrigerated isothermal boxes to the microbiology laboratory at the Instituto de Ciências do Mar/LABOMAR/UFC and analyzed immediately. Physico-chemical parameters such as pH, salinity and water temperature were determined using standard methods (2).

Bacterial Isolation

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⁻¹ of ampicillin (GSPA) added.

For *Aeromonas* enumeration the DP method was used by spreading 0.1 mL of each dilution (10⁻¹ - 10⁻³) onto the surface of two GSPA agar plates and incubating at 28°C for 24 h. After 24 h presumptive colonies (yellow colonies of 2-3 mm, surrounded by a yellow-zone) were counted. For *Aeromonas* P/A test the trypticase soy broth (TSB, Difco) added with 20 µg mL⁻¹ 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°C for 24 h. After incubation, portions from each tube were streaked onto the surface of two GSPA agar plates and re-incubated. The P/A test was carried out in duplicate.

Aeromonas isolates were counted using a Phoenix mod. EC 550A counter from plates with between 25-250 colonies. *Aeromonas* spp were initially identified based on being amylase positive, yellow in colour and showing a clear halo around each colony.

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.* (28). The positive control was *Aeromonas* ATCC 7966.

Antimicrobial susceptibility test

The antimicrobial susceptibilities of *Aeromonas* isolates were tested *in vitro* according to standard procedures CLSI (4) with the following antibiotics: ceftriaxone 30µg; cephalothin 30µg; chloramphenicol 30µg; ciprofloxacin 5µg; nalidixic acid 30µg; nitrofurantoin 300µg; sulfamethoxazole-trimethoprim 23.75/1.25µg and tetracycline 30µg.

RESULTS AND DISCUSSION

Of the 30 water samples, 23 (77%) contained culturable

Aeromonas spp; this observation is similar to Dumonter *et al.* (6) who found *Aeromonas* spp in 62% of water samples and marine sediments from along 900 km of the Italian coast. Fuzihara *et al.* (9) analysed treated and untreated water samples collected in the interior of São Paulo, and reported the presence of aeromonas in 6.3% and 55.3% of the samples, respectively.

CFU counts for *Aeromonas* spp varied from < 10 to 1.4 x 10⁴ CFU mL⁻¹ (Table 1). We note that a CFU's of 10 mL⁻¹ is very low and is likely to reflect experimental technique for a non enriched medium using 0.1 mL sample is very close to the minimal detection limits for *Aeromonas* spp from this environment. TSBA broth enriches for *Aeromonas* spp and was used in the presence absence tests combined with 10 mL samples, given that samples were enriched, they were not counted. Maalej *et al.* (21) studying the seasonal dynamics of *Aeromonas* in treated urban effluent and in surface marine waters along the coast of Sfax (Mediterranean Sea, Tunisia), found counts varying from 1.48 x 10⁵ to 2.2 x 10⁸ CFU 100 mL⁻¹ for the effluent and 7.9 x 10³ CFU 100 mL⁻¹ for the marine waters.

While there is no specific legislation which stipulates limits concerning the numbers of these microorganisms, we must not forget that their presence in this area of the estuary is of social importance. These waters are used for leisure activities and they are also used for oyster fishing, which is normally eaten raw. Generally, *Aeromonas* spp are the most commonly found contaminants in fish and marine products, which to an extent is explained by their ubiquitous nature in aquatic environments Hänninen *et al.* (10). In rivers, they form part of the normal microbiota being able to multiply under normal environmental conditions.

The microbiological properties of waterways are altered depending on source, type and quantity of pollutant entering them. That noted, the presence of *Aeromonas* spp can not always be directly related to fecal pollution Dumonter *et al.* (6). In this study, it is important to highlight that upstream of the water sample site the river flows through several shanty-towns without any basic sanitation system, thus increasing environmental contamination.

Table 1. Quantification of *Aeromonas* species from the Cocó River estuary (Fortaleza, Brazil) by presence/absence (P/A) and direct plating (CFU mL⁻¹) methods

Collect	P/A	*Species (n° of strains)	CFU/mL	*Species (n° of strains)
1°	A	-	1.4 x 10 ⁴	As, Avs
2°	A	-	1.2 x 10 ⁴	Avs, Ac
3°	A	-	1.7 x 10 ³	Ac
4°	P	Avs, Ac	< 10	Ah, Avs
5°	A	-	< 10	-
6°	P	Avv	3.2 x 10 ³	Avs
7°	A	-	2.9 x 10 ³	As
8°	A	-	< 10	-
9°	P	Ac, Avs	1,0 x 10 ³	Ac
10°	A	-	5.0 x 10 ²	Avs
11°	A	-	2.1 x 10 ³	Ac
12°	A	-	3.3 x 10 ²	Am, Ac
13°	P	Ac, At	3.5 x 10 ²	Ac, Avs
14°	P	Asp, At, Ac	< 10	-
15°	A	-	1,6 x 10 ²	Avv
16°	A	-	3.0 x 10 ²	Ac, At
17°	P	Ac	1.7 x 10 ²	Avv, Ac
18°	P	Ac, At	< 10	-
19°	A	-	2.1 x 10 ³	Ac
20°	P	Avv	< 10	-
21°	A	-	< 10	-
22°	P	Ah	< 10	-
23°	P	Avs	< 10	Asp
24°	A	-	< 10	-
25°	A	-	< 10	-
26°	A	-	9.9 x 10 ²	Ac, Avs
27°	A	-	< 10	Am
28°	A	-	< 10	-
29°	P	Ac	< 10	Am
30°	A	-	< 10	-

P (presence); A (absence)

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

Table 1 identifies and quantifies the isolates obtained by direct plating and presence absence tests from the water samples. With the direct plating method, 19 (63%) samples tested positive for *Aeromonas* spp, while the P/A method 11 (37%) samples were positive for *Aeromonas* isolates. Combining the results resulted in detection of *Aeromonas* in 23 samples (77%).

TSBA was chosen as an enrichment medium because authors including Hänninen *et al.* (12) found it to be excellent for detecting the presence of *Aeromonas*. Nevertheless, direct counting of CFU's of *Aeromonas* spp proved more successful in detecting this genus. Seven species of *Aeromonas* were identified from the samples analysed, the most common being

A. caviae detected in 14 samples (47%), *A. veronii* biovar *sobria* in 09 (30%), *A. veronii* bv. *veronii* and *A. trota* in 04 (13%), *A. media* in 03 (10%) and *A. hydrophila*, *A. sobria* e *Aeromonas* sp. in 02 (07%) (Table 1). Although it is known that *A. caviae* predominates in marine waters, it can also be found in water contaminated by sewage (34). The indigenous nature of *Aeromonas* spp in aquatic environments highlights that these organisms can play an important role as opportunistic pathogens (20). Chopra & Houston (3) reported that the species, *A. hydrophila*, *A. veronii* bv. *sobria* and *A. caviae* are commonly isolated from human infections and demonstrated their capacity to produce a variety of biologically active extra-cellular products.

Among the 69 strains isolated using both isolation methods, the following were identified *A. caviae* in 29 (42%), *A. veronii* bv. *sobria* in 13 (19%), *A. veronii* bv. *veronii* in 08 (11%), *A. trota* in 06 (07%), *A. media* in 05 (07%), *A. sobria* in 04 (06%) *Aeromonas* spp and *A. hydrophila* in 02 (03%) (Table 2). The species *A. caviae* as well as *A. hydrophila* and *A. sobria* are capable of causing diarrhea; especially if contaminated water is ingested by susceptible individuals (28).

Aeromonas hydrophila and *A. sobria* have been described as the most virulent phenospecies among the mesophile *Aeromonas* (13). In Brazil, an acute diarrhea outbreak, with 2170 cases, occurred between January and July, 2004, in São Bento do Una, Pernambuco. In this case, *Aeromonas* species were the most frequent (19.5%) and the main isolates were *A. caviae* (9.8%), *A. veronii* biovar *sobria* (3.9%) and *A. veronii* biovar *veronii* (2.6%) (14).

Table 2. Percentage of *Aeromonas* isolated from the Cocó River estuary (Fortaleza, Brazil), by presence/absence (P/A) and direct plating (CFU mL⁻¹) methods.

Species	P/A (%)	CFU mL ⁻¹ (%)	Total identified strains (%)
<i>A. caviae</i>	12 (46)	17 (40)	29 (42)
<i>A. hydrophila</i>	01 (04)	01 (02)	02 (03)
<i>A. media</i>	no detected	05 (12)	05 (07)
<i>A. sobria</i>	no detected	04 (09)	04 (06)
<i>A. trota</i>	05 (19)	01 (02)	06 (09)
<i>A. veronii</i> bv. <i>sobria</i>	03 (12)	10 (23)	13 (19)
<i>A. veronii</i> bv. <i>veronii</i>	04 (15)	04 (09)	08 (11)
<i>Aeromonas</i> sp.	01 (04)	01 (02)	02 (03)
Total	26 (100)	43 (100)	69 (100)

Table 3 shows the results of resistance to eight antimicrobial agents for the 38 strains of *Aeromonas* spp tested. Of the 38 strains tested 23 (60%) showed resistance to at least one of the antimicrobials. Antimicrobial resistance is a fact which is increasingly worrying health authorities, due to its increasing occurrence each year (29). According to the literature, the majority of *Aeromonas* are susceptible to tetracycline, aminoglycosides, third generation cephalosporins and the quinolones (16), although studies have shown high resistance to tetracycline (17), and combined oxytetracycline (OTC) and sulphadiazine/trimethoprim (36).

Aeromonas strains were shown to be less sensitive to trimethoprim-sulfamethoxazole, with 21 (55%) of the strains showing resistance to this antimicrobial. *Aeromonas caviae* was the species which presented the highest resistance (29%),

followed by *A. media* (08%), *A. veronii* both biogroups (05%) and *A. media* and *A. trota* with 03%.

In this study, all *A. trota*, *A. hydrophila*, *A. sobria* and *A. veronii* bv. *veronii* strains were shown to be sensitive to this antibiotic. *A. caviae*, *A. veronii* bv. *sobria* and *A. media* strains were sensitive to tetracycline in 92, 90 and 33% of tests respectively. Similar results were found by Rall *et al.* (32) who observed sensitivity to this antimicrobial from 100% of *A. sobria* strains and 93% of *A. caviae* strains. Limited/rate resistance to tetracycline from strains of *Aeromonas* spp is hypothesised as being related to acquired resistance through mobile genetic elements/plasmids (13).

Nalidixic acid is an antimicrobial which can be applied with great success in the treatment of "traveller's diarrhoea" caused by these microorganisms (7). In this study, only 13% of

the strains showed resistance to this antimicrobial. The resistance to quinolones and nalidixic acid is considered to be chromosomally mediated, as a result of drug resistant isolates selective pressure (11), or even multiplication of resistant clones (18). The relation between the increasing number of aquatic bacteria resistant to antibiotics and their ability to uptake and transfer resistance genes, is a well known fact (33).

Multiple resistance was observed in *A. caviae*, *A. media*, *A. sobria* e *A. veronii* bv. *sobria*. The strains of *A. caviae* were multi-resistant to four (50%) of the antimicrobials tested, followed by *A. sobria* and *A. veronii* bv. *sobria*, which were

resistant to three (37%), *A. media*, which was resistant to two (25%), and *A. hydrophila*, *A. trota* and *A. veronii* bv. *veronii*, which were each resistant to one (12%). The isolation of multi-resistant aquatic *Aeromonas* species (including trimethoprim-sulphamethoxazole and nalidixic acid) from freshwater in other parts of the world (10) along with our own findings warrant the need to take proper measures to prevent the introduction of resistant aeromonas into water sources used by humans, because the ingestion of contaminated fish may result in resistance gene transfer from fish to the human intestinal microbiota.

Table 3. Antibiotics susceptibility of *Aeromonas* spp strains isolated from from estuary of the Cocó river (Fortaleza, Brazil).

Antibiotics* Species	N° (%) susceptible to							
	TE	F	AK	CIP	CRO	SXT	C	NA
<i>A. caviae</i> (n=13)	12(92)	13(100)	12(92)	13(100)	13(100)	02(15)	13(100)	11(85)
<i>A. media</i> (n=03)	01(33)	03(100)	03(100)	03(100)	03(100)	0,0(0)	03(100)	03(100)
<i>A. sobria</i> (n=2)	02(100)	02(100)	01(50)	02(100)	02(100)	01(50)	02(100)	01(50)
<i>A. hydrophila</i> (n=2)	02(100)	02(100)	02(100)	02(100)	02(100)	02(100)	02(100)	01(50)
<i>A. trota</i> (n=4)	04(100)	04(100)	04(100)	04(100)	04(100)	03(75)	04(100)	04(100)
<i>A. veronii</i> bv. <i>sobria</i> (n=10)	09(90)	10(100)	10(100)	10(100)	10(100)	08(80)	10(100)	09(90)
<i>A. veronii</i> bv. <i>veronii</i> (n=4)	04(100)	04(100)	04(100)	04(100)	04(100)	02(50)	04(100)	04(100)
Total (n=38)	34(89)	38(100)	36(95)	38(100)	38(100)	18(47)	38(100)	33(87)

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

Physical-chemical parameters of the water, including temperature, pH and salinity were recorded. Temperature varied from 28° to 32°C (Table 3). In the seven collections where bacteria were not recovered, the temperature was found to be in the 30° to 32°C range. Sautour *et al.* (35) observed that a temperature of 30°C and pH 7.0 are ideal environmental parameters for the growth of *A. hydrophila*. The occurrence of blank results of the water samples may have been related to the fact that they were collected from water to a depth of 30 cm/sea surface during the hottest part of the day (12:00 h). The water sampling did not follow the tide regime, and sometimes took

place in the presence of strong sunlight which may have raised water temperatures.

The pH of the water varied from 6.4 to 8.0 (Table 4), a range considered ideal by Khadori & Fainstein (17) provided that the temperature is 28°C. During the period of the study the water temperature was only above the 30°C range in 06 (20%) samples of which 02 (07%) samples of the samples did not yield bacteria (Table 4). One highly significant factor in understanding infections is knowledge of an organism's ecological niche, as this has an influence on the thermal scale of its growth and its ability to proliferate at the temperature of

the host body. *Aeromonas* spp can produce enterotoxins at different temperatures (19).

The values for salinity varied from not detected to 3.6% (Table 4). This fact justifies the high percentage of positive samples obtained in the current study; as Palumbo *et al.* (27) demonstrated that *Aeromonas* spp grow in a maximum concentration of 4.5% sodium chloride (NaCl). However there was a decrease in the scores of these microorganisms, mainly from the 18th collection, coinciding with the dry season when an increase in salinity and temperature could be noted. Knochel (19) observing the growth of 80 strains of *Aeromonas* spp in different concentrations of salt, noted that at a temperature of

25°C and pH 7.3, all the strains grew at 0.5% and 2% of NaCl, with only a few of them growing in the medium containing 4 and 6% of NaCl.

The potential risk from *Aeromonas* spp to human health from aquatic environments has been stressed by several researchers, principally in infections caused by these microorganisms when related to recreation and other activities in aquatic environments. In the face of the results obtained, the presence of *A. hydrophila*, *A. caviae*, *A. veronii* and *A. veronii* bv. is highlighted as it is known that these species are involved in diverse outbreaks of gastroenteritis.

Table 4. Physical-chemical (temperature, pH e salinity) parameters determined from 30 water samples in the estuary of the Cocó river, Brazil.

Samples	Temperature (°C)	pH	Salinity (‰)	Samples	Temperature (°C)	pH	Salinity (‰)
1 ^a	32	6.7	no detected	16 ^a	28	6.9	32
2 ^a	29	7.4	1.0	17 ^a	30	7.6	17
3 ^a	30	7.5	2.0	18 ^a	29	7.8	15
4 ^a	29	6.8	18	19 ^a	27.5	7.3	20
5 ^a	30	8.0	35	20 ^a	29	7.6	28
6 ^a	28	7.5	34	21 ^a	30	7.2	27
7 ^a	28	6.4	1.0	22 ^a	27	7.7	25
8 ^a	28	6.5	7.0	23 ^a	31	7.5	29
9 ^a	29	6.5	2.0	24 ^a	31	8.0	20
10 ^a	30	6.5	2.5	25 ^a	32	7.0	24
11 ^a	28	5.9	2.5	26 ^a	28	7.4	12
12 ^a	29	7.0	no detected	27 ^a	27.5	7.5	36
13 ^a	27	7.3	8.0	28 ^a	30	7.4	33
14 ^a	31.5	8.0	9.0	29 ^a	31	7.5	25
15 ^a	28	7.2	22	30 ^a	30	7.9	20

CONCLUSION

Current major obstacles to human health in developing regions are well understood and a large component relates to unsafe water, poor sanitation and inappropriate hygiene. In this work the widespread presence of three pathogenic species genus of *Aeromonas* known to be involved in outbreaks of gastroenteritis, shows that despite our knowledge, pathogens continue to be a major issue for human health, and particularly so in developing regions. Monitoring pathogen distribution in public environments allied with educational programs that

explain risk continues to be an important activity in developing regions.

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