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AEROMONAS ASSOCIATED DIARRHOEAL DISEASE IN SOUTH BRAZIL: PREVALENCE, VIRULENCE FACTORS AND ANTIMICROBIAL RESISTANCE

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ABSTRACT

Aeromonas were isolated from 27 (6.6%) of 408 patients admitted with acute gastroenteritis in two hospitals at Rio Grande do Sul, Brazil. Isolates were classified as *A. hydrophila* (51.8%), *A. caviae* (40.8%), and *A. veronii* biotype *sobria* (7.4%). The highest prevalence of *Aeromonas* associated infections occurred in lactants and children. Virulence genes (*aerA* -aerolysin/hemolysin, *ahpA* -serine-protease, *satA* -glycerophospholipid-cholesterol acyltransferase, *lipA* -lipase, and *ahyB* -elastase) and virulence factors (hemolytic, proteolitic, lipolitic activities, and biofilm formation) were identified in most *A. hydrophila* and *A. veronii* biotype *sobria* isolates, with lower frequencies on *A. caviae*. All *Aeromonas* isolates were resistant to ampicillin, ticarcillin/clavulanic acid, cephalotin, and cephazolin, and most of them (>70%) exhibited resistance to imipenem, carbenicillin, amoxillin/sulbactan, and piperacillin. Multiple-resistance, more than four antibiotics, was evidenced in 29.6% of the isolates. The most efficient antibiotics were the quinolones (ciprofloxacin and norfloxacin), and the aminoglycosides (amikacin and netilmicin).

Key words: *Aeromonas*, diarrhea disease, virulence factors, antimicrobial resistance

INTRODUCTION

Diarrhea disease is an important cause of morbidity and mortality in developing countries, particularly in infants and elders. Diarrheas are caused by viral, bacterial, and parasitic infections, as well as food intolerances, reaction to medicines, and other physiological and immunological disorders (2). Bacterial infections are responsible for 20-40% of diarrhea illness, and several bacterial species have been frequently ascribed to diarrhea episodes, including *Campylobacter jejuni*, *Escherichia coli*, *Salmonella* spp., *Vibrio cholerae*, *Yersinia enterocolitica*, *Aeromonas* spp., and *Plesiomonas* spp.

In the last decades, *Aeromonas* have been increasingly recognized as relevant etiological agents in gastrointestinal infections (14,24,30), as well as extraintestinal infections such as cellulitis, wound infections, septicemia, urinary tract infections, among others (2). Three *Aeromonas* species, *A. hydrophila*, *A. caviae*, and *A. veronii* biotype *sobria* are considered of clinical significance (2,14).

The virulence of *Aeromonas* is multifactorial, including adhesions, S-layer, lipopolyssaccharides, siderophores, and an array of exoenzymes and exotoxins, i.e. aerolysin/hemolysin, lipases, proteases, among others (24,26,32). Genes encoding these virulence factors have been isolated and sequenced allowing the detection of signature regions of these genes and the evaluation of their presence in *Aeromonas* clinical and environmental isolates (5,6). Moreover, the regulation and secretion processes of viralence factors, as well as the host response alter the pathogenicity of *Aeromonas* (10,27,28).

Antimicrobial resistance among enteric pathogens is a serious problem in developing countries where there is a high frequency of gastroenteric illness and many antibiotics fall routinely into inadequate use. Antibiotic resistance is particularly relevant in pathogenic *Aeromonas* species in which,

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besides the classical resistance to β-lactamic antibiotics, multiple-resistance has been frequently identified (11,15,29,30). These bacteria can receive and transfer antibiotic resistance genes to other Gram negative bacteria (17).

The aim of this study was to determine the prevalence of *Aeromonas* species among patients hospitalized with diarrhea disease in Rio Grande do Sul, Brazil, as well as to determine the antibiotic resistance patterns and the presence of virulence factors among *Aeromonas* isolates.

MATERIALS AND METHODS

A total of 408 stool specimens were collected from patients admitted with diarrhea disease, between January 1999 and Dezember 2000, in two hospitals of Rio Grande do Sul, Brazil: Hospital Geral of Caxias do Sul, and Hospital São Lucas of Porto Alegre. A diarrheic subject was identified as someone suffering three or more episodes of watery or loose discharges in a 24 h period prior to admission in the hospital. As control, 70 stool samples form infants (0 to 2 years), 50 from children (2 to 10 years), 50 form adults (< 60 years), and 42 from elderly, were collected from non-hospitalized apparently healthy subjects. Clinical reports of all patients and controls were recorded on a standard form, including personal data, clinical history, diarrhea intensity and overt clinical conditions (fever, nausea, vomiting and dehydration status).

Stool specimens were diluted (0.5g/10ml) in alkaline peptone water (1% peptone, 1% NaCl, pH 8.6). Aliquots (0.1ml) were plated on Ampicillin Sheep-blood Agar medium (ASA-blood agar base supplemented with 5% sheep blood and 10mg/L ampicillin) and Yersinia Selective Agar medium (YSA), and incubated at 28ºC for 24 h and 48h. Hemolytic colonies on ASA medium with a characteristic creamy aspect and light brown color, or purple colonies with a deep purple halo in YSA medium, were streaked on Trypticase Soy Agar (TSA) and incubated for 24 h at 37ºC. These isolates were tested for oxidase activity, and if proved positive were stored in TSA slants at room temperature, and in Brain Heart Infusion (BHI) supplemented with 25% glycerol at -70ºC. All culture media were purchased from Merck, except BHI, from Difco.

Biochemical tests for identification of *Aeromonas* species were performed according to the AerokeyII system (4). All samples were examined for other common enteric pathogens following standard procedures (13).

The following *Aeromonas* reference strains were used: ATCC 7966 and CECT 398 (*A. hydrophila*), ATCC 43979 (*A. veronii* biotype *sobria*), ATCC 15468 and ATCC 14486 (*A. caviae*), ATCC 35624 (*A. veronii* var. *veronii*), ATCC 33907 (*A. media*), and ATCC 23309 (*A. eucrenophila*).

Antibiotic susceptibility testing was performed by the disk diffusion method (7). *Aeromonas* strains were examined for resistance against a panel of 24 antibiotics, including representatives of the most important classes: amikacin, ampicillin, amoxillin/sulbactam, ticarcillin/clavulanic acid, piperacillin, aztreonam, carbenicillin, cephalothin, cephazolin, cefuroxime, cefoperazone, cefotaxime, ciprofloxacin, ceftazidime, ceftriaxone, tetracycline, chloramphenicol, trimethoprim/sulfamethoxazole, streptomycin, gentamycin, imipenem, netilmicin, norfloxacin, and tobramycin. Zones of inhibition were measured after 24 h incubation at 37ºC, and susceptibility/resistance interpretation were performed according to CLSI for enterobacteria (7). *Aeromonas* strains ATCC 7966, ATCC 43979, and ATCC 15468, and *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923), were included as quality controls.

Hemolytic positive isolates were identified by the presence of clear (β-hemolysis) or diffuse (α-hemolysis) halos around the colonies grown at 37ºC for 24 h on TSA agar (Difco) containing 5% rabbit or 5% human blood. To determine the proteolytic activity *Aeromonas* isolates were striped on TSA agar containing gelatin (1%), grown for 24 h at 30ºC, and developed by the addition of a saturate solution of ammonium sulfate. Lipase activity was assay by the Tween-calcium method (19), and biofilm formation was evaluated by the method proposed by Heilmann *et al*. (12).

The presence of the genes *aerA* (aerolysin/hemolysin), *ahpA* (serine-protease), *satA* (GCTA- glycerophospholipid-cholesterol acyltransferase), *lipA* (lipase), and *ahyB* (elastase) was evaluated by PCR amplification using the primers and procedures previously proposed (5,6).

RESULTS AND DISCUSSION

Potentially gastroenteric bacteria, as well as other bacteria, were isolated from 47 (11.5%) out of 408 stool specimens obtained from patients admitted with acute diarrhea in two hospitals of Rio Grande do Sul, Brazil. *Aeromonas* prevalence corresponded to 6.6% with a total of 27 isolates, significantly different from 0.0% in the 212 control stool samples evaluated. Twenty one samples exhibited just *Aeromonas*, and six samples showed *Aeromonas* associated with *E. coli* (2 samples), *Salmonella* (2 samples), *P. aeruginosa* (1 sample) and *Klebsiella* (1 sample). *Escherichia coli*, *Salmonella* sp., and other bacteria (*Shigella*, *Pseudomonas*, and *Klebsiella*) were identified in 2.7%, 1.5%, and 0.7% of the samples, respectively. The prevalence of *Aeromonas* is consistent with that previously reported (16,20,22,30). However, the frequency of *Aeromonas* in comparison with other enteric bacteria was higher to that previously reported in several countries, including Brazil (22). The presence of more than one enteric bacteria was evidenced in 15% of the patients, a frequency lower than that reported in other South-American countries (23).

The highest prevalence of *Aeromonas* (14.3%) was observed in lactants (< 1 years old), decreasing to 9.5% in 1-2 years old children, to 5.7% in 2-10 years old children, 2.7% in adults, and

7.3% in elders (Table 1). High frequency of *Aeromonas* in infants and elder were reported by several authors (9,16,22,31). Although not completely understood, this fact has been attributed to intrinsic physiological and immunological characteristics of infants and elder, associated with the presence of *Aeromonas* in food and beverages (2,18).

Three species of *Aeromonas* were identified among the 27 isolates: 14 (51.8%) were classified as *A. hydrophila*, 11 (40.8%) as *A. caviae*, and 2 (7.4%) as *A. sobria*. These data different from those obtained at Rio de Janeiro (8,9), and at Goiânia (22), both in the tropical region of Brazil, in which *Aeromonas caviae* was the most prevalent species.

Analyzing the clinical reports, 51.9% of the cases associated with *Aeromonas* could be classified as toxigenic (watery diarrhea with rare abdominal cramps, nausea and vomiting), and 48.1% as dysenteric (loose and bloody stools, strong abdominal cramps, fever, nausea and vomiting), which is consistent with other reports (3,16,30).

Table 1. Frequency of *Aeromonas* strains from fecal specimens according to patients age.

Patient age	Nº of cases evaluated	Nº of *Aeromonas* positive cases	(%)
Under 1 year	56	8	14.3
1-2 years old	63	6	9.5
2-10 years old	87	5	5.7
Adults (up to 60 years)	147	4	2.7
Elderly	55	4	7.3
Total	408	27	6.6

The gene encoding the aerolysin/hemolysin (cytotoxic enterotoxin) was detected in 20 isolates (74.1%), with no significant difference at the species level (Table 2). This frequency is similar to that previously reported on clinical isolates of these bacterial species (6). All the isolates that harbor the *aerA* gene exhibited hemolytic activity on human blood. However, despite the presence or absence of the *aerA* gene, all the isolates showed β-hemolytic activity on rabbit blood, fact that may be associated to the presence of other hemolysin genes such as *hlyA* (32).

All the isolates obtained from patients with toxigenic diarrhea harbor the *aerA* gene, supporting the idea that aerolysin is an important virulence factor associated with watery diarrhea caused by *Aeromonas* species (3).

The serine protease gene (*ahpA*) was detected in 63.0% of the isolates (Table 2), a frequency 10% lower than that previously reported (6). The serine protease has been associated with the activation of aerolysin (2) and other extracellular enzymes, thus affecting the overall virulence of *Aeromonas* strains. In this sense it is important to note that all the *aerA* positive isolates associated with toxigenic diarrhea were also positives for the serine protease gene, whereas the *ahpA* gene was present in only 23% of the isolates obtained from dysenteric samples.

Moreover, twenty-four isolates (88.9%) were positives for the *ahyB* gene that codes for a metalloprotease (elastase), considered one of the most important virulence factors (5). Lipase and glycerophospholipid-cholesterol acyltransferase genes (*lipA* and *satA*) were detected in 96.3% of the isolates (Table 2). Glycerophospholipid-cholesterol acyltransferases and lipases have been associated with intestinal damage and interaction with human leukocytes, respectively (6).

Biofilm formation, a character that can influence the was detected in 71.4% of *A. hydrophila* isolates. *Aeromonas*

Table 2. Prevalence of virulence factors and genes among *Aeromonas* isolates.

	***A. hydrophila* (n=14)**	***A. caviae*(n=11)**	***A. veronii* biovar. *sobria*(n=2)**
Virulence factors			
Hemolytic activity (human blood)	12 (85.7%)	6 (54.5%)	2 (100%)
Hemolytic activity (rabbit blood)	14 (100%)	8 (72.7%)	2 (100%)
Proteolytic activity	14 (100%)	11 (100%)	2 (100%)
Lipolytic activity	14 (100%)	10 (91%)	2 (100%)
Biofilm	10 (71.4%)	3 (27.3%)	1 (50%)
Virulence genes			
Aerolysin (*aerA*)	11 (78.6%)	7 (63.6%)	2 (100%)
Serine protease (*ahpA*)	11 (78.6%)	5 (45.4%)	1 (50%)
Elastase (*ahyB*)	12 (85.7%)	10 (91%)	2 (100%)
Lipase (*lipA*)	14 (100%)	10 (91%)	2 (100%)
GCAT (*satA*)	14 (100%)	10 (91%)	2 (100%)

biofilms are associated with putative virulence proteins implicated in polar flagellar assembly and bacterial adhesion to host tissues (33).

The expression of several putative virulence factors and genes in all the isolates can be seen as an indicative of pathogenic potential of these clinical isolates. Comparisons between clinical and environmental isolates of *Aeromonas* have shown that the formers present a higher frequency of virulence genes and exhibited a higher proportion of virulence factors (6,8).

Considering the importance of antibiotic resistance in the treatment of diarrhea diseases, the 27 *Aeromonas* isolated in this study were tested for antibiotic susceptibility. As can be observed in Table 3, all the isolates were sensitive to amikacin, norfloxacin, netilmicin, and ciprofloxacin. *Aeromonas* susceptibility to these drugs has been previously reported (15,29). Most of the isolates (>80%) were susceptible to aztreonam, cefoperazone, ceftriazime, gentamicin, and chloramphenicol.

Conversely, all the isolates were resistant to ampicillin, cephalotin, cephazolin, and ticarcillin/clavulanic acid (Table 3), and most of them were also resistant to amoxillin/subactam (92.6%), piperacillin (81.5%), imipenem (77.8%), and carbenicillin (74%). The large spectra of resistance to β-lactam antibiotics, the resistance to imipenem (a drug highly stable to serine- β-lactamases), as well as the inefficiency of clavulanic acid and sulbactam to inhibit *Aeromonas* β-lactamases, corroborate previous results indicating the chromosomally mediated production of metallo- β-lactamases by these bacteria (21,25). Furthermore, beyond the penicillins and first generation cephalosporins, some isolates (18.5%) showed resistance to several new extended-spectrum cephalosporins, and one isolate of *A. hydrophila* exhibited resistance to aztreonam and all the penicillins and cephalosponins.

Some isolates could be considered as multi-resistant, as they stand to more than four antibiotics other than the β-lactam. In this sense, one isolate exhibited resistance to seven antibiotics including three aminoglycosides, tetracycline, chloramphenicol, and trimethoprim/sulfamethoxazole), two were resistant to streptomycin, tetracycline, chloramphenicol and trimethoprim/sulfamethoxazole, two were resistant to four aminoglycosides, and three were resistant to tetracycline and trimethoprim/sulfamethoxazole. Similar multiple antibiotic resistance patterns in *Aeromonas* were reported (8,11,15,29), indicating that this is a common and concerning feature of these bacteria, specially considering their ability to efficiently receive and transmit antibiotic resistance genes to other enteric species (17).

In conclusion, the data obtained in this study strongly convey the need to consider *Aeromonas* as an important causative agent of acute diarrhea disease in South Brazil, particularly among infants and children. The presence of several virulence factors and genes, and the occurrence of multidrug resistance in *Aeromonas* isolates, highlights the necessity to implement routine identification of *Aeromonas* species, and continuous monitoring their antibiotic resistance pattern.

Table 3. Antibiotic resistance of 27 isolated *Aeromonas* strains.

CLASS	ANTIBIOTICS	NR	Resistant (%)
β- lactam antibiotics			
1. Penicillins	CBN	20	74.1
	AMN	27	100.0
	AXS	25	92.6
	CXT	27	100.0
	PPN	22	81.5
2. Monobactams	AZM	1	3.7
3. Cephalosporins	CEF (1st generation)	27	100.0
	CZN (1st generation)	27	100.0
	CXN (2nd generation)	9	33.3
	CRE (2nd generation)	4	14.8
	CPN (3th generation)	1	3.7
	CRO (3th generation)	1	3.7
	CAE (3th generation)	5	18.5
	CDE (3th generation)	2	7.4
4. Carbapenem	IMI	21	77.8
Aminoglycosides	AMI	0	0
	GEN	4	14.8
	NET	0	0
	TOB	9	33.3
	STR	8	29.6
Tetracycline	TET	14	51.8
Chloramphenicol	CLO	4	14.8
Quinolones	CIP	0	0
	NOR	0	0
Sulphonamides	SXT	13	48.1

NR: number of antimicrobial resistant strains
CBN= carbenicillin; AMN= ampicillin; AXS= amoxillin/sulbactam; CXT= ticarcillin/clavulanic acid; PPN= piperacillin; AZM= aztreonam; CEF= cephalothin; CZN= cephazolin; CXN= cefoxitin; CRE= cefuroxime; CPN= cefoperazone; CRO= ceftriaxone; CAE= cefotaxime; CDE= ceftazidime; IMI= imipenem; AMI= amikacin; GEN= gentamicin: NET= netilmicin: TOB= tobramycin; STR= streptomycin; TET= tetracycline: CLO= chloramphenicol: CIP= ciprofloxacin; NOR= norfloxacin; SXT= trimethoprim/sulfamethoxazole.

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RESUMO

Aeromonas associadas a diarréias no sul do Brasil: prevalência, fatores de virulência, e resistência a antibióticos

Aeromonas foram isoladas de 27 (6.6%) dos 408 pacientes admitidos com gastroenterite aguda em dois hospitais do Rio Grande do Sul, Brasil. Os isolados foram classificados com *A. hydrophila* (51.8%), *A. caviae* (40.8%), e *A. veronii* biotype *sobria* (7.4%). A maior prevalência de *Aeromonas* ocorreu em lactantes e crianças. Genes (*aerA* -aerolisina/hemolisina, *ahpA* -serina-protease, *satA* - glicerofosfolipidio-colesterol aciltransferase, *lipA* -lipase, e *ahyB* -elastase) e factores (atividade hemolítica, proteolítica, lipolítica, e formação de biofilme) de virulência foram identificados na maioria dos isolados de *A. hydrophila* e *A. veronii* biotype *sobria*, com freqüências menores em *A. caviae*. Todos os isolados de *Aeromonas* apresentaram resistência a ampicilina, ticarcilina/ácido clavulânico, cefalotina e cefazolina, e a maior parte (>70%) exibiram resistência a imipenem, carbenicilina, amoxacilina/sulbactam e piperacilina. Resistência múltipla foi evidenciada em 29,6% dos isolados. Os antibióticos mais eficientes foram as quinolonas (ciprofloxacina e norfloxacina) e os aminoglicosídicos (amicacina e netilmicina).

Palavras-chave: *Aeromonas*, diarréia, fatores de virulência, resistência antimicrobiana

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