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Prevalence and virulence properties of *Vibrio cholerae* non-O1, *Aeromonas* spp. and *Plesiomonas shigelloides* isolated from Cambé Stream (State of Paraná, Brazil)

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A. GIBOTTI, H.O. SARIDAKIS, J.S. PELAYO, K.C. TEGLIARI AND D.P. FALCÃO. 2000. The incidence of *Vibrio cholerae*, *Aeromonas* spp. and *Plesiomonas shigelloides* was determined in water samples from Cambé Stream. The samples were collected from seven different sites. The serogroups, virulence markers and drug resistance profiles were also evaluated. Twelve *Aer. hydrophila*, 12 *Aer. caviae*, eight *Aer. sobria*, seven *Ple. shigelloides* and two *V. cholerae* non-O1 were isolated. They belonged to different serogroups and all produced haemolysis in different assays. Five of the *Aeromonas* strains and one of *V. cholerae* non-O1 were positive for enterotoxin activity. Haemagglutination and its inhibition, using erythrocytes of different origins, was variable for *Aeromonas* spp. and *V. cholerae*, while none of the *Ple. shigelloides* haemagglutinated in association with any type of erythrocyte. All isolates exhibited multiple drug resistance. These results indicate that the occurrence of *V. cholerae* non-O1, *Aeromonas* spp. and *Ple. shigelloides*, in water used for vegetable irrigation, human recreation and animal consumption, among others, represents a potential risk for humans.

INTRODUCTION

Aeromonas and *Vibrio* species, and *Plesiomonas shigelloides*, have been associated with human diarrhoeal diseases and extra-intestinal infections (Janda *et al*. 1995; McLaughlin 1995; Bravo *et al*. 1998).

The varied clinical nature of *Aeromonas* infection, and gastroenteric illness in particular, suggests that complex pathogenic mechanisms exist in the aeromonads. In *Aeromonas* species, the occurrence of degradative extracellular enzymes such as proteases, lipases, and elastases have been previously described, as have the production of amonabactin and enterobactin siderophores, the production of exotoxins including α and β haemolysins, thermo-stable and thermo-labile enterotoxins, invasins and adhesins (Janda 1991; Majeed and MacRae 1991; Byers and Arceneaux 1993; Kirov 1997). Plasmids are not commonly detected in human diarrhoea-associated *Aeromonas* isolates, but they have been found in up to 95% of the isolates from patients with bacteraemia (Vadivelu *et al*. 1995; Kirov 1997).

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Ple. shigelloides has been described as capable of producing cholera-like enterotoxin (Gardner *et al*. 1987), thermostable enterotoxin (ST) (Matthews *et al*. 1988) and thermolabile enterotoxin (LT) (Sears and Kaper 1996). The production of β-haemolysin by *Ple. shigelloides* strains was observed in erythrocytes from different mammals (Janda and Abbott 1993). Little is known about intestinal colonization and mucosal interactions by *Plesiomonas*. Some strains reportedly adhere to HeLa and HEp-2 cells and invade them (Kirov 1997). Holmberg *et al*. (1986) reported that patients with *Plesiomonas* tended to display typical symptoms of an invasive pathogen. Large plasmids have been detected in strains from patients with *Plesiomonas*-associated colitis (Kirov 1997).

Amongst the pathogenic species belonging to the genus *Vibrio*, only *V. cholerae* and *V. mimicus* may be found in fresh water. *V. cholerae* possesses about 155 serogroups based on somatic antigens. *V. cholerae* non-O1 is biochemically indistinguishable from *V. cholerae* O1. However, it does not agglutinate in the presence of polyvalent O1 antiserum (McLaughlin 1995). Some *V. cholerae* non-O1 strains can produce various toxins such as cholera-toxin (CT) and others, similar to ST produced by enterotoxigenic *Escherichia coli* (ETEC) or to Shiga-toxin produced

by *Shigella dysenteriae* type I, plus cytolysins, haemolysins and haemagglutinins (Kaper *et al*. 1995).

The presence of *Vibrio, Aeromonas and Plesiomonas* isolates has been reported by different authors in Brazil, in different types of water (Gibotti 1996). Falcão *et al*. (1998), studying the presence of *Ple. shigelloides*, *Aeromonas* spp. and *V. cholerae* in fresh water from different sources in Araraquarara, SP-Brazil, isolated different species of *Aeromonas* and *V. cholerae* non-O1, but no strains of *Ple. shigelloides* were detected. They observed that all strains of *Aeromonas* or *V. cholerae* non-O1 produced β-haemolysis and some strains of *Aeromonas* exhibited cytotoxic activity.

The Cambé Stream crosses the region of Londrina and Cambé, located in the state of Paraná, Brazil. This stream has its origins in the rural area of Cambé and flows through the region of Londrina, bisecting the central area of the city and continuing throughout all of northern Londrina. There are no previous reports on the isolation of *V. cholerae, Ple. shigelloides and Aeromonas* spp. in this area, either from clinical materials or from the environment.

The objectives of this study were to determine the prevalence of *Aeromonas* spp., *Vibrio* spp. and *Ple. shigelloides* in water samples from Cambé Stream collected in rural and urban areas of both cities, as well as to study some virulence factors and drug resistance profiles of the isolates.

MATERIALS AND METHODS

Sampling

A total of 70 freshwater samples from different sites along the Cambé Stream were collected from April 1992 to March 1993. Samples were taken from seven different sites, 10 samples (in duplicate) from each location. The first site (site one) was situated in the rural area of Cambé close to the stream's nascent spring. Sites two, five and six were on the periphery of Londrina, three and four in the central area, and seven in a rural area, again in Londrina. Site two was located just below a tannery from which waste is discharged *in natura* into the water. Sites two, three, four and five were located in the area where the stream was transformed into a lake by the construction of a dam (Igapó Lake). The lake is bordered by a public playground and a social club (sites three and four) and small rural properties where the water is used for vegetable irrigation and animal consumption (site five). On the border, close to site six, there is an ecological park with wild animals, while site seven is located just below the outlet for the city's treated sewage.

On each occasion, two 500 ml water samples were collected in sterile flasks, transferred immediately into an ice bath and transported back to the laboratory for processing within 6 h.

Analysis

The two water samples were concentrated by centrifugation under refrigeration (4 °C) at 3675 *g* for 30 min (Freitas *et al*. 1987).

One sediment was resuspended in 10 ml peptone water, pH 7·2, and incubated at 37 °C for 24 h for the enrichment of *Aeromonas* and *Plesiomonas* (Neves *et al*. 1990). The second sediment was resuspended in 10 ml alkaline peptone water, pH 8·6, with subsequent incubation at 25 °C for 24 h for *Vibrio* enrichment (Furniss *et al*. 1979).

V. cholerae isolation was performed on thiosulphate–citrate–bile salts–sucrose agar (TCBS) with an incubation at 25 °C for 18 h (Furniss *et al*. 1979).

Aeromonas isolation was achieved with MacConkey agar incubated for 24 h at 37 °C and on *Pseudomonas–Aeromonas* selective agar base (GSP) with ampicillin (10 mg l^{-3}) incubated for 24–48 h at 37 °C (Kielwein 1969).

Ple. shigelloides isolation was performed on MacConkey agar with incubation at 37 °C for 24 h, and on inositol brilliant green–bile salts agar (IBB) with incubation at 37 °C for 24 h (Schubert 1984).

Suspect colonies from the selective agars were identified as previously described (Furniss *et al*. 1979; Carnahan *et al*. 1991; Abbott *et al*. 1992; Janda *et al*. 1995; McLaughlin 1995).

Dr Toshio Shimada (Laboratory of Enteric Infections, National Institute of Health, Tokyo, Japan) serotyped *V. cholerae, Aeromonas and Plesiomonas* isolates.

The isolates were submitted to the following virulence tests.

(i) Haemolysin production employing the following techniques: sheep blood agar plate (Morgan *et al*. 1985); microplate using sheep erythrocytes (Burke *et al*. 1986); agar overlay (only for *Ple. shigelloides*), using human (type A), bovine, sheep, rabbit and guinea pig erythrocytes (Janda and Abbott 1993).

(ii) Enterotoxin production by suckling mouse assays (Dean *et al*. 1972).

(iii) Haemagglutination using human (type O), horse, and guinea pig erythrocytes (Elbashier and Millership 1989).

(iv) Inhibition of haemagglutination in the presence and absence of D-mannose and D-galactose (Atkinson and Trust 1980).

All the isolates were also tested for drug resistance using the disc diffusion technique (Bauer *et al*. 1966). The following antimicrobial drugs were used: ampicillin (A), penicillin (P), amikacin (Am), gentamicin (G), tobramycin (Tb), cephalothin (C), carbenicillin (Cr), thrimetropin-sulphamethoxazole (S), neomycin (N), chloramphenicol (Co), norfloxacin (Nr), tetracycline (Tc) and nitrofurantoin (Ni).

Table 1 *Aeromonas* spp. and *Vibrio cholerae* non-O1 distribution, virulence markers and drug resistance profiles

		Haemolysin		Ent.	Haemagglutination			Inhibition of haemagglutination						
								Gp		H		Ho		
Site no.	Isolates	Ba	M		Gp	H	Ho	m	g	m	g	m	g	Resistance profiles
3	*Aer. hydrophila* O14	β	+	−	+	+	−	+	−	+	−	nt	nt	APCCrS
2	*Aer. hydrophila* O16	β	+	+	+	+	+	+	−	−	−	+	+	APAAmGTbCCrSN
7	*Aer. hydrophila* O16	β	+	−	+	+	+	+	−	+	−	+	+	APCr
2	*Aer. hydrophila* O25	β	+	−	−	+	−	nt	nt	+	−	nt	nt	APCCr
1	*Aer. hydrophila* O25	β	+	−	−	+	−	nt	nt	+	−	nt	nt	APTbCCr
1	*Aer. hydrophila* O26	β	+	−	+	+	−	+	−	+	−	nt	nt	APAmCCrS
2	*Aer. hydrophila* O29	β	−	−	−	+	−	nt	nt	+	−	nt	nt	APCCrN
1	*Aer. hydrophila* O35	β	−	−	+	+	−	+	−	+	−	nt	nt	APCCrS
1	*Aer. hydrophila* O54	β	+	+	+	+	+	+	−	+	−	+	+	APCCrSN
4	*Aer. hydrophila* O54	β	+	−	+	+	−	+	−	+	−	nt	nt	APCrS
6	*Aer. hydrophila* NAG	β	+	−	+	+	−	+	+	+	+	nt	nt	APCCrS
3	*Aer. hydrophila* NAG	β	+	−	+	+	+	+	−	+	−	+	+	APCr
5	*Aer. caviae* O14	α	−	−	+	+	−	+	−	+	−	nt	nt	PCCrSN
6	*Aer. caviae* O14	α	−	−	+	+	+	−	−	+	−	+	+	APCCrSN
3	*Aer. caviae* O16	α	−	−	+	+	−	+	−	+	−	nt	nt	APCCrSN
7	*Aer. caviae* O16	α	−	−	+	+	−	+	+	+	−	nt	nt	PC
7	*Aer. caviae* O16	α	−	−	−	−	−	−	nt	nt	nt	nt	nt	APCCrS
7	*Aer. caviae* O16	α	+	+	−	−	−	nt	nt	nt	nt	nt	nt	APCCrS
1	*Aer. caviae* O34	α	−	−	+	+	−	+	+	+	−	nt	nt	APCCr
4	*Aer. caviae* O34	α	+	−	+	+	−	+	−	+	−	nt	nt	APCCrS
1	*Aer. caviae* O35	α	+	−	+	+	−	+	−	+	−	nt	nt	APCCrS
2	*Aer. caviae* O38	α	−	−	+	+	−	nt	nt	+	−	nt	nt	APCCRs
4	*Aer. caviae* O54	α	+	−	+	+	+	+	+	+	+	+	+	APCCr
2	*Aer. caviae* NAG	α	−	−	+	+	−	+	−	+	−	nt	nt	APCCrS
6	*Aer. sobria* O6	β	+	−	+	+	+	+	−	+	−	+	+	APCCr
5	*Aer. sobria* O16	β	+	−	+	+	+	+	−	+	−	+	+	APCCrSN
5	*Aer. sobria* O16	β	+	+	+	+	+	+	−	+	−	+	+	APCCrS
6	*Aer. sobria* O26	β	−	−	+	+	+	+	−	+	−	+	+	APCrS
3	*Aer. sobria* O35	β	−	−	+	+	+	+	−	+	−	+	+	APCCr
3	*Aer. sobria* O35	β	−	−	+	+	+	+	−	+	−	+	+	APCCr
6	*Aer. sobria* O54	β	−	−	+	+	+	+	−	+	−	+	+	APCrSN
1	*Aer. sobria* O56	β	+	+	+	+	+	+	−	+	−	+	+	APCr
7	*V. cholerae* O62	β	+	−	+	−	+	+	+	nt	nt	+	+	APCrS
4	*V. cholerae* O152	β	+	+	+	+	+	+	−	+	+	+	+	APCrN

NAG: nonagglutinable; Ent.: enterotoxin in suckling mouse model; Ba: sheep blood agar; M: microplate; Gp: guinea pig; H: human; Ho: horse; m: d-mannose; g: d-galactose; nt: not tested.

A: ampicillin; P: penicillin; Am: amikacin; G: gentamicin; Tb: tobramycin; C: cephalothin; Cr: carbenicillin; S: trimethoprim-sulfamethoxazole; N: neomycin; β: beta haemolysis; α: alpha haemolysis; +: positive; − negative.

Results were interpreted as susceptible or resistant according to the diameter of growth inhibition compared with standard values (discs and table from Cefar Farmaco Diagnostica Ltda, São Paulo, Brazil).

RESULTS

Twelve *Aer. hydrophila*, 12 *Aer. caviae* and eight *Aer. sobria* belonging to different serogroups were isolated across all seven sampling sites. Seven *Ple. shigelloides* of different serotypes were isolated at sampling sites one, two, three, six and seven. One strain of *V. cholerae* O62 was found at site seven and another strain, *V. cholerae* O152, at site four.

Two strains of *Ple. shigelloides* were isolated only on IBB agar and seven *Aeromonas* spp. only on GSP agar.

All of the isolates were haemolytic on blood agar plates. The haemolysis was partial (α-haemolysin) for all *Aer. caviae* and *Ple. shigelloides*. These results were variable when using the microplate technique. *Ple. shigelloides* was β-haemolytic with all types of erythrocytes tested when examined by the agar overlay technique.

The suckling mouse assay (Dean Test) for the detection of fluid accumulation was positive for two isolates of *Aer. hydrophila*, one isolate of *Aer. caviae*, two isolates of *Aer. sobria* and one isolate of *V. cholerae*. However, heat treatment at 56 °C for 10 min inhibited the effect of fluid accumulation in this test.

The results of haemaglutination and its inhibition using human, horse and guinea pig erythrocytes were variable for *V. cholerae* and *Aeromonas* spp.; *Ple. shigelloides* isolates did not haemagglutinate with any type of the erythrocytes tested.

All isolates were resistant to three or more drugs and were susceptible to chloramphenicol, norfloxacin, tetracycline and nitrofurantoin.

Table 1 shows the serogroups, virulence characteristics and drug resistance profiles of the *Aeromonas* spp. and *V. cholerae*, and Table 2 displays these characteristics as exhibited by *Ple. shigelloides*.

DISCUSSION

The water samples examined in this study, although collected from a single stream, presented variable features. Different sampling points represent different situations in terms of water quality but at all points, including those supposedly non-polluted, it was possible to isolate those *Aeromonas* spp. (*Aer. hydrophila, Aer. caviae and Aer. sobria*) most frequently associated with human disease (Ko and Chuang 1995). *Ple. shigelloides* was isolated from sites one, two, three, six and seven and *V. cholerae* non-O1 was found at site four, which is a central area, and site seven where the disposal of treated sewage is carried out.

It is important to emphasize that the highest number of isolates occurred at site one, presumed to be non-polluted because of the proximity of the stream's source. At this site, the three species of *Aeromonas* and *Ple. shigelloides* were isolated. These results confirm data reported by other authors who observed that pathogenic bacteria are isolated in higher numbers in non-polluted environments (Leite *et al.* 1989; Falcão *et al.* 1993).

The value of the selective media used in this study should also be emphasized. Seven strains of *Aeromonas* were isolated only on GSP agar, and the same occurred

Table 2 *Plesiomonas shigelloides* distribution, virulence markers and drug resistance profiles

Site no.	Isolates	Haemolysin		Agar overlay					Ent.	Haemagglutination			Resistance profiles
		Ba	M	H	B	S	R	Gp		Gp	H	Ho	
3	*Ple. shigelloides* O4:H3	α	+	β	β	β	β	β	–	–	–	–	PCrN
6	*Ple. shigelloides* O34:H11	α	+	β	β	β	β	β	–	–	–	–	APAmGCrN
7	*Ple. shigelloides* O35:H2	α	–	β	β	β	β	β	–	–	–	–	APCr
1	*Ple. shigelloides* O41:H1	α	–	β	β	β	β	β	–	–	–	–	APAmGCrN
2	*Ple. shigelloides* O44:H1	α	+	β	β	β	β	β	–	–	–	–	PamCr
3	*Ple. shigelloides* O44:H1	α	+	β	β	β	β	β	–	–	–	–	APAmCrN
1	*Ple. shigelloides* O52:H2	α	–	β	β	β	β	β	–	–	–	–	APCrN

Ba: sheep blood agar; M: microplate; Ent.: enterotoxin in suckling mouse model; H: human; B: bovine; S: sheep; R: rabbit; Gp: guinea pig; Ho: horse.
A: ampicillin; P: penicillin; Am: amikacin; G: gentamicin; Tb: tobramycin; C: cephalothin; Cr: carbenicillin; S: trimethoprim-sulfamethoxazole; N: neomycin.
β: beta haemolysis; α: alpha haemolysis; +: positive; – negative.

with two isolates of *Ple. shigelloides* on IBB agar, thus demonstrating the importance of using more than one medium for optimizing the isolation of these bacteria.

Different *Ple. shigelloides* serotypes were identified without any one being prevalent.

Among the *Aeromonas* species, a variety of serogroups was detected. However, serogroup O16 was isolated most frequently (25%). According to Janda *et al.* (1996), this serogroup, together with O11 and O34, is most frequently associated with human clinical cases.

Although the *V. cholerae* isolates did not belong to serogroup O1, the occurrence of these micro-organisms, mainly in recreational waters and at points of discharge of treated sewage, is of potential concern, as these bacteria are responsible for a series of clinical manifestations (McLaughlin 1995). It is known that in addition to *V. cholerae* non-O1 strains that produce cholera enterotoxin, there are strains that do not produce this toxin but can elicit disease. *Vibrio cholerae* non-O1 haemolysin is similar to that produced by *V. cholerae* O1 El Tor (Yamamoto *et al.* 1986). It is also described as an enterotoxic factor capable of inducing fluid accumulation in the ileal loop of rabbits and mice (Ichinose *et al.* 1987).

One of the present *V. cholerae* isolates produced a positive response in the suckling mouse model similar to ST enterotoxin, and both produced β-haemolysin and haemagglutinins.

With regard to the *Aeromonas* isolates, it is important to reiterate the significance of the present data as 45% of the water samples contained haemolytic and haemagluting *Aeromonas* spp. Five strains of *Aeromonas* also showed a positive response in the suckling mouse model similar to ST, indicating their potential pathogenicity.

Fujii *et al.* (1998) also reported that *Aer. sobria* induced a positive response in the suckling mouse test and in haemolytic assays. The enterotoxins produced by five *Aeromonas* isolates in the suckling mouse model were inactivated after 10 min of heating. All five *Aeromonas* strains were also haemolytic. Janda (1991) reported that a 5 min heat treatment at 56 °C is sufficient to inactivate the enterotoxic β-haemolysin of *Aeromonas*.

In Brazil, reports of the isolation of *Ple. shigelloides* from clinical cases are rare. According to Mondino *et al.* (1991), the low incidence of gastroenteritis due to *Ple. shigelloides* partly reflects inadequate laboratory diagnostics, as lactose fermenters and non-fermenters are frequently classified as enterobacteria, especially when an oxidase test is not included in the identification scheme. All *Ple. shigelloides* isolates showed a positive reaction in the haemolysin test, which is one of the most important virulence markers for *Ple. shigelloides,* but were negative for haemagglutinin and enterotoxin production as tested in the suckling mouse model.

When the isolates were tested for susceptibility to antibacterial drugs, all strains displayed multiple drug resistance. Most *Aeromonas* spp. are resistant to ampicillin (Janda *et al.* 1995) but two of these isolates were susceptible. This is important because some culture media recommended for *Aeromonas,* including GSP and blood agar with ampicillin, contain this antibiotic, which could lead to a lower isolation rate of the micro-organism.

There has been no previous systematic search for non-O1 *V. cholerae, Aeromonas* spp. and *Ple. shigelloides* in the region studied. Therefore, it is difficult to establish a correlation between the occurrence of diarrhoea and these bacteria. However, the results indicate that the occurrence of these micro-organisms in water used for vegetable irrigation, human recreation and animal consumption represents a potential risk for humans, mainly due to virulence markers related to diarrhoea being present in these bacteria.

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