Occurrence of *Plesiomonas shigelloides* in Water Environments of Rio de Janeiro City

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Fresh and salt water samples analyzed in Rio de Janeiro city showed the presence of Plesiomonas shigelloides. *Forty-six strains were isolated from both environments. A high incidence of* P. shigelloides *was achieved in polluted fresh and salt waters as well as in samples from non-polluted streams.* P. shigelloides *isolates had biochemical characteristics similar to those already described in the literature. None of the isolates analyzed produced enterotoxin in the suckling mouse assay. Hemolytic activity against sheep and human type A erythrocytes was detected in the strains tested. The results of the antibiotic susceptibility tests indicated that all the isolates were susceptible to the cephalosporins, penicillins combinated with a ß-lactamase inhibitor, aminoglycosides, imipenem, norfloxacin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole. All the isolates were resistant to the penicillins.*

Key words: *Vibrionaceae* - *Plesiomonas shigelloides* occurrence - water environments

The genus *Plesiomonas*, inserted in the family *Vibrionaceae* has as the only species *P. shigelloides*. Like most members of this family, this species is widespread in the environment and most often associated with fresh and salt waters (Zakhariev 1971, Miller & Koburger 1986). These environments may have an important role in the direct transmission of these microorganisms to man and also contaminate sea foods particularly oysters (Rutala et al. 1982) and fish (Arai & Ikejima 1980). So, *P. shigelloides* may be considered as a food and waterborne pathogen being implicated in diseases such as gastroenteritis (Reinhardt & Lance George 1985, van Loon et al. 1989), septicemia (Ingram et al. 1987, Nolte et al. 1988), meningitis (Pathak et al. 1983) and cholecystitis (Körner et al. 1992).

The purpose of this study was to investigate the presence of *P. shigelloides* in water environments of Rio de Janeiro city, using a simple scheme for the isolation and identification. In addition, virulence factors such as heat-stable enterotoxin and hemolysin production, were investigated. Antimicrobial susceptibility test was performed on the isolates.

MATERIALS AND METHODS

Water samples - Four 1000 ml water samples were collected from each of six aquatic environments of Rio de Janeiro city, (four salt and two fresh) during six month from March to August 1990. They were collected in 1000 ml sterile flasks, transported immediately in an ice bath to the laboratory and processed within 1 hr. At the laboratory, from each 1000 ml water sample, four 100 ml portion were separated aseptically for isolation of *P. shigelloides*.

Isolation - Water samples were centrifuged at 10000 x g for 30 min, at 4°C (Freitas et al. 1987). The sediment was directly cultured onto plates containing inositol brilliant green bile salt agar (IBB) (Schubert 1984) and in two tubes with alkaline peptone water (APW). Plates and tubes were incubated at 37°C for 24 hr. After this period, cultures were spreaded onto IBB plates and incubated at 37°C for 24 hr. *P. shigelloides* standard strain was obtained from Culture Collection of Oswaldo Cruz Institute (FIOCRUZ, RJ, Brazil).

Identification - Whitish and red colonies from IBB agar were submitted to inositol fermentation, oxidase, fermentation of glucose and sensitivity to 150 µg of the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (0/129) tests. Gram negative rods, inositol fermentative, oxidase positive, glucose fermentative and sensible to the vibriostatic agent, were submitted to final identification with the API 20 E system (Analytab Products International, SA).

Enterotoxin assay - Enterotoxin was assayed according to the mouse system (Dean et al. 1972)

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except that the medium utilized for the production of enterotoxin was trypticase soy broth (TSB-OXOID) containing 1% inositol (Sanyal et al. 1980).

Hemolysin assay - Ten strains of *P. shigelloides* were screened for hemolytic activity in the agar overlay (AO) assay (Daskaleroz et al. 1991) against sheep and human type A erythrocytes. Briefly, the surface of an L agar (Janda & Abbott 1993) plates were inoculated by spotting with a 5 µl suspension of an overnight tryptone culture of each strain, and incubated at 37°C for 18 hr. After this period, each plate was overlaid with 5 ml of a soft agar suspension (phosphate-buffered saline - PBS - and 0.65% agar) containing 1% washed of each type of erythrocytes. After allowing the overlay to solidify, plates were reincubated at 37°C and were read to hourly intervals for signs of hemolysis, during a 4 hr period.

Antimicrobial susceptibility testing - The antibiotic susceptibilities of 46 isolates of *P. shigelloides* were studied with 12 antibiotics and antibiotic-ß-lactamase-inhibitor combination. Minimum inhibitory concentrations (MICs) were determined by the standard agar dilution method of the National Committee for Clinical Laboratory Standards (1990). All *P. shigelloides* isolates were tested for ß-lactamase production by the hydrolysis of nitrocefin (Montgomery et al. 1979).

Analysis of coliforms - Water pollution was evaluated through the total and faecal coliform counts by using a 5 tube Most Probable Number technic (APHA 1980).

RESULTS

Of a total of 24 water samples obtained from six sites, four of them were positive for *P. shigelloides*. Thirty-six strains were isolated from polluted and non polluted fresh water environments and ten strains were isolated from polluted salt water. None of the strains were isolated from non polluted salt water (Table I).

The strains isolates from polluted salt and fresh water were recovered only after enrichment in alkaline peptone water (APW) whereas two strains isolated from non polluted fresh water were recovered directly from IBB agar without enrichment.

Biochemical characteristics of our environmental isolates were similar to those reported by von Graevenitz and Altwegg (1991). The final identification in the API 20 E system resulted in the profile number 7-144-204, an excellent identification for *P. shigelloides*.

All *P. shigelloides* tested for hemolytic activity were positive for sheep and human type A erythrocytes, within the first hour of incubation. The lysis was beta-hemolitic with clear zones surrounding individual overlaid colonies. Hemolytic activity was not seen when colonies were grown on the surface of blood agar plates.

None of the 46 environmental *P. shigelloides* isolates were positive for *Escherichia coli* ST-like enterotoxin using the infant mouse test.

The result of testing the 46 *P. shigelloides* isolates against 12 antibiotics and antibiotic-ß lactamase-inhibitor combination are shown in Table II. The aminoglycosides, cephalosporins, imipenem, norfloxacin, tetracycline, chloramphenicol and trimethoprim-sulfamethoxazole showed very good activity. In contrast, all isolates were resistant to both ampicillin and amoxicillin. The addition of the ß lactamase-inhibitor clavulanate to amoxicillin reduced the amoxicillin MIC at least fourfold. The 46 *P. shigelloides* isolates, produced a ß lactamase as detected with the nitrocefin-hydrolysis method.

TABLE I

Distribution of *Plesiomonas shigelloides* from salt and fresh water environments at Rio de Janeiro city

Sampling local	Type of water	No. of strains isolated	Total coliforms 100 ml	Fecal coliforms 100 ml	Temperature (0^0C)	Salinity (‰ NaCl)
Fundão Estuary	Salt	5	4.7×10^5	1.2×10^5	29	24.25
Tijuca Lake	Salt	5	2.1×10^5	8.6×10^4	21	18.5
Barra da Tijuca Ocean Waters	Salt	-	4.5×10^1	4.5×10^1	28	34.0
Recreio dos Bandeirantes Ocean Waters	Salt	-	$>10^1$	$>10^1$	22	34.0
Tijuca National Park	Fresh	8	1.5×10^3	5.2×10^2	18	-
Boa Vista River	Fresh	28	2.8×10^5	8.0×10^4	19	-

TABLE II

Antibiotic MICs for the 46 *Plesiomonas shigelloides* isolates

Antibiotic	Breakpoint concn. (µg/ml)	MIC (µg/ml)[a] Range	50%	90%	% Susceptible
Ampicillin	< 8	8-256	32	128	0
Amoxicillin	< 8	8-64	16	64	0
Amoxicillin-clavulanate	< 8/4	2/1 - 4/2	4/2	4/2	100
Imipenem	< 4	0.125-0.25	0.25	0.25	100
Cephalothin	< 8	2-4	2	4	100
Cefotaxime	< 8	0.07	0.07	0.07	100
Amikacin	< 16	2-16	8	8	100
Gentamicin	< 4	1-4	4	4	100
Chloramphenicol	< 8	0.25-0.5	0.5	0.5	100
Tetracycline	< 4	0.5-1	1	1	100
Sulfamethoxazole-Trimethoprim	< 2/38	0.06/1.18-1/19	0.25/4.75	1/19	100
Norfloxacin	< 4	>0.015-0.03	0.03	0.03	100

[a] : 50% and 90%, MIC for 50 and 90% of isolates tested, respectively

DISCUSSION

P. shigelloides has been implicated as a waterborne enteropathogen (Tsukamoto et al. 1978). The inicial goal of this study was to verify the environmental aquatic occurrence and distribution of *P. shigelloides* in the Rio de Janeiro city. Very little data exist in Brasil regarding this microorganism (Leitão & Silveira 1991). The high incidence of isolates confirms the reported prevalence in fresh water (Arai & Ikejima 1980, Miller & Koburger 1986). Few data are available in the literature with respect to the isolation of this microorganism from salt water (Zakhariev 1971).

The highest numbers of *P. shigelloides* were obtained in water environments where the coliform counts were also very high.

The source of *P. shigelloides* in Rio de Janeiro water environments is unknown. The isolation from polluted water suggests that the contamination may be due to excretion by human and domestic animals (Arai & Ikejima 1980). The source of *P. shigelloides* found in non polluted fresh water from Tijuca National Park may be due to excretion by wild animals (Davis et al. 1978, Glunder 1989, Winsor et al. 1991). Despite the source, these results reforced the obvious public health implication of water resources contaminated with *Plesiomonas*.

The recovery of *P. shigelloides* without enrichment from non polluted fresh water, confirmed other studies which demonstrated that the isolation of *P. shigelloides* is good in pure culture (Millership & Chattopadhyay 1984), without contaminant interference and that the utilization of APW as enrichment media and IBB agar as selective media proved efficacious for recovering *P. shigelloides* (von Graevenitz & Bucher 1983).

Although *P. shigelloides* ferments inositol, colonies can appear small and white. In such cases they are difficult to identify on IBB agar. The use of inositol fermentation as screening test facilitated our scheme for identification particularly with respect to highly contaminated water. This procedure may be helpful in the presumptive identification of *P. shigelloides* in the clinical microbiology laboratory.

The enterotoxin test using the infant mouse model has detected several enterotoxins originated from *Yersinia enterocolitica*, *Aeromonas* spp. and *Escherichia coli*. Sanyal et al. (1980), using the same cultivation media for preparation of filtrates, obtained positive results in strains from human origin. Our isolates were negative when tested in this animal model. Attempts to identify other virulence factors in these strains are currently under consideration.

Production of hemolysin plays an important role in iron acquisition *in vivo* via the lysis of erythrocytes, liberating hemoglobin. Although *P. shigelloides* was non hemolytic on the surface of blood agar plates, hemolysin production could be detected by an overlay method, in agreement with the results of Janda and Abbott (1993). Reduce oxygen tension may influence this expres-

sion. Further studies are required for the characterization of this hemolysin and to evaluate its importance as a virulence factor.

The study of antibiotic susceptibility of 46 environmental isolates of *P. shigelloides* showed that there are a large number of antimicrobial agents that are protentially useful for eradicating both systemic and gastrointestinal *P. shigelloides* infections; with exceptions of penicillins. All of our strains isolated produced a ß-lactamase as detected by the nitrocefin method. Our findings were similar to those reported by other authors (Kain & Kelly 1989, Clark et al. 1990), in antibiotic susceptibilities studies of *P. shigelloides* recovered from human sites and environmental sources.

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