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Incidence & enteropathogenicity of *Aeromonas* spp in children suffering from acute diarrhoea in Chennai

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A total of 200 stool samples from children below 10 yr suffering from diarrhoea were screened for enteric pathogens with special interest on *Aeromonas*. *Aeromonas* spp were isolated from 6.5 per cent of the patients, comprising 4 per cent *A. hydrophila*, 2 per cent *A. sobria* and 0.5 per cent *A. caviae*. Among the 13 isolates obtained, 10 isolates produced enterotoxin in ligated rabbit ileal loops, and 11 produced cytotoxin in HEp 2 cells. Many of the *Aeromonas* isolates exhibited resistance to commonly used antibiotics such as trimethoprim, sulphdiazine, chloramphenicol and tetracycline. None of the stool samples obtained from 52 age matched control children yielded *Aeromonas* species. Four isolates of *Salmonella typhi*, 7 of *S. paratyphi* A, 6 of *Shigella flexneri*, 4 of *Sh. dysenteriae* and 3 isolates of *Vibrio cholerae* (Ogawa) were also recovered during the study. Among the samples analyzed, one from a 7 yr old female patient, had *A. hydrophila* with *S. paratyphi* A. The results of this study indicate that drug resistant enteropathogenic *Aeromonas* is also an important etiological agent of childhood diarrhoea in Chennai.

Key words *Aeromonas* - cytotoxin - diarrhoea - enterotoxin

Aeromonas spp are ubiquitous water-borne microorganisms, and are reported to be involved in various illnesses in humans such as wound infections including cellulitis, myonecrosis *etc.*, septicaemia, osteomyelitis, peritonitis, respiratory infections and gastroenteritis[1]. These organisms are being increasingly recognized as an important cause of gastroenteritis world-wide[2-4]. Although the only human volunteer study to demonstrate the enteropathogenicity of *Aeromonas* spp was not encouraging[5], these organisms have been recognized as enteropathogens considering the epidemiological data on their association with gastroenteritis[6,7]. Despite being recognized as an important causative agent of extraintestinal and intestinal illnesses, reports from India are scanty[8-12] . The present study was undertaken to get an insight into the association of aeromonads in cases of childhood gastroenteritis in Chennai city, the species affecting children, the virulence factors associated with the isolates and their response to commonly used antibiotics.

Material & Methods

The study was conducted from July to December 1996 at Chennai. Rectal swabs were collected in duplicate from 200 children below 10 yr of age suffering with acute watery diarrhoea admitted to the Institute of Child Health and Hospital for Children, Chennai. Stool samples were collected from 52 preschool healthy children in the age group of 2½ to 3½ yr, who served as control population for this study. The samples were transported in Cary Blair

medium to the laboratory. One swab was plated onto MacConkey's agar (MA), Salmonella Shigella agar (SSA) and thiosulphate citrate bile salts sucrose (TCBS) medium, and the other swab was inoculated into alkaline peptone water for enrichment. All these culture media were obtained from Himedia, Mumbai. After overnight enrichment at 37°C, a loopful was inoculated on to blood agar (BAA) supplemented with *Aeromonas* selective agent[13] (ampicillin 10 µg/ml, Himedia). The non lactose fermenting colonies (NLFs) on MA and SSA were screened routinely for *Salmonella, Shigella* and the colonies on TCBS for *Vibrio*. The NLFs on MA and SSA and both haemolytic and non-haemolytic colonies on BAA were tested for oxidase and Gram reaction. Colonies of Gram negative bacilli, positive for oxidase were further tested for resistance to the vibriostatic agent[14] O/129 (2,4 diamino, 6,7-diisopropyl pteridine; Sigma). *Aeromonas* isolates were identified and speciated on the basis of biochemical reactions[15] such as esculin hydrolysis, production of acetyl methyl carbinol (VP), production of indole, reaction in triple sugar iron agar, mannitol motility medium, fermentation of sucrose and arabinose.

Toxin studies

(*i*) Preparation of culture supernatant — From the overnight pure cultures of each isolate grown on blood agar, 2-3 colonies were inoculated into 10 ml of BHI broth and incubated in a shaking water bath at 37° C for 18 h. The culture broths were centrifuged at 15000 rpm for 30 min at 4°C and filtered through 0.22 µ filters (Millipore). The culture supernatants were stored in two aliquots at -20° C till further testing, one for enterotoxin and the other for cytotoxin assay.

(*ii*) Enterotoxin assay — Enterotoxin assay was performed by rabbit ileal loop technique[16]. Albino rabbits weighing 1.7 to 2.2 kg were used. The rabbits were starved for 24 h preceding the test with only water being provided during this period. The animals were anaesthetized with 3 per cent sodium pentobarbitone. The abdomen was opened and a series of 4-5 cm long ileal loops starting from the caecum and separated by spacer loop of 2 cm, were made using cotton thread taking care to avoid blood vessels. 1ml of culture supernatant was injected into each loop through the antemesentric border. Positive control (*V. cholerae*) and negative control (sterile BHI) were included in each test. The ileum was replaced in the abdomen which was closed and stitched. The animals were kept under observation and after 16 h, the abdomen was opened and the ileal loops examined for distension and accumulation of watery fluid.

Cytotoxin assay : Cytotoxin assay was performed in triplicate using HEp-2 cell lines[17]. Monolayer of HEp-2 cells was prepared by culturing the cells in 96 well tissue culture plates (Laxbro, India), in minimal essential medium (Eagle's, modified, Flow Labs, UK) supplemented with 10 per cent foetal calf serum for 48 h. The culture filtrates were added to give a final dilution of 1:10. Positive control (*Shigella dysenteriae* type 1) and negative control (sterile BHI) were included in each batch. Positive cytotoxic activity was taken as 50 per cent cell rounding and detachment.

Antibiotic sensitivity of the *Aeromonas* isolates was assessed by disc diffusion[18]. Antibiotics tested include ampicillin and streptomycin (10 µg each); trimethoprim (5 µg) and sulphadiazine, kanamycin, chloramphenicol, nalidixic acid and tetracycline (30 µg each).

Results

Isolation rate and speciation : Of the 200 stool samples analyzed, *Aeromonas* spp were recovered from 13 cases, with an isolation rate of 6.5 per cent, comprising *A. hydrophila* (4%), *A. sobria* (2%) and *A. caviae* (0.5%). The number of isolates of *A. hydrophila* and *A. sobria* from children below 2 yr of age was relatively higher (Table I). The biochemical characteristics of these isolates are given in Table II. We could not recover *Aeromonas* spp from any of the 52 stool samples collected from the healthy controls. In addition to the *Aeromonas* isolates, we could also recover *Salmonella typhi* from 4 patients, *S. paratyphi* A from 7, *Sh. flexneri* from 6, *Sh. dysenteriae* from 4 and *Vibrio cholerae* (Ogawa) from 3 patients. Among the 7 patients from whom *S. paratyphi* A was recovered, a stool sample obtained from a 7 yr old girl also yielded *A. hydrophila* (Table I).

Table I. Isolation of *Aeromonas* species and other enteropathogens from children with acute diarrhoea

Age group, yr	No. of patients	*Aeromonas* species isolated			Other enteropathogens isolated				
		A. hydrophila	*A. sobria*	*A. caviae*	*S. typhi*	*S. paratyphi*-A	*Sh. flexneri*	*Sh. dysenteriae*	*V. cholerae*
< 1	48	1	1	1	-	1	1	1	-
1-2	63	4	2	-	2	3	3	2	1
3-5	57	2	1	-	1	1	2	1	1
5-7	1	1	-	-	1	2	-	-	1
Total	200	8	4	1	4	7	6	4	3

Table II. Biochemical characteristics of *Aeromonas* isolates

Test	*A. hydrophila*		*A. sobria*		*A. caviae*	
	AH 19	Isolates	AS 3	Isolates	AC 36	Isolate
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Indole	+	+	+	+	+	+
Reaction in TSI	A/A, G	A/A, G	K/A, G	K/A, G	A/A	A/A
VP	+	+	+	+	-	-
LDC	+	+	+	+	-	-
ADH	+	+	+	+	+	+
ODC	-	-	-	-	-	-
Esculin hydrolysis	+	+	-	-	+	+
Sensitivity to O/129	-	-	-	-	-	-
Acid from						
Glucose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Arabinose	+	+	-	-	+	+
Mannitol	+	+	+	+	+	+
Lactose	-	-	-	-	-	-

A, acid; K, alkaline; G, gas; TSI, triple sugar iron agar; VP, Voges Proscaur's test; LDC, lysine decarboxylase; ADH, arginine dihydrolase; ODC, ornithine decarboxylase; O/129, 2,4 diamino 6,7 diisopropyl pteridine; AH19, AS3 and AC36 are standard strains from Dr J.M. Janda

Of the 13 isolates, 10 produced enterotoxin as determined by their ability to produce secretory response in the ligated rabbit ileal loops (RILs). Seven isolates of *A. hydrophila* and 3 isolates of *A. sobria* caused significant fluid accumulation in the rabbit ileal loops. *A. caviae* did not induce diarrhoeagenic response in the rabbit ileal loops (Table III). Eight of the 13 isolates produced enterotoxin and cytotoxin.

Table III. Toxin profile of *Aeromonas* isolates

Toxin(s)	*A. hydrophila* (8)	*A. sobria* (4)	*A. caviae* (1)
E+C+	6	2	0
E-C+	1	1	1
E+C-	1	1	0

E, enterotoxin; C, cytotoxin

Table IV. Resistance of *Aeromonas* isolates to antibiotics

Species (no. tested)	No. of isolates resistant to							
	A	Su	Tr	S	K	C	Na	T
A. hydrophila (8)	8	7	8	6	5	5	3	3
A. sobria (4)	4	4	3	3	2	2	2	2
A. caviae (1)	1	1	-	-	1	-	-	-

A, ampicillin; Su, sulphadiazine; Tr, trimethoprim; S, streptomycin; K, kanamycin; C, chloramphenicol; Na, nalidixic acid; T, tetracycline

Cytotoxin assay of the 13 isolates revealed that 11 isolates inclusive of 7 isolates of *A. hydrophila* and three isolates of *A. sobria* were found to be cytotoxic to HEp-2 cells as observed by rounding and detachment of cells. The single isolate of *A. caviae* elaborated only the cytotoxin (Table III).

Antibiogram of the isolates revealed that all the isolates were resistant to ampicillin. More than 90 per cent of the isolates were resistant to sulphadiazine and trimethoprim. Resistance to aminoglycoside antibiotics, chloramphenicol and tetracycline was also seen in a high percentage of isolates (Table IV).

Discussion

In the present study, the isolation rate of *Aeromonas* was found to be 6.5 per cent, inclusive of *A. hydrophila* (4%), *A. sobria* (2%) and *A. caviae* (0.5%). Although several other species such as *A. schuberti, A. veronii, A. trota etc.,* have been reported to occur in stool samples, we could not recover these species from the stool samples analyzed. Non recovery of *A. trota* could be attributed to use of blood agar containing ampicillin during primary isolation[19]. However, this species would have grown on MA or SSA, if present in the samples. Studies on the association of *Aeromonas* in diarrhoeal disease in various cities in India have shown wide variations in the isolation rates ranging from 0.2 per cent in Vellore[9] to 11.96 per cent in Calcutta[20]. Similar isolation rates have been reported from other parts of the world[2,3].

Eight of the 13 isolates obtained in the present study have been found to elaborate one or more virulence factors. In a study conducted at Pondicherry[21], 92.5 per cent of strains of *Aeromonas* produced one or more toxins irrespective of their source and all the 45 isolates of *Aeromonas* obtained by them were reported to be enterotoxigenic in ligated rabbit ileal loops and also haemolytic. It was also reported that *A. hydrophila* was more toxigenic than the other two species. Although our single isolate of *A. caviae* was not found to be enterotoxigenic in the RILs, its cytotoxic activity on the HEp-2 cells supports its role as a gastrointestinal pathogen[17]. Moreover, recently it has been reported that[22] isolates irrespective of the source can be potentially enteropathogenic, since the non-enterotoxigenic isolates of *Aeromonas* produce diarrhoeagenic response upon 1-3 passages in the RILs. Activities such as enterotoxigenicity, cytotoxicity and ability to haemolyze erythrocytes are most likely to be different manifestations of a single toxin and that different strains produced different variations[23].

Aeromonas species are reported to be resistant to penicillin and related antibiotics and sensitive to second and third generation cephalosporins[24] and aminoglycosides, chloramphenicol, tetracycline, trimethoprim-sulphamethoxazole and quinolones. However, the isolates obtained during the present study appear to have assumed resistance to many commonly used antibiotics including the aminoglycosides to which earlier reports had showed susceptibility of aeromonads[11,24].

It can be inferred from this study that gastroenteritis associated with drug resistant, enteropathogenic strains of *Aeromonas* occurs in the Chennai region in about 6.5 per cent instances. However, a large number of patients need to be studied over a period of at least a year to understand the prevalence and seasonal occurrence of *Aeromonas* sp.

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