

PREVALENCE, IN-VITRO SECRETORY ACTIVITY, AND CYTOTOXICITY OF *AEROMONAS* SPECIES ASSOCIATED WITH CHILDHOOD GASTRO- ENTERITIS IN CHENNAI (MADRAS), INDIA

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SUMMARY: An investigation on the prevalence of *Aeromonas* in gastrointestinal illnesses of pediatric inpatients 1 month to 3 years of age was conducted from February 1997 through January 1998 in Madras. Sixteen *Aeromonas* spp. were isolated from 11 male and five female children among the 341 pediatric inpatients suffering from acute diarrhoea. *A. caviae*, which was isolated from nine cases, was found to be the most predominant isolate, followed by *A. veronii* biovar *sobria*, isolated from six cases, and *A. hydrophila*, isolated from one case. *Shigella flexneri* was recovered along with *Aeromonas veronii* biotype *sobria* serotype 035 from one 5-month-old female child. We did not notice any seasonal pattern in the association between *Aeromonas* and childhood gastroenteritis. None of the 147 stool samples obtained from age-matched non-diarrhoeic control children yielded *Aeromonas* spp. Isolation of *Aeromonas* spp. from patients suffering from gastroenteritis was found to be significant ($\chi^2 = 7.1312$; $P = 0.008$, < 0.01). Among the 16 *Aeromonas* isolates, seven isolates of *A. caviae* and two isolates of *A. veronii* biovar *sobria* induced a secretory response in rabbit intestinal mucosa mounted in Ussing chambers as demonstrated by a significant increase in the short circuit current. Nine of the 16 *Aeromonas* isolates, including three isolates of *A. caviae*, five isolates of *A. veronii* biovar *sobria*, and the solitary isolate of *A. hydrophila*

were also cytotoxic to CHO cells. Five of the six isolates of *A. veronii* biovar *sobria* and the *A. hydrophila* isolate produced hemolysin. The results of this study indicate that *Aeromonas* species are important causative agents of diarrhoea in childhood gastroenteritis and are prevalent throughout the year in Madras.

INTRODUCTION

Aeromonas species have also been implicated in a variety of infections in humans such as wound infections (cellulitis), septicemia, and gastroenteritis (1). During the past several years, *Aeromonas* spp. have often been reported as etiological agents of gastroenteritis, especially among young children and the aged (1,2). Increased levels of neutralizing antibodies have been reported in patients convalescing after a bout of *Aeromonas* gastroenteritis (3). The epidemiological data showing increased incidence of toxigenic *Aeromonas* in gastroenteritis support their role in human intestinal infections (4,5). Incidences of travellers' diarrhoea among people travelling from the developed world to developing countries are also often associated with *Aeromonas* spp (6). The *Aeromonas* group of bacteria produce a variety of factors such as hemolysins, enterotoxins, adhesins, siderophores, S-layers and LPS etc., which endow them with virulence and pathogenicity (5). *Aeromonas* has been reported to occur in from about less than 1% to as high as 27% of patients suffering from gastroenteritis worldwide (7). Some studies on the prevalence of these organisms have indicated that they occur more frequently in the summer months in cases associated with gastroenteritis (8,9). Since similar studies from this geographical region were lacking, this study was undertaken to determine the prevalence of *Aeromonas*-associated gastroenteritis, the species involved, and their seasonal occurrence. We also report the in vitro secretory response induced by the *Aeromonas* isolates in intestinal mucosa by employing Ussing chambers.

MATERIALS AND METHODS

Patients: The patients admitted to the diarrhoea ward of the Institute of Child Health and Hospital for Children were included in this study. In most cases, stool samples or rectal swabs were collected from patients before starting antibiotic therapy. The samples were collected in Cary Blair's medium, transported to the laboratory within 2 hr of collection, and immediately processed after reaching the laboratory. Stool samples were also obtained from 147 non-diarrhoeic healthy preschool children in the 2.5 - 3.5 year age group and processed for *Aeromonas* spp.

Clinical data: A questionnaire was developed, and details regarding

patient identification, age, sex, duration of illness, vomiting, number of loose bowel movements a day, stool consistency, blood and mucus in the stool, and abdominal pain were obtained by interviewing the patients. Other details, regarding fever, medication, etc., were obtained from the hospital records.

Isolation and identification of bacteria: Stool samples were inoculated onto MacConkey's Agar (MA), Salmonella-Shigella agar (SSA), and alkaline peptone water and incubated at 37 C for 24 hr. Alkaline peptone water was subcultured onto Inositol Brilliant green Bile salts agar (IBB) and 5% sheep blood agar (SBA). The non-lactose fermenting colonies on MA and SSA were biochemically identified (10). *Salmonella*, *Shigella* and *Vibrio cholerae* were confirmed by slide agglutination using specific antisera. Non-inositol-fermenting colonies exhibiting characteristics of *Aeromonas* spp. were subcultured on nutrient agar and incubated overnight at 37 C. Hemolytic or non-hemolytic colonies on SBA and those subcultured from IBB on nutrient agar were tested for oxidase activity, and the oxidase-positive isolates were further tested for sensitivity to 150 μ g of vibriostatic compound O/129 (2,4-diamino, 6,7-diisopropyl pteridine) (Sigma, St. Louis, MO) and glucose fermentation. Oxidase-positive, glucose-fermenting gram-negative bacteria resistant to O/129 were regarded as *Aeromonas* and were further identified to the species level using Aerokey II (11) based on esculin hydrolysis, gas production in TSI, production of indole and acetyl methyl carbinol, fermentation of sucrose and arabinose, and susceptibility to 30 μ g cephalothin. *A. hydrophila* ATCC 7966, *A. sobria* CIP 224 and *A. caviae* ATCC 13137, were used as control organisms for growth characteristics and biochemical identification of *Aeromonas* species obtained during the study.

Serotyping: All *Aeromonas* isolates were serotyped at the National Institute of Infectious Diseases, Tokyo by Dr. T. Shimada (12).

Toxin assays:

Preparation of culture filtrates: *Aeromonas* isolates along with appropriate controls were grown in brain heart infusion broth (BHI) in 5 ml volumes at 37 C for 24 hr. Cultures were centrifuged at 10,000 rpm at 4 C for 20 min, and the culture supernatants were filtered through 0.22 μ m filters (Millipore, Bedford, MA). These culture filtrates were stored at -20 C and used for the following assays.

Production of hemolysin: The *Aeromonas* isolates were tested for ability to hemolyse sheep erythrocytes in microtiter plates as described earlier (13). Briefly, 50 μ l of BHI grown culture filtrates prepared as above were mixed with 50 μ l of 1% suspension of sheep RBCs in PBS in round bottom 96-well microtiter plates, incubated at 37 C for 1 hr, and

then at 4 C for another 1 hr. Supernatants producing a minimum of 50 % hemolysis were considered positive, and those showing a well formed button of RBCs at the bottom were considered negative.

Secretary response in Ussing chambers: The experiment was performed as described earlier (14). Rabbits weighing 1.5-2.0 kg were sacrificed by cranial shock and the abdomen was opened. About 30 cm of ileum was removed, and the contents were first washed thoroughly in water to remove the particulate intestinal contents and then with Ringer's solution. The ileum was opened along the mesenteric border while immersed in Ringer's solution with aeration. Pieces of ileum about 2.5 cm long were mounted in the Ussing Chambers (World Precision Instruments, City and State, USA), bathed in Ringer's solution at 37 C and aerated with a gaseous mixture of 95% oxygen and 5 % CO₂. The equipment was balanced, and 400 ml of culture filtrates prepared as above were added on both the serosal and mucosal sides. *V. cholerae* Ogawa culture filtrate was used as a positive control, and sterile LB medium was used as a negative control. Variations in the transmembrane potential difference (PD) and short circuit current (I_{sc}) were measured every 15 min for up to 90 min. Each isolate producing a positive response was tested three times.

Tissue culture assay: Cytotoxicity of the *Aeromonas* isolates against CHO cell monolayers was tested by the method of Guerrant et al (15). The CHO cell monolayers were grown in Modified Eagle's minimum essential medium (MEM)(ICN Biomedicals Inc., Thame, Oxfordshire, UK) supplemented with 10% fetal calf serum in 96-well microtiter plates. To each well, 25 μ l of the culture filtrates of test organisms prepared as above were added to the monolayers in microtiter plates in triplicate. A cytotoxic laboratory strain of *Vibrio cholerae* was used as a positive control, while sterile BHI medium served as a negative control. Microtiter plates were incubated at 37 C, and the cell lines were observed up to 48 hr for characteristic elongation as described by earlier workers (14).

RESULTS

Clinical Features and Etiologic Agents

Stool samples were processed from 341 patients reporting with diarrhoea for *Aeromonas* species and other enteropathogenic bacteria *Salmonella*, *Shigella* and *Vibrio cholerae*, and 97% of the patients were in the 1-month to 3-year age group. *Aeromonas* spp. were isolated from 16 children, comprising 11 males and five females. Fifteen of these children from whom *Aeromonas* spp. were recovered were in the 3- to 12-month age group, and one isolate of *A. caviae* serotype O27 was recovered from a 4-year-old female child. *A. caviae* was isolated from the stool samples of

nine children, *A. veronii* biovar *sobria* from six cases, and *A. hydrophila* from one case. During this investigation, we also recovered *Salmonella paratyphi* A from six cases and *Vibrio cholerae* from five cases (Table I). In one case, *Shigella flexneri* was recovered from the stool sample of a 5-month-old female child along with *Aeromonas veronii* biovar *sobria* serotype O35.

None of the 147 stool samples obtained from the healthy children yielded *Aeromonas* species. Comparison of the isolation rates between the two groups indicated that isolation of aeromonads from the diarrhoeic

Table I. Isolation of *Aeromonas* spp. and other enteric pathogens from patients with diarrhoea

Pathogen	Number of isolates
<i>Aeromonas hydrophila</i>	1
<i>Aeromonas veronii</i> biovar <i>sobria</i>	6
<i>Aeromonas caviae</i>	9
<i>Salmonella typhi</i>	2
<i>Salmonella paratyphi</i> A	6
<i>Salmonella</i> sp	1
<i>Vibrio cholerae</i> (Ogawa)	4
<i>Vibrio cholerae</i> (O139)	1

Table II. Clinical features of 16 patients with *Aeromonas*

Associated diarrhoea	
Features	Number of patients
Fever: mild	6
moderate	10
Vomiting	12
Abdominal pain	1
Type of stool:	
watery	15
blood	0
semi-solid	1
Duration of diarrhoea (days)	2-7
Number of stools/day	4-20
Extent of dehydration:	
mild	5
moderate	6
severe	4
nil	1

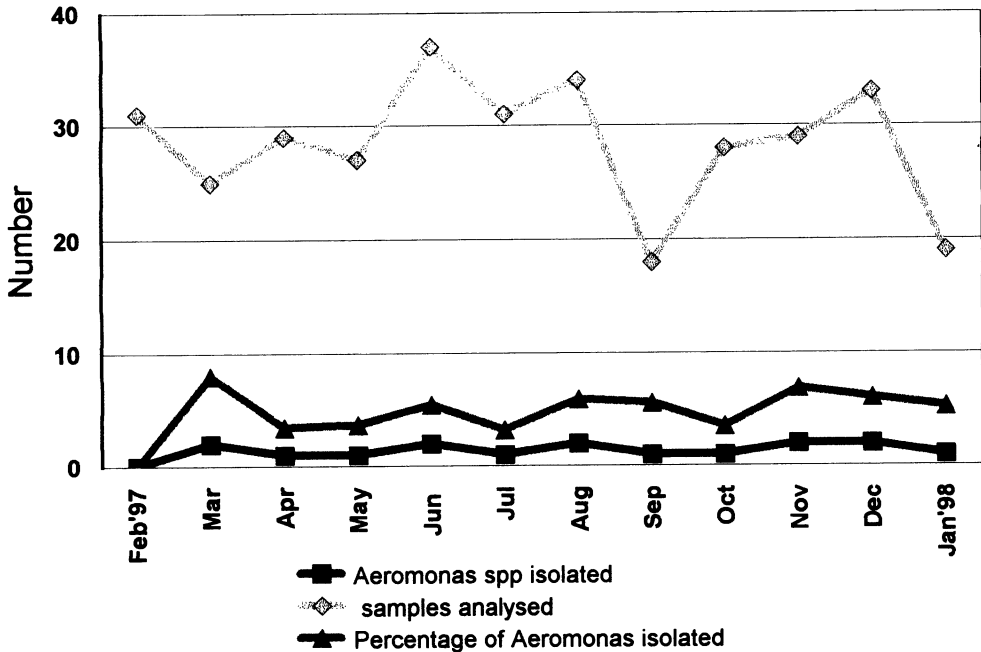


Fig. 1. Prevalence of *Aeromonas*-associated gastroenteritis in children

stool samples was significant ($\chi^2=7.1312$; $P=0.008$, <0.1). All 16 *Aeromonas* species were recovered on SBA and IBB as moderate to heavy growth after enrichment in APW. Only four of these isolates could be obtained on primary plating on MA, indicating need for their enrichment. Isolates obtained from both primary plating and upon enrichment in APW and subsequent subculture on IBB or BA were regarded as one isolation.

Clinical features: Patients from whom *Aeromonas* spp. were recovered had mild to moderate fever and diarrhoea ranging between 2 to 7 days. Most patients had watery diarrhoea, passing 4-20 watery loose stools a day. Twelve of the 16 patients reported vomiting associated with diarrhoea. Four patients were severely dehydrated, and the rest of them showed mild to moderate signs of dehydration (Table II). One 5-month-old female patient whose stool sample yielded *Shigella flexneri* along with *A. veronii* biovar *sobria* was severely dehydrated, suggesting probable synergistic action of both enteropathogens.

Prevalence: The *Aeromonas* spp. were isolated from the stool samples of children suffering from acute diarrhoea more or less uniformly throughout the year from February 1997 to January 1998 (Fig. 1).

Virulence Characteristics

Hemolysin production: Five *A. veronii* biovar *sobria* isolates and the *A. hydrophila* isolate produced hemolysis according to the results of the microplate method. These organisms were also found to produce hemolysin on the SBA.

Ussing chambers: There was a significant increase in the short circuit current (Δ I_{sc}) of the toxigenic laboratory strain of *Vibrio cholerae* and the nine *Aeromonas* isolates after 15 min. Peak activity was generally noticed at 45 min. In contrast, no significant variations in tissue conductance were noted. Eight of the nine *Aeromonas* isolates induced a significant increase in the short circuit current (Δ I_{sc}) of more than 17 μ A, and one *A. veronii* biovar *sobria* isolate induced an increase up to 41 μ A (Fig. 2).

Cytotoxin production: The culture filtrate from the *Vibrio cholerae* laboratory strain used as a positive control showed significant cytotoxicity as evidenced by cell elongation, cell detachment, and cell disintegration, while the plain medium which was used as negative control did not show any cytotoxic activity. Ten of the culture filtrates of the test strains of *Aeromonas* spp., consisting of five isolates of *A. veronii* biovar *sobria*, four isolates of *A. caviae*, and a culture filtrate of *A. hydrophila*, showed pronounced cytotoxic activity (Table III).

Among the 16 isolates of *Aeromonas* spp. isolated from the stool samples obtained from the children suffering from acute diarrhoea, one isolate of *A. veronii* biovar *sobria*, and three isolates of *A. caviae* did not produce any of the virulence factors tested for above.

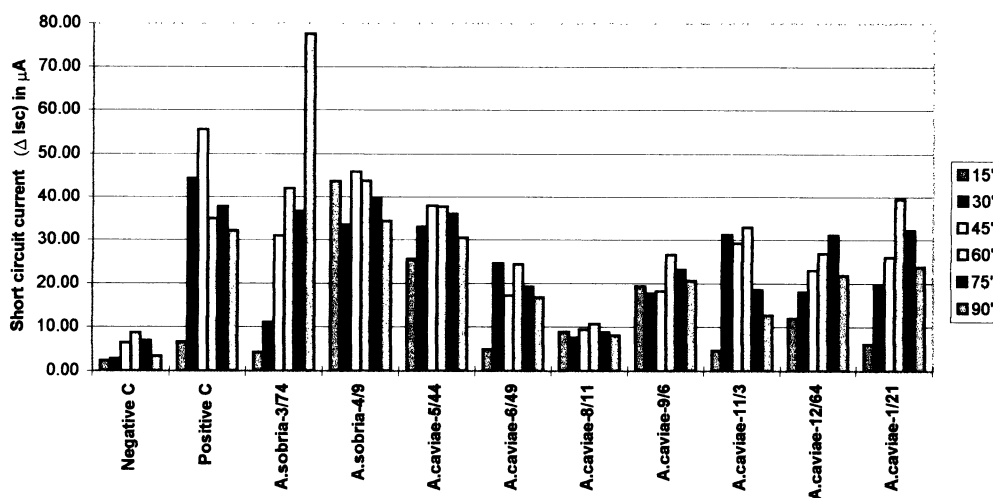


Fig. 2. Secretory response of *Aeromonas* isolates in Ussing Chambers

Table III. Virulence characteristics of *Aeromonas* isolates

<i>Aeromonas</i> species (Isolate Number)	Serotype	Hemolysin	Secretory Response in Ussing Chambers	Toxicity to CHO cells
<i>A. hydrophila</i> (8/143)	O21	+	—	+
<i>A. sobria</i> (3/8a)	O35	+	—	+
<i>A. sobria</i> (3/59)	O27	—	—	—
<i>A. sobria</i> (3/74)	O35	+	+	+
<i>A. sobria</i> (4/9)	R	—	+	+
<i>A. sobria</i> (6/143)	O32	+	—	+
<i>A. sobria</i> (8/11)	O13	+	+	+
<i>A. caviae</i> (5/44)	O14	—	+	—
<i>A. caviae</i> (6/49)	O16	—	+	+
<i>A. caviae</i> (9/6)	R	—	+	+
<i>A. caviae</i> (11/3)	UK	—	+	—
<i>A. caviae</i> (11/54)	R	—	—	—
<i>A. caviae</i> (12/33)	R	—	—	—
<i>A. caviae</i> (12/35)	ND	—	—	—
<i>A. caviae</i> (12/64)	ND	—	+	—
<i>A. caviae</i> (1/21)	ND	—	+	+

R: Rough type; UK: Unknown; ND: No data.

DISCUSSION

During this study, we were able to recover aeromonads from nearly 5 % of the children, mostly below the age of one year, suffering from acute watery diarrhoea in this city. Earlier studies have showed rates of aeromonal gastroenteritis ranging from <1 % to 12.8% in the other localities in India (7, 8, 16), whereas the incidence has been reportedly quite low in the developed countries (7).

Although our primary objective in this study was to assess the prevalence of *Aeromonas*-associated gastroenteritis, we also looked for some of the recognized enteropathogens such as *Salmonella*, *Shigella*, and *Vibrio*. Due to certain practical difficulties, we could not include protocols for detection of rotavirus, *Clostridium difficile*, *Yersinia*, *Campylobacter*, and other enteropathogens. The cumulative isolation rate of *Salmonellae*, *Vibrio*, and *Shigellae* was about 4.1% *vis-a-vis* 4.7% for *Aeromonas* species in the present study.

Aeromonal gastroenteritis has been reported to be especially prevalent during the summer months (8, 9, 17). However, in the current study we failed to notice such a pattern, and the aeromonal gastroenteritis cases were found to be prevalent almost throughout the entire year.

Colonization of the gastrointestinal tract occurs during the first week of life in 23.1% of the newborns with *Aeromonas* spp. (18), and they can serve as source of infection and disease. Our preliminary studies on the occurrence of *Aeromonas* in drinking water sources from several locations in the city have revealed that nearly 30% of the samples yielded *Aeromonas* spp. (unpublished data). Considering the fact that aeromonads are natural inhabitants of aquatic environments, consumption of untreated water and even contaminated food appears to be the source of *Aeromonas* infection in these individuals, as observed earlier (19).

All the aeromonal gastroenteritis cases recorded during the present investigation were of chronic, acute type of diarrhoea. We could not recover any aeromonads from dysenteric cases, although aeromonads are also known to be involved in the dysenteric type of gastroenteritis (1).

In recent years, new species have been added to the list of *Aeromonas* species (20). Presently, the *Aeromonas* species includes 12 legitimate species and 14 genomospecies (21). Identification of genomospecies by using phenotypic characteristics has been fraught with difficulty, since there appears to be no correlation between the phenotypic characteristics and the various genomospecies, although some schemes to identify genomospecies have been proposed (22). However, the Aerokey II, which uses eleven phenotypic characteristics, makes it possible to identify seven commonly encountered mesophilic *Aeromonas* species (11). In the present study, we found it convenient to use this scheme, and we were able to demonstrate the three most common *Aeromonas* species encountered in clinical samples. *A. hydrophila*, *A. caviae*, and *A. sobria* have been reported to be the predominant aeromonads associated with gastroenteritis (23, 24), and *A. caviae*, in particular, is known to be the predominant species affecting children under 2 years of age (25). The present study has confirmed these findings in this geographic region as well.

Among the nine *Aeromonas* spp. which evoked a secretory response in the rabbit intestinal mucosa mounted in Ussing chambers, culture supernatants of two isolates of *A. veronii* biovar *sobria* and four isolates of *A. caviae* evoked a significant increase in the short circuit current (ΔI_{sc}) within a short time and peak activity was generally seen after 45 min of addition of the culture filtrates. For the first time we have shown the enteropathogenicity of culture filtrates of *Aeromonas* strains by means of increase in I_{sc} in Ussing chambers.

Cytotoxic strains of *Aeromonas* spp. have usually been reported to be associated with infections in older humans (20). In the present instance, nine of the 16 aeromonads isolated from children under 1 year were found to be cytotoxic to CHO cells. Six of these cytotoxic isolates were also

hemolytic. Incidentally, six of the cytotoxic isolates produced a secretory response in the Ussing Chambers as well. It was reported earlier that *Aeromonas* spp. associated with childhood gastroenteritis are enterotoxigenic, while those isolated from healthy individuals rarely show enterotoxigenic properties (9). The fact that *Aeromonas* spp. were recovered from 16 children suffering from acute watery diarrhoea and that 12 of these isolates exhibited one or more virulence features such as production of hemolysin, secretory activity in vitro, and toxicity to CHO cells suggests that motile aeromonads are among the important etiologic agents of childhood gastroenteritis in this geographic region.

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