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Enterotoxigenic *Aeromonas* spp and *Plesiomonas shigelloides* in HIV AIDS Patients Reporting with Diarrhoea

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An investigation on the role of *Aeromonas* and *Plesiomonas shigelloides* in the gastrointestinal illnesses of HIV AIDS patients was conducted. Out of the 34 HIV AIDS patients reporting with diarrhoea, *Aeromonas veronii* biovar *sobria* was isolated from the stool samples of two patients, and *Aeromonas caviae* and *Plesiomonas shigelloides* from one patient each. Other enteric pathogens isolated from the HIV infected gastroenteritis patients included one isolate each of *Salmonella typhi, Salmonella paratyphi* A, *Salmonella* sp. and *Vibrio cholerae* (Ogawa). Culture filtrates of the isolates of *Aeromonas* and *Plesiomonas* stimulated enterotoxigenic response in the ligated rabbit ileal loops. Culture filtrates of the three *Aeromonas* - isolates also induced secretory response in the rabbit intestinal mucosa mounted in the Ussing chambers as revealed by increase in the short circuit current indicating enterotoxigenic nature of these isolates. The *Pl. shigelloides* isolate failed to show similar effect in Ussing Chambers. All the three *Aeromonas* isolates were also cytotoxic to Vero and CHO cells while *Pl. shigelloides* was non cytotoxic. Only one of the two *A. veronii* biovar *sobria* isolates produced haemolysin. The study indicated association of *Aeromonas* species and *Pl. shigelloides* as causative agents of diarrhoea in HIV AIDS patients.

Key words: *Aeromonas* spp, HIV AIDS, *Plesiomonas shigelloides*, diarrhoea, enterotoxigenicity, gastroenteritis.

In the HIV AIDS patients, diarrhoea is a major complication and occurs in nearly 90% of the cases in developing countries (1). The most common pathogens reported to be associated with diarrhoea and dysentery in the AIDS cases are parasites like *Cryptosporidium*, *Giardia*, and bacteria such as *Salmonella*, *Shigella*, *Campylobacter* and *Vibrio* (2-4). During the past several years, *Aeromonas* spp have been often reported as etiological agents of gastroenteritis especially among young children and aged (5,6). *Aeromonas* associated diarrhoea has been reported from several cities in India (7-9). *Aeromonas* group of bacteria by virtue of their haemolysins, enterotoxins, adhesins, iron sequestering siderophores, S-layers and LPS assume pathogenicity (10). Studies on the association *Aeromonas* and *Plesiomonas* in HIV-positive individuals suffering from gastroenteritis are scanty (11). Hence, this investigation was undertaken with the objective to study frequency of association of enterotoxigenic *Aeromonas* in gastrointestinal illnesses of HIV AIDS patients suffering from acute diarrhoea.

Materials and Methods

Patients: The Government Hospital for Chest Diseases, Chennai (Madras), admits patients suffering from HIV and pulmonary tuberculosis. The HIV cases are confirmed by western blot test. Among these cases, 34 patients complaining of more than 3 loose stools d^{-1} for at least two days were selected for this study. Stool samples from patients suffering from diarrhoea were collected in sterile containers and transported to the laboratory within 1 h of collection. Immediately the samples were examined for *Aeromonas* and *Plesiomonas* and other enteropathogenic bacteria *Salmonella, Shigella* and *Vibrio cholerae.*

Stool samples were also collected from 31 healthy HIV negative adults inclusive of 13 females and 18 males between the age group of 23 to 40 and examined for enteropathogens particularly for *Aeromonas* species.

Clinical data: Details of patents identification, age, sex, duration of illness, vomiting, number of loose motions per day, consistency of stool, blood and mucous in the stool and abdominal pain were obtained by interviewing the patients and other information regarding fever, underlying illnesses, opportunistic infections, medication etc. were obtained from the hospital records. Of the 34 HIV patients, 28 were males and 6 were females. Of the 28 male patients, 3 were 45-51 years and 25 were between 25-37 The 6 female patients were in age group 21-35 years.

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Isolation and identification of bacteria: Stool samples were inoculated onto MacConkey agar (MA), Salmonella Shigella agar (SSA), thiosulphate citrate bile salts sucrose agar (TCBS) and alkaline peptone water (APW); and incubated at 37°C for 24 h. A loopful from APW was subcultured onto inositol brilliant green bile salts agar (IBB) and 5% sheep blood agar (SBA). MA, SSA, TCBS, IBB and Blood agar base were purchased from HiMedia, Mumbai, India. The non-lactose fermenting colonies on MA and SSA were biochemically identified by conventional methods (12). *Salmonella, Shigella* and *Vibrio cholerae* were confirmed by slide agglutination using specific polyvalent antisera. Both inositol fermenting and non-inositol fermenting colonies on IBB appearing like *Plesiomonas shigelloides* and *Aeromonas* spp., respectively were subcultured on nutrient agar and incubated overnight at 37°C. Haemolytic or non-haemolytic colonies on SBA and those subcultured from IBB on nutrient agar were tested for oxidase reaction. The oxidase positive isolates were further tested for sensitivity to 150 μg of vibriostatic compound O/129 (2,4-diamino, 6,7-diisopropyl pteridine) (Sigma) and glucose fermentation. Oxidase positive, glucose fermenting Gram negative bacteria resistant to O/129 were regarded as *Aeromonas* and were further identified to species level using Aerokey II (13) based on the esculin hydrolysis, gas production in TSI, production of indole, acetyl methyl carbinol, fermentation of sucrose and arabinose and susceptibility to 30 μg cephalothin. *A. sobria* CIP 224, *A. caviae* ATCC 13137, and *Plesiomonas shigelloides* 12726 were used as control organisms for growth characteristics and biochemical identification of the isolates of *Aeromonas* and *Plesiomonas*. Inositol fermenting colonies on IBB medium were further confirmed for their ability to ferment inositol by Hugh and Leifson's method (12). Isolates found sensitive to 150 μg of O/129, positive to lysine, ornithine decarboxylase and arginine dihydrolase and negative to mannitol were regarded as *Plesiomonas shigelloides* (14).

Toxin assays

Preparation of cell free culture filtrates: Aeromonas-isolates along with appropriate controls were grown in 5 ml of brain heart infusion broth (BHI) at 37°C for 24 h. Cultures were centrifuged at 10,000 rpm at 4°C for 20 min and the culture supernatants were passed through 0.22 μm (Millipore) filters. These culture filtrates were stored at -20°C and used for following assays.

Production of haemolysin: The *Aeromonas* isolates were tested for haemolysis of sheep erythrocytes in microtitre plates as described earlier (15). Briefly, 50 μl of BHI grown culture filtrate prepared as above was mixed with 50 μl of 1% suspension of sheep RBCs in phosphate buffered saline (PBS, pH 7.4) in 96 U-well microtitre plates (Tarsons, Calcutta, India). Incubated at 37°C for 1 h and then at 4°C for overnight. Haemolysin production was indicated by release of red pigment into the upper suspension with a small RBC pellet at the bottom of the well. Negative reaction was indicated by a well-formed button of RBCs at the bottom with colorless supernatant.

Rabbit ileal loop assay : Aeromonas and *Plesiomonas* isolates were tested for production of enterotoxin by injecting 1 ml of the culture filtrates into the ligated rabbit ileal loops (RIL) (16). Albino rabbits weighing about 2.0 kg were used for the assay. An enterotoxigenic laboratory strain of *V. cholerae* (Ogawa) was included as a positive control. Sterile BHI medium was used as a negative control.

Detection of enterotoxin in Ussing chambers : The experiment was performed as described by Fasano and co-workers (17). Rabbits weighing 1.5-2.0 kg were sacrificed by cranial shock and the abdomen was opened. About 20 cm of ileum was removed and the contents were washed thoroughly in Ringer's solution. The ileum was opened along the mesenteric border while keeping immersed in Ringer's solution with aeration. Rectangular pieces of ileum of about 2 cm were mounted in the Ussing Chambers (World Precision Instruments, USA), bathed in Ringer's solution at 37°C and aerated with a gaseous mixture of 95% oxygen and 5% CO_2. The equipment was balanced and 400 μl of culture filtrates prepared as above were added on both serosal and mucosal sides. *V. cholerae* Ogawa culture filtrate was used as positive control and sterile BHI medium was taken as a negative control. Variations in the short circuit current (ΔIsc) and transmembrane potential difference (PD) were measured every 15 min in a period of 90 min. Each isolate was tested thrice in this system.

Cytotoxicity assay: Cytotoxicity of the *Aeromonas* and *Plesiomonas* isolates on Vero and CHO cell monolayers

was performed following the methods described earlier (18, 19). Vero and CHO cell monolayers were grown in modified Eagles minimum essential medium (ICN Biomedicals Inc, Costa Mesa, UK) supplemented with 10% fetal calf serum in 96 well microtitre plates. To each well, 10 µl of the culture filtrates of test organisms prepared as above were added to the monolayers in microtitre plates in triplicate. Cytotoxic laboratory strains of *S. dysenteriae* and *V. cholerae* (Ogawa) were used as a positive controls for vero cell and CHO cell cytotoxicity respectively, while sterile BHI served as a negative control. Microtitre plates were incubated at 37°C for 24 h and Vero cell lines were observed for cell rounding and detachment, while CHO cell monolayers were examined for elongation, detachment disintegration of cells.

Enteric parasites: The stool samples were fixed in 4% formalin. Stool smears were microscopically examined for the pink spherical oocysts of *Cryptosporidium* species by modified Kinyoun's acid fast staining (20), and other enteric parasites such as *Giardia lamblia*, *Entamoeba histolytica* and *Ascaris lumbricoides* by conventional iodine staining.

Results and Discussion

Enteric bacteria: In this study, *Aeromonas* species were recovered from the stool samples of 3 of the 34 HIV infected patients suffering from gastroenteritis. *A. veronii* biovar *sobria* (A3) was isolated on IBB medium upon enrichment in APW from a 51-year-old man, who was severely dehydrated with high fever, accompanied by rigors. The patient passed more than 6 loose stools a d-1 for over two weeks and excreted blood and mucous in the stools. Stool sample from another 25-year-old male patient complaining of chronic intermittent watery diarrhoea for two years yielded *A. veronii* biovar *sobria* (A4) upon enrichment in APW and subculture on the IBB medium. The patient passed more than 10 stools d^{-1} and did not complain of vomitting, abdominal pain or fever. *A. caviae* (A6) was isolated on IBB medium from a 28-year-old male patient complaining of diarrhoea for over two months, passing more than 10 stools d^{-1} with severe dehydration. *Pl. shigelloides* (A5) was also isolated on the IBB medium after enrichment in APW from a 37-year-old male patient suffering from watery diarrhoea along with abdominal pain for 4 d, passing stools more than 6 times d^{-1}, accompanied by vomiting. The patient was afebrile and had no abdominal pain. The stool samples of these patients were negative for enteric parasites.

During this study, *Salmonella* sp., *S. typhi, S. paratyphi A* and *V. cholerae* were also isolated from the stool samples of four other HIV patients.

Enteric parasites: In this study, *Cryptosporidium* species could be implicated as the sole pathogen in four patients, *Giardia lamblia* in one case, *Ascaris lumbricoides* and *Entamoeba histoloytica* in two cases each. In one case, *G. lamblia* was detected along with *V. cholerae* and in another case, *A. lumbricoides* was observed in the stool sample of a patient from whom *Salmonella typhi* was recovered. Previous studies on the etiologic agents of diarrhoeal illness in AIDS patients have indicated the predominance of *Cryptosporidium* sp. (1, 21). The stool samples from the 31 healthy control subjects did not yield any enteropathogenic bacteria. Stool sample of one person had cysts of *G. lamblia*.

Enterotoxigenicity: During recent past, enterotoxigenicity of enteric pathogens such as *Shigella*, enteroaggregative *Escherichia coli*, and parasites like *Cryptosporidium* has been demonstrated *in vitro* in Ussing chambers (22-24). The three *Aeromonas* spp isolated during this study evoked notable increase in the ΔIsc within a short time and showed peak activity in 60 min of addition of the culture filtrates in Ussing Chambers (Fig 1). These isolates were also enterotoxigenic in the *in vivo* RIL model. The culture filtrates of *Aeromonas* isolates induced secretory

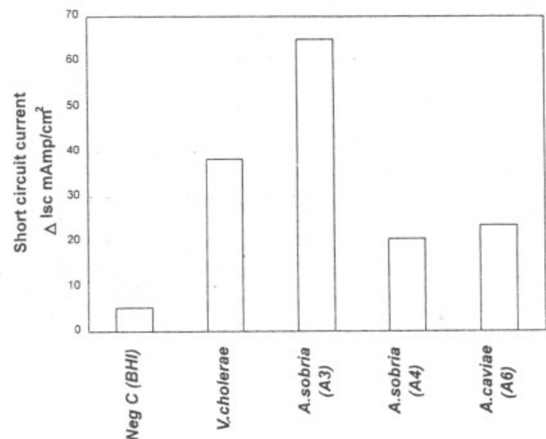

Fig 1. Average increase in short circuit current (ΔIsc) upon exposure of rabbit ileal tissue mounted in Ussing Chambers to culture filtrates of *Aeromonas* spp.

Table 1. Virulence characteristics of *Aeromonas* and *Plesiomonas* isolates

Isolate	Production of β-haemolysin	Cytotoxicity		Secretory response in	
		Vero	CHO	RIL	Ussing chambers
Aeromonas veronii biovar *sobria* (A3)	+	+	+	+	+
Aeromonas veronii biovar *sobria* (A4)	+	+	+	+	+
Aeromonas caviae (A6)	-	+	+	+	+
Plesiomonas shigelloides (A7)	-	+	-	+	-

response with fluid accumulation ranging from 1.07 to 1.16 ml cm^{-1} of the loop. Among the three *Aeromonas*-isolates and one *Pl. shigelloides* isolate, only *A. veronii* biovar *sobria* (A3) was β-haemolytic on SBA. In the microtitre haemolysin assay, this isolate was found to show haemolytic activity at the titre of 1:16. Other *A. veronii* biovar *sobria* (A4) also produced haemolysin in microtitre assay, but with a relatively low titre (1:8). *A. caviae* (A6) and *Pl. shigelloides* (A5) isolates did not produce haemolysin in the microtitre assay. The culture filtrates of *Aeromonas* isolates also showed pronounced cytotoxic activity. The single isolate of *Pl. shigelloides* caused Vero cell rounding followed by cell death, while it was non-cytotoxic to CHO cell monolayers (Table 1). Although *A. caviae* evoked relatively less secretary response in the Ussing chambers compared to the *V. cholerae* control isolate, the classical RIL experiment and the cell culture toxicity assay confirmed enteropathogenicity of this isolate. These results correlate well with watery diarrhoea suffered by the patients.

So far only a few studies have shown the association of aeromonads with immuno-compromised individuals. Patients with malignancies and those with acute leukemia or hepatic involvement are reported to be predisposed to *Aeromonas* and *Plesiomonas* infections (11). San Joaquin and Piquet (25), reported isolation of *A. hydrophila*, *A. caviae* and *A. sobria* from one immunosuppressed child during separate episodes of diarrhoea. In another study, isolation of *Aeromonas* sp. has also been reported from the stool and blood samples of a patient suffering from acute gastroenteritis and septicemia following partial gastrectomy (26).

Many of the infections in AIDS patients are caused by opportunistic organisms which have low virulence, that were considered to be of little clinical importance in the normal host (4). In view of the increasing reports on the association of *Aeromonas* spp. in human disease, especially, isolation of enterotoxigenic *Aeromonas* and *Plesiomonas* strains from the patients suffering from diarrhoea reinforces their role in gastroenteritis of immunosuppressed patients. The results of this study indicate that aeromonads are also enteropathogens associated with gastroenteritis in HIV patients.

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