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Aeromonas spp. and Their Association with Human Diarrheal Disease

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Between January 1988 and December 1989 *Aeromonas* species were isolated from 45 (1.8%) of 2,480 patients with acute gastroenteritis. No other bacterial enteric pathogens were found in any of these 45 patients. Of the 45 *Aeromonas* isolates, 35 strains (77.8%) were *Aeromonas hydrophila*, 7 (15.5%) were *Aeromonas sobria*, and 3 (6.7%) were *Aeromonas caviae*. Most of the patients were under 5 years of age. No bacterial enteric pathogens, including *Aeromonas* species, were isolated from 512 age- and sex-matched control subjects. Examination of the *Aeromonas* isolates for exotoxin production (enterotoxin and hemolysin) indicated that all strains, irrespective of species, were enterotoxin positive (rabbit ileal loop model) and hemolysin positive (rabbit erythrocyte model). These results suggest that *Aeromonas* species are potential enteric pathogens in our geographical region.

Motile aeromonads have been recognized with increasing frequency as human enteric pathogens. Several reports have implicated *Aeromonas* species as a cause of gastroenteritis in children and adults (1–4, 7, 8, 10, 12, 14–18, 26, 27, 29, 31, 33, 36, 38, 41). However, the role of *Aeromonas* species in gastrointestinal infection remains controversial. Several virulence factors such as enterotoxins, hemolysins, cytotoxins, and adhesins have been described (21, 22, 24, 37, 40). Several recent studies suggest a correlation between phenotypic characteristics of *Aeromonas* species and gastrointestinal disease (20, 21, 40). Therefore, the present study was undertaken to assess the incidence and significance of *Aeromonas* species in cases of acute gastroenteritis. The isolates were tested for exotoxin production, i.e., enterotoxin and hemolysin production.

MATERIALS AND METHODS

Over a 2-year period (January 1988 to December 1989), stool samples or rectal swabs from 2,480 patients with acute gastroenteritis were screened for *Aeromonas* spp. as well as for other bacterial enteropathogens such as vibrios, *Salmonella* spp., *Shigella* spp., enteropathogenic and enterotoxigenic *Escherichia coli*, *Yersinia* spp., *Campylobacter* spp., and *Plesiomonas* spp. No attempts were made to study rotavirus. Most of the patients were <5 years old (n = 2,033). Of 2,480 patients, 61 were outpatients and others had been admitted to pediatric and medical wards of our institution. The patients were from a low socioeconomic group.

Stool specimens were received in sterile McCartney bottles (9), and rectal swabs were received in Cary-Blair transport medium. The specimens were processed within 2 h of collection. During the same period, stool samples from 512 age- and sex-matched control patients (admitted to our hospital for illnesses other than gastroenteritis) were also studied.

Isolation and identification of *Aeromonas* strains. For isolation of *Aeromonas* spp., all fecal samples were plated directly on ampicillin (10 μg/ml)-sheep blood agar. Subcultures were made on the same agar after overnight enrichment in alkaline peptone water (pH 8.6) at 37°C. The plates were incubated for 48 h at 37°C. Presumptive identification of *Aeromonas* sp. was made on the basis of oxidase positivity and resistance to vibriostatic agent 0/129. Oxidase-positive colonies were identified and confirmed as *Aeromonas* sp. by conventional biochemical methods (25). *Aeromonas* isolates were identified to the species level by the following biochemical tests, proposed by Popoff (32): Voges-Proskauer, esculin hydrolysis, growth in potassium cyanide broth, gas production from glucose and glycerol, acid production from salicin and arabinose, L-histidine and L-arginine utilization, and production of hydrogen sulfide from cysteine. All tests were performed at 37°C. Results were read daily for 5 days. All strains were tested for hemolytic activity on sheep blood agar, and plates were incubated at 37°C for 48 h. *Aeromonas* strain ATCC 7966 was used as a control for biochemical identification and exotoxin studies.

Isolation of other bacterial pathogens. All fecal samples were also plated on MacConkey agar, salmonella-shigella agar (Oxoid), thiosulfate-citrate-bile salt-sucrose agar, cefsulodin-Irgasan-novobiocin agar (Oxoid), and Campy-BAP medium (Oxoid). Enteric media except Campy-BAP were incubated overnight at 37°C. Campy-BAP plates were incubated in an atmosphere of 5% O_2–10% CO_2–85% N_2 at 42°C for 48 h. All media were screened for potential enteric pathogens by standard bacteriological methods. Comparison of results for *Aeromonas*-infected and control groups of patients was done statistically by the chi-square test (23).

Exotoxin assays. Culture filtrates of *Aeromonas* strains were tested for the production of enterotoxin and hemolysin. The culture filtrates were prepared as follows. The organisms were cultivated in 50 ml of Trypticase soy broth (BBL Microbiology Systems) supplemented with 0.6% yeast extract in 250-ml Erlenmeyer flasks. The flasks were incubated at 37°C in a shaking water bath (120 oscillations per min) for 18 h. The cultures were centrifuged at 22,000 × g for 30 min at 4°C. The supernatants were filtered through membrane filters (0.45-μm pore size; Millipore Corp.), stored at 4°C, and tested within 24 h.

Rabbit ileal loop assay. The rabbit ileal loop assay, was performed with live cells and culture filtrates of all strains of *Aeromonas* spp. in ileal loops of adult albino rabbits weigh-

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ing 1.5 to 2.0 kg as described by Sanyal et al. (37). The inoculum was 1 ml of a 10^{-3} saline dilution of a live 3- to 4-h culture of the organism in peptone water or 1 ml of a cell-free culture filtrate. Live cells or culture filtrate of the toxigenic *Vibrio cholerae* 569B served as a positive control, and isotonic saline or Trypticase soy broth was the negative control. The animals were sacrificed 8 h after inoculation, and the reaction in the loops was noted. The length of the each loop and the volume of the fluid in it were measured to determine the accumulation of fluid per centimeter of gut. Fluid accumulation of >0.5 ml/cm of gut was considered positive. Each strain or culture filtrate was tested in duplicate. The results were analyzed statistically, using Student's *t* test (23). For histopathological study, each loop was cut open through the entire length and fixed in 10% Formalin (38).

Effect of temperature. Culture filtrates were heated in a water bath at 56 and 60°C for 5, 10, 15, 20, 25, and 30 min. The enterotoxic activity of the heat-inactivated culture filtrates was tested by the rabbit ileal loop assay as described above.

Hemolysin assay. A hemolysin assay was performed in microtiter plates by the method described by Burke et al. (4). In brief, doubling dilutions of culture filtrates were made in phosphate-buffered saline (pH 7.4). An equal volume of a 1% suspension of fresh, washed rabbit erythrocytes was added. Trypticase soy broth and phosphate-buffered saline blanks were included with each test. Trays were incubated at 37°C for 1 h and then at 4°C for 1 h. The hemolysin titer was recorded as the highest serial dilution showing 50% hemolysis of the erythrocytes (20). A hemolysin titer of >1:4 dilution was considered positive. Each culture filtrate was tested in duplicate.

RESULTS

A total of 45 *Aeromonas* strains were isolated from 2,480 patients with acute diarrhea, an isolation rate of 1.8%. No *Aeromonas* species was isolated in the control group of 512 patients ($\chi^2 = 9.56$; $P < 0.01$). Of the 45 strains isolated, 35 (77.8%) were identified as *Aeromonas hydrophila*; 7 (15.5%), as *A. sobria*; and 3, (6.7%), as *A. caviae*. All *Aeromonas* isolates were detected in large numbers on ampicillin (10 μg/ml)-sheep blood agar on primary isolation. No additional isolates were detected following overnight enrichment in alkaline peptone water. Aeromonads were not associated with any other known bacterial agents of diarrhea in any of these 45 patients. Three-fourths of the fecal samples positive for *Aeromonas* spp. ($n = 34$) were obtained during the summer months of February to May in Bombay.

Other bacterial enteropathogens isolated from 2,480 patients with diarrhea were 819 strains of *V. cholerae*, 521 enteropathogenic *E. coli*, 273 enterotoxigenic *E. coli*, 102 *Salmonella* species, 43 *Shigella* species, 38 *Campylobacter jejuni*, 25 NAG vibrios, 2 *Plesiomonas shigelloides*, and 2 *Yersinia enterocolitica*. No bacterial enteropathogens were isolated in the control group of patients.

Live cells and culture filtrates of all motile strains of *Aeromonas* caused accumulation of fluid in the ranges of 0.8 to 1.8 and 0.6 to 2.0 ml/cm of gut, respectively. However, all three strains of *A. caviae* caused fluid accumulation after two or three serial passages through rabbit ileal loops, indicating potential enteropathogenicity. Cell-free culture filtrates of *A. hydrophila* and *A. sobria* caused significantly ($P < 0.001$) more fluid accumulation than that of *A. caviae*. No histological changes were noted in ileal loops exposed to live cells or culture filtrates, and the epithelium was intact. The enterotoxic activity of the culture filtrates was lost after inactivation at 60°C for 10 min.

All isolates of *A. hydrophila* and *A. sobria* had high hemolysin titers ranging from 256 to 2,048. *A. caviae* had low hemolysin titers of between 32 and 64.

Associated clinical features. All patients yielding *Aeromonas* spp. were symptomatic and had watery diarrhea of 3 to 7 days in duration. The number of stools ranged from 10 to 12 per day. Microscopic examination of the stool showed absence of leukocytes and erythrocytes. Some 20% of the patients had vomiting and low-grade fever. None of the patients had an underlying illness or received antibiotics within 2 weeks prior to the onset of illness. Males and females were affected equally in all age groups. All patients were hospitalized and recovered uneventfully with parenteral rehydration. Remission of symptoms in all of these patients was associated with subsequent disappearance of *Aeromonas* spp. from their stools. Two weeks after primary culture, repeat fecal cultures were negative for *Aeromonas* spp.

DISCUSSION

Aeromonas species are environmental bacteria which are widely distributed in both fresh and saltwater and among fish and invertebrate species of animals. Intestinal and extraintestinal infections caused by these organisms are being recognized with increasing frequency. Diarrheal diseases caused by these organisms have been reported from all over the world (1, 14, 16–18, 21, 26, 31, 36). Despite these reports, the role of *Aeromonas* as a potential human enteric pathogen is still unclear. This may be due to the lack of its clinical significance, confusion over the taxonomy of the *Aeromonas* genus, and difficulty in identifying *Aeromonas* isolates with various virulence factors.

In developed countries, the clinical spectrum varies from toxigenic diarrhea to colitis, whereas in developing countries *Aeromonas*-induced diarrhea is mainly toxigenic. *Aeromonas* spp. have been reported as an important cause of acute diarrhea in children (1, 12, 16, 17, 29) and in adults, especially in those older than 60 years (10, 15, 33). The overall incidence of *Aeromonas* spp. in diarrheal disease varies from 1 to 27% (1, 3, 8, 16, 26, 27, 31, 36). However, the frequency of isolation of *Aeromonas* spp. in adults was less (15) than in children (16). In a recent study from Thailand, an isolation rate of 31% has been reported from individuals with traveler's diarrhea (11). In some studies, *Aeromonas* was found to be associated with other enteric pathogens (7, 8, 27). In our study, *Aeromonas* spp. were isolated as the sole bacterial enteropathogen in 1.8% of patients with acute diarrhea, in concurrence with reports by others (1, 36). In our series, as well as in those of other investigators (1, 26, 39), no *Aeromonas* strains were isolated from nondiarrheal controls. However, others have reported a low frequency of *Aeromonas* spp. from normal stools (41). von Graevenitz and Zinterhofer reported an asymptomatic fecal carriage rate of 3.2%, mostly in adults (42). In a hospital study of 815 patients, *Aeromonas* sp. was isolated from two healthy persons and from one baby (27). In most countries, the asymptomatic fecal carriage rate in children varies from 0.5 to 2.1% (2, 4–6, 12, 16, 35, 36). In contrast, a high fecal carriage rate of about 27% has been reported in Thai children (31). A recent study from Bangladesh also showed a high fecal carriage rate in a control population (34). In fact, most of the studies have not included a matched control group of

individuals because of the known low rates of isolation of *Aeromonas* spp. from feces (17, 21, 29). As shown previously (4, 16), *Aeromonas* spp. were detected in significantly higher numbers in children with diarrhea than in controls. However, others have found no significant difference in the frequency of isolation of *Aeromonas* spp. from individuals with and without diarrhea (12, 31).

The results of the several recent studies suggest that *A. hydrophila* and *A. sobria* are potential enteric pathogens (4, 18, 20, 34, 40). However, others have shown clinical and epidemiological evidence that *A. caviae* also may cause diarrhea by production of enterotoxin and cytotoxin and by adherence to human epithelial cells (29, 30). In our study, all strains of *Aeromonas* produced a heat-labile enterotoxin, as shown previously (24). However, *A. hydrophila* and *A. sobria* caused significantly more fluid accumulation as compared with *A. caviae*, indicating their higher enterotoxic activity. A significant correlation was found between the isolation of enterotoxin-producing *Aeromonas* in feces and diarrhea (16). However, enterotoxin production is not always associated with clinical disease (31). A correlation between enteropathogenicity and in vitro production of enterotoxin does exist for other bacterical enteropathogens (13). Consequently, enterotoxin production by *Aeromonas* spp. could serve as the virulence mechanism in this setting as well.

In recent years, it has been suggested that enterotoxin is produced mostly by beta-hemolytic strains of *Aeromonas* (4, 18). Burke et al. (4) proposed hemolysin production as a criterion to distinguish *A. hydrophila* and *A. sobria* from *A. caviae*. However, the results in the present study suggest that hemolysin production is not confined to *A. hydrophila* and *A. sobria* but also may include *A. caviae*.

In the present study, the peak period for isolation of *Aeromonas* spp. was between February and May and was related to summer months in this geographical area. The summer peak of *Aeromonas*-associated gastroenteritis might be related to waterborne infections (4).

It has been shown that host factors may play an important role in *Aeromonas*-associated gastroenteritis (14). This is further strengthened by the finding that only 2 of 57 healthy volunteers developed diarrhea after oral administration of high doses of *Aeromonas* strains (28). It is possible that younger children and the elderly are more susceptible to gastrointestinal tract infections. This is supported by the fact that we, as well as others (1, 2, 29), have shown an increased frequency of isolation of *Aeromonas* spp. in children. Moreover, the results in the present study suggest a correlation between diarrhea and presence of aeromonads in large numbers (1, 29) in stool.

In conclusion, our results clearly indicate that motile *Aeromonas* strains are potential enteric pathogens in this geographical region, especially in younger children and in adults over 60 years in age. This is further supported by the following findings: the organism was present in large numbers on primary isolation from patients with watery diarrhea, no other known bacterial enteric pathogen was isolated from *Aeromonas*-infected patients, repeat fecal cultures were negative after 2 weeks of primary culture, and all patients showed clinical improvement with the disappearance of the organisms from their stools. We have further shown that *Aeromonas* spp. possess a virulence mechanism that may operate in the gastrointestinal tract to provoke a diarrheal syndrome.

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