JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2000, p. 3785–3790 Vol. 38, No. 10
0095-1137/00/$04.00+0

Prevalence of Enterotoxin Genes in *Aeromonas* spp. Isolated From Children with Diarrhea, Healthy Controls, and the Environment

M. JOHN ALBERT,[1]* M. ANSARUZZAMAN,[1] KAISAR A. TALUKDER,[1] ASHOK K. CHOPRA,[2] INGER KUHN,[3] MOTIUR RAHMAN,[1] A. S. G. FARUQUE,[1] M. SIRAJUL ISLAM,[1] R. BRADLEY SACK,[4] AND ROLAND MOLLBY[3]

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka-1000, Bangladesh[1]; Department of Microbiology and Immunology, University of Texas Medical Branch at Galveston, Galveston, Texas 77555-1070[2]; Microbiology and Tumor Biology Centre, Karolinska Institute, S-171 77 Stockholm, Sweden[3]; and Department of International Health, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland[4]

Received 12 June 2000/Returned for modification 6 July 2000/Accepted 1 August 2000

Aeromonads are causative agents of a number of human infections. Even though aeromonads have been isolated from patients suffering from diarrhea, their etiological role in gastroenteritis is unclear. In spite of a number of virulence factors produced by *Aeromonas* species, their association with diarrhea has not been clearly linked. Recently, we have characterized a heat-labile cytotonic enterotoxin (Alt), a heat-stable cytotonic enterotoxin (Ast), and a cytotoxic enterotoxin (Act) from a diarrheal isolate of *Aeromonas hydrophila*. Alt and Ast are novel enterotoxins which are not related to cholera toxin; Act is aerolysin related and has hemolytic, cytotoxic, and enterotoxic activities. We studied the distribution of the *alt*, *ast*, and *act* enterotoxin genes in 115 of 125 aeromonads isolated from 1,735 children with diarrhea, in all 27 aeromonads isolated from 830 control children ($P = 7 \times 10^{-4}$ for comparison of rates of isolation of aeromonads from cases versus those from controls), and in 120 randomly selected aeromonads from different components of surface water in Bangladesh. *Aeromonas* isolates which were positive only for the presence of the *alt* gene had similar distributions in the three sources; the number of isolates positive only for the presence of the *ast* gene was significantly higher for the environmental samples than for samples from diarrheal children; and isolates positive only for the presence of the *act* gene were not found in any of the three sources. Importantly, the number of isolates positive for both the *alt* and *ast* genes was significantly higher for diarrheal children than for control children and the environment. Thus, this is the first study to indicate that the products of both the *alt* and *ast* genes may synergistically act to induce severe diarrhea. In 26 patients, *Aeromonas* spp. were isolated as the sole enteropathogen. Analysis of clinical data from 11 of these patients suggested that isolates positive for both the *alt* and *ast* genes were associated with watery diarrhea but that isolates positive only for the *alt* gene were associated with loose stools. Most of the isolates from the three sources could be classified into seven phenospecies and eight hybridization groups. For the first time, *Aeromonas eucrenophila* was isolated from two children, one with diarrhea and another without diarrhea.

Aeromonads are ubiquitous organisms found in aquatic environments; food items, including meat, fish, and vegetables; and the intestines of apparently healthy humans and humans with diarrhea (18). There is circulation of strains between humans and the environment. Aeromonads are causative agents of a number of infections, including bacteremia, meningitis, wound infections, and lung infections in humans (22). The etiological role of aeromonads in human diarrheal disease is unclear. It is believed that the difficulty in assigning an unequivocal role to the causation of diarrhea is because aeromonads are heterogeneous and because certain subgroups may be only pathogenic (26). The limitation lies in our inability to identify these pathogenic subgroups. Recent advances in the taxonomy of aeromonads may contribute to the identification of the pathogenic subgroups. Despite the identification of a variety of virulence factors in *Aeromonas* spp., including enterotoxins, cytotoxins, hemolysins, aerolysins, proteases, hemagglutinins, and the ability to adhere to and invade tissue culture cell lines (34), the linkage of these factors to the diarrheagenic ability of the isolates has not been clearly demonstrated. In one of our laboratories (that of A. K. Chopra), three distinct genes encoding enterotoxins from a diarrheal isolate of *Aeromonas hydrophila* have been identified (8, 9, 10). One encodes a cytotoxic enterotoxin (Act), and two encode cytotonic enterotoxins, one of which is heat labile at 56°C (Alt) and the other of which is heat stable at this temperature (Ast). Both Alt and Ast are novel enterotoxins unrelated to cholera toxin (CT). Act is a single-chain polypeptide of 52 kDa that is aerolysin related and has hemolytic, cytotoxic, and enterotoxic activities (8). The role of Act in the overall virulence of the organism has been clearly demonstrated by determinations of 50% lethal doses and the inability of culture filtrates from the *act* isogenic mutants to evoke a fluid-secretory response and to cause tissue damage in mouse ligated ileal loops (37). Both Alt and Ast consist of single polypeptide chains, with Alt exhibiting a size of 44 kDa (9, 10). Alt caused elevation of cyclic AMP (cAMP) and prostaglandin (e.g., PGE_2) levels in Chinese hamster ovary (CHO) and intestinal epithelial cells, resulting in fluid-secretory responses in rat ligated ileal loops. Alt exhibits intriguing homology with lipases and phospholipase C (9). Expression of the *ast* gene in *Escherichia coli* using the bacteriophage T7 RNA polymerase-promoter system demonstrated the presence of two [^{35}S]methionine-labeled bands of 33 and 67 kDa (A. K. Chopra, unpublished data), and the crude preparations of Ast elevated cAMP levels in CHO cells (10). The detailed mechanism of action of Act leading to tissue damage and fluid secretion has recently been delineated (11). In the

* Corresponding author. Present address: Department of Microbiology, Faculty of Medicine, Kuwait University, P.O. Box 24923, Safat, 13110 Kuwait. Phone: (965) 533 2719. Fax: (965) 531 8454. Email: john@hsc.kuniv.edu.kw.

TABLE 1. Distribution of various *Aeromonas* spp. (and HGs[a]) in three sources

Source	Total no. of isolates	No. of isolates (%) positive for:								
		A. hydrophila		*A. veronii* biotype *sobria* (HG8)	*A. caviae* (HG4)	*A. media* (HG5)	*A. eucrenophila* (HG6)	*A. trota* (HG13)	*A. jandeii* (HG9)	Unknown *Aeromonas* sp.
		HG1	HG2							
Diarrheal children	115	11 (9.6)	3 (2.6)	21 (18.3)	39 (33.9)	14 (12.2)	1 (0.9)	25 (21.8)	0 (0.0)	1 (0.9)
Control children	27	1 (3.7)	0 (0.0)	4 (14.8)	11 (40.7)	1 (3.7)	1 (3.7)	6 (22.2)	1 (3.7)	2 (7.4)
Environment	120	18 (15.0)	0 (0.0)	16 (13.3)	7 (5.8)	2 (1.7)	0 (0.0)	72 (60.0)	4 (3.3)	1 (0.8)
Total	262	30 (11.5)	3 (1.2)	41 (15.7)	57 (21.8)	17 (6.5)	2 (0.8)	103 (39.3)	5 (1.9)	4 (1.5)

[a] Also known as genomospecies.

present study, we examined the distribution of the cytotoxic enterotoxin gene *act* (encoding Act) and the cytotonic enterotoxin genes *alt* (encoding Alt) and *ast* (encoding Ast) in aeromonad isolates from children with diarrhea, healthy matched controls, and the environment. The objectives of the study were to determine whether an association exists between these enterotoxin genes and diarrheal isolates and to identify a new virulence property or properties that may, possibly in combination with other virulence traits, contribute to diarrhea.

MATERIALS AND METHODS

Environmental samples. Surface water, sediment, aquatic plant, phytoplankton, and zooplankton samples were collected from four different areas of Bangladesh every fortnight from May 1997 until June 1998. This environmental sampling was part of a study to environmentally monitor cholera. The four areas were Bakergonj, Chattack, Chaugacha, and Matlab. Bakergonj is situated approximately 300 km south, Chattack is 400 km northeast, Chaugacha is 300 km southwest, and Matlab is 50 km southeast of the capital, Dhaka.

Plants were collected in sterile polyethylene bags, water samples were collected in sterile Nalgene plastic bottles, and sediment samples were collected by a core sampler (made at the International Centre for Diarrhoeal Disease Research, Bangladesh [ICDDR,B]) in sterile glass bottles. Phytoplankton and zooplankton were collected in sterile glass bottles using plankton nets with 20- and 64-μm mesh sizes. All samples were transported to the microbiology laboratory in an insulated box with a cool pack. Samples were processed within 6 h of collection.

Roots of plants (*Eichhornia crassipes*) were washed in sterile physiological saline. Ten grams each of the washed root and sediment were mixed separately with 90 ml of sterile physiological saline and homogenized in a commercial waring blender (model 32BL79; Dynamics Corporation of America, New Hartford, Conn.) at 22,000 rpm for 2 min. Five milliliters each of phytoplankton and zooplanton samples were homogenized in a glass homogenizer (Wheaton Scientific, Millville, N.J.) using a StedFast stirrer (model 300; Fisher Scientific, Loughborough, Leicestershire, United Kingdom). Tenfold dilutions of the above-described samples were made in sterile physiological saline for culturing for aeromonads.

Patients and controls. Patients were children up to 5 years of age with acute diarrhea seen at the Clinical Research and Service Centre of the ICDDR,B, located in Dhaka, Bangladesh, during March 1997 through May 1998. They were part of a routine 2% (reduced from the original 4%) surveillance sampling of all patients with diarrhea seen at the Clinical Research and Service Centre (33). Stool samples from patients were immediately transported to the microbiology laboratory. Controls were healthy children from the same neighborhood as that of the patients. Rectal swabs from matched controls were collected within 2 weeks of collection of samples from patients and transported to the laboratory in Cary-Blair medium at ambient temperature within a few hours of collection. Control children had not taken antibiotics in the previous 2 weeks.

Microbiology. Stool and rectal swab samples were analyzed for bacterial pathogens as described previously (2). The pathogens sought were *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, *Vibrio cholerae* O1 and O139, diarrheagenic *E. coli* (enterotoxigenic, enteropathogenic enteroinvasive, enterohemorrhagic, enteroaggregative, and diffusively adherent *E. coli*), *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and rotavirus.

For detection of aeromonads, stool (0.5-g) or rectal swab samples were enriched in 5 ml of bile peptone broth (29). After overnight incubation at 37°C, the broth was subcultured onto taurocholate-tellurite-gelatin agar (TTGA) (20, 28). Tenfold dilutions of the environmental samples were also cultured on TTGA. After overnight incubation at 37°C, the plates were examined for characteristic colonies resembling aeromonads. Gray, oxidase-positive colonies without a black center but with a zone of opacity were further tested for different *Aeromonas* spp. and hybridization groups (HGs) by a battery of biochemical tests as described previously (1, 23). Reference cultures were obtained from S. W. Joseph, University of Maryland, College Park, Baltimore, Md.

All *Aeromonas* spp. isolates were stocked in Luria broth with 25% glycerol at −70°C for further study.

Preparation of DNA probes. The recombinant plasmid pXHC95, which contains a 2.8-kb *Bam*HI fragment from *A. hydrophila* strain SSU and harbors the *act* gene (37), was used as a template DNA to generate a 0.8-kb fragment which represents an internal segment of the *act* gene (8). The plasmid was digested with *Bst*XI restriction enzyme, which cuts twice within the coding region of the *act* gene, to generate a 0.8-kb fragment. The fragment was excised from a 0.8% agarose gel, extracted with phenol-chloroform, precipitated with ethanol (3), and purified with a GeneClean II kit (Bio 101, Vista, Calif.).

Plasmid pSL24, which contains a 4.0-kb *Sal*I DNA fragment containing the *alt* gene, was used as a template DNA to amplify the coding region of the *alt* gene as described previously (10). Briefly, a Geneamp reagent kit with AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was used for PCR by using 40 ng of the template DNA and a set of primers (30-mer, 1 mM each) representing the 5′ and 3′ ends of the *alt* gene (9). For PCR, the DNA was first denatured for 3 min at 94°C, followed by 25 cycles of 1 min each at 55°C (annealing) and 72°C (extension), followed by a final extension at 72°C for 7 min. The PCR product was excised from the gel and purified as described for the *act* gene-containing DNA fragment.

The *ast* gene was originally localized to a 4.8-kb *Sal*I/*Bam*HI DNA fragment of *A. hydrophila* SSU and was subcloned into pBluescript to generate a recombinant plasmid, pSBS32 (10). The cell lysate from this clone exhibited CHO cell elongation activity which was stable at 56°C for 20 min. By *Bal*31 digestion and addition of polylinkers (3), we generated another clone, pSBS33, which contained an approximately 3.5-kb *Sal*I/*Bam*HI DNA fragment and had a level of CHO cell activity similar to that of the pSBS32 clone. Expression of these DNA fragments in plasmid pT7-6 with a T7 polymerase-promoter system resulted in protein products of 33 and 67 kDa after [^{35}S]methionine labeling (9). This DNA fragment was excised from the agarose gel and purified as described above.

Labeling of DNA probes. Purified DNA probes were labeled with digoxigenin (DIG)-dUTP (Boehringer Mannheim Gmbh, Mannheim, Germany) using a random primer DNA labeling kit (Boehringer Mannheim) according to the instructions of the manufacturer. DIG-labeled probes were recovered by ethanol precipitation, resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and stored at −20°C until used. Immediately prior to use, the probes were denatured to single-stranded DNA by boiling for 10 min and then chilling on ice to prevent renaturation.

Preparation of colony blots and hybridization. Aeromonad isolates were inoculated onto gelatin agar plates (1% NaCl, 3% gelatin, 1% tryptone, 1.6% agar [pH 7.4]) (40 colonies per plate, plus a positive and a negative control) in a grid pattern. After overnight incubation at 37°C, a Hybond-N$^+$ positively charged nylon membrane (version 2; Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom) was placed over the surface of the plate. The membrane was removed after 1 min and placed with the colony side up on a pad of absorbent filter paper soaked with a denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 min to lyse the bacteria and denature the DNA. The membrane was then placed with the colony side up on a pad of absorbent filter paper soaked in a solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 8.0], 0.001 M EDTA) for 3 min to neutralize the alkalinity. The neutralization procedure was repeated with another soaked pad. The membrane was washed in 2× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and air dried. DNA material was fixed to the membrane by UV cross-linking by placing the side of the membrane containing the colony blot down on a transilluminator (model T2201; Sigma, St. Louis, Mo.) for 5 min.

Hybridization of the colony blots and development of the blots with anti-DIG-alkaline phosphatase were performed according to the instructions provided in a DIG DNA labeling and detection kit (Boehringer Mannheim). The *A. hydrophila* strain SSU, which contains all three toxin genes, was used as a positive control, and *E. coli* K-12 was used as a negative control in the hybridization studies.

The chi-square test and Fisher's exact test were used to compare differences in

TABLE 2. Presence of toxin genes in *Aeromonas* spp. isolated from diarrheal children

Species (HG)	No. of isolates	No. (%) of isolates positive for the gene(s) encoding:		
		Alt[a]	Ast[b]	Alt + Ast
A. hydrophila				
(HG1)	11	0 (0.0)	0 (0.0)	6 (54.6)
(HG2)	3	0 (0.0)	1 (33.3)	0 (0.0)
A. veronii biotype *sobria* (HG8)	21	2 (9.5)	6 (28.6)	8 (38.1)
A. caviae (HG4)	39	11 (28.2)	0 (0.0)	26 (66.7)
A. media (HG5)	14	5 (35.7)	1 (7.1)	8 (57.1)
A. eucrenophila (HG6)	1	0 (0.0)	1 (100.0)	0 (0.0)
A. trota (HG13)	25	0 (0.0)	9 (36.0)	16 (64.0)
Unknown	1	1 (100.0)	0 (0.0)	0 (0.0)
Total	115	19 (16.5)	18 (15.7)	64 (55.7)

[a] Heat-labile cytotonic enterotoxin.
[b] Heat-stable cytotonic enterotoxin. No isolate was positive for the gene encoding cytotoxic enterotoxin (Act) only, the genes encoding Alt and Act, or the genes encoding Ast and Act; one *A. hydrophila* (HG1) isolate was positive for the genes encoding Alt, Ast, and Act.

TABLE 3. Presence of toxin genes in *Aeromonas* spp. isolated from control children

Species (HG)	No. of isolates	No. (%) of isolates positive for the gene(s) encoding:		
		Alt[a]	Ast[b]	Alt + Ast
A. hydrophila (HG1)	1	0 (0.0)	0 (0.0)	0 (0.0)
A. veronii biotype *sobria* (HG8)	4	1 (25.0)	1 (25.0)	0 (0.0)
A. caviae (HG4)	11	4 (36.4)	0 (0.0)	5 (45.5)
A. media (HG5)	1	1 (100.0)	0 (0.0)	0 (0.0)
A. eucrenophila (HG6)	1	1 (100.0)	0 (0.0)	0 (0.0)
A. trota (HG13)	6	1 (16.7)	4 (66.7)	1 (16.7)
A. jandeii (HG9)	1	1 (100.0)	0 (0.0)	0 (0.0)
Unknown	2	0 (0.0)	2 (100.0)	0 (0.0)
Total	27	9 (33.3)	7 (25.9)	6 (22.2)

[a] Heat-labile cytotonic enterotoxin.
[b] Heat-stable cytotonic enterotoxin. No isolate was positive for the gene encoding cytotoxic enterotoxin (Act) only, the genes encoding Alt and Act, or the genes encoding Ast and Act; the *A. hydrophila* isolate was positive for genes encoding Alt, Ast, and Act.

the levels of prevalence of toxin genes between groups of isolates. A P value of ≤ 0.05 was considered significant.

RESULTS

A total of 1,735 children with diarrhea were studied for aeromonads and other enteric pathogens, and a total of 830 control children were studied for aeromonads alone. Aeromonads were isolated from 125 diarrheal children (7.2%) and from 27 control children (3.3%) ($P = 7 \times 10^{-4}$). Of the 125 diarrheal children positive for aeromonads, 28 children were infected with *Aeromonas* spp. alone and the remainder had mixed infections with other enteric pathogens: 66 children had infections with an *Aeromonas* sp. and another pathogen; 26 children had infections with an *Aeromonas* sp. and two other pathogens; and 5 children had infections with an *Aeromonas* sp. and three other pathogens. Among the 66 children with dual infections, 19 children were coinfected with rotavirus and enteropathogenic or enterotoxigenic *E. coli* (9 children each). Among the 26 children infected with three pathogens, the predominant combination was enterotoxigenic *E. coli* and *C. jejuni*, being found in 7 children.

A total of 2,120 environmental samples were cultured for *Aeromonas* spp. Of these, 600 samples were positive for aeromonads (28.3%). Every fifth isolate was selected for further study, which constituted 120 isolates. One hundred fifteen isolates from diarrheal children (10 isolates were lost) and 27 isolates from control children were selected for further study. The species and HG distributions of isolates from diarrheal children, control children, and the environment are shown in Table 1. Among isolates from diarrheal children and control children, *Aeromonas caviae* predominated, but among isolates from the environment, *Aeromonas trota* was found most often. All *A. trota*, *A. caviae*, and *Aeromonas veronii* biotype *sobria* isolates belonged to HG13, -4, and -8, respectively. *A. hydrophila* isolates from children with diarrhea belonged to HG1 and HG2; isolates from control children and the environment belonged to HG1 only. There were less prevalent species that belonged to other HGs. Of note was the isolation of *Aeromonas eucrenophila*, from one diarrheal child and from one control child.

The distribution of the cytotonic enterotoxin genes among the diarrheal isolates is shown in Table 2. The *alt* or *ast* gene was singly distributed in approximately 16% of total isolates. However, up to 56% of total isolates had both the genes. None of the isolates had the *act* gene. One isolate of *A. hydrophila* had all three toxin genes.

The distribution of the toxin genes in control isolates is shown in Table 3. The *alt* and *ast* genes either singly or in combination had a similar distribution. None of the isolates was positive for the *act* gene. One isolate of *A. hydrophila* was positive for all three toxin genes.

Among the environmental isolates, 17 and 30% of isolates were positive for *alt* and *ast* genes, respectively, and about one-third of the isolates were positive for both. None of the isolates was positive for the *act* gene. Two *A. hydrophila* isolates were positive for all three genes (Table 4).

The distributions of the toxin genes in isolates from the three sources were compared (Table 5). They were similar for the *alt* gene, but the *ast* gene had a significantly higher prevalence among the environmental isolates than among the isolates from diarrheal children. The occurrence of both the *alt* and *ast* genes was significantly higher among diarrheal isolates

TABLE 4. Presence of toxin genes in *Aeromonas* spp. isolated from the environment

Species (HG)	No. of isolates	No. (%) of isolates positive for the gene(s) encoding:		
		Alt[a]	Ast[b]	Alt + Ast
A. hydrophila (HG1)	18	10 (55.6)	0 (0.0)	2 (11.1)
A. veronii biotype *sobria* (HG8)	16	5 (31.3)	1 (6.3)	5 (31.3)
A. caviae (HG4)	7	3 (42.9)	0 (0.0)	3 (42.9)
A. media (HG5)	2	0 (0.0)	0 (0.0)	0 (0.0)
A. trota (HG13)	72	1 (1.4)	35 (48.6)	30 (41.7)
A. jandeii (HG9)	4	0 (0.0)	0 (0.0)	0 (0.0)
Unknown	1	1 (100.0)	0 (0.0)	0 (0.0)
Total	120	20 (16.7)	36 (30.0)	40 (33.3)

[a] Heat-labile cytotonic enterotoxin.
[b] Heat-stable cytotonic enterotoxin. No isolate was positive for the gene encoding cytotoxic enterotoxin (Act) only, the genes encoding Alt and Act, or the genes encoding Ast and Act; two *A. hydrophila* (HG1) isolates were positive for the genes encoding Alt, Ast, and Act.

TABLE 5. Prevalence of three toxin genes in *Aeromonas* spp. isolated from various sources

Source	No. of isolates	No. (%) of isolates positive for the gene(s) encoding:		
		Alt[a]	Ast[b]	Alt + Ast
Diarrheal children	115	19 (16.5) A	18 (15.7) D	65 (55.7) G
Control children	27	9 (33.3) B	7 (25.9) E	6 (22.2) H
Environment	120	20 (16.7) C	36 (30.0) F	40 (33.3) I

[a] Alt, heat-labile cytotonic enterotoxin; Ast, heat-stable cytotonic enterotoxin. A versus B, $P = 0.087$; A versus C, $P = 0.884$; D versus E, $P = 0.259$; D versus F, $P = 0.014$; G versus H, $P = 0.004$; G versus I, $P = 0.001$.

than isolates from control children and the environment. Other comparisons were not significant.

The complete demographic and clinical presentation data were available for 11 of 26 patients who had aeromonads isolated as the only enteropathogen. These data are presented in Table 6, along with information on the toxin genes in the isolates. The consistencies of stools were watery (very thin with little fecal matter) for eight patients and loose (able to assume the shape of the container but with enough fecal matter) for three patients. All seven patients who had isolates positive for both the *alt* and *ast* genes had watery diarrhea. Three patients who had isolates positive for the *alt* gene had only loose stools.

DISCUSSION

The role of aeromonads as significant diarrheal disease agents is unclear. These organisms have been epidemiologically linked to acute diarrhea in some controlled studies (2, 4, 17, 31) but not in others (13, 32). One oral-challenge study performed thus far with aeromonads failed to show significant diarrhea in adult volunteers (30). However, *Aeromonas* strains of questionable suitability to challenge the volunteers were used and therefore it is difficult to draw any meaningful conclusions from this study (15). Several case reports support a role for aeromonads in the etiology of diarrheal disease. Diarrheal diseases in certain individuals have been associated with excretion of organisms as pure or predominant cultures, serological responses to the organisms, and resolution of symptoms and pathology with the disappearance of the organisms from the stool (22). Our study has revealed a significant association with diarrhea for aeromonads, and this result confirms an earlier finding from our center (2). Another finding of the present study and of a previous study from our center (2) is a high prevalence of mixed infections of *Aeromonas* sp. with other pathogens. A similar high frequency of coinfecting enteropathogens was found in Peruvian infants with *Aeromonas*-associated diarrhea (31). Since aeromonads are present in food and water, it can be argued that they are mere passengers in the intestinal tract and not true pathogens. It is also possible that multiple pathogens act synergistically to produce diarrhea.

Aeromonads are currently divided into 14 DNA HGs, genomospecies, or genospecies and 14 phenospecies (22). The HGs are identified by a variety of methods, including DNA-DNA hybridization, PCR amplification, ribosomal DNA restriction, restriction fragment length polymorphism analysis, and pulsed-field gel electrophoresis (22). However, these methods are relatively complex and not amenable to use in many laboratories. Fortunately, it has been reported that >98% of aeromonads can be accurately identified to the genospecies level by a battery of biochemical tests (1, 22). Therefore, we employed these biochemical tests for the identification of aeromonads to the genospecies and species levels in our study. The majority of our isolates were classified by these tests. Most aeromonads irrespective of source could be classified into the four species *hydrophila*, *veronii* biotype *sobria*, *caviae*, and *trota*. There were some isolates of *Aeromonas media*, *Aeromonas eucrenophila*, *Aeromonas jandeii*, and unknown species. In several studies, it has been found that >85% of clinical isolates are represented by the *Aeromonas* species *hydrophila*, *veronii* biotype *sobria*, and *caviae* (21). The unique finding in the present study is the high isolation rate of *A. trota* from the environment as well as humans compared to those of previous studies (13, 14, 18, 27). This finding may be related to the isolation technique used in our study and our geographical location in south Asia. Likewise, the lower prevalence of the *act* gene in *Aeromonas* spp. may be related to geographical location. Our recent study in which *Aeromonas* spp. isolated from diarrheal children (approximately 100 isolates) in developed countries were examined indicated the presence of all three toxin genes (*act*, *alt*, and *ast*) in 50% of the isolates, while only the *alt* and *ast* genes were detected in all isolates. Those isolates with the *act*, *alt*, and *ast* genes caused bloody diarrhea in patients (Chopra, unpublished). In our study, aeromonads were isolated by an enrichment technique followed by plating on TTGA. Neither

TABLE 6. Demographic and clinical data for 11 of 26 patients who had an *Aeromonas* sp. as the only pathogen and the toxin gene(s) of the *Aeromonas* spp.

Patient	Age (mo)	Gender[a]	Stool consistency	No. of stools in 24 h	No. of erythrocytes in stool[b]	No. of leukocytes in stool[b]	Abdominal pain	Vomiting	Fever[c]	Dehydration status[d]	*Aeromonas* sp. isolated	Toxin gene(s) of *Aeromonas* sp.
12057	48	M	Watery	15–20	0	0–10	+	+	−	Moderate	*A. caviae*	*alt*, *ast*
12780	24	F	Watery	6–10	0	11–20	−	−	−	Mild	*A. caviae*	*alt*, *ast*
14980	29	F	Watery	6–10	0	0–10	−	+	−	Mild	*A. caviae*	*alt*, *ast*
17718	22	M	Watery	6–10	0	11–20	−	−	−	Mild	*A. caviae*	*alt*, *ast*
18750	54	F	Loose	6–10	1–10	11–20	−	+	+	Mild	*A. veronii* biotype *sobria*	*alt*
24161	6	M	Loose	6–10	1–10	15–20	+	−	+	Mild	*A. caviae*	*alt*
32317	5	M	Loose	6–10	0	11–20	−	−	−	Mild	*A. media*	*alt*
939	11	F	Watery	>20	0	11–20	+	+	−	Mild	*A. hydrophila*	*alt*, *ast*
10776	18	M	Watery	6–10	0	0–10	−	+	−	Mild	*A. caviae*	*alt*, *ast*
14732	36	F	Watery	11–15	0	0–10	−	−	−	None	*A. caviae*	None
30340	44	F	Watery	3–5	0	11–20	−	+	−	Moderate	*A. veronii* biotype *sobria*	*alt*, *ast*

[a] M, male; F, female.
[b] Number per high-power microscopy field.
[c] Defined as ≥37.7°C.
[d] Defined as per reference 36.

the enrichment broth nor the plating medium contained ampicillin, which was incorporated in the selective media for the isolation of aeromonads in other studies (13, 18, 27). However, *A. trota* is a unique species which is ampicillin susceptible, and most of the isolates identified in the first study originated in either south or southeast Asia (5). The predominant species identified in children both with and without diarrhea was *A. caviae*. *Aeromonas veronii* biotype *sobria* and *A. trota* were next in prevalence, followed by both *A. hydrophila* and *A. media*. With the exception of *A. trota*, this type of prevalence for species has been reported previously for human diarrheal cases (21). All the aeromonad isolates identified to the species level could be assigned to a genomospecies. Thus, irrespective of source of isolation, *A. veronii* biotype *sobria* belonged to HG8, *A. caviae* belonged to HG4, *A. trota* belonged to HG13, *A. media* belonged to HG5, and *A. eucrenophila* belonged to HG6. All the environmental isolates and the single control human isolate of *A. hydrophila* belonged to HG1; diarrheal isolates belonged to either HG1 or HG2. These results are in agreement with previous reports for human and environmental strains (1, 23, 26, 27) but at variance with a report from Finland for environmental isolates (18). In the Finnish study, the majority of environmental isolates of *A. hydrophila* belonged to HG2 and -3, *A. caviae* belonged to HG5B, and *A. veronii* biotype *sobria* belonged to HG7 (18). These results may be related to differences in the geographical distributions of HGs. *A. eucrenophila* has never been previously reported from human infection (22). Ours is the first report of isolation of this species from a human source.

Aeromonads produce more than one type of enterotoxin (7). Cytotoxic enterotoxin is also known as aerolysin and hemolysin. The cytotoxic enterotoxin (Act) and the gene encoding it (*act*) have been compared with aerolysins and hemolysins from other aeromonads and were found to be different (7). Similarly, there are reports of at least four cytotonic enterotoxins from aeromonads (7). They all have a mechanism of action similar to that of CT in that they cause fluid accumulation in animal intestinal loops, elongation of CHO cells, rounding of Y1 adrenal tumor cells, and, in a number of cases, increases in the intracellular cAMP levels. Some of these toxins have been reported to cross-react with CT, while others do not. Alt and Ast differ from each other based on DNA sequence analysis (Chopra, unpublished). Using biological models, such as animals and tissue culture systems, some previous studies have documented the distribution of cytotoxins and enterotoxins among aeromonads from various sources (13, 19, 35). These virulence factors were found to be distributed in isolates from all sources; thus, an association with diarrhea could not be established (12, 13, 24, 25, 32, 35). This finding is not surprising in light of recent evidence of the existence of multiple types of enterotoxins and cytotoxins which produce similar effects in various assay systems (7), thus making the interpretation of results difficult. We have overcome this limitation in our study by investigating the distribution of three distinct toxin genes by a DNA hybridization technique and not by biological assay systems. In our study, the distributions of the *alt* gene were not significantly different between isolates from diarrheal children and control children. Although the *ast* gene had a significantly higher prevalence among the environmental isolates than among the diarrheal isolates, this negative correlation was not considered further, as the prevalences of the gene among the diarrheal isolates and that among the control isolates were not significantly different. However, a significantly higher proportion of isolates from patients with diarrhea harbored both the *alt* and *ast* genes than did isolates from control children and the environment. These findings suggest that the product of either gene alone may evoke less severe diarrhea in most hosts but that together they may synergistically act to induce severe diarrhea. Our data suggest that enterotoxigenic aeromonads possessing both the *alt* and *ast* genes may be true diarrheal pathogens in south Asia. This seems to confirm some previous reports of an association of enterotoxigenic aeromonads with diarrhea in which toxigenic aeromonads were detected by biological assay systems (4, 6, 16). Although the number of patients is limited, clinical data from seven patients whose isolates were positive for both the *alt* and *ast* genes revealed that all of them had watery diarrhea, which is consistent with the presence of cytotonic enterotoxin genes in these isolates. This finding needs to be confirmed with a larger series of patients. However, ours is the first study to clearly show the distribution of specific enterotoxin genes among aeromonads from different sources and the association of two combined enterotoxin genes with diarrhea.

ACKNOWLEDGMENTS

This research was supported by a grant awarded to the ICDDR,B (INT-ICDDR,B-HN-01-AV) by the Swedish Agency for Research Cooperation with Developing Countries and by grants awarded to Johns Hopkins University and the ICDDR,B (RO1AI39129-01A1) and to the University of Texas Medical Branch at Galveston (RO1A41611) by the U.S. National Institutes of Health. The ICDDR,B is supported by agencies and countries which share its concern for the health problems of developing countries.

We thank M. A. Malek for help with data analysis and M. Atiqullah for secretarial assistance.

REFERENCES

1. **Abbott, S. L., W. K. W. Cheung, S. Kroske-Bystrom, T. Malekzadeh, and J. M. Janda.** 1992. Identification of *Aeromonas* strains to the genospecies level in the clinical laboratory. J. Clin. Microbiol. **30:**1262–1266.
2. **Albert, M. J., A. S. G. Faruque, S. M. Faruque, R. B. Sack, and D. Mahalanabis.** 1999. Case-control study of enteropathogens associated with childhood diarrhea in Dhaka, Bangladesh. J. Clin. Microbiol. **37:**3458–3464.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley and Sons, Inc., New York, N.Y.
4. **Burke, V., M. Gracey, J. Robinson, D. Peck, J. Beaman, and C. Bundell.** 1983. The microbiology of childhood gastroenteritis: *Aeromonas* species and other infective agents. J. Infect. Dis. **148:**68–74.
5. **Carnahan, A. M., T. Chakraborty, G. R. Fanning, D. Verma, A. Ali, J. M. Janda, and S. W. Joseph.** 1991. *Aeromonas trota* sp. nov., an ampicillin-susceptible species isolated from clinical specimens. J. Clin. Microbiol. **29:** 1206–1210.
6. **Challapalli, M., B. R. Tess, D. G. Cunningham, A. K. Chopra, and C. W. Houston.** 1988. *Aeromonas*-associated diarrhea in children. Pediatr. Infect. Dis. J. **7:**693–698.
7. **Chopra, A. K., and C. W. Houston.** 1999. Enterotoxins in *Aeromonas*-associated gastroenteritis. Microb. Infect. **1:**1129–1137.
8. **Chopra, A. K., C. W. Houston, J. W. Peterson, and G.-F. Jin.** 1993. Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. Can. J. Microbiol. **39:**513–523.
9. **Chopra, A. K., J. W. Peterson, X.-J. Xu, D. H. Coppenhaver, and C. W. Houston.** 1996. Molecular and biochemical characterization of a heat-labile cytotonic enterotoxin from *Aeromonas hydrophila*. Microb. Pathog. **21:**357–377.
10. **Chopra, A. K., R. Pham, and C. W. Houston.** 1994. Cloning and expression of putative cytotonic enterotoxin-encoding genes from *Aeromonas hydrophila*. Gene **139:**87–91.
11. **Chopra, A. K., X.-J. Xu, D. Ribardo, M. Gonzalez, K. Kuhl, J. W. Peterson, and C. W. Houston.** 2000. The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. Infect. Immun. **68:**2808–2818.
12. **Cumberbatch, N., M. J. Gurwith, C. Langston, R. B. Sack, and J. L. Brunton.** 1979. Cytotoxic enterotoxin produced by *Aeromonas hydrophila*: relationship of toxigenic isolates to diarrheal diseases. Infect. Immun. **23:**829–837.
13. **Figura, N., L. Marri, S. Verdiani, C. Ceccherini, and A. Barberi.** 1981. Prevalence, species differentiation, and toxicity of *Aeromonas* strains in cases of childhood gastroenteritis and in controls. J. Clin. Microbiol. **23:**595–599.
14. **Fiorentini, C., E. Barbieri, L. Falzano, P. Matarrese, W. Baffone, A. Pianetti, M. Katouli, I. Kuhn, R. Mollby, F. Bruscolini, A. Casiere, and G. Donelli.** 1998. Occurrence, diversity, and pathogenicity of mesophilic *Aeromonas* in

estuarine waters of the Italian coast of the Adriatic sea. J. Appl. Bacteriol. **85:**501–511.
15. **Gosling, P. J.** 1996. The genus *Aeromonas*, p. 245–265. *In* B. Austin, M. Altwegg, P. J. Gosling, and S. Joseph (ed.), Pathogenic mechanisms. John Wiley & Sons, New York, N.Y.
16. **Gracey, M., V. Burke, and R. Robinson.** 1982. *Aeromonas*-associated gastroenteritis. Lancet **2:**1304–1306.
17. **Hanninen, M. L., S. Salmi, L. Mattila, R. Taipalinen, and A. Siitonen.** 1995. Association of *Aeromonas* spp. with travellers' diarrhoea in Finland. J. Med. Microbiol. **42:**26–31.
18. **Hanninen, M.-L., and A. Siitonen.** 1995. Distribution of *Aeromonas* phenospecies and genospecies among strains from water, foods or from clinical samples. Epidemiol. Infect. **115:**39–50.
19. **Haque, Q. M., A. Sugiyama, Y. Iwada, Y. Midorikawa, and T. Yamaguchi.** 1996. Diarrheal and environmental isolates of *Aeromonas* spp. produce a toxin similar to Shiga-like toxin 1. Curr. Microbiol. **32:**239–245.
20. **Islam, M. S., M. J. Alam, and S. Tzipori.** 1992. Abundance of *Aeromonas* spp. in various components of pond ecosystems in Dhaka, Bangladesh. Int. J. Environ. Stud. **39:**297–304.
21. **Janda, J. M.** 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. Clin. Microbiol. Rev. **4:**397–410.
22. **Janda, J. M., and S. L. Abbott.** 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. Clin. Infect. Dis. **27:**332–344.
23. **Janda, J. M., S. L. Abbott, S. Khashe, G. H. Kellogg, and T. Shimada.** 1996. Further studies on biochemical characteristics and serologic properties of the genus *Aeromonas*. J. Clin. Microbiol. **34:**1930–1933.
24. **Kindschuch, M., L. K. Pickering, T. B. Cleary, and G. R. Palacios.** 1987. Clinical and biochemical significance of toxin production by *Aeromonas hydrophila*. J. Clin. Microbiol. **25:**916–925.
25. **Krovacek, K., V. Pasquale, S. B. Baloda, V. Soprano, M. Conte, and S. Dumontet.** 1994. Comparison of putative virulence factors in *Aeromonas hydrophila* strains isolated from the marine environment and human diarrheal cases in southern Italy. Appl. Environ. Microbiol. **60:**1379–1382.
26. **Kuhn, I., M. J. Albert, M. Ansaruzzaman, N. A. Bhuiyan, S. A. Alabi, G. Huys, M. S. Islam, P. Janssen, K. Kersters, P. K. B. Neogi, and R. Mollby.** 1997. Characterization of *Aeromonas* spp. isolated from humans with diarrhea, from healthy controls and from surface water in Bangladesh. J. Clin. Microbiol. **35:**369–373.
27. **Kuhn, I., G. Allestam, G. Huys, P. Janssen, K. Kersters, K. Krovacek, and T.-A. Stenstrom.** 1997. Diversity, persistence, and virulence of *Aeromonas* strains isolated from drinking water distribution systems in Sweden. Appl. Environ. Microbiol. **63:**2708–2715.
28. **Monsur, K. A.** 1961. A highly selective gelatin taurocholate tellurite medium for isolation of *Vibrio cholerae*. Trans. R. Soc. Trop. Med. Hyg. **55:**440–445.
29. **Monsur, K. A.** 1963. Bacteriological diagnosis of cholera under field condition. Bull. W. H. O. **28:**387–389.
30. **Morgan, D. R., P. C. Johnson, H. L. Dupont, T. K. Satterwhite, and L. V. Wood.** 1985. Lack of correlation between known virulence properties of *Aeromonas hydrophila* and enteropathogenicity of humans. Infect. Immun. **50:**62–65.
31. **Pazzaglia, G., R. B. Sack, E. Salazar, A. Yi, E. Chea, R. Leon-Barua, C. E. Guerrero, and J. Palomino.** 1991. High frequency of coinfecting enteropathogens in *Aeromonas*-associated diarrhea of hospitalized Peruvian infants. J. Clin. Microbiol. **29:**1151–1156.
32. **Pitarangsi, C., P. Echeverria, R. Whitmore, C. Tirapat, S. Formal, G. J. Dammin, and M. Tingtalapong.** 1982. Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*: prevalence among individuals with and without diarrhea in Thailand. Infect. Immun. **35:**666–673.
33. **Stoll, B. J., R. I. Glass, M. I. Huq, M. U. Khan, J. E. Holt, and H. Banu.** 1982. Surveillance of patients attending a diarrheal disease hospital in Bangladesh. Br. Med. J. **285:**1185–1188.
34. **Thorney, J. P., J. G. Shaw, I. A. Gryllos, and A. Eley.** 1997. Virulence properties of clinically significant *Aeromonas* species: evidence for pathogenicity. Rev. Med. Microbiol. **8:**61–72.
35. **Turnbull, P. C. B., J. V. Lee, M. D. Miliotis, S. V. D. Valle, H. J. Koornhof, L. Jeffery, and T. N. Bryant.** 1984. Enterotoxin production in relation to taxonomic grouping and source of isolation of *Aeromonas* species. J. Clin. Microbiol. **19:**175–180.
36. **World Health Organization.** 1990. A manual for the treatment of diarrhea, p. 6–7. World Health Organization, Geneva, Switzerland.
37. **Xu, X.-J., M. R. Ferguson, V. L. Popov, C. W. Houston, J. W. Peterson, and A. K. Chopra.** 1998. Role of a cytotoxic enterotoxin in *Aeromonas*-associated infections: development of transposon and isogenic mutants. Infect. Immun. **66:**3501–3509.