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

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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 cytotoxic enterotoxin (Alt), a heat-stable cytotoxic 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 × 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 significantly higher for diarrheal children than for control children and the environment.

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 cytotoxic 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., PGE₂) 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 [³⁵S]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
present study, we examined the distribution of the cytotoxic enterotoxin gene act (encoding Act) and the cytotoxic 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 Bagergon, Chattack, Chauqaha, and Matlab. Bagergon is situated approximately 330 km southwest, Chattack is 400 km northeast, Chauqaha is 300 km southeast, 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. Ten grams each of the washed root and sediment were mixed separately with 90 ml of sterile physiological saline and homogenized in a commercial glass homogenizer (Wheaton Scientific, Millville, N.J.) using a StedFast stirrer (model 300; Fisher Scientific, Pittsburgh, Pa.). Tenfold dilutions of the environmental samples were also cultured on TTGA.

Preparation of DNA probes. The recombinant plasmid pXHC95, which contains a 2.8-kb BamHI 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 represented the internal segment of the act gene (8). The plasmid was digested with BstXI 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 pBl24, which contains a 4.0-kb SalI DNA fragment containing the alt gene, was used as a template DNA to amplify the coding region of the act 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 act 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), 2 min each at 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 SalI/BamHI DNA fragment of A. hydrophila SSU and was subcloned into pBluescript to generate a recombinant plasmid, pSB322 (10). The cell lysis from this clone exhibited CHO cell elon-gation activity which was stable at 56°C for 20 min. By Bal31 digestion and addition of polylinkers (3), we generated another clone, pSB333, which contained an approximately 3.5-kb SalI/BamHI DNA fragment and had a level of CHO cell activity similar to that of the pSB322 clone. Expression of these DNA fragments in plasmid pBl7-t with a T7 polymerase-promoter system resulted in protein products of 33 and 67 kDa after [35S]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 inactivated 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 [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 (Amersham Life Science, Little Chalfont, Buckingham-shire, 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 down 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: 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 the proportions of the percentage-positive isolates for each gene among the sources and subcategories. Differences were considered significant if the probability values were less than 0.05.

### TABLE 1. Distribution of various Aeromonas spp. (and HGs) in three sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Total no. of isolates</th>
<th>A. hydrophila (HG1)</th>
<th>A. veronii biotype sobria (HG2)</th>
<th>A. caviae (HG4)</th>
<th>A. media (HG5)</th>
<th>A. enuresphila (HG6)</th>
<th>A. trota (HG13)</th>
<th>A. jandeii (HG9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrheal children</td>
<td>115</td>
<td>11 (9.6)</td>
<td>3 (2.6)</td>
<td>21 (18.3)</td>
<td>39 (33.9)</td>
<td>14 (12.2)</td>
<td>1 (0.9)</td>
<td>25 (21.8)</td>
</tr>
<tr>
<td>Control children</td>
<td>27</td>
<td>1 (3.7)</td>
<td>0 (0.0)</td>
<td>4 (14.8)</td>
<td>11 (40.7)</td>
<td>1 (3.7)</td>
<td>2 (7.4)</td>
<td>1 (3.7)</td>
</tr>
<tr>
<td>Environment</td>
<td>120</td>
<td>18 (15.0)</td>
<td>0 (0.0)</td>
<td>16 (13.3)</td>
<td>7 (5.8)</td>
<td>2 (1.7)</td>
<td>0 (0.0)</td>
<td>72 (60.0)</td>
</tr>
<tr>
<td>Total</td>
<td>262</td>
<td>30 (11.5)</td>
<td>3 (1.2)</td>
<td>41 (15.7)</td>
<td>7 (21.8)</td>
<td>17 (6.5)</td>
<td>2 (0.8)</td>
<td>103 (39.3)</td>
</tr>
</tbody>
</table>

*Also known as genospecies.
the predominant combination was enterotoxigenic among the 26 children infected with three pathogens, the dual infections, 19 children were coinfected with rotavirus and sp. and three other pathogens. Among the 66 children with pathogens; and 5 children had infections with an Aeromonas spp. Of these, 600 samples were positive for aero-jejuni, being found in 7 children.

The distributions of the toxin genes in isolates from control 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 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.

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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.

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 × 10⁻⁵). 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 cytotoxenterotoxin genes among...
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, genospecies, 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 jandei*, 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* sp. may be related to geographical location. Our recent study in which *Aeromonas* sp. 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.

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

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of isolates</th>
<th>No. (%) of isolates positive for the gene(s) encoding:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diarrheal children</td>
<td>115</td>
<td>Alt&lt;sup&gt;a&lt;/sup&gt; 19 (16.5) A 18 (15.7) D 65 (55.7) G</td>
</tr>
<tr>
<td>Control children</td>
<td>27</td>
<td>Alt&lt;sup&gt;a&lt;/sup&gt; 9 (33.3) B 7 (25.9) E 6 (22.2) H</td>
</tr>
<tr>
<td>Environment</td>
<td>120</td>
<td>Alt&lt;sup&gt;a&lt;/sup&gt; 20 (16.7) C 36 (30.0) F 40 (33.3) I</td>
</tr>
</tbody>
</table>

<sup>a</sup> Alt, heat-labile cytotoxic enterotoxin; Ast, heat-stable cytotoxic enterotoxin.

**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* sp.**

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

<sup>a</sup> M, male; F, female.

<sup>b</sup> Number per high-power microscopy field.

<sup>c</sup> Defined as ≥37.7°C.

<sup>d</sup> 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 cytotoxic 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. All and Act 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, 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 biochemical 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 had had watery diarrhea, which is consistent with the presence of cytotoxic 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.

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