Clinical and Epidemiologic Aspects of Members of Aeromonas DNA Hybridization Groups Isolated from Human Feces

ED J. KUIJPER,1* P. BOL, M. F. PEETERS,3 ARNOLD G. STEIGERWALT,3 H. C. ZANEN,1 and DON J. BRENNER1

Department of Medical Microbiology, University of Amsterdam Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam; and Regional Public Health Laboratory, 5000 AS Tilburg, The Netherlands, and Molecular Biology Laboratory, Meningitis and Special Pathogens Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333

Received 29 December 1988/Accepted 6 April 1989

Between June 1982 and May 1987 Aeromonas species were isolated from 208 of 34,311 (0.61%) fecal samples submitted to a Regional Public Health Laboratory in The Netherlands. Aeromonas isolates were found most frequently in summer and rarely in winter. Of 169 Aeromonas isolates that were available for further study, 19% were isolated from patients with a mixed infection, 5% from patients with underlying diseases, and 15% from patients who used medication that could predispose the intestinal tract to colonization with Aeromonas species. Aeromonas species that produced cytotoxins to Vero cells (cytotoxigenic) were found in hybridization groups 1 (11% of all isolates), 2 (1%), 3 (2%), and 8 (25%) and were identified phenotypically as A. hydrophila or A. sobria. Aeromonas species that did not produce cytotoxins to Vero cells (noncytotoxigenic) were found in hybridization groups 4 (57%) and 5A (4%) and were identified phenotypically as A. caviae. Distribution of Aeromonas species by age showed a predominance of noncytotoxigenic strains in children under the age of 5 years (46% of all noncytotoxigenic strains), while cytotoxigenic strains were mainly cultured from patients aged 50 years or older (54% of all cytotoxigenic strains). Significant correlations were found between cytotoxigenic strains and hospitalization, foreign travel, and contact with surface water. Cytotoxigenic strains were isolated significantly more often than noncytotoxigenic strains from patients with diarrhea, but in a multivariate analysis including age, previous medication, underlying disease, and foreign travel, this association was not significant.

An increasing number of studies report on the possible significance of some Aeromonas species as enteric pathogens (8–10, 15, 20, 21, 27, 31). However, the role of Aeromonas species in gastrointestinal disease remains controversial. The production of hemolysins, cytotoxins, and enterotoxins by these Aeromonas species has been considered as a virulence marker for enteropathogenicity (5, 6, 10, 17, 21, 26). The absence of outbreaks of Aeromonas species-associated diarrhea and the report that only 2 of 57 healthy volunteers developed diarrhea after oral administration of a high dose of enterotoxin-producing Aeromonas strains foster doubts as to the enteropathogenicity of Aeromonas species (30).

Problems in assessing the clinical significance of Aeromonas species have been greatly compounded by the inability to correctly identify Aeromonas species. Seven Aeromonas species have been identified on the basis of biochemical characteristics and polynucleotide sequence relatedness: A. hydrophila, A. sobria, A. caviae, A. salmonicida, A. medica, A. veronii, and A. schubertii (1, 13, 14, 34). Several of these species are difficult or impossible to distinguish phenotypically (22; J. J. Farmer III, F. W. Hickman-Brenner, G. R. Fanning, M. J. Arduino, and D. J. Brenner, Abstr. Int. Workshop on Aeromonas, p. 1–2, 1986). In addition, some as yet unnamed DNA hybridization groups (HG) are phenotypically inseparable from named species (22).

Members of 5 of 11 hybridization groups recognized within the genus Aeromonas have been found in human feces, and these belong to groups 1 (genospecies, A. hydrophila), 2 (unnamed), 3 (A. salmonicida), 4 (A. caviae), 5 (A. medica), and 8 (A. veronii) (15, 22). These reports serve to emphasize the problem of identifying Aeromonas species, since other studies indicate a high isolation rate of phenotypically identified A. sobria (HG 7) strains for human feces (8, 17, 27, 31).

We biochemically and genetically characterized Aeromonas strains recovered from human fecal samples that were submitted to the Public Health Laboratory in Tilburg, The Netherlands, over a 5-year period.

MATERIALS AND METHODS

Isolation and phenotypic identification of Aeromonas strains. The Regional Public Health Laboratory in Tilburg, The Netherlands, serves an area with 600,000 people and receives specimens for bacteriological investigation from general practitioners as well as from four general hospitals. Between 1 June 1982 and 31 May 1987, we cultured all fecal samples (n = 34,311) submitted to our laboratory for Aeromonas identification. Specimens were diluted in 0.9% NaCl (1 g/10 ml). and 5 μl of this solution was inoculated onto sheep blood agar containing 10 μg of ampicillin per ml (10). After 48 h of incubation at 37°C, all oxidase- and catalase-positive gram-negative rods which reduced nitrates, fermented mannitol, and did not grow in broth containing 6% NaCl were identified further by using conventional biochemical methods. Aeromonas isolates were identified to the species level by using biochemical tests recommended by Popoff (34) and Janda et al. (17). Extracellular production of

* Corresponding author.
hemolysins to rabbit erythrocytes and cytotoxins to Vero cells was also determined (22).

Isolation of other enteropathogenic bacteria. Fecal samples were also cultured for Salmonella, Shigella, Yersinia, and Campylobacter species by placing the diluted feces onto salmonella-shigella agar (Oxoid Ltd., Hampshire, United Kingdom), deoxycholate-citrate agar (Oxoid), deoxycholate-citrate agar of Wauters (Oxoid) (G. Wauters, Ph.D. thesis, University of Louvain, Louvain, Belgium, 1970), Campy-Bap (Oxoid), bismuth sulfite agar (Oxoid), and in the enrichment selenite medium (E. Merck AG, Darmstadt, Federal Republic of Germany) as well as selenite malachite green medium (selenite medium plus 0.0007% malachite green). Inoculated deoxycholate-citrate agar, salmonella-shigella agar, bismuth sulfite agar, and selenite medium were incubated for 18 h at 37°C. Deoxycholate-citrate agar of Wauters plates were incubated for 48 h at 22°C. Selenite malachite green medium was held at 22°C for 72 h. Campy-Bap plates were held at 42°C in a microaerophilic atmosphere for 48 h. Bacteria were identified by conventional bacteriological methods. Microscopical examinations for parasitic protozoal infections were performed on fecal samples only when requested.

Fecal cytotoxins. To detect Aeromonas and Clostridium difficile cytotoxins, feces were diluted in Hanks balanced salt solution (0.5 g/3 ml) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) and were mixed by agitation in a Vortex mixer (The Vortex Manufacturing Co., Cleveland, Ohio). After centrifugation (2,500 × g, 20 min), the supernatant was decanted, filtered (0.45-μm pore size; Millipore Corp., Bedford, Mass.), and transferred (0.2 ml) together with fresh tissue culture medium (0.8 ml) to monolayers of human embryonic lung fibroblasts and vero cells. Cells were microscopically examined after 3, 16, and 48 h of incubation at 34°C. Fecal samples containing cytotoxic activity were tewfold serially diluted from an initial dilution of 1:5. A toxin-neutralizing assay (Clostridium sordellii antitoxin; Wellcome Diagnostics, Dartford, England) was used to detect C. difficile toxin. Serum from a patient with a 1:640 neutralizing activity against A. hydrophila and A. sobria cytotoxins present in broth cultures filtrates was used in the neutralizing assay for Aeromonas cytotoxins in feces. Neutralization was done by incubating undiluted human serum or antitoxin with the filtered fecal sample for 1 h at 37°C.

DNA hybridization studies. A total of 142 human fecal Aeromonas strains were tested for DNA relatedness to Aeromonas strains representing the 11 DNA HG (3, 22; J. J. Farmer II et al., 1986).

Viral cultures. Viral cultures were done from fecal samples containing Aeromonas species by the use of vero cells. Chang conjunctiva cells, and primary African green monkey kidney cells (Flow Laboratories Ltd., Irvine, Scotland). Viruses were identified by using characteristic cytopathic effects and virus-neutralizing antisera (25). An enzyme immunoassay (Abbott, Amstelveen, The Netherlands) was used to test for the presence of rotavirus in Aeromonas spp.-positive fecal samples. Electron microscopy was not performed.

Clinical and epidemiological data. For each Aeromonas isolate, clinical data were obtained, including symptoms of diarrhea, contact with surface water, underlying diseases, medication, and occurrence of diarrhea in the immediate environment of the patient. Follow-up fecal culture was also requested. Additional data were obtained on the severity and duration of diarrhea, consistency of the stools and number per day, presence of blood and mucus, occurrence of abdominal pain or cramps, the presence of nausea, vomiting, or fever, exposure to surface water, and recent travel. Daily charts were used to follow the clinical course of patients in St. Elisabeth Hospital (Tilburg, The Netherlands), as were discharge letters and questionnaires from patients in other hospitals.

Statistical analysis. Statistical analysis was performed with the chi-square test (including Yates’ correction when necessary), the odds ratio test, and binomial 95 and 99% confidence intervals. To determine the relationships among four variables, a log-linear model using the Biomedical Computer Program (BMDP-4F) was used (7). A delta factor of 0.5 was added to each of the 24 cells in the log-linear analysis.

RESULTS

Aeromonas species were found in 208 of 34,311 samples, an isolation rate of 0.61%. Of 142 isolates characterized biochemically and by DNA relatedness, 87 (61%) were identified phenotypically as A. caviae, 29 (21%) were identified as A. sobria, and 26 (18%) were identified as A. hydrophila. By DNA relatedness, most strains were in DNA HG 4 (57%), 8 (25%), and 1 (11%), with a few strains in HG 5A (4%), 3 (2%), and 2 (1%).

Of strains identified phenotypically as A. hydrophila, 35% were in DNA HG that represent species other than A. hydrophila, and 10% of strains identified phenotypically as A. sobria were not confirmed by hybridization studies with the remaining 90% constituting a biogroup of A. veroni (HG 8). Of the strains identified phenotypically as A. caviae, 2% were in an HG that represents A. veroni. Because of the poor correlation between phenotypic identification of Aeromonas species and DNA HG, we chose to use the term cytotoxigenic for strains belonging to HG 1, 2, 3, and 8, since 96% of these strains produced cytotoxins to Vero cells, and the term noncytotoxigenic for groups 4 and 5A, of which 96% did not produce cytotoxins. Cytotoxigenic strains were identified phenotypically as A. hydrophila or A. sobria, and noncytotoxigenic strains were identified phenotypically as A. caviae. Cytotoxigenic strains differed phenotypically from the noncytotoxigenic strains in the production of gas and acetoin from glucose, production of H2S, decarboxylation of L-lysine, and production of hemolysins to rabbit erythrocytes.

Enteropathogens isolated from fecal samples during this same 5-year period included Campylobacter jejuni (6.9%), Salmonella spp. (3.6%), Shigella spp. (0.3%), and Yersinia enterocolitica (0.67%). Isolated concomitantly with 32 (19%) of 169 Aeromonas isolates were 17 strains of Salmonella, 12 C. jejuni, 2 C. difficile, and 1 enterovirus. Enteropathogenic bacteria were isolated equally with cytotoxigenic and noncytotoxigenic Aeromonas strains.

A total of 84 fecal samples from patients with Aeromonas-associated diarrhea were examined for cytotoxic effects to Vero cells and to human lung fibroblasts. Human lung fibroblasts were used to detect cytotoxins of C. difficile. Samples from 2 children less than 1 year old, one with an Aeromonas strain from HG 8 and one with a strain from HG 4, contained C. difficile cytotoxins. Cytotoxic effects not due to C. difficile were found for 20 strains of Aeromonas strains from HG 8 (6 of 14 patients), from HG 1 (2 of 12 patients), and from HG 4 (7 of 58 patients). Titers of fecal cytotoxic effects varied from 1:5 to 1:40. Cytotoxic effects were not removed by incubation of the filtered supernatants with Aeromonas cytotoxins neutralizing human serum.

Aeromonas isolates were found throughout the year but were most common in the months June through September.
(for months 6, 7, and 8, the expected percentage of Aeromonas isolates was 25.1%; the observed percentage was 40.3% [99% confidence interval, 31.2 to 49.2%]). The lowest incidence occurred in the winter months (for months 12, 1, and 2, the expected percentage was 24.7%; the observed percentage was 13% [99% confidence interval, 7.6 to 20.1%]).

Distribution of Aeromonas species by age (Fig. 1) showed a predominance of noncytotoxigenic strains in children under the age of 5 years (46% of all noncytotoxigenic strains), while cytotoxigenic strains were mainly cultured from patients aged 50 years or older (54% of all cytotoxigenic strains; Table 1). In all age groups, diarrhea was more frequent in patients with cytotoxigenic strains than in patients with noncytotoxigenic strains. Aeromonas strains in individuals with and without diarrhea were distributed equally among males and females in all age groups.

A strong association was observed (Table 2) between the presence of cytotoxigenic Aeromonas strains in human feces and clinical symptoms of gastroenteritis or enterocolitis, if other variables for age and predisposing factors were not included in the statistical analysis (see log-linear analysis). Of 48 patients with cytotoxigenic Aeromonas strains, 44 had diarrhea, which differed significantly from the percentage of noncytotoxigenic strains isolated from patients with diarrhea ($\chi^2 = 7.95$, $P < 0.005$). Information from questionnaires completed by 55 (33%) of 169 patients studied is shown in Table 3.

Two weeks after primary culture, all repeat fecal cultures ($n = 38$) were negative for Aeromonas strains (23 noncytotoxigenic and 15 cytotoxigenic strains).

Cytotoxigenic strains were more frequently present in fecal samples of patients with traveler's diarrhea than were noncytotoxigenic strains ($\chi^2 = 4.26$, $P < 0.05$) (Tables 1 and 2). Of 10 patients with traveler's diarrhea, seven traveled to

TABLE 1. Relation between age, predisposing factors, enterotoxin, and diarrhea

<table>
<thead>
<tr>
<th>Predisposing factor</th>
<th>No. of patients ($n = 48$) with cytotoxigenic strains by age (yr [%])</th>
<th>Total (100)</th>
<th>No. of patients ($n = 89$) with noncytotoxigenic strains by age (yr [%])</th>
<th>Total (100)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (8%)</td>
<td>1–49 (38%)</td>
<td>&gt;50 (54)</td>
<td>Total (100)</td>
</tr>
<tr>
<td>None</td>
<td>1/1</td>
<td>6/8 (75)</td>
<td>13/14 (93)</td>
<td>20/23 (87)</td>
</tr>
<tr>
<td>Medication</td>
<td>1/1</td>
<td>2/3</td>
<td>9/9 (100)</td>
<td>12/13 (92)</td>
</tr>
<tr>
<td>Underlying disease</td>
<td>0/0</td>
<td>2/2</td>
<td>0/0</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Foreign travel</td>
<td>2/2</td>
<td>5/5 (100)</td>
<td>0/0</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>4/4 (100)</td>
<td>15/18 (83)</td>
<td>25/26 (96)</td>
<td>44/48 (92)</td>
</tr>
</tbody>
</table>

* Predisposing factors (underlying disease, medication, and foreign travel) were more common in patients with cytotoxigenic strains (52%) than noncytotoxigenic strains (21%) ($\chi^2 = 13.51$, $P = 0.001$).

* The fraction represents the number of patients with diarrhea divided by the total number of patients. Percentages of patients with diarrhea are in parentheses.
The Netherlands from countries in southern Europe, two from countries in Africa, and one from Sri Lanka.

The occurrence of cytotoxigenic *Aeromonas* strains in feces was strongly associated with contact with surface water (Table 2), such as swimming, surfing, or fishing during the week preceding the illness. All patients who recently had contact with surface water and whose feces contained *Aeromonas* strains had diarrhea.

Ten patients with diarrhea associated with cytotoxigenic strains were hospitalized because of their symptoms; in two of these patients, *Aeromonas* strains were also isolated from blood (Table 2). Of the 10 patients, 7 had an underlying illness: an 82-year-old male with prostatic carcinoma and metastases; a 75-year-old male with amyotrophic lateral sclerosis (who subsequently died from a combination of cardiac asthma, bronchopneumonia, and ascending cholangitis); an 87-year-old male with pneumonia treated with amoxycillin; a 64-year-old female with cerebral carcinoma stage IIb treated with radical hysterectomy and radiation therapy; a 67-year-old male with chronic bronchitis, rheumatoid arthritis, and hypertension; a 78-year-old female with senile dementia, diabetes mellitus (type II), and hypertension; and a 22-year-old male with rectal adenocarcinoma. Six of the hospitalized patients recovered after antibiotic treatment. In three patients, conservative therapy alone resulted in clinical improvement. Diarrhea generally showed an acute onset and disappeared within 1 week of hospitalization.

None of the eight hospitalized patients with noncytotoxigenic strains was known to have an underlying illness. Blood cultures were not done. Seven of the hospitalized patients were under 6 months of age, representing 29% of all patients younger than 6 months of age with noncytotoxigenic *Aeromonas* strains ($\chi^2 = 12.88, P < 0.0005$). One patient had acute watery diarrhea, whereas the seven others suffered from chronic gastrointestinal complaints. Reflecting the mild course of the gastrointestinal disease, only two patients received antibiotic treatment. All eight hospitalized patients recovered uneventfully, and after 2 weeks, repeat fecal cultures were always negative.

The relationship between DNA HG and clinical symptoms of gastrointestinal infection is shown in Table 4. It was assumed that *Aeromonas* strains were not the causative agents in cases of mixed infection. Strains from HG 3 (100%), 8 (78%), and 1 (73%) were isolated more frequently from patients with *Aeromonas* species-associated gastroenteritis than from healthy individuals or from patients with mixed infections. Strains from HG 4 (53%) and 5A (50%) were isolated approximately equally from patients with *Aeromonas* species-associated gastroenteritis, from healthy individuals, and from patients with mixed infections. The single strain from HG 2 was isolated from a patient with a mixed infection.

To study the relationship between cytotoxigenic strains with diarrhea more carefully, the data from Tables 1 and 2 were separated into 24 categories for a log-linear analysis (6.2 samples per cell, including a delta factor of 0.5) with four variables: age (0, 1 to 49, and >50 years), predisposing factors (present and absent), cytotoxigenic strains (positive and negative), and diarrhea (present and absent). The significant association between cytotoxigenic strains and presence of diarrhea was absent in the multivariate analysis ($\chi^2 = 1.43, P = 0.23$), apparently due to influence of two confound-
The presence of watery diarrhea in 57% of patients with cytotoxigenic strains suggests enterotoxin involvement. We could not demonstrate Aeromonas-specific cytotoxins in fecal samples, despite the presence of cytotoxic effects in 8 (31%) of 26 fecal samples containing cytotoxigenic strains. It is possible that cytotoxic enterotoxins of other enteropathogenic bacteria caused diarrhea in these patients, especially since stools from 7 (12%) of 56 patients with noncytotoxigenic strains in their feces also produced fecal cytotoxic effects. It is also possible that either our cytotoxin-neutralizing assay is not sufficiently sensitive to detect fecal Aeromonas cytotoxins or the cytotoxic effects are nonspecific. We did not investigate the presence of cytotoxins in fecal samples, although Aeromonas enterotoxins have also been described as cytotoxic (6, 35).

We found two patients with Aeromonas bacteremia caused by cytotoxigenic Aeromonas strains. One of these patients suffered from an underlying illness (prostatic carcinoma with metastases), and severe diarrhea was present before bacteremia occurred in both patients. The occurrence of Aeromonas bacteremia caused primarily by cytotoxigenic strains in patients with underlying illnesses has also been described (16, 19, 36, 38). In most cases the gastrointestinal tract was suspected as the source of the bacteremia, and patients were neutropenic due to hematological malignancies or treatment with immunosuppressive drugs.

Aquatic environments favor Aeromonas species and could serve as a source of transmission for human infections. In Switzerland, Aeromonas species are isolated most often from surface water during summer months when the water temperature fluctuates between 15 and 25°C. Cytotoxigenic strains are isolated more often from surface water than are noncytotoxigenic strains (12). The number of cytotoxic Aeromonas strains in surface water varies up to 10^3 CFU/ml depending on the geographic location. In our study, recreational contact with surface water, for example by swimming, fishing, or surfing, was more frequent in patients with cytotoxigenic strains than with noncytotoxigenic strains. Of patients with fecal cytotoxigenic Aeromonas strains, 21% had contact with surface water in the week before the onset of diarrhea, in contrast to 3% of patients with noncytotoxigenic strains. Recreational contact with surface water may also play a role in the significance of Aeromonas species as a cause of traveler’s diarrhea, as shown in 7 of 10 patients with this condition. In our study, 10 of 169 (6%) patients with Aeromonas (mainly cytotoxigenic) strains in their feces and 10 of 78 (13%) patients with diarrhea had traveler’s diarrhea. In the study of George et al. (9), recent travel abroad preceded Aeromonas species-associated diarrhea in 4 of 80 (5%) patients. The significance of Aeromonas species as a cause of traveler’s diarrhea was also shown by Gracey et al. (11) and Pitargangi et al. (33).

It is possible that the presence of Aeromonas species in the intestinal tract reflects the occurrence of an opportunistic pathogen attacking an intestine predisposed to infection by a lack of (local) immunity, by an enteric pathogen, by medication, by an underlying disease, by an alteration of the normal enteric flora, or by nutritional factors. This hypothesis is supported by the finding that 66 of 169 (39%) Aeromonas isolates were from patients who had either a mixed infection or an underlying disease. These patients used prior medication that could predispose the gastrointestinal tract to colonization with Aeromonas strains. The age-related occurrence of Aeromonas species-associated diarrhea suggests that host immunity to Aeromonas species is acquired at an early age but may be lost in later life, since fecal Aeromonas
strains were frequently found in children less than 1 year of age (28% of all nontoxigenic isolates) and in adults aged 50 years or older (54% of all cytotoxigenic isolates). Hospitalized patients with cytotoxigenic strains were predominantly elderly people with an underlying illness, whereas most hospitalized patients with nontoxigenic strains were younger than 6 months of age. In young children, it is difficult to dismiss the possibility that nontoxigenic strains are pathogenic (2).

Initial analysis of our results suggested a significant association between cytotoxigenic strains and clinical symptoms of diarrhea. Log-linear analysis showed that the positive correlation between cytotoxigenic strains and the presence of diarrhea was not significant, due to interaction with two variables, age and predisposing factors. Although these variables have been recognized by other investigators, multivariate analysis was never used to study relationships among them. Our results suggest that Aeromonas species-associated diarrhea cannot be attributed solely to known virulence properties of the bacteria but that it is also strongly associated with host factors. This might explain why only 2 of 57 healthy volunteers developed diarrhea after oral administration of high doses of Aeromonas strains (30).

ACKNOWLEDGMENTS

We thank Brian Pliskayits for critically reviewing the manuscript. This study was supported by a grant from the Ter Meulen-Foundation of the Royal Netherlands Academy of Arts and Sciences.

LITERATURE CITED