Aeromonas hydrophila in Acute Diarrheal Disease: Detection of Enterotoxin and Biotyping of Strains

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Eleven isolates of *Aeromonas* species from human stool cultures were found to produce enterotoxin activity as determined by assay of culture filtrates in rabbit intestinal loops and rabbit skin and on adrenal Y1 cells. Hemolysin(s) and a cytotoxic protein were found to interfere in all three assay systems but could be inactivated upon heating at 56°C or by specific antihemolysin. Biotyping of each isolate was performed with a conventional test system and with API 50E and APIZYM kit systems (Analytab, Inc.). No single test of the more than 70 biochemical reactions investigated was found to correlate with enterotoxigenicity in the strains of *Aeromonas hydrophila* examined. All strains were found to belong to ideal phenotypes of *A. hydrophila*, but each strain possessed its own biochemical profile.

Besides the causative agent of classical cholera (*Vibrio cholerae*), other members of the *Vibrio* genus have recently been found to cause acute diarrheal disease by producing an extracellular enterotoxin (11). Apart from many studies concerning enterotoxinogenic *Escherichia coli* causing a cholera-like disease in both children and adults (22), very few investigations have dealt with the possibility that other members of *Enterobacteriaceae* or oxidase-positive organisms such as *Aeromonas* and *Pseudomonas* produce enterotoxins (11, 17, 25).

Four reports from Asian countries (1, 4, 5, 25) and one unpublished study from Ceylon (see reference 9) have recently described *Aeromonas hydrophila* as a possible causative agent of acute diarrheal disease. A few reports from the Western Hemisphere have also indicated that *A. hydrophila* can be found in stools from normal individuals as well as in stools from patients with gastrointestinal symptoms, especially during summer months (18, 30, 31). Some reports have emphasized the role of this organism in septicemia or other human infections in debilitated patients (29). Only in an Indian study (25) were these bacteria found to give a positive test upon inoculation into rabbit intestinal loops, i.e., the classical cholera test system.

Enterotoxinogenic bacteria were isolated from 33% of 354 children with gastrointestinal symptoms in a recent study from the Ethio-Swedish

Pediatric Clinic in Addis Ababa, Ethiopia (32). Only one-third of these isolates were found to be *E. coli*, the others belonging to other species of *Enterobacteriaceae*, except 11 strains of oxidase-positive organisms identified as *A. hydrophila*.

The first report on detection of enterotoxin in culture filtrates of these organisms was published recently (33). In addition, problems concerning the detection of enterotoxin in such fluids containing other factors with cytotoxic properties, which interfere with different bioassay systems, were discussed.

The aim of the present investigation concerned further biochemical testing of these 11 strains classified as *A. hydrophila* by a conventional biochemical typing system and a more extensive testing with the API 50 *Enterobacteriaceae* (API 50E) and the APIZYM systems (Analytab, Inc. System, La Balme-les-grottes, France) to see if a correlation between the enterotoxinogenic property and biotype would exist.

MATERIALS AND METHODS

Bacterial strains. Eleven oxidase-positive enterotoxinogenic strains from stool cultures from Ethiopian children, identified as *A. hydrophila* by the test system recommended by Ewing and Hugh (9), were selected for this study. Sixteen strains isolated from patients and the environment (e.g., water supplies) in India were kindly sent to us by B. D. Chatterjee, School of Tropical Medicine, Calcutta, India (4). The two strains of *Plesiomonas shigelloides* were kind gifts from J. Vandepitte, St. Rafael University Hospital, Leuven, Belgium (27).

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Cultivation and enterotoxin assays. Each strain was cultivated in shake flasks in brain heart infusion broth (Difco) at 24°C, and samples were taken for enterotoxin determinations as previously described (33). Enterotoxin was assayed in three bioassay systems (i) the rabbit intestinal loop test in two rabbits (20); (ii) the rabbit skin test in two rabbits (14); and (iii) the adrenal Y1 test on two separate occasions (7, 32). A strain was considered enterotoxinogenic if a positive result was obtained in both rabbits with either the loop or the skin test and/or in the adrenal cell test on both occasions.

Neutralizing antisera. A rabbit antiserum against *A. hydrophila* hemolysin with a neutralization titer of 64 U was used to inhibit hemolytic activity in culture fluids (34). Rabbit antisera against cholera enterotoxin were kindly supplied by W. Ungar, Swiss Serum Institute, Basel, Switzerland, and R. Thomson, Wellcome Research Laboratories, Beckenham, Kent, England. An antiserum produced in our laboratory against enterotoxin from *E. coli* strain 853/67 was also used for inhibition experiments (26).

Biotyping and other assays. The biotyping of the 11 enterotoxinogenic *Aeromonas* strains was performed on API 50E and APIZYM, according to the instructions of the manufacturer. API 50E consists of 50 biochemical tests. APIZYM comprises enzymatic tests performed on freeze-dried substrates for the following: alkaline and acid phosphatases, esterase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin- and chymotrypsin-like proteases, phosphoamidase, α -galactosidase, β -galactosidase, β -glucosaminidase, α -glucosidase, β -glucosidase, β -glucuronidase, α -mannosidase, and α -fucosidase.

The utilization of L-histidine and L-arginine (28) as sole carbon sources and growth in KCN medium were also tested. Production of extracellular enzymes and toxins by each isolate was assayed by sensitive agar plate methods: protease on skim milk agar, elastase on elastin agar, deoxyribonuclease on deoxyribonucleic acid agar, phospholipase on lecithin agar, and lipase on tributyrin agar. The last two enzymes were also determined on egg yolk agar. Production of hemolysin was detected on agar plates containing horse, ox, or rabbit erythrocytes. All these plate methods have been described in detail in a previous publication (19).

RESULTS

Enterotoxinogenicity. Five of the 11 Ethiopian *Aeromonas* strains were positive in all three enterotoxin assay systems. Four strains were positive in the skin and adrenal cell tests; two strains were positive only in the skin or cell test. The results of the skin and adrenal cell tests were confirmed after incubation of the test samples with an antiserum to extracellular hemolysin (34) to prevent dermonecrosis in the skin test and cytotoxic effects in the adrenal cell test. Enterotoxigenicity could also be shown after inactivation of the cytotoxic factors at

56°C for 10 min (33). Each test became negative when the samples were heated at 80°C for 15 min before testing (32). All 16 Indian strains were negative when culture filtrates were tested in the three assay systems. Two *P. shigelloides* grown and tested under the same conditions as the *A. hydrophila* strains were positive only in the adrenal Y1 cell test.

Nonconcentrated extracellular proteins from 9 of the 11 enterotoxinogenic *Aeromonas* strains gave one immunoprecipitate in double immunodiffusion in agarose gel against two different cholera enterotoxin antisera (13). However, in the rabbit intestinal loop test, cholera enterotoxin antiserum did not neutralize the crude *Aeromonas* toxin even at concentrations 10 times the neutralization titer for one mean effective dose of cholera enterotoxin (14, 32), nor did *E. coli* enterotoxin antiserum neutralize the activity. Crude ganglioside mixture (Sigma) did not neutralize the *Aeromonas* toxin preparations when used in five times the concentration necessary to inhibit cholera or *E. coli* enterotoxin (13).

Biotyping. All 11 enterotoxinogenic strains examined produced acid from glucose, ribose, D-(-)-levulose, D-(+)-mannose, maltose, D-(+)-trehalose, N-acetylglucosamine, dextrin, starch, glycogen, mannitol, and glycerol, but all were negative for D-(-)-arabinose, D-(+)-xylose, L-(-)-xylose, L-(-)-sorbose, rhamnose, D-(+)-melezitose, amylose, inulin, amygdalin, methylxyloside, methyl-D-mannoside, adonitol, erythritol, dulcitol, and meso-inositol.

L-Histidine and L-arginine were utilized as sole carbon and energy sources by the enterotoxigenic strains. Growth in KCN medium was positive for all these strains (strain K206 did not grow in KCN medium within 24 h but had grown after 48 h). The results for variable characters are presented in Table 1.

TABLE 1. Variable characters for the 11 enterotoxinogenic strains of *A. hydrophila*

Fermentation of:	Negative strains
L-(+)-Arabinose	K194
Galactose	K206
Sorbitol	All except K118, K137, K140, K142, K144
Methyl-D-glucoside	K141, K206
Arbutin	K194
Esculin	K194
Salicin	K195, K194
D-(+)-Cellobiose	K138, K139, K141, K185
Lactose	All except K139, K140, K141, K142, K206
D-(+)-Melibiose	All except K194
Sucrose	K138, K141
D-(+)-Raffinose	All except K194
Glucose with gas	K118, K206

On APIZYM, the following enzymatic profile was observed with the enterotoxigenic strains: presence of alkaline and acid phosphatases, esterase, lipase, leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin-like protease, phosphoamidase, α -galactosidase, β -galactosidase, β -glucosaminidase, α -glucosidase (except strain K106), and β -glucosidase (except strain K194); absence of chymotrypsin-like protease, β -glucuronidase, α -mannosidase, and α -fucosidase. Each strain was examined twice on both API 50E and APIZYM, once in Sweden (Å.L.) and once in France (M.P.). The reproducibility of each reaction was satisfactory.

The results of the agar plate tests for various enzymes and hemolytic patterns are given in Table 2. Each strain produced at least some of the different extracellular proteins described in a previous study (19). Each strain (except strain K206) also produced hemolysin(s).

The biochemical profiles of the 16 Indian strains of *A. hydrophila* as tested by the API 50E system and the enzyme plate assays were not found to differ from the 11 enterotoxigenic Ethiopian strains. A subculture of strain K140 which had lost its enterotoxinogenicity had not changed its biochemical profile in the API 50E system.

DISCUSSION

Recent studies from Brazil and Ethiopia have shown the possible etiological role of enterotox-

igenic strains of different species other than vibrios and *E. coli* in acute diarrheal disease in childhood (12, 32). The 11 oxidase-positive strains among the enterotoxinogenic strains from the Ethiopian study were identified as *A. hydrophila* by conventional systems. Using the taxonomic scheme proposed by one of us (21), these 11 strains of *Aeromonas* could also be assigned to *A. hydrophila*, but each strain had its own biochemical profile (except strains K140 and K142) and, thus, on epidemiological grounds, could not represent strains from a single outbreak at the Ethio-Swedish Pediatric Clinic during the period of the epidemiological survey (Table 3; 32). This is substantiated by the good reproducibility upon biotyping of each strain twice in separate laboratories. These commercial test kits are, in our opinion, useful in epidemiological studies of *Aeromonas* isolates provided the test conditions are well standardized (2).

The recent taxonomic study on the *A. hydrophila*-*A. punctata* group showed that the latter designation was an illegitimate synonym for the first nomenclature, whereas a new species, *A. sobria*, was proposed for a class of strains in which a comparatively smaller number of substrates supported growth (21). No strains of *A. sobria* were isolated from the 354 Ethiopian children. It is therefore an open question as to whether or not this species can also produce enterotoxin.

All 11 strains were classified as enterotoxino-

TABLE 2. Agar plate assays for different extracellular enzymes and hemolysins produced by the 11 enterotoxinogenic strains of *A. hydrophila*

Extracellular enzymes and hemolysins	Assay of <i>A. hydrophila</i> strain:										
	K118	K137	K138	K139	K140	K141	K142	K144	K185	K194	K206
Protease (caseinolytic)	+	+	+	+	-	+	+	+	+	+	-
Deoxyribonuclease	+	+	+	+	+	+	+	+	+	+	+
Lecithinase	+	+	+	+	+	+	+	+	+	-	+
Elastase	-	-	+	+	-	+	-	+	+	-	+
Lipase	+	+	+	+	-	+	+	+	+	+	-
Egg yolk	+	+	+	+	+	+	+	+	+	+	+
Hemolysin horse erythrocytes	+	+	+	+	+	+	+	+	+	+	-
Hemolysin ox erythrocytes	+	+	+	+	+	+	+	+	+	+	-
Hemolysin rabbit erythrocytes	+	+	+	+	+	+	+	+	+	+	-

TABLE 3. Biotyping of the 11 enterotoxinogenic strains of *A. hydrophila*

Character	Biotyping of <i>A. hydrophila</i> strain:										
	K118	K137	K140	K142	K144	K138	K139	K141	K185	K194	K206
Sorbitol	+	+	+	+	+	-	-	-	-	-	-
Lactose	-	-	+	+	-	-	+	+	-	-	+
Glucose with gas	-	+	+	+	+	+	+	+	+	+	-
Elastase	-	-	-	-	+	+	+	+	+	-	+
Salicin	+	+	+	+	+	+	+	+	-	-	+
Sucrose	+	+	+	+	+	-	+	-	+	+	+

genic; the 2 strains positive only in one test system were positive upon repeated testing in rabbits or Y1 cell cultures, respectively (32). It is known that even freshly isolated cholera vibrios can be negative in the classical intestinal loop test, probably due to the low sensitivity of this test system compared with that of the skin test or different cell tests (11, 22). On the other hand, a collection of 16 Indian strains of *A. hydrophila* were all negative in the three enterotoxin tests. In contrast to strains of *V. cholerae* and toxinogenic non-cholera vibrios with chromosomal control of toxin synthesis (11), preliminary data indicate that the enterotoxin property is lost upon secondary subculturing of *A. hydrophila* strains and upon treatment of growing cultures with chemicals, such as acriflavine, known to be plasmid-curing agents (10; Å. Ljungh, unpublished data).

The present investigations failed to demonstrate any correlation between any of the more than 70 biochemical properties or extracellular factors produced and the ability of strains to elaborate enterotoxin. In contrast, recent studies on enterotoxinogenic strains of *E. coli* have often been shown to lack the ability to ferment sucrose compared to ordinary *E. coli* isolates (8, 23).

Further studies on *Aeromonas* strains isolated in different geographic areas must be performed to determine the relative importance of this genus in acute diarrheal disease. It should, however, be emphasized that *Aeromonas* is much underdiagnosed in stool cultures and is probably often misdiagnosed as *E. coli* and *Klebsiella* (9, 31). The oxidase test in screening for *Aeromonas*, *Pseudomonas*, and *Vibrio* should be performed more generally on stool cultures, as pointed out in previous studies on *Aeromonas* (4, 9).

The cytotoxic properties of most *Aeromonas* culture filtrates make the detection of enterotoxin difficult in all three assay systems (33). Cell lysis in the tissue culture and dermonecrosis in the skin test prevent the use of these tests without special precautions, whereas the loop test seems to be less susceptible to these toxic reactions. Similar problems were also encountered in the detection of enterotoxin in *Vibrio parahaemolyticus*. Indeed, there are still different opinions as to existence of an enterotoxin in the latter species (3, 15).

The greater heat sensitivity of the cytotoxins makes it possible to detect enterotoxin after heating of culture fluid, in both the skin and cell tests (33). Inhibition with antisera also confirmed that the enterotoxin gave a classical skin reaction and rounding of adrenal cells as described for cholera and *E. coli* enterotoxins

(33). It is thus likely that the *Aeromonas* enterotoxin should be classified among the cytotoxic enterotoxins (11) and not among the cytotoxic enterotoxins, e.g., *Shigella* enterotoxin (16).

The inability of antiserum against cholera and *E. coli* enterotoxins to neutralize the enterotoxin from *Aeromonas* indicates that this enterotoxin might belong to a separate group of enterotoxins. However, preliminary studies with partially purified *Aeromonas* enterotoxin have shown that the mode of action is probably similar to that of cholera toxin, since it causes release of corticosteroids in Y1 adrenal cells as described for cholera enterotoxin (6; Ljungh, unpublished data).

P. shigelloides, previously in the *Aeromonas* genus, has been isolated from animals and humans with diarrhea, e.g., from 41 patients with diarrhea over an 8-year period in Zaire (27). The two *P. shigelloides* strains investigated in this study gave a positive adrenal cell test but negative rabbit intestinal loop and skin tests. This indicates either that a very low quantity of enterotoxin was produced, which was only detectable in the sensitive cell test, or that this toxin had another range of activity in different bioassay systems as recently described for *Salmonella* enterotoxin (24), which had a very high specific activity in the rabbit skin test and a very low activity in intestinal loop tests in different animals species.

A single report on enterotoxin formation by a human stool isolate of *Pseudomonas aeruginosa* has been published (17). More studies should be performed to indicate the relative importance of enterotoxinogenic oxidase-positive organisms other than cholera and non-cholera vibrios in acute diarrhea in different geographic locations in both humans and animals, since certain species cause a variety of infections in different animal species (9).

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