Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*: Prevalence Among Individuals With and Without Diarrhea in Thailand

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To evaluate the enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*, the rate of isolation of these organisms was compared among individuals with and without diarrhea in Thailand. In two groups of American travelers, *A. hydrophila*, but not *P. shigelloides*, was associated with episodes of travelers diarrhea more often than when individuals did not have diarrhea ($P < 0.025$). Among three populations of Thais, *A. hydrophila* and *P. shigelloides* were isolated with similar frequencies from individuals with and without diarrhea. The biochemical characteristics, production of cytotoxin, and ability to distend suckling mouse intestine were similar among *A. hydrophila* isolates from individuals with and without diarrhea. However, cytotoxic *A. hydrophila* strains distended rabbit and suckling mouse intestine and produced destructive lesions in intestinal mucosa of both species of animal. *P. shigelloides* strains produced neither cytotoxin nor distended intestine. Oral administration of whole cultures (10^9) of cytotoxic *A. hydrophila* or *P. shigelloides* failed to cause diarrhea in rhesus monkeys. Volunteer studies or intestinal biopsies of patients with diarrhea may be required to establish whether *A. hydrophila* is a gastrointestinal pathogen in humans.

Although the role of *Aeromonas hydrophila* and *Plesiomonas shigelloides* as enteric pathogens has not been proven conclusively, diarrheal diseases thought to be caused by *A. hydrophila* (14, 16, 22) and *P. shigelloides* have been described (21). In the United States, Von Graevenitz and Mensch (22) reported two cases of diarrhea associated with *Aeromonas* isolates in the stool, but they also recovered these organisms from the stools of five asymptomatic individuals. In recent years numerous authors have published reports of acute, occasionally severe diarrheal syndromes associated with the recovery of *Aeromonas* species in the stool (16, 21, 22). These illnesses were usually self-limited, and in most cases other possible etiologies were excluded. A recently reported case of a patient with prolonged diarrhea associated with repeated isolation of *A. hydrophila* from feces in the absence of other enteric pathogens, with recovery after appropriate antimicrobial therapy, provides further evidence to support the enteropathogenicity of this organism (14). On the

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Indian subcontinent, *Aeromonas* species have been implicated in episodes of cholera-like disease (3, 17), and *P. shigelloides* strains have been implicated in two large epidemics of diarrheal disease in Japan (21). Although the rate of asymptomatic bowel carriage has been reported to be very low (2, 11, 17), there have been few attempts to compare the prevalence of aeromonads in patients with diarrhea with the prevalence in controls without gastrointestinal symptoms.

To determine the enteropathogenicity of *A. hydrophila* and *P. shigelloides*, we compared the prevalence of these organisms in different populations of Thais and Americans in Thailand. Isolates from patients with and without diarrhea were examined with assays previously used to evaluate enteropathogenicity of other enteric bacteria.

MATERIALS AND METHODS

Source of isolates. Three Thai populations and two groups of American Peace Corps volunteers who had recently arrived in Thailand were studied. The characteristics of these populations are given in Table 1. A study of travelers diarrhea among the first group of

TABLE 1. Characteristics of populations cultured in Thailand

Individuals with diarrhea	Description	Individuals without diarrhea	Description
Bangkok			
Children's Hospital			
105 children	Children 0–2 yr of age admitted to the rehydration ward; May–July 1979	74 convalescent children	Same children cultured 3–4 wk after their episode of diarrhea
		100 children	Children 0–2 yr of age without diarrhea for 2 wk seen at the outpatient department; May–July 1979
Phra Mongkutklao Hospital			
18 newborns	Newborns less than 1 wk of age with diarrhea; May–July 1979	66 newborns	Newborns cultured within 7 days after delivery May–July 1979
Soongnern			
Soongnern Hospital			
30 children 0–2 yr of age	Children admitted to the hospital for rehydration; May–July 1980	101 children 0–2 yr of age	Children without diarrhea for the previous 2 wk living in villages in Soongnern; May–July 1980
7 children, 2–10 yr of age	Children admitted to the hospital for rehydration; May–July 1980	51 children 2–10 yr of age	Children without diarrhea for the previous 2 wk living in villages in Soongnern; May–July 1980
9 children, 10–20 yr of age	Children admitted to the hospital for rehydration; May–July 1980		None cultured
56 adults (>20 yr of age)	Adults admitted to the hospital for rehydration; May–July 1980	51 adults (>20 yr of age)	Adults without diarrhea for the previous 2 wk living in villages in Soongnern; May–July 1980
American Peace Corps volunteers			
Group I			
39 episodes	Episodes of travelers diarrhea among 35 volunteers during their first 5 wk in Thailand	35	Volunteers after 5 wk in Thailand
Group II			
14 episodes	Episodes of travelers diarrhea among 35 volunteers during their first 5 wk in Thailand	26	Volunteers after 2 wk in Thailand
		33	Volunteers after 5 wk in Thailand

Peace Corps volunteers has previously been published (8). Among Thais, an episode of diarrhea was defined as twice the number of normal bowel movements accompanied with stools assuming the shape of the container occurring for less than 48 h. In studies of diarrhea among American Peace Corps volunteers, three or more watery stools or two watery stools accompanied by nausea, vomiting, abdominal cramping, fever, or prostration within a 24-h period was defined as an episode of travelers diarrhea. Formed stools were collected from asymptomatic volunteers in the first group on arrival and after they had been in Thailand for 5 weeks. Similar specimens were collected from the second group on arrival and after they have been in Thailand for 2 and 5 weeks. No volunteers were receiving antibiotics during the study period.

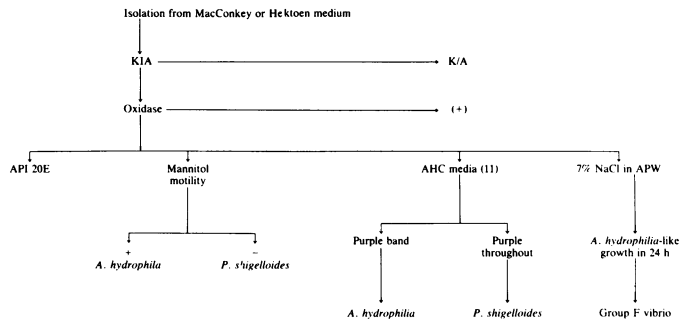**Bacteriology.** Stool specimens were cultured directly

FIG. 1. Identification of A. hydrophila and P. shigelloides. KIA, Kligler iron agar; APW, alkaline peptone water.

on MacConkey and Hektoen media (Difco Laboratories, Detroit, Mich.) at 37°C for 24 h. Colonies that did not ferment lactose on MacConkey medium, flat green (P. shigelloides), convex green, or yellow-or-ange colonies (A. hydrophila) on Hektoen medium, or colonies which did not ferment lactose on deoxy-cholate medium were identified by methods summarized in Fig. 1 (12). Also, any of 10 lactose-positive colonies, selected from the MacConkey medium, which were indole negative, metabolized citrate, and were methyl red negative were further identified. Colonies which initially appeared to be A. hydrophila were inoculated into alkaline peptone water with 7% sodium chloride to distinguish A. hydrophila from Vibrio fluvalis (group "F" or EF6 vibrios) (10).

In vitro test. Twofold dilutions in phosphate-buffered saline, pH 7.4, of sterile filtrates of aeromonads grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 0.6% yeast extract (Difco) at 37°C on a roller drum (8 rpm) for 18 h were tested for hemolysis of sheep and rabbit erythrocytes and cytotoxicity in Y-1 adrenal cell tissue cultures (4). A. hydrophila and P. shigelloides were tested for mannose-resistant hemagglutination of human (group A) and bovine erythrocytes (9).

P. shigelloides isolates were tested for agglutination in Shigella grouping antisera (Difco). Isolates that agglutinated in Shigella antisera and cytotoxic A. hydrophila strains were tested in the Sereny test (20).

In vivo tests. Whole cultures (10^9/ml) of A. hydrophila or P. shigelloides were tested for their ability to distend ligated rabbit ileal loops after 7 and 18 h (15) and 3- to 4-day-old suckling mouse intestine after 4 h (6). Tissue sections of suckling mouse and rabbit ileal loops were fixed in 10% Formalin, Giemsa stained, and examined microscopically.

After 24 h of starvation, groups of five adult rhesus monkeys were fed 10^9 P. shigelloides isolated from a patient with severe diarrhea or 10^9 cytotoxic A. hydrophila from another patient with watery stools and abdominal cramps. The inocula were administered by nasogastric tube 20 min after each animal received 3 g of $NaHCO_3$ in 20 ml of distilled water. Monkeys were observed four times a day for 5 days, and rectal temperatures were measured twice daily. Another group of five monkeys was pretreated for 5 days with 40 mg of trimethoprim and 200 mg of sulfamethoxazole twice daily, starved for 24 h, and fed 10^9 cytotoxic A. hydrophila 20 min after receiving sodium bicarbonate.

RESULTS

Prevalence of Aeromonas sp. A. hydrophila and P. shigelloides were uncommon in Thai infants cultured within the first week of life, and the isolation rate among asymptomatic inhabitants of Soongnern increased with age. The percentage of Thais with and without diarrhea from whom A. hydrophila and P. shigelloides were isolated were not significantly different (Table 2). In the first group of 35 Peace Corps volunteers, A. hydrophila was isolated significantly more frequently from individuals during episodes of travelers diarrhea than when volunteers were well after 5 weeks (12 of 39 versus 3 of 35; P < 0.025). One volunteer had two separate episodes of travelers diarrhea associated with A. hydrophila. This pathogen was isolated from two individuals who were not infected with viral, bacterial, or parasitic pathogens within 24 h of the onset of travelers diarrhea. P. shigelloides strains were not statistically associated with episodes of travelers diarrhea more often than when volunteers were asymptomatic after 5 weeks in Thailand (13 of 39 versus 5 of 35).

In the second group of 33 volunteers, A. hydrophila was isolated during episodes of travelers diarrhea more frequently than when individuals were well and submitted formed stools after 2 and 5 weeks in Thailand (8 of 14 versus 9 of 59; P < 0.001). In seven episodes of travelers diarrhea, A. hydrophila was the only possible enteric pathogen found. Again, P. shigelloides was not isolated during episodes of travelers diarrhea more often than when volunteers were well (6 of 14 versus 15 of 59). Tenesmus and anorexia were the most common symptoms of nine volunteers with travelers diarrhea from whom A. hydrophila was isolated as the sole potential enteric pathogen (Table 3).

Biochemical characteristics. The biochemical characteristics of A. hydrophila isolated from patients with diarrhea and others without gastrointestinal symptoms were similar. There was, however, a correlation between cytotoxicity and

TABLE 2. Prevalence of *A. hydrophila* and *P. shigelloides* in populations in Thailand

Indigenous population	A. hydrophila		Signifi-cance[a]	P. shigelloides		Signifi-cance
	Diarrhea	Controls		Diarrhea	Controls	
Children's Hospital						
0–2 yr	9/105 (9)[b]	6/74 (9)[c]	NS	4/105 (4)	3/74 (4)	NS
		8/90 (8)	NS		2/90 (2)	NS
Soongnern district						
0–2 yr	6/30 (20)	8/101 (8)	NS	4/30 (13)	3/101 (3)	NS
2–10 yr	1/7 (14)	8/51 (16)	NS	3/7 (43)	5/51 (10)	NS
10–20 yr	2/9 (22)	NC[d]	NT	3/9 (33)	NC	NT
Adults	19/56 (34)	14/51 (27)	NS	20/56 (38)	12/51 (24)	NS
Phramongkutklao Hospital						
Newborns	0/18 (0)	1/84 (1)	NS	0/18 (0)	0/84 (0)	NS

[a] NS, Not significant; NT, not tested.

[b] Number from whom organism was cultured/total (percentage in parentheses).

[c] Seventy-four of the 105 children with diarrhea cultured 3 to 4 weeks after their acute episodes.

[d] NC, No controls.

lysine decarboxylase, the Voges-Proskauer reaction, and metabolism of citrate in the API 20E system.

Assays for enterotoxigenicity. Seventy-one percent (30 of 42) of culture supernatants of *A. hydrophila* isolated from individuals with diarrhea and 64% (18 of 28) isolated from those who were passing formed stools were cytotoxic to Y-1 adrenal cells and hemolyzed rabbit erythrocytes. Whole cultures of 38% (16 of 42) of strains

isolated from individuals with diarrhea and 29% (8 of 28) from those without gastrointestinal complaints distended suckling mouse intestine (gut/remaining body weight ratio, >0.083). Sterile supernatants of 13 of 24 whole cultures of *A. hydrophila* which distended suckling mouse intestine were also positive in the mouse assay. Distension of mouse intestine did not occur when cultures were heated at 100°C for 10 min. The results of these assays are summarized in Table 4.

None of 44 *A. hydrophila* or 11 *P. shigelloides* isolates tested caused mannose-resistant agglutination of human or bovine erythrocytes. Histological examination of mouse intestine infected with six cytotoxic *A. hydrophila* strains did not reveal bacteria adhering to small intestinal brush borders. Cytotoxic strains of *A. hydrophila* caused blunting of villi and degeneration of enterocytes, but no intracellular organisms were

TABLE 3. Summary of travelers diarrhea in nine volunteers from whom *A. hydrophila* was found as the only potential enteric pathogen

Characteristics of patients with travelers diarrhea	No. of volunteers
Stools per day	
2	3
3–5	4
6–10	2
Symptoms	
Headache	3
Photophobia	1
Anorexia	7
Malaise	6
Chills	2
Fever	3
Temp > 37.8°C	1
Abdominal cramps	7
Nausea	3
Vomiting	1
Prostration	2
Flatulence	3
Duration of diarrhea (days)	
1–2	3
3–4	2
5–6	2
>7	2

TABLE 4. In vitro tests with *A. hydrophila*

Test	No. positive/no. of isolates[a] (%)	
	Diarrhea	No diarrhea
Cytotoxin	30/42 (71)[b,c]	18/28 (64)
Hemolysis of rabbit erythrocytes	30/42 (71)[b]	18/28 (64)
Distension of mouse intestine		
Whole culture	16/42 (38)[c]	8/28 (29)
Culture filtrate	9/42 (21)[c]	4/28 (14)

[a] Number of isolates = one isolate per patient per episode of diarrhea.

[b] All isolates which were cytotoxic to Y-1 adrenal cells hemolyzed rabbit erythrocytes.

[c] All isolates which distended suckling mouse intestine were cytotoxic to Y-1 adrenal cells.
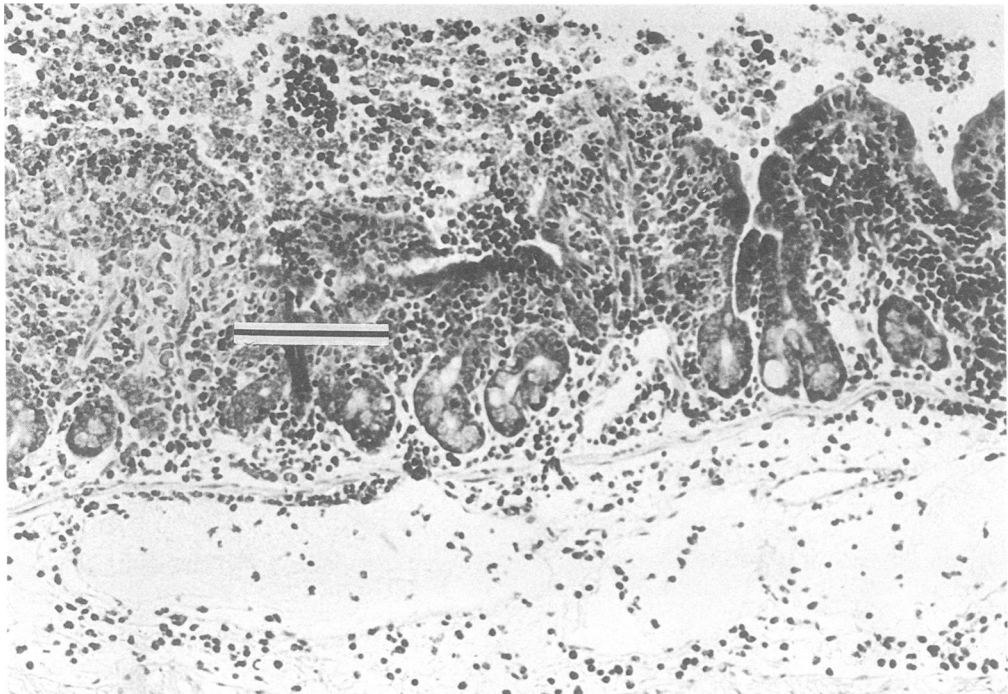
FIG. 2. Tissue section of a rabbit ileal loop 7 h after challenge with a cytotoxic *A. hydrophila* strain (6Y). The villus on the right is broad and blunt, has no mucus-containing cells, and has a hypercellular lamina propria. The cellular infiltrate, consisting largely of polymorphonuclear cells, extends through muscularis mucosa and into a widened edematous submucosa in which there are dilated lymphatics and numerous polymorphonuclear cells. Epithelial damage of the villi on the left is extensive, with an overlay of this portion by a hemorrhagic and leukocytic exudate. Damage extends to and into the crypt level of the mucosa. There is margination of the submucosal lymphatics by polymorphonuclear cells. Giemsa stain; bar, 100 μm.

seen in mouse intestine. Ten cytotoxic *A. hydrophila* strains were also negative in the Sereny test (20).

Eight of nine cytotoxic and none of five noncytotoxic *A. hydrophila* strains caused distension in ligated rabbit ileal loops after 18 h. Two of the cytotoxic strains which distended rabbit intestinal loops after 18 h also caused distension after 7 h. Exposure of rabbit intestine to eight cytotoxic *A. hydrophila* strains caused epithelial damage of the villi associated with a polymorphonuclear infiltrate extending through the muscularis mucosa (Fig. 2). Bacteria were found in the subepithelial space under intact mucosa (Fig. 3). The one cytotoxic *A. hydrophila* strain which did not distend rabbit intestine also did not cause epithelial damage of the mucosa.

Monkeys fed 10^9 cytotoxic *A. hydrophila* after 24 h of starvation and oral administration of sodium bicarbonate failed to develop diarrhea over the ensuing 5 days. To decrease any interference by the normal aerobic stool flora, another five monkeys were treated with trimethoprim and sulfamethoxazole twice daily for 5 days and

then infected with 10^9 cytotoxic *A. hydrophila*. None of these monkeys developed diarrhea.

None of 27 *P. shigelloides* strains tested was cytotoxic to Y-1 adrenal cell tissue cultures, hemolyzed rabbit or bovine erythrocytes, distended suckling mouse intestine after 4 h, or distended ligated rabbit intestinal loops after 18 h. An isolate from an individual with severe diarrhea did not cause diarrhea in five rhesus monkeys. Twenty-six percent (7 of 27) of *P. shigelloides* isolates did agglutinate in group A and D (2 isolates), C (1 isolate), or C and D (4 isolates) *Shigella* grouping antisera; however, none of the cross-agglutinating isolates was positive in the Sereny test (20).

DISCUSSION

Aeromonas spp. are frequently isolated from tropical or subtropical water supplies contaminated with nonfecal material (1, 14–16, 19), and extraintestinal infections are often acquired from such water sources (5, 11). Presumably, *Aeromonas* spp. found in stools of patients in Thailand were acquired from water since 17
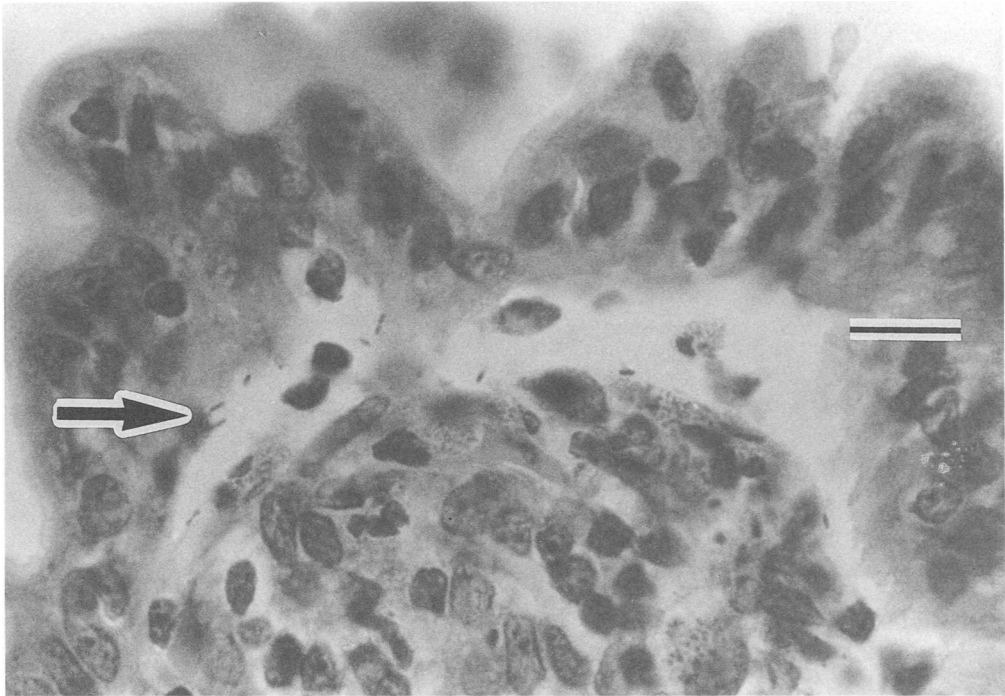
FIG. 3. Tissue section of a rabbit ileum 7 h after infection with a cytotoxic *A. hydrophila* strain (6Y). The epithelium is intact and consists of cuboid and columnar cells. There is a cellular infiltration of the epithelium and lamina propria, predominantly mononuclear, with subepithelial edema and bacteria (arrow) in the subepithelial space. Giemsa stain; bar, 20 μm.

(74%) of 23 canals (klongs) and all 15 drinking water jars (ongs) cultured in Soongnern contained *Aeromonas* spp. (unpublished data). It has been suggested that since *A. hydrophila* is frequently found in water the isolation of this organism from patients with diarrhea may be a concomitant variable reflecting ingestion of contaminated water along with another enteric pathogen (4). Evidence from epidemiological studies of patients with diarrhea failed to prove or disprove the role of *A. hydrophila* or *P. shigelloides* as an enteric pathogen in Thailand. However, among U.S. Peace Corps volunteers who presumably had less prior exposure to aeromonads than did Thai populations, there was epidemiological evidence to support the role of *A. hydrophila* as an enteric pathogen.

Although there were no significant differences in the prevalence of cytotoxic *A. hydrophila* among Thais or Americans with diarrhea and those with no gastrointestinal symptoms, eight of nine cytotoxic and none of five noncytotoxic *A. hydrophila* strains distended rabbit intestinal loops. Distension of intestine was associated with shortening of villi and destruction of enterocytes. Others have also found that most cytotoxic strains cause distension of rabbit in-

testine loops (4, 21). It is unclear whether this effect is due to cytotoxin (23) produced by a specific strain or to the presence of another unidentified enterotoxin.

Wretlind et al. (24) purified two hemolysins by isoelectric focusing from the culture supernatant of an isolate of *A. hydrophila*. Both hemolysins were cytotoxic to HeLa cells and human fibroblasts and caused dermonecrosis in rabbits. In a later publication, this group identified two different toxins in culture supernatants from 11 strains isolated from children with diarrhea in Ethiopia (24). They found that neither the rabbit skin test nor the adrenal cell assay could be interpreted for enterotoxin activity since dermonecrosis or cytotoxicity produced by hemolytic or cytotoxic factors or both interfered with interpretation of the tests. Heating culture supernatants at 56°C for 10 min inhibited hemolytic and cytotoxic activity of the test sample and permitted detection of enterotoxin, presumably the cytotonic effect of heat-labile *Escherichia coli* or *Vibrio cholerae* toxin. Donta and Haddow (7), however, were unable to demonstrate that *A. hydrophila* supernatants stimulated adenyl cyclase. *A. hydrophila* isolates from Thailand did not demonstrate a cytotonic, as compared with a

cytotoxic, effect even after supernatants were heated at 56°C for short periods of time. One of the cytotoxic strains which distended rabbit and mouse intestine was examined with a radiolabeled fragment of DNA encoding for *E. coli* heat-labile enterotoxin to search for homologous DNA sequences (13). No common nucleotide sequences were detected (S. Moseley, personal communication).

When infected intestinal rabbit mucosa was examined histologically, *A. hydrophila* appeared to be enteroinvasive; however, sterile filtrates of *A. hydrophila* cultures also distended animal intestine, indicating that the cytotoxin as well as the ability of the organism to invade intestinal mucosa plays a role in the enteropathogenicity of this organism. Although both enteroinvasive capacity and cytotoxigenicity of this organism cause intestinal lesions in animal intestinal mucosa, it is unclear which is responsible for causing diarrhea in humans. The whole culture of one cytotoxic isolate of *A. hydrophila* did not distend rabbit ileal loops. Whether this was due to less cytotoxin formed by this particular strain or to the lack of some other enteropathogenic factor is unclear.

Attempts to induce diarrhea in primates were unsuccessful. Since *A. hydrophila* and *P. shigelloides* have frequently been isolated from monkeys in our animal facilities in Bangkok, it is possible that the animals used in these experiments had acquired some form of immunity to infections with *Aeromonas* spp.

P. shigelloides isolates from patients with diarrhea and well controls were inert in the assays used to identify enteropathogenicity. Sanyal et al. (18) also tested *P. shigelloides* isolates from patients with diarrhea and found that their isolates did not distend rabbit intestinal loops. Whether this species of organism causes diarrheal disease by an unexplained mechanism is still uncertain.

No differences in biotypes were found between *A. hydrophila* or *P. shigelloides* isolated from patients with or without diarrhea. Among isolates from three groups, we found a correlation between cytotoxicity and the lysine decarboxylase and Voges-Proskauer reactions and the ability to metabolize citrate. One group of investigators (4) found good correlation between "toxigenicity" and lysine decarboxylase and Voges-Proskauer-positive reactions.

Human volunteer studies or intestinal biopsies of patients with diarrhea from whom *Aeromonas* spp. are isolated will probably be required to definitely established whether *A. hydrophila* and *P. shigelloides* are gastrointestinal pathogens. There is little question, however, that *A. hydrophila* is enteroinvasive in rabbits, and this organism can produce a cytotoxic substance(s) which distends animal intestine and causes destruction of intestinal mucosa. The pathogenesis of cytotoxic *A. hydrophila* may be similar to that of *Shigella dysenteriae* or *Clostridium perfringens*, although the toxins produced by these three species of bacteria are immunologically unrelated (7).

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