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Lack of Correlation between Known Virulence Properties of *Aeromonas hydrophila* and Enteropathogenicity for Humans

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Five strains of *Aeromonas hydrophila* were selected for use in volunteer challenge trials. All five strains produced cytotoxin, hemolysin enterotoxin, lysine decarboxylase, acetylmethylcarbinol, and DNase. Two strains hydrolyzed esculin. All strains produced purulent hemorrhagic fluid accumulation in rabbit ileal loops, but failed to induce keratoconjunctivitis in guinea pigs. None of the strains produced mannose-resistant hemagglutinins. In challenge studies, diarrhea was demonstrated in only 2 of 57 human volunteers with doses ranging from 10^4 to 10^{10} CFU. One person experienced mild diarrhea with 10^9 CFU of strain 6Y. A second person developed moderate diarrhea with 10^7 CFU of strain 3647. At higher doses, no diarrhea was seen in any of the volunteers. The other three strains (B158, SSU, 3284) failed to cause diarrhea and were not recovered from stools of volunteers. Additional virulence properties of *A. hydrophila* need to be sought before enteropathogenicity for humans can be established.

Diarrheal diseases are a major cause of morbidity and mortality. The etiology of a large percentage of acute diarrhea remains undefined, yet these cases frequently respond to antimicrobial therapy (9, 11). This suggests that undefined or poorly defined bacterial agents may be responsible for acute infectious gastroenteritis.

Various strains of *Aeromonas hydrophila* possess characteristics associated with virulence of better-defined enteropathogenic bacteria (8). Most *A. hydrophila* strains produce a heat-labile cytotoxin and have enterotoxin activity (22, 28, 33). Recent reports indicate that a cholera-like toxin may be produced also (17). Some *A. hydrophila* strains are capable of mannose-resistant hemagglutination of human erythrocytes (1, 2). Among pathogenic *Escherichia coli* this property is associated with colonization fimbriae that are able to bind to human epithelial cells.

A. hydrophila is currently considered a human pathogen (6, 14, 19, 32, 35), producing infection mainly in immunocompromised patients. Recently strains of *A. hydrophila* have been associated with cases of diarrhea in both children and adults (3, 10, 22, 28, 30). However, *A. hydrophila* is an ubiquitous organism which is frequently found in water and in stools of healthy persons (15, 18, 20, 28, 33, 35). Although a statistical relationship exists between illness and fecal recovery of *A. hydrophila* (28), definite proof of the enteropathogenicity of this organism for humans is lacking. Oral administration of whole cultures to rhesus monkeys, an animal model felt to correlate with human infections, failed to cause diarrhea (28). This study, therefore, was designed to determine whether *A. hydrophila* is capable of causing diarrhea in humans.

MATERIALS AND METHODS

Bacterial strains. Five isolates of *A. hydrophila* from humans were selected for study from among more than 50 tested, based on the presence of well-characterized virulence properties or because of an apparently clear relationship to diarrhea in human subjects. All strains were lyophilized upon receipt and stored at room temperature. A new lyophilized sample of the same lot was used for each study. The five strains were tested for susceptibility, by the standard Kirby-Bauer method, to the following antimicrobial agents: ampicillin, tetracycline, trimethoprim-sulfamethoxazole, gentamicin, furazolidone, doxycycline, and sulfisoxazole.

Biochemical characterization. All strains were confirmed as *A. hydrophila* with the API 20E identification system (Analytab Products, Plainview, N.Y.). In addition, strains were plated on DNase test agar (Difco Laboratories, Detroit, Mich.) supplemented with oxgall (Difco) and crystal violet (Difco), as previously described (24), and on bile esculin agar (Remel Media, Lenexa, Kans.).

Toxin assays. Hemolysin activity was assayed with sheep and rabbit blood agar plates (Remel Media). Strains were inoculated onto both types of blood agar plates. After overnight incubation, the plates were examined for beta-hemolysis.

Cytotoxin activity was measured in Y-1 adrenal cells (YAC). Cell-free supernatants of *A. hydrophila*, prepared as previously described (24), were added in 100-μl amounts to confluent YAC monolayers. After an 18- to 24-h incubation, the monolayers were examined for cytotoxin activity (i.e., presence of detached cells). Only supernatants which caused 100% cytotoxicity were considered positive.

Enterotoxin activity was measured as previously described with cell-free culture supernatants in the suckling mouse assay (24) and rabbit ileal loop assay (12). Cholera toxin cross-reactive factor was measured in a ganglioside enzyme-linked immunosorbent assay (23), using purified cholera toxin to produce a standard curve.

Invasiveness assays. The ability to invade tissues was assayed by the Sereny method (31). Overnight Casamino Acids (Difco)-yeast extract broth (13, 24) cultures (0.1 ml) were inoculated into the eyes of adult Hartley strain guinea pigs (Charles River Breeding Laboratories, Wilmington, Mass.). The animals were examined for the development of keratoconjunctivitis over 8 days.

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TABLE 1. Sources of strains of *A. hydrophila*

Strain	Location	Site of infection
6Y	Bangkok, Thailand	Stool (healthy)
B158	Perth, Australia	Wound
3647	Perth, Australia	Stool (illness)
SSU	United States (Centers for Disease Control)	Stool (illness)
3284	Perth, Australia	Stool (illness)

A second method to measure potential invasiveness was used. Overnight CYE broth cultures (1.0 ml) were injected into ligated rabbit ileal loops. The animals were sacrificed after 18 h, and the ligated loops were examined for evidence of invasion and fluid secretion. In both assays, virulent *Shigella sonnei* 53GI was used as a positive control.

Hemagglutination assays. Mannose-sensitive and mannose-resistant hemagglutination patterns were determined by the method of Evans et al. (13). Human (type A), bovine (Flow Laboratories, McLean, Va.), chicken (Flow), monkey (Flow), and guinea pig (Flow) erythrocytes were tested in the presence and absence of D-mannose (Sigma Chemical Co., St. Louis, Mo.).

Volunteer challenge studies. Volunteer protocols were approved by the institutional review boards of the University of Texas Health Science Center, Baylor College of Medicine, The Methodist Hospital, and the General Clinical Research Center. Consenting healthy adults were admitted and confined to Baylor College of Medicine's General Clinical Research Center at the Methodist Hospital. A prechallenge admission stool was cultured for enteropathogens as previously described (24). Volunteers abstained from eating and drinking for 90 min before and after oral challenge with *A. hydrophila* cells. In a double-blind study, groups of three or four volunteers were given 2 g of sodium bicarbonate in 150 ml of sterile distilled H_2O as follows: (i) 120 ml of the solution was rapidly swallowed; (ii) after 60 s, the remaining 30 ml, which contained viable organisms at predetermined levels, was rapidly swallowed. The challenge strains were grown in 10 ml of Casamino Acids-yeast extract broth in 50-ml Erlenmeyer flasks. The broth was shaken at 200 rpm for 17 h at 37°C. The culture was washed by centrifugation (12,000 × *g*); the pellet was suspended in the bicarbonate mixture, and the concentration was adjusted by optical density measurement. The volunteers were carefully monitored for symptoms and signs of gastroenteritis (defined as two or more unformed stools in 24 h with an additional symptom of enteric infection). Daily physical examinations were performed on each volunteer. All stools passed were collected and cultured for *A. hydrophila*.

RESULTS

Biochemical characterization and antibiograms. All five strains were confirmed as *A. hydrophila* biochemically. The sources of these strains are given in Table 1. Several biochemical properties were noted as potential indicators of virulence (Table 2). All of the strains gave a positive Voges-Proskauer reaction and produced lysine decarboxylase and DNase. Only two of the five strains (3647, SSU) were capable of hydrolyzing esculin. Three of the five strains (B158, 3647, 3284) were resistant to ampicillin (10 μg), whereas all five strains were susceptable to the remaining antimicrobial agents tested.

Toxin production. The five strains were examined for hemolysis, cytotoxin, and enterotoxin production (Table 3).

TABLE 2. Biochemical characterization of strains of *A. hydrophila*

Strain	Voges-Proskauer reaction	Lysine decarboxylase production	Esculin hydrolysis	DNase production
6Y	+	+	−	+
B158	+	+	−	+
3647	+	+	+	+
SSU	+	+	+	+
3284	+	+	−	+

All strains were hemolytic for sheep and rabbit erythrocytes, produced cytotoxin in YAC cells, and produced cholera toxin cross-reactive factor as measured in a ganglioside enzyme-linked immunosorbent assay. Only three strains (6Y, B158, and 3647) produced fluid accumulation in both the suckling mouse and rabbit ligated ileal loop assays.

Invasion. None of the five strains produced keratoconjunctivitis in guinea pigs. All five strains produce hemorrhagic, purulent fluid accumulation in ligated ileal loops. This positive response with whole culture challenge may be due to cytotoxin activity rather than to invasiveness.

Hemagglutination. All five strains were tested for mannose-sensitive and mannose-resistant hemagglutination of human, bovine, chicken, monkey, and guinea pig erythrocytes. All strains tested failed to show mannose-resistant hemagglutination of erythrocytes from any of the animal species. Only strain B158 showed mannose-sensitive hemagglutination of guinea pig erythrocytes.

Challenge studies. Preenrollment stool analysis revealed no asymptomatic carriage of *A. hydrophila* (or any other bacterial enteropathogen) by the 57 volunteers. With the five *A. hydrophila* strains, we failed to demonstrate the development of diarrheal illness in 55 of 57 volunteers even with doses of 10^{10} CFU for three of the strains (Table 4). Three strains (B158, SSU, and 3284) were not recovered from the stools of the volunteers. Strain 6Y was recovered from 11 of the 20 volunteers challenged. One of the volunteers developed mild diarrhea (passage of six unformed stools over 12 h associated with a brief period of nausea, vomiting, anorexia, and malaise) 48 h after ingesting 3×10^9 CFU. The patient remained afebrile, was not treated, and failed to develop a progressive enteric illness. No fecal leukocytes were present in the patient's stools. A small bowel biopsy was normal histologically. No illness occurred among the four persons ingesting a higher dose of 6Y (4×10^{10} CFU), casting doubt on the importance of the mild illness seen at the lower dose in the one volunteer. Strain 3647, initially isolated from a diarrheal stool from a patient in Australia, was recovered from 3 of the 16 volunteers challenged. One volunteer

TABLE 3. Toxins produced by strains of *A. hydrophila*

Strain	Hemolysin	Cytotoxin	Enterotoxins		
			Suckling mouse assay	Rabbit assay	CTCRF[a]
6Y	+	+	+	+	+
B158	+	+	+	+	+
3647	+	+	+	+	+
SSU	+	+	−	−	+
3284	+	+	−	−	+

[a] CTCRF, Cholera toxin cross-reactive factor.

receiving a dose of 10^7 CFU passed three unformed stools over an 18-h period (48 to 72 h after challenge). The patient remained afebrile with mild abdominal cramps and failed to excrete the test organism. Illness did not occur as the dose was increased and administered to additional volunteers.

DISCUSSION

A. hydrophila, an organism found commonly in the environment, is capable of causing disease in humans (6, 14, 19, 32, 35). Most of the earlier reports described *A. hydrophila* in association with septicemia and skin infection (19, 32). Recent reports have associated *A. hydrophila* with acute diarrhea (3, 10, 22, 28, 30). In this study, we failed to induce diarrhea in volunteers when as many as 10^{10} CFU were administered orally.

Five strains of *A. hydrophila* were selected for the volunteer challenge trials based on their possession of well-characterized virulence properties. Three of the strains (6Y, B158, 3284) may fall into the group sometimes referred to as *Aeromonas sobria* (4, 5, 16, 29), a subgroup which has not gained universal acceptance as a separate species (16). We included an isolate from a wound infection which produced similar virulence properties to determine whether *A. hydrophila* might show host tissue specificity. All five strains were hemolytic and cytotoxic with variable enterotoxigenicity. Previous studies have shown that production of enterotoxin alone by enterotoxigenic *E. coli* is not sufficient to cause disease (7). Perhaps the strains of *A. hydrophila* tested lacked the necessary adhesion factors to initiate colonization, the first step in pathogenesis. Even though some strains of *A. hydrophila* possess hemagglutinins which may be associated with fimbriae, these fimbriae may not be intestinal epithelial cell adhesins. The strains of *A. hydrophila* tested failed to demonstrate the ability to invade tissue by the Sereny test; however, all five strains caused severe tissue destruction in the rabbit ileal loop model. Perhaps this tissue response is attributable to cytotoxin activity rather than to invasiveness. Selection of potentially enteropathogenic clones for future testing may be advantageous. Several approaches are possible: selection for high-titer cytotoxin-producing clones, subculture of lesions within positive ileal loops, and selection of highly fimbriated clones. Perhaps challenge with a homogeneous suspension (i.e., from a highly virulent clone) would be fruitful. Although care was taken in our laboratory to retain virulence properties of the bacteria, subculture by primary isolation laboratories may be responsible for the failure to reproduce gastroenteritis in volunteers.

Previously recommended biochemical testing (16, 33) was not useful in defining virulence among the strains. These observations are in agreement with and extend previously published data (22, 36). In addition, two of five strains were susceptible to ampicillin. This might pose a problem when *A. hydrophila* is sought on primary isolation media containing ampicillin (3, 25, 34). Having examined more than 50 isolates, we have found that all strains do produce DNase (D. R. Morgan, unpublished data). Therefore, we have chosen DNase agar supplemented with oxgall and crystal violet (24) as the optimal primary isolation medium. This medium has the advantage of direct oxidase detection applicability as well.

One of two conclusions will undoubtedly prove to explain the results obtained in the present study: virulence properties of *A. hydrophila* as we now understand them (i.e., biochemical characteristics and production of hemolysin, cytotoxin, and enterotoxins) are insufficient to explain virulence for humans; or widespread immunity to *A. hydrophila* exists among adults from Houston, Tex. We feel that the latter is not a reasonable explanation for failure to produce illness in view of the rarity of isolating *A. hydrophila* from diarrheal stools of infants and children from Houston studied by our group over the past 10 years (26, 27). Also, we know that enteropathogenic *E. coli*, which are normally pathogenic only for infants, can produce diarrhea in adults when given in the doses employed in the present study (21). *A. hydrophila* may be confirmed as an enteropathogen for humans under certain conditions. Additional virulence properties of *A. hydrophila* strains need to be sought and characterized before future volunteer studies are likely to be rewarding.

TABLE 4. Administered dose and excretion of *A. hydrophila* in volunteers

Strain	Challenge dose (CFU)	No. of volunteers	No. shedding test strain	No. with diarrhea[a]
6Y	2×10^4	4	1	0
	1×10^6	4	1	0
	7×10^7	4	4	0
	3×10^9	4	3	1
	4×10^{10}	4	2	0
B158	6×10^4	4	0	0
	2×10^7	4	0	0
3647	1×10^7	4	0	1
	4×10^7	4	0	0
	2×10^9	4	2	0
	3×10^{10}	4	1	0
SSU	4×10^8	4	0	0
	5×10^{10}	3	0	0
3284	3×10^8	3	0	0
	1×10^{10}	3	0	0

[a] ≥2 unformed stools in 24 h with systemic or enteric symptoms.

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