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Invasiveness of *Aeromonas* spp. in Relation to Biotype, Virulence Factors, and Clinical Features

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Of 69 fecal isolates of *Aeromonas* spp., 18 had the ability to invade HEp-2 cells. Invasiveness correlated with biotype; of the 18 invasive strains, 16 were *A. sobria* and 2 were *A. hydrophila*. No invasive strains were found among the *A. caviae*. Of the 18 invasive strains, 13 were enterotoxigenic. Of the enterotoxigenic and invasive strains, 12 were *A. sobria*, but enterotoxicity was also more common among noninvasive strains of *A. sobria*. Fucose-resistant hemagglutination was also more common in *A. sobria*, but invasive strains were equally divided between fucose-resistant hemagglutination and other patterns. Detailed clinical information was available for 27 of the 69 strains. All 15 strains of *A. sobria* or *A. hydrophila* associated with diarrhea were enterotoxigenic; 6 of the 10 strains of *A. sobria* were also invasive. Blood was present in the stool samples of five of the six patients with invasive *A. sobria* and in none of the patients with noninvasive strains. Although limited, these observations suggest that dysenteric symptoms may be produced by invasive *Aeromonas* spp.

Adherence to intestinal mucosa, toxin production, and invasion are recognized virulence factors associated with bacterial pathogens that cause diarrhea. *Vibrio cholerae* is typical of bacteria which do not invade enterocytes but cause watery diarrhea by the production of enterotoxin after adhering to intestinal mucosa. In contrast, with *Salmonella* and *Shigella* spp., invasion is essential in pathogenesis, and strains which are not invasive are avirulent (7). Diarrhea associated with these bacteria usually contains blood and mucus.

Many invasive enteric pathogens also produce toxins, e.g., *Shigella* (13) and *Salmonella* spp. (20), *Yersinia enterocolitica* (15), and *Campylobacter jejuni* (19), but the role of these toxins in the pathogenesis of diarrhea is not clear. It has been suggested for *C. jejuni* that enterotoxicity predominates in patients with watery diarrhea, and invasiveness predominates in those with dysenteric symptoms (19).

The situation may be analogous with *Aeromonas* spp. Watery diarrhea is the most common symptom associated with the isolation of enterotoxigenic *Aeromonas* spp., but ca. 20% of patients have dysenteric symptoms (8). Although *Aeromonas* spp. have not been found to be invasive with the Séreny test (10), this method is not adequate for detecting invasiveness of *Salmonella* spp. (7), and some *Aeromonas* spp. have the ability to invade cultured HEp-2 cells (12). We therefore investigated the invasiveness of fecal isolates of *Aeromonas* spp. in HEp-2 cells and related the results to biotype, hemagglutination patterns, and enterotoxicity, as we have previously found correlations between these properties (5).

MATERIALS AND METHODS

Bacterial strains. There were 69 strains of *Aeromonas* spp. included in this study. All were fecal isolates from adults or children in Western Australia (40 strains), Thailand (5 strains), Indonesia (20 strains), or the United States (4 strains). Western Australian strains were isolated from patients of three metropolitan hospitals and from patients not attending hospitals but referred for investigation to two microbiology laboratories, one in the city and one in a country center. *Aeromonas* spp. were classified as described by Popoff (17); there were 36 *A. sobria*, 21 *A. hydrophila*, and 12 *A. caviae* strains.

Bacterial preparations. Strains of *Aeromonas* spp. were stored in maintenance medium containing 5 g of sodium chloride, 2.5 g of Bacto-Peptone 0118 (Difco Laboratories, Detroit, Mich.), 2.5 g of peptone L 34 (Oxoid Ltd., Basingstoke, England) in 200 ml of phosphate buffer (containing 2.8 g of Na_2HPO_4 in 134 ml of distilled water and 1.3 g of K_2HPO_4 in 66 ml of distilled water), and 800 ml of distilled water at pH 6.7.

For enterotoxin assays, 5 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 0.6% yeast extract in 25-ml Erlenmeyer flasks was inoculated with the *Aeromonas* spp. strain to be tested and incubated at 37°C and 300 rpm on an environmental incubator-shaker (New Brunswick Scientific Co., New Brunswick, N.J.) for 24 h. Cell-free preparations were made by centrifuging the cultures at 7,000 × *g* for 10 min at 4°C, followed by filtration through a membrane filter (pore size, 0.45 μm; type HA; Millipore Corp., Bedford, Mass.). Supernatant fluids were stored at 4°C and tested within 1 day.

For invasiveness assays, strains to be tested were grown overnight on blood agar plates (Oxoid Ltd.), inoculated into 7 ml of brain heart infusion broth (Oxoid Ltd.), and incubated overnight at 37°C in stationary culture. Growth on blood agar after serial dilution was used to assess the concentration of bacteria attained in brain heart infusion broth. Results were discarded unless this concentration was at least 10^7 bacteria per ml.

HEp-2 cell culture. HEp-2 cells (Commonwealth Serum Laboratories, Melbourne, Australia) were maintained in

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TABLE 1. Invasiveness in relation to hemagglutination pattern, enterotoxicity, and species

Organism	No. of strains[a]			
	Invasive		Noninvasive	
	FRHA	Other[b]	FRHA	Other[b]
A. sobria	8 (8)	8 (4)	8 (8)	12 (9)
A. hydrophila	1 (0)	1 (1)	0	19 (8)
A. caviae	0	0	0	12 (0)

[a] Number in parentheses = number of enterotoxigenic strains.
[b] Fucose-sensitive hemagglutination or no agglutination.

Eagle minimum essential medium (Flow Laboratories, North Ryde, Australia) with 10% fetal calf serum containing 50 IU of penicillin per ml, 200 IU of streptomycin per ml, and 100 μg of neomycin per ml.

Invasiveness assay. Confluent monolayers of HEp-2 cells grown in 24-well trays (Nunc, Kampstrup, Denmark) for 18 h were washed in situ three times with 1 ml of Hanks balanced salt solution (Flow Laboratories). Bacterial cultures, diluted 1:500 in Eagle minimum essential medium with 1% fetal calf serum to give a concentration of ca. 5×10^5 cells per ml, were added in volumes of 1 ml to each well and tested in duplicate on at least two occasions. Bacterial concentration was determined by serial dilution and plating onto blood agar. Infected monolayers were incubated at 37°C for 3 h. Cells were washed three times in situ with 1 ml of Hanks balanced salt solution each time, and 1 ml of Eagle minimum essential medium containing 5% fetal calf serum and gentamicin (10 μg/ml) was added to each well to kill extracellular bacteria (24). Monolayers were incubated for a further 2 h at 37°C, the washing procedure was repeated, and 1 ml of Eagle minimum essential medium containing 5% fetal calf serum but no antibiotics were added. After washing three times with Hanks balanced salt solution, the integrity of the monolayer was checked, and 1 ml of lysing solution (16) containing 0.01 M NaH_2PO_4, Tween 20 (1% [vol/vol]), and 0.025% trypsin (wt/vol) at pH 8.0 was added to each well and incubated for 30 min at 37°C. Bacterial counts in the lysate were determined by serial dilution and plating of 10-μl volumes onto blood agar.

Strains were classified as noninvasive if bacteria were not recovered or were recovered only from the undiluted lysate; growth did not exceed 2×10^3 CFU/ml. Strains with growth of $\geq 5 \times 10^6$ CFU/ml were classified as invasive. Of eight strains recovered in concentrates of $>2 \times 10^3$ and $\leq 5 \times 10^5$ CFU/ml, retesting showed seven to be clearly negative, with no bacteria recovered; one strain consistently showed growth of $\geq 5 \times 10^5$ cells upon retesting. We therefore considered cell growth between 2×10^3 and 5×10^5 as an indication for retesting. The same two known positive and negative strains were included in duplicate as controls in each assay.

Suckling mouse test. The suckling mouse assay detects the nondialyzable, heat-labile enterotoxin of *Aeromonas* spp. and was carried out as previously described (4).

Hemagglutination. Patterns of hemagglutination of human group O cells with fucose, galactose, and mannose added were determined as previously described (1, 5).

Biochemical characteristics. A biochemical profile was determined as described by Popoff (17), with hydrolysis of arabinose, arbutin, esculin, and salicin at 30°C detected by the method of Cruikshank (6). The production of elastase was determined by the method of Scharmann (21).

RESULTS

Eighteen strains of *Aeromonas* spp. were invasive in the HEp-2 cell system. Sixteen strains were *A. sobria*, 2 were *A. hydrophila*, and there were no invasive *A. caviae*. Except for two strains of *A. sobria* from Indonesia, one *A. sobria* from Thailand, and one *A. hydrophila* from the United States, invasive strains were fecal isolates from Western Australia.

Invasive strains were predominantly enterotoxigenic; 13 of the 18 invasive *Aeromonas* spp. produced enterotoxins detected in the suckling mouse test. Twelve of the enterotoxigenic invasive strains were classified as *A. sobria*; the remaining strain was *A. hydrophila*. Seventeen of 20 noninvasive *A. sobria* and 8 of 19 noninvasive *A. hydrophila* produced enterotoxins. No *A. caviae* were enterotoxigenic.

Sixteen (44.4%) of the *A. sobria* strains showed fucose-resistant hemagglutination (FRHA) which was found with only 1 (4.8%) of the *A. hydrophila* and none of the *A. caviae*. With *A. sobria*, eight invasive strains showed FRHA, and eight showed no agglutination or fucose-sensitive hemagglutination. Eight noninvasive *A. sobria* showed FRHA, and 12 did not. The only FRHA strain of *A. hydrophila* was invasive as was one of the 20 fucose-sensitive hemagglutination strains. Among strains of *A. sobria*, there was no significant association between invasiveness and FRHA ($P > 0.05$; χ^2 test). Table 1 shows the hemagglutination pattern and enterotoxicity in relation to invasiveness and species.

Detailed clinical information was available for 27 of the *Aeromonas* spp. isolated in Western Australia, i.e., 15 *A. sobria*, 7 *A. hydrophila*, and 5 *A. caviae*. Diarrhea was associated with the isolation of *Aeromonas* spp. in 10 patients in the first group and 5 in both the second and third groups (Table 2). All *A. sobria* and *A. hydrophila* associated with diarrhea were enterotoxigenic; none of the *A. caviae* produced enterotoxins. Of the 10 enterotoxigenic *A. sobria*, 6 were also invasive, and 5 of these invasive strains were associated with blood in the stool samples. There were no other invasive strains in patients for whom clinical data was adequate. Blood was not present in the stool samples of patients from whom noninvasive *Aeromonas* spp. were isolated (Table 2).

DISCUSSION

Although the production of keratoconjuctivitis in guinea pigs detects invasive strains of *Shigella* and *Escherichia coli*, the Séreny test is unsuitable for discriminating invasive *Salmonella* spp. (7); thus, cell culture systems have been used as an alternative (18). With the recognition that aminoglycosides kill extracellular but not intracellular bacteria (23), several investigators have utilized this property to demonstrate the invasiveness of *Salmonella* (11), *Shigella* (14), *Yersinia* (22), and *Proteus* spp. (16). This technique has

TABLE 2. Enterotoxicity and invasiveness related to symptoms in 27 Western Australian patients

Biotype	No. of strains	No. of strains associated with:			
		Diarrhea	Enterotoxins	Invasion	Blood in stools
A. sobria	15	10	10	6	5
A. hydrophila	7	5	5	0	0
A. caviae	5	5	0	0	0

demonstrated invasiveness in strains of *Aeromonas* spp. isolated from fecal samples (12), whereas the results of Séreny tests in our laboratory have agreed with other investigators (10) who failed to produce keratoconjunctivitis with *Aeromonas* spp. The present study has confirmed the invasiveness of *Aeromonas* spp. for HEp-2 cells.

Invasiveness in cell culture correlated with biotype in that 16 of 18 invasive *Aeromonas* spp. (88.9%) were classified as *A. sobria*. Earlier studies suggest that invasiveness in *Aeromonas* spp. is chromosomally mediated (12). This would be consistent with the correlation with biotype found in the present study. We have previously shown (2) that enterotoxin production also correlates with biotype. In the present study, 29 of the 38 enterotoxigenic strains were *A. sobria*; of these, 12 were invasive and 17 were noninvasive.

In our experience, FRHA of human O cells is significantly associated with the *A. sobria* biotype (5). All but 1 of these FRHA strains were *A. sobria*; of the 18 invasive strains, however, 9 showed FRHA and 9 did not. For noninvasive *A. sobria*, 8 of 20 strains showed FRHA. The limited data available suggest that invasiveness is most common in *A. sobria* but within this species is not correlated with FRHA or enterotoxin production.

We have analyzed biotype, enterotoxicity, and invasiveness in relation to clinical data in 27 of the patients from Western Australia for whom adequate information was available. The proportion of *Aeromonas* spp. from patients without diarrhea does not reflect our overall experience. Of the seven strains not associated with diarrhea, four were not enterotoxigenic and three were isolated from the enrichment of fecal samples in nutrient broth, a technique we have found to be too sensitive to correlate with the presence of diarrhea (17a). In our experience, *A. caviae* occurs with equal frequency in patients with and without diarrhea so that the five strains chosen which were associated with diarrhea probably do not indicate a causal relationship. However, we chose this spectrum of *Aeromonas* spp. to include the three species, diarrheal and nondiarrheal strains, and variation in possible virulence factors (enterotoxicity, cytotoxicity, and hemagglutination patterns).

Of these 27 isolates, all 15 enterotoxigenic strains, whether *A. sobria* or *A. hydrophila*, were associated with diarrhea. Six *A. sobria* were invasive as well as enterotoxigenic, and in five patients from whom these strains were isolated, there had been blood in the stool samples. Although limited, these observations suggest that dysenteric symptoms may be associated with invasive strains of *Aeromonas* spp. in a manner that is analogous to possible mechanisms of pathogenesis for *C. jejuni* (19).

The strains of *A. caviae* from patients for whom clinical data were adequate were associated with diarrhea but did not produce enterotoxins, did not show FRHA, and were not invasive. This is in agreement with our experience of *A. caviae* in Western Australia. The isolation rate is not increased in diarrheal stool samples, and we have not yet found an enterotoxigenic or invasive strain of *A. caviae*. Decreased virulence of *A. caviae* has previously been reported (24), and Janda et al. (9) considered this species to be less invasive, as it was rarely associated with bacteremia in their patients.

Invasiveness should now be considered as a mechanism of virulence in *Aeromonas* spp. in addition to enterotoxicity and adherence. Larger studies are necessary to demonstrate whether the observed association between invasiveness and blood in the stool samples is consistent.

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