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Correlation of Enterotoxicity with Biotype in *Aeromonas* spp.

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Enterotoxin production correlated with biotype in a study of 686 strains of *Aeromonas* spp. from Indonesia, Thailand, the United States, and Western Australia. Most strains were isolated from feces but nonfecal human isolates and environmental strains were also included. More than 80% of Voges-Proskauer (VP)-positive strains, classified as *A. hydrophila*, were enterotoxigenic in the suckling mouse assay as were 90% of VP-positive, arabinose-negative strains. An association between positive VP, arabinose fermentation, and failure to produce enterotoxins was found only with environmental strains. VP-negative strains which did not oxidise gluconate or produce gas from glucose were classified as *A. punctata* subsp. *caviae*. Only 2 of the 286 strains produced enterotoxins, and both were from Indonesian fecal samples. There were few remaining VP-negative strains, classified as *A. punctata* subsp. *punctata* and, of these, about half were enterotoxigenic. Regardless of source and species, 97% of *Aeromonas* spp. were correctly classified in relation to enterotoxin production with a hemolysin assay. A combination of biochemical testing and hemolysin assay should be suitable for diagnostic laboratories to identify enterotoxigenic *Aeromonas* spp. which, in children, are associated with diarrhea, unlike non-enterotoxigenic strains.

We have suggested previously that enterotoxigenic *Aeromonas* spp. can be discriminated from non-enterotoxigenic strains by using a scheme of classification similar to that proposed by Schubert (7) combined with hemolysin assay (1). We have now examined a larger number of strains from several geographical sources in relation to the proposed classification.

MATERIALS AND METHODS

Bacterial strains. Of 686 *Aeromonas* strains investigated, 256 were from fecal samples in Indonesia, 214 were from feces of children in Western Australia, and 100 were from fecal samples in Thailand. Of 30 strains from New York, 13 were of fecal origin, 2 were from bile, 10 were from wounds, 2 were from sputum, 1 was from a nasal swab, 1 was from urine, and 1 was from an unknown source. There were 96 strains isolated from water samples in Western Australia. Strains previously investigated (1) were not included in this study. Table 1 shows the source of the strains and their classification based on the scheme of Schubert (7).

Bacterial preparations. Strains of *Aeromonas* spp. were stored in maintenance medium which consisted of 5 g of agar, 5 g of sodium chloride, 2.5 g of peptone 0118 (Difco Laboratories, Detroit, Mich.), 2.5 g of peptone L34 (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 exotoxin 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* 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 10,000 × *g* for 30 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 after preparation.

Exotoxin assays. (i) Suckling mouse test. Test solutions (100 μl) containing 2 drops (ca. 0.02 ml) of 2.5% Pontamine sky-blue dye per ml were administered perorally into the stomachs of suckling mice 2 to 4 days old with a fine polyethylene tube (external diameter, 0.6 mm) connected to a 1-ml syringe. At least three mice were used in each test. After incubation for 3 h at 28°C, the animals were killed by cervical dislocation, and total small and large intestines were removed. Intestinal weight and remaining body weight were measured, and the intestinal weight/body weight ratio was calculated. The presence of diarrhea, made obvious by blue staining of blotting paper under the animals, was also recorded. Weight ratios and amounts of diarrhea were both used to classify positive strains as described elsewhere (2). The assay used

TABLE 1. *Aeromonas* spp. from different geographic sources

Source	Subspecies[a]		
	hydrophila[b]	*punctata*	*caviae*
Indonesia[c]	51	4	201
Thailand[c]	65	0	35
New York[d]	21	3	6
Western Australia			
Feces	159	3	42
Water	96	0	0

[a] Based on the classification of Schubert (7).
[b] Includes subsp. *hydrophila* and *anaerogenes*.
[c] All strains of fecal origin.
[d] 13 strains of fecal origin, 4 subsp. *caviae*, 9 subsp. *hydrophila*.

detects the nondialyzable, heat-labile enterotoxin of *Aeromonas* spp.

(ii) Hemolysin assay. Volumes (100 μl) of doubling dilutions of cell-free broth in phosphate-buffered saline were added to equal volumes of a 1% suspension of rabbit erythrocytes in microtiter trays (Linbro, Hamden, Conn.). Hemolysis was recorded after incubation for 1 h at 37°C and then after incubation for 1 h at 4°C. Hemolysis of 50% of the erythrocytes was considered the endpoint, and results were expressed as the $\log_2$ of the reciprocal of the greatest dilution showing hemolysis. Values of >2 were considered positive.

Biochemical characteristics. Biochemical properties were determined as described previously (1) with the Microbact MB24E system (Disposable Products, Adelaide, South Australia). This system contains tests for the following: lysine decarboxylase; ornithine decarboxylase; H_2S production; fermentation of glucose, mannitol, and xylose; *o*-nitrophenyl-β-D-galactopyranosidase; indole production; urease; Voges-Proskauer reaction (VP); citrate utilization (Simmons); tryptophan deaminase; gelatin liquefaction; malonate; inositol; sorbitol; rhamnose; sucrose; lactose; arabinose; adonitol; raffinose; salicin; and arginine dihydrolase. All carbohydrates were D-(+) forms, except for L-(+)-arabinose and L-(+)-rhamnose. Tests were read after 24 h, except for gelatin liquefaction which was read at 48 h.

We have previously shown (1) that results with this multitest system agree with those found using API20E (Analytab Products, La Balme-Les-Grottes, Montalieu-Verceiu, France).

The following tests were used as described by Cowan (3): oxidation of gluconate, production of gas from glucose, and esculin hydrolysis.

TABLE 2. Correlation between enterotoxin tests and hemolysin assay[a]

Hemolysin assay result	Suckling mouse test	
	Positive	Negative
Hemolysin positive	316	4
Hemolysin negative	15	351

[a] χ^2 test, $P < 0.05$.

RESULTS

Exotoxin assays. There were 331 enterotoxigenic and 355 nonenterotoxigenic strains of *Aeromonas* spp. Of the 331 enterotoxigenic strains, 316 were positive in the hemolysin assay; 4 non-enterotoxigenic strains produced hemolysin (Table 2). This difference is significant by the χ^2 test ($P < 0.05$).

If hemolysin assays were used to discriminate enterotoxigenic strains, all but 19 of 686 strains (2.8%) would have been correctly classified.

Biochemical characteristics. Table 1 shows the distribution of subspecies, based on the classification of Schubert (7). Strains were considered to be *A. hydrophila* if they were VP positive. VP-negative strains were classified as *A. punctata* subsp. *caviae* if they failed to produce gas from glucose and did not oxidase gluconate. Other VP-negative strains were considered to be *A. punctata* subsp. *punctata*.

Table 3 shows the results of suckling mouse tests in relation to this classification. Of the 686 strains tested, 284 were classified as *A. punctata* subsp. *caviae*. Most of these (201) came from Indonesia. Only two of these strains, both from Indonesia, were positive in the suckling mouse test, and both also produced hemolysins.

There were 392 strains classified as *A. hydrophila*. The proportion of strains positive in the suckling mouse test varied in relation to their source. Overall there were 320 enterotoxigenic

TABLE 3. Enterotoxin tests in relation to species and source of isolates

Source and species (no.)	Suckling mouse test	
	Positive	Negative
Indonesia		
A. hydrophila (51)	39	12
A. punctata subsp. *punctata* (4)	2	2
A. punctata subsp. *caviae* (201)	2	199
Thailand		
A. hydrophila (65)	54	11
A. punctata subsp. *caviae* (35)	0	35
New York		
A. hydrophila (21)	13	8
A. punctata subsp. *punctata* (3)	3	0
A. punctata subsp. *caviae* (6)	0	6
Western Australia		
Feces		
A. hydrophila (159)	146	13
A. punctata subsp. *punctata* (3)	2	1
A. punctata subsp. *caviae* (42)	0	42
Water		
A. hydrophila (96)	68	28

TABLE 4. *A. hydrophila* positive in the suckling mouse test in relation to source of isolates

Source	No. (%) of enterotoxigenic *A. hydrophila*
Indonesia (n = 57)	39 (76.4)
Thailand (n = 65)	54 (83.1)
New York (n = 21)	13 (61.9)
Western Australia	
Feces (n = 159)	146 (91.8)
Water (n = 96)	68 (70.8)
All strains (n = 392)	320 (81.6)

and 72 non-enterotoxigenic strains of *A. hydrophila*, that is, 81.6% of these strains produced enterotoxins. The proportions of enterotoxigenic strains, according to source, were as follows: from Thailand, 83.1%; from Indonesia, 76.4%; from New York, 61.9%; from fecal samples in Western Australia, 91.8%; and from water in Western Australia, 70.8% (Table 4). If only strains isolated from feces were considered, 85.6% of the *A. hydrophila* were enterotoxigenic.

Enterotoxin production correlated with failure to ferment arabinose, except in strains not isolated from feces; 90% of arabinose-negative fecal strains were enterotoxigenic. However, the correlation between arabinose fermentation and failure to produce enterotoxins was not remarkable, and overall, only 37.4% of strains which fermented arabinose were not enterotoxigenic. However, there were differences in relation to the source of the isolates and for strains isolated from water, 82.1% of non-enterotoxigenic strains were arabinose positive (Table 5).

Both VP and LDC results correlated with results of toxin testing because the VP-positive strains classified as *A. hydrophila* were mainly enterotoxigenic. As 95.7% of these strains, but only 3% of VP-negative strains gave positive results for lysine decarboxylase in the multitest system, this test also correlated with results in the suckling mouse assay. No other biochemical test correlated with enterotoxin production.

Strains classified as *A. punctata* subsp. *punctata* were uncommon and made up only 1.5% of the 686 strains included in this study. Of the 10 strains of *A. punctata* subsp. *punctata*, 7 were enterotoxigenic and produced hemolysin. The remaining three strains were negative in both suckling mouse and hemolysin assays.

DISCUSSION

Our earlier investigation of 174 strains of *Aeromonas* spp. suggested that enterotoxin production was associated with biotype (1). The present study has examined 686 additional strains of *Aeromonas* spp. from Indonesia, Thailand, New York, and Western Australia and has included fecal and nonfecal human isolates as well as environmental strains.

Although there are some differences in relation to geographical source and fecal or nonfecal origin, we have confirmed that 97% of strains can be correctly classified with regard to enterotoxin production without the need for enterotoxin assays.

Strains are first grouped on the basis of VP testing. This classification is closely related to that suggested by Schubert (7). However, this author has recognized rare strains of *A. hydrophila* which were VP negative. We have simplified this scheme so that the Schubert (7) VP-negative *A. hydrophila* strains are not included in the classification as *A. hydrophila*. VP-positive strains are classified as *A. hydrophila* and may be subsp. *hydrophila* or *anaerogenes*. VP-negative strains may be *A. punctata* subsp. *caviae* or *A. punctata* subsp. *punctata* and are distinguished by gluconate oxidation and production of gas from glucose.

This step is particularly useful in identifying *A. punctata* subsp. *caviae* which are rarely enterotoxigenic. In the present study, only 2 of 686 strains were enterotoxigenic *A. punctata* subsp. *caviae*, and these were of fecal origin from Indonesia. We have found no enterotoxigenic *A. punctata* subsp. *caviae* on examination of about 200 Australian strains. Jánossy and Tárjan (6) have reported enterotoxin production in this subspecies, but few of their strains were of fecal origin.

Schubert (8) considered that *A. punctata* subsp. *caviae* was not pathogenic. Although Fritsche et al. (4) have suggested this organism to be a cause of diarrhea, this was based on the report of a single patient with diarrhea in whom *A. punctata* subsp. *caviae* was found in the feces. In our investigation of about 1,000 children with diarrhea and age- and sex-matched controls, only 1% of children with diarrhea and

TABLE 5. Classification of *A. hydrophila* in relation to suckling mouse assay, fermentation of arabinose and source of isolates[a]

Source	Ent^+		Ent^-	
	Ara^+	Ara^-	Ara^+	Ara^-
Indonesia	2	37	4	8
Thailand	7	47	3	8
New York	9	4	6	2
Western Australia				
Feces	22	124	7	6
Water	32	36	23	5
Total	72	248	43	29

[a] Ent^+, Enterotoxigenic; Ent^-, non-enterotoxigenic. Ara^+ isolates ferment arabinose, and Ara^- isolates do not ferment arabinose.

0.7% of controls were found to have *A. punctata* subsp. *caviae*, whereas 10% of patients with diarrhea and 0.6% without diarrhea had enterotoxigenic *Aeromonas* spp. in their stools (V. Burke, M. Gracey, J. Robinson, D. Peck, J. Beaman, and C. Bundell, J. Infect. Dis., in press). The evidence suggests that *A. punctata* subsp. *caviae* is not of clinical importance as an enteric pathogen. We therefore recommend that fecal *Aeromonas* spp. identified as belonging to this subspecies should not be further investigated as possible enterotoxin producers. Hemolysin assay correctly classified all *Aeromonas punctata* subsp. *caviae* in relation to enterotoxin production, including the only two strains which were enterotoxigenic.

A. punctata subsp. *punctata*, the other VP-negative group, is uncommon, but in our experience, more than half the strains are enterotoxigenic. Results of hemolysin assay and enterotoxin testing have correlated in all the strains we have studied, and hemolysin assay seems suitable for discrimination of enterotoxin producers in this group.

Most enterotoxigenic *Aeromonas* spp. are included in the VP-positive group classified here as *A. hydrophila*. In our previous study, we found an association between enterotoxin production and failure to ferment arabinose as well as between fermentation of arabinose and failure to produce enterotoxin. The present study has confirmed that most arabinose-negative strains are enterotoxigenic, but the relationship between fermentation of arabinose and lack of enterotoxin production was not significant. There was variation in this relationship depending on the geographical source of the isolate, but the most striking difference was seen in nonfecal isolates. Of enterotoxin-negative strains isolated from water, 82% were arabinose positive, as were 80% of enterotoxin-negative nonfecal strains, but only 50% of enterotoxin-negative fecal strains from New York showed a similar biotype.

In our previous study, less than one-half of the enterotoxin-negative, arabinose-positive group were of fecal origin, and it would seem that our previous finding of a good correlation between these properties was affected by the inclusion of nonfecal strains.

However, arabinose-negative strains are predominantly enterotoxigenic, and, in the present study, 90% of enterotoxigenic strains isolated from feces would have been correctly classified in this way.

Hemolysin assay is the most reliable method of discriminating enterotoxigenic *Aeromonas* spp. and is applicable to both fecal and nonfecal isolates. With this assay alone, 97% of strains would have been correctly classified both in the present study and in the one reported previously. Stationary cultures are inadequate for hemolysin assay. If an environmental incubator-shaker is not available, a water bath shaking at 100 oscillations per min may be used.

We suggest that the following approach is suitable for diagnostic microbiology laboratories wishing to identify enterotoxigenic strains of *Aeromonas* spp. Strains should first be separated in relation to VP tests. VP-negative strains which do not produce gas from glucose or glycerol and do not oxidise gluconate are classified as *A. punctata* subsp. *caviae* and considered to be non-enterotoxigenic. Other VP-negative strains, classified as *A. punctata* subsp. *punctata* need the hemolysin assay to distinguish enterotoxigenic strains.

VP-positive strains, classified as *A. hydrophila*, will require hemolysin assay to identify enterotoxigenic strains, although arabinose-negative strains can be assumed to be enterotoxigenic with an error of 10%.

The present study has confirmed the usefulness of biochemical tests in association with the hemolysin assay for identification of enterotoxigenic *Aeromonas* spp. Since we have found a significant difference between isolation of enterotoxigenic *Aeromonas* spp. but not enterotoxin-negative strains in children with and without diarrhea (5), it is relevant for diagnostic microbiology laboratories to be able to identify enterotoxigenic *Aeromonas* spp. The scheme we suggest allows correct classification of 97% of strains of *Aeromonas* spp. without the need for expensive and specialized tests for *Aeromonas* enterotoxin.

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