Biochemical Characteristics of Enterotoxigenic *Aeromonas* spp.

VALERIE BURKE, JENNIFER ROBINSON, H. MAX ATKINSON, AND MICHAEL GRACEY

Gastroenterological Research Unit, Princess Margaret Children's Medical Research Foundation, Perth, 6001, Western Australia, and School of Pharmacy, South Australian Institute of Technology, Adelaide, South Australia

Received 15 June 1981/Accepted 6 August 1981

Biotypes of *Aeromonas* spp. correlated well with enterotoxin production in a study of 174 strains. Using biochemical characteristics determined by conventional methods and multitest systems, we correctly classified 93% of the strains with regard to enterotoxin production. Most of the enterotoxigenic strains were Voges-Proskauer (VP) positive and did not hydrolyze arabinose, but VP-positive strains which hydrolyzed arabinose were mainly non-enterotoxigenic. *Aeromonas punctata* subsp. *caviae*, which is VP negative and does not oxidize gluconate or produce gas from glucose, was non-enterotoxigenic. Although the number of other VP-negative strains was small, most were enterotoxigenic. Discrimination was improved so that 97% of the strains were correctly classified if the hemolysin assay was used either for all strains or for only the VP-positive, arabinose-positive and VP-negative, non-*A. punctata* subsp. *caviae* strains. Because the proposed classification system does not require facilities for carrying out in vivo assays such as suckling mouse or ileal loop methods, the identification of enterotoxigenic *Aeromonas* strains should be possible in diagnostic laboratories and will facilitate epidemiological studies of the role of these organisms in acute diarrhea.

*Aeromonas hydrophila* has been implicated as a cause of diarrhea in several countries (12). The finding that some strains of *A. hydrophila* are enterotoxigenic (9) provided further evidence for considering this organism to be an enteric pathogen. However, epidemiological studies of the role of enterotoxigenic *Aeromonas* spp. in acute diarrhea have been limited by difficulties in isolating these organisms (12) and in identifying enterotoxigenic strains.

*Aeromonas* spp. may produce three types of exotoxins, namely, enterotoxins, hemolysins, and cytotoxins, which are not invariably associated in a given strain. The sucking mouse test (3), rabbit ileal loop assay (9), and perfusion of rat jejunum in vivo (11) have all been used to demonstrate changes in intestinal function produced by enterotoxins, but these methods are unsuitable for use in routine diagnostic microbiology laboratories. Cell culture systems have also been used to assay *Aeromonas* toxins, but cytotoxicity is not identical with enterotoxigenicity. We have found that many non-enterotoxigenic strains may produce cytotoxins, and there is greater correlation between hemolysin production and enterotoxigenicity (V. Burke, J. Robinson, H. M. Atkinson, M. Dibley, R. J. Berry, and M. Gracey, Aust. J. Exp. Biol. Med. Sci., in press).

The present study describes a possible scheme for the identification of enterotoxigenic *Aeromonas* strains, making use of methods available in diagnostic microbiology laboratories.

**MATERIALS AND METHODS**

**Bacterial strains.** The 174 *Aeromonas* strains used were obtained from the following: Department of Microbiology, Princess Margaret Hospital, Perth, Western Australia; South Australian Institute of Technology, Adelaide, South Australia; Christian Medical College, Vellore, India; and International Centre for Diarrhoeal Disease Research, Bangladesh. Most strains were of fecal origin, but there were six from water, one from blood, one from a tracheal aspirate, and two from wound swabs.

**Bacterial preparations.** *A. hydrophila* strains were stored in maintenance medium consisting 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 Na2HPO4 in 134 ml of distilled water and 1.3 g of K2HPO4 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 (V. Burke, J. Robinson, R. J. Berry, and M. Gracey, J. Med. Microbiol., in press) 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
TABLE 1. Number (percent) of strains positive for the MB24E tests and other tests

<table>
<thead>
<tr>
<th>Test</th>
<th>No. (%) positive&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All strains (n = 174)</td>
</tr>
<tr>
<td>MB24E</td>
<td></td>
</tr>
<tr>
<td>LDC</td>
<td>161 (92.5)</td>
</tr>
<tr>
<td>Glucose</td>
<td>171 (98.3)</td>
</tr>
<tr>
<td>Mannitol</td>
<td>168 (96.6)</td>
</tr>
<tr>
<td>ONPG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>164 (94.3)</td>
</tr>
<tr>
<td>Indole</td>
<td>169 (97.1)</td>
</tr>
<tr>
<td>VP</td>
<td>152 (87.4)</td>
</tr>
<tr>
<td>Citrate</td>
<td>79 (45.9)</td>
</tr>
<tr>
<td>Gelatin</td>
<td>123 (70.7)</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>4 (2.3)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>155 (89.1)</td>
</tr>
<tr>
<td>Lactose</td>
<td>12 (6.9)</td>
</tr>
<tr>
<td>Arabinose</td>
<td>34 (19.5)</td>
</tr>
<tr>
<td>Salicin</td>
<td>9 (5.2)</td>
</tr>
<tr>
<td>Arginine dihydrodase</td>
<td>174 (100)</td>
</tr>
</tbody>
</table>

Other

<table>
<thead>
<tr>
<th>Test</th>
<th>No. (%) positive&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Production of H&lt;sub&gt;2&lt;/sub&gt;S from cysteine</td>
<td>132 (75.9)</td>
</tr>
<tr>
<td>Esculin hydrolysis</td>
<td>87 (50.0)</td>
</tr>
<tr>
<td>Growth on L-arginine</td>
<td>162 (93.1)</td>
</tr>
<tr>
<td>Growth on L-histidine</td>
<td>171 (98.3)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentages are based on the total number of strains tested in each category of strains (n). SM<sup>+</sup>, Positive in sucking mouse assay; SM<sup>-</sup>, negative in sucking mouse assay. No strains were positive for the following tests: ornithine decarboxylase, xylose, urease, tryptophan deaminase, malonate, inositol, sorbitol, adonitol, and raffinose.

<sup>b</sup> P < 0.05 between SM<sup>+</sup> and SM<sup>-</sup> strains.

<sup>c</sup> ONPG, o-Nitrophenyl-β-D-galactopyranoside.

μm; type HA; Millipore Corp., Bedford, Mass.). Supernatant fluids were stored at 4°C and tested within 1 day after being prepared.

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 diarrhoea, made obvious by blue staining of blotting paper under the animals, was also recorded. Weight ratios and amounts of diarrhoea were both used to classify positive strains as described elsewhere (Burke et al., J. Med. Microbiol., in press). The assay used detects the non-dialyzable, heat-labile enterotoxin of Aeromonas spp. (Burke et al., Aust. J. Exp. Biol. Med. Sci., in press).

(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 (Limbro, 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 log2 of the reciprocal of the greatest dilution showing hemolysis. Values of >2 were considered positive.

Biochemical characteristics. Biochemical profiles were determined with the Microbact MB24E system (Disposable Products, Adelaide, South Australia). This system contains tests for the following: lysine decarboxylase (LDC); ornithine decarboxylase; H2S production; fermentation of glucose, mannitol, and xylose; o-nitrophenyl-β-D-galactopyranosidase; indole production; urease; Voges-Proskauer reaction (VP); citrate utilization (Simmon's); tryptophan deaminase; gelatin liquefaction; malonate; inositol; sorbitol; rhamnose; sucrose; lactose; arabinose; adonitol; raffinose; salicin; and arginine dihydrodase. All carbohydrates were pH forms, except for L+ arabinose and L+ rhamnose. Tests were read after 24 h. Results for 50 strains were compared with those from the API 20E system (Analytab Products, La Balme-Les-Grottes, Montalieu-Vercieu, France) either by us or by P. Mugg (Institute of Medical and Veterinary Science, Adelaide, South Australia).

The following tests were used as described by Cowan (1): oxidation of gluconate, production of gas.
Aeromonas strains would have been classified incorrectly. Of the 131 enterotoxigenic strains fermented at least four of these carbohydrates. These differences were statistically significant ($P < 0.05$) by the Wilcoxon's rank sum test for categorical data (6).

Although no single test classified suckling mouse assay-positive strains, 93% of the strains were correctly classified as enterotoxigenic or non-enterotoxigenic when biochemical characteristics similar to those in the scheme proposed by Schubert (10) were considered.

**RESULTS**

Exotoxin assays. A total of 131 enterotoxigenic and 43 non-enterotoxigenic *Aeromonas* strains were detected with the suckling mouse assay. Of the 131 enterotoxigenic strains, 127 produced hemolysin, but only 2 of the 43 non-enterotoxigenic strains produced hemolysin.

If hemolysin production were used as the only marker of enterotoxigenicity, 6 (3.5%) of 174 *Aeromonas* strains would have been classified incorrectly.

Biochemical characteristics. Table 1 shows the numbers of strains positive for the various tests in the MB24E system and for other tests. A comparison of results for enterotoxigenic and non-enterotoxigenic strains showed that there were significant differences in the numbers positive ($P < 0.05$ by the chi-square test) for the following tests: LDC, VP, citrate, rhamnose, sucrose, and arabinose. However, no single test differentiated between enterotoxin-positive and -negative strains.

There were also significant differences ($P < 0.05$) between suckling mouse assay-positive and -negative strains for production of H$_2$S from cysteine and esculin hydrolysis (Table 1), but neither test was useful in classifying individual strains. Most *Aeromonas* strains used L-arginine or L-histidine as the sole carbon source.

Suckling mouse assay-negative strains fermented more carbohydrates from glucose, mannitol, sucrose, rhamnose, arabinose, salicin, and esculin than did suckling mouse assay-positive strains. A total of 36 (83%) of the 43 non-enterotoxigenic strains and 51% of the enterotoxigenic strains fermented at least four of these carbohydrates. These differences were statistically significant ($P < 0.05$) by the Wilcoxon's rank sum test for categorical data (6).

Although no single test classified suckling mouse assay-positive strains, 93% of the strains were correctly classified as enterotoxigenic or non-enterotoxigenic when biochemical characteristics similar to those in the scheme proposed by Schubert (10) were considered.

**Classification of enterotoxigenic *Aeromonas* spp. by biochemical characteristics.** *Aeromonas* strains were subdivided into VP-positive and VP-negative groups (Fig. 1). VP-positive strains (group A) were further subdivided into arabinose-negative (group A, type 1) and arabinose-positive (group A, type 2) strains. There were 125 VP-positive, arabinose-negative strains; 120 were enterotoxigenic, and 5 were non-enterotoxigenic. In the VP-positive, arabinose-positive group, 19 strains were non-enterotoxigenic, and 6 were enterotoxigenic.

VP-negative strains were classified into those which did not oxidize gluconate or produce gas from glucose (group B) and those which had one or both of these properties and did not produce gas from glycerol (group C). None of the 18 strains of group B, which corresponds to *Aeromonas punctata* subsp. *caviae*, as defined by Schubert (10), produced enterotoxins. Of the remaining six VP-negative strains, which correspond to *Aeromonas punctata* subsp. *punctata*, five were enterotoxigenic.

The results of the API 20E and MB24E tests used in this classification agreed for all strains. Reevaluation of the results of biochemical tests which had shown significant differences between enterotoxigenic and non-enterotoxigenic strains revealed that most of these differences were accounted for by *A. punctata* subsp. *caviae* strains. Elimination of these 18 strains...
from the suckling mouse assay-negative group showed that the only significant differences remaining were accounted for by hydrolysis of rhamnose, arabinose, and esculin.

Classification by biotyping and hemolysin assay. Whereas the hemolysin assay alone yielded the correct classification of 97% of the strains, other biochemical testing resulted in the correct classification of 93%. Discrimination was improved to 96% accuracy in classifying strains in group A, type 2 by the hemolysin assay, as shown in Table 2, and to 97% if the VP-negative strains of group C were also classified by the hemolysin assay.

The biochemical classification scheme described corresponds closely to that used by Schubert (10) in the taxonomy of Aeromonas spp. The characteristics used by Popoff and Véron (8) for classifying Aeromonas spp., including esculin hydrolysis, growth in L-histidine or L-arginine, fermentation of salicin, and H₂S production from cysteine, did not result in the correct classification of individual enterotoxigenic strains.

Table 2 summarizes the numbers of strains misclassified in each group and relates our suggested classification scheme to that proposed by Schubert (10).

**DISCUSSION**

This study has shown that enterotoxin production in Aeromonas spp. is associated with biotype. Using biochemical characteristics alone, we correctly classified 93% of 174 Aeromonas strains with regard to enterotoxin production. Of 131 enterotoxigenic strains, 120 were VP positive and did not hydrolyze arabinose. Of 43 non-enterotoxigenic strains, 19 were VP positive and hydrolyzed arabinose, and 18 strains, none of which produced enterotoxins, were identified as A. punctata subsp. caviae. Of six VP-negative strains identified as A. punctata subsp. punctata, five produced enterotoxins; greater numbers of such strains need to be investigated to establish the validity of this observation. In our experience, A. punctata subsp. punctata strains were rare.

Cumberbatch et al. (2) reported the association of cytotoxic A. hydrophila strains with positive tests for VP and LDC. Although cytotoxicity is not invariably associated with enterotoxin production, these characteristics are correlated, particularly in enterotoxigenic strains (Burke et al., Aust. J. Exp. Biol. Med. Sci., in press). Our experience suggests that biochemical differences are related to the inclusion of A. punctata subsp. caviae in the non-enterotoxigenic group. After exclusion of this subspecies, there were no significant differences between the numbers of LDC- or VP-positive strains which produced enterotoxins.

Ljungh et al. (7) failed to correlate biotype with enterotoxin production measured in rabbit intestinal loops and Y1 adrenal cells. However, these authors invested only 11 A. hydrophila strains, 5 of which caused secretion in intestinal loops, and did not report their results in sufficient detail to allow comparison with our findings.

When hemolysin production alone was used for the characterization of Aeromonas spp., 97% of the strains were correctly classified with regard to enterotoxin production. Eddy (4) included production of filterable hemolysins as a characteristic useful in the taxonomy of the genus Aeromonas. We have established this relationship for hemolysins produced during shaking of Trypticase soy broth with 0.6% yeast extract at 300 rpm on a gyratory incubator-shaker. Because such equipment is not available in all routine microbiology laboratories, we have investigated alternative conditions for hemolysin production. When cultures shaken in a water bath at 100 oscillations per minute at 37°C overnight were used, results agreed in all cases with those obtained when the incubator-shaker was used.

Correct classification of 97% of the strains was also possible if the hemolysin assay was
used only with VP-positive, arabinose-positive strains and *A. punctata* subsp. *punctata*. This necessitated a hemolysin assay for only 31 of our 174 strains and may be a more suitable approach in routine microbiology laboratories.

Our scheme for biotyping enterotoxigenic strains resembles the scheme proposed by Schubert (10) but modified by the subdivision of VP-positive strains on the basis of fermentation of arabinose. The classification scheme described by Popoff and Véron (8) is not applicable to the identification of enterotoxigenic *Aeromonas* strains.

Because biochemical characteristics, including hemolysin production, resulted in the misclassification of only 3% of *Aeromonas* spp., these tests are suitable for use in microbiology laboratories without facilities for enterotoxin testing such as suckling mouse or ileal loop assays. The MB24E multittest system used in our study is locally made and much less expensive for us than alternative imported systems. However, we found the API 20E system to be equally satisfactory in classifying *Aeromonas* spp. Application of these biochemical methods simplifies epidemiological studies and allows the processing of large numbers of samples so that the role of enterotoxigenic *Aeromonas* spp. in acute diarrhea may be clarified.

**ACKNOWLEDGMENTS**

This work was supported by TVW Telethon Foundation, Perth, Western Australia; the Wellcome Trust, London, England; and the National Health and Medical Research Council of Australia.

We thank I. Huq, A. M. Molla, and V. I. Mathan for supplying *Aeromonas* isolates and Peter Worthy, June O’Connor, Jan Beaman, Sally-Ann Dalton-Morgan, and Doug Peck for skilled technical assistance.

**LITERATURE CITED**


