BACTERIAL PATHOGENICITY

A putative heat-labile enterotoxin expressed by strains of *Aeromonas media*

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Fifteen isolates of *Aeromonas media* (seven from diarrhoeal stools, four from water and four from superficial skin ulcers of catfish) were examined for enterotoxin production. Ten of these isolates (six diarrhoeal, one from water and three from fish) caused accumulation of fluid in the initial rabbit ileal loop (RIL) tests. Isolates from diarrhoeal stools and fish caused relatively more fluid accumulation than those from water. Those strains that caused little or no fluid accumulation in the initial experiments became enterotoxin producers after one passage through RILs, regardless of source, and also showed gradual enhancement of fluid pouring after each subsequent passage. Inocula of c. 1 × 10^4 viable cells and 0.25 ml of culture filtrate (CF) caused fluid accumulation similar to that of toxigenic *Vibrio cholerae* 569B. The enterotoxin factor(s) were inactive when held at 56°C for 20 min or 65°C for 10 min and showed biological activity over a wide range of pH values. These results suggest that strains of *A. media*, whether from diarrhoeal stools, water or infected fish, are potentially enterotoxigenic and may have the potential to produce a heat-labile and pH-stable diarrhoeagenic factor in the same way as other known heat-labile and pH-stable enterotoxins.

Introduction

*Aeromonas* spp., often originating from water, have been implicated as causes of diarrhoea in man [1, 2]. The production of heat-labile enterotoxin by clinical and environmental isolates of *Aeromonas* was first demonstrated in the ligated rabbit ileal loop (RIL) model [3–5], and subsequently in various other animal and tissue culture assays [6–11]. Of the 13 currently recognised hybridisation groups (HGIs) of *Aeromonas*, *A. hydrophila*, *A. veronii* bv *sobria* and *A. caviae*, and more recently *A. veronii* bv *veronii*, *A. trota*, *A. jandaei* and *A. eucrenophila*, have been shown to produce enterotoxin [12–16]. Strains of *A. media* have been isolated from patients with diarrhoea [17, 18] and from the environment [19]. However, little is known about their pathogenic potential in terms of the expression of an enterotoxin and the role they may play in the causation of diarrhoea, except that this species belongs to a different ribotype [17] and that the suicidal phenomenon may be prevented by stimulation of bacterial metabolism [18]. Therefore, in the present study a number of diarrhoeal, water and fish isolates of *A. media* were examined for enterotoxigenic activity in the experimental animal and, if found, the enterotoxin was characterised and assessed for correlation, if any, with the source of isolation.

Materials and methods

Bacterial strains

Of the 15 isolates of *Aeromonas* included in the study, seven were from diarrhoeal stools and four each from water and superficial skin ulcers of infected catfish. These isolates were identified as *A. media* on the basis of the criteria of Abbott *et al.* [20]. The strains were maintained in peptone agar stab culture at room temperature and did not undergo more than three subcultures before the experiments.

Enterotoxicity test

Live cells and culture filtrates (CFs) of *A. media* strains were tested for enterotoxin production in ligated ileal loops of adult rabbits (Belgian strain) by the method of De and Chatterji [21]. Briefly, bacteria grown for 3 h in Brain Heart Infusion Broth (BHB; Difco) were diluted 10-fold in the same medium and inoculated into a RIL.
in a 1.0-ml dose containing \(10^3–10^6\) cfu. BHIB cultures of toxigenic \(V.\) cholerae strain 569B and BHIB served as positive and negative controls, respectively. CFs in 1.0-ml amounts were also tested in the same way. Each test was done in two rabbits, 8–10 loops being ligated in each. The rabbits were killed after 8 h.

**Passage through RIL**

Those strains of \(A.\) media that caused little or no accumulation of fluid in the initial tests were passaged through RILs by the method of Sanyal et al. [22]. Briefly, each strain was cultured aseptically from a RIL on to nutrient agar and, after overnight incubation, five or six colonies were inoculated into BHIB, incubated for 3 h and 1.0 ml of the 10-fold diluted culture was inoculated again into a RIL. The process was continued until a positive response was obtained.

**Multiplication of the organism in ileal loops**

The method described by Sanyal et al. [23] was used to determine the multiplication of the organism in the RIL. Briefly, a 3–4 h culture in BHIB was diluted 10-fold in the same medium and 1 ml from each dilution was inoculated into a RIL. After laparotomy, the fluid was collected aseptically and a visible count was made in duplicate by the pour plate method. Colonies were identified by means of biochemical tests. Where no fluid accumulated in the RILs, fluid for viable counts was obtained by removing the loop and washing the lumen twice with 10 ml of sterile isotonic saline.

**Preparation of culture filtrate**

Culture filtrates of those \(A.\) media isolates that gave positive ileal loop reactions were prepared by the method of Annapurna and Sanyal [5]. Briefly, 10 ml of BHIB in a 50-ml conical flask was inoculated with five or six smooth colonies grown overnight on nutrient agar. The flasks were incubated at 37°C in a water-bath with shaking at 80–120 oscillations/min for 16–18 h. The cultures were centrifuged at 22,000 \(\text{g}\) for 20 min at 4°C, and the supernates were filtered through membrane filters (Millipore, 0.22 \(\mu\)m) and stored at 4°C in small volumes. These CFs were tested for enterotoxic activity in RIL.

**Determination of the minimum reacting dose of culture filtrate in ileal loops**

Culture filtrates prepared in BHIB with three strains of \(A.\) media in amounts ranging from 0.1 to 1.0 ml were inoculated into different loops of each rabbit. The animals were killed after 8 h and the accumulation of fluid in ml/cm of gut was measured. The minimal amount of CF that caused maximum fluid accumulation was regarded as the minimal reacting dose. All experiments were done in duplicate.

**Effect of temperature on enterotoxic activity of culture filtrates**

Culture filtrates were heated at different temperatures (56–65°C) for various time periods (10–20 min) in a water bath and 1.0 ml of each was then inoculated into a RIL. The animals were killed after 8 h and the reaction was noted. Each CF was tested in two rabbits.

**Effect of pH on enterotoxic activity of culture filtrates**

To examine the effect of pH on enterotoxic activity, the pH of each CF was adjusted to 5.0 and 6.0 by the addition of 0.1N HCl and to 8.0 and 9.0 by the addition of 1.0 M ammonia solution. The CFs were kept overnight at 4°C and, after re-adjustment of the pH to 7.2, tested for enterotoxic activity in RIL.

**Results**

The isolates of \(Aeromonas\) were identified as \(A.\) media by the criteria of Abbott et al. [20] after 28 standard biochemical tests had been performed by conventional methods. When examined at 37°C, all 15 isolates were found to be resistant to the vibriostatic agent \(O(129; 50 \mu g\) and 150 \(\mu g\) and uniformly positive for oxidase, indole, motility, asculin and arginine hydrolysis. Most isolates were positive for haemolysis on 5% sheep blood agar plates. All these strains fermented glycerol, D-mannose, maltose, D-glucose, D-mannitol, sucrose and salicin, but most did not ferment cellobiose or arabinose. They were all negative for lysine and ornithine decarboxylation. None of the strains produced gas from glucose or \(H_2S\) from cysteine, but all utilised citrate as their sole source of carbon and were resistant to ampicillin. These characteristics clearly distinguish these strains from other species of \(Aeromonas\) and they were designated as \(A.\) media.

In the initial tests, live cells of 10 of the 15 strains of \(A.\) media caused fluid accumulation. Six of these strains were from diarrhoeal stools, one from water and three from fish sources. Isolates from diarrhoeal stools and fish caused more fluid accumulation than those from water. With organisms from all sources, there were strain variations in the volume of fluid accumulation, and also variation between individual loops inoculated with the same strain (Table 1).

The remaining isolates from all sources that caused little or no fluid accumulation in the initial test did so after a single passage through RIL. However, ATCC strain 35950 required two serial passages to do so. These strains also showed gradual enhancement of the outpouring of fluid after each passage. Culture filtrates prepared only after they showed secretory activity also caused fluid accumulation. The volumes of fluid accumulated were similar to those produced by live
The minimal loop reacting dose of the CFs was 0.25 ml with all the three strains tested, as there was no enhancement of secretory response with increasing doses, and lower doses caused either no or less fluid accumulation.

CFs prepared with the strains of *A. media* caused fluid accumulation after being held at 56°C for 10 min (Table 3) and little or no fluid accumulation was observed after the filters were held at 56°C for 20 min or 65°C for 10 min. The optimal pH at which maximal fluid accumulation was observed was 7.2 and there was proportionally less accumulation of fluid at more acid or alkaline pH (Table 4).

**Discussion**

These results show that not only the diarrheal isolates but also water and fish isolates of *A. media* may produce an enterotoxin, as detected by fluid accumulation in the RIL, the traditional model for detection of enterotoxin production. The differences in fluid accumulation between strains may be due to variation in the amount of toxin released. This observation suggests that *A. media* produces an enterotoxin that may have the potential to cause diarrhoea in man, which is consistent with the isolation of *A. media* from the faeces of such cases in this study.

However, the observation that strains of diarrhoeal and fish origin caused relatively more fluid accumulation suggests their higher enterotoxic potential, which may explain the less frequent detection of enterotoxic activity in water isolates. A probable explanation of this difference in enterotoxic activity may be the observation that, when compared with water isolates, strains of diarrhoeal and fish origin produced haemolysin in the initial tests, which may be correlated with higher enterotoxicity [12, 14].

Five of the 15 isolates of *A. media* from all sources,

<table>
<thead>
<tr>
<th>Fluid accumulation (ml/cm of RIL) and passage number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source and strain no.</td>
</tr>
<tr>
<td>Diarrhoea</td>
</tr>
<tr>
<td>D-22</td>
</tr>
<tr>
<td>D-58</td>
</tr>
<tr>
<td>D-88</td>
</tr>
<tr>
<td>D-Pappu</td>
</tr>
<tr>
<td>D-Suchi</td>
</tr>
<tr>
<td>D-Surita</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>W-4</td>
</tr>
<tr>
<td>W-13</td>
</tr>
<tr>
<td>W-20</td>
</tr>
<tr>
<td>W-56</td>
</tr>
<tr>
<td>Catfish</td>
</tr>
<tr>
<td>CF-7</td>
</tr>
<tr>
<td>CF-40</td>
</tr>
<tr>
<td>CF-45</td>
</tr>
<tr>
<td>CF-75</td>
</tr>
<tr>
<td>ATCC 35950</td>
</tr>
<tr>
<td><em>V. cholerae</em> 569B</td>
</tr>
<tr>
<td>BHIIB</td>
</tr>
</tbody>
</table>

ND not detected (lower limit for detection >0.20 ml/cm); NT, not tested.

*Mean volume of fluid accumulated in ileal loops of two rabbits.

Positive control.

bacterial cells, and comparable to that produced by the toxigenic *V. cholerae* 569B.

Table 2 shows that the three representative strains, one each from diarrheal stool, water and infected fish, caused no fluid accumulation when inoculated at a dose of c. 1 × 10^7 cfu, a small accumulation at c. 1 × 10^8 cfu. There was no further enhancement of fluid accumulation when the inoculum size was increased to 1 × 10^9 cfu or more (data not shown). The bacteria multiplied by c. 10^2–10^5-fold in all loops, irrespective of the fluid accumulated in RIL.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Source</th>
<th>Viable count of inoculum (cfu/ml)</th>
<th>Viable count after incubation for 8 h (cfu/ml)</th>
<th>Fluid accumulation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-88</td>
<td>Diarrhoea</td>
<td>4.0 × 10^7</td>
<td>3.1 × 10^9</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.1 × 10^8</td>
<td>5.3 × 10^9</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.3 × 10^9</td>
<td>2.3 × 10^10</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.7 × 10^9</td>
<td>1.8 × 10^10</td>
<td>0</td>
</tr>
<tr>
<td>W-20</td>
<td>Water</td>
<td>2.2 × 10^8</td>
<td>2.7 × 10^7</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.4 × 10^8</td>
<td>1.9 × 10^7</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.4 × 10^8</td>
<td>2.2 × 10^8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.8 × 10^8</td>
<td>1.7 × 10^8</td>
<td>0</td>
</tr>
<tr>
<td>CF-7</td>
<td>Catfish</td>
<td>8.1 × 10^8</td>
<td>6.9 × 10^9</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.6 × 10^9</td>
<td>4.6 × 10^10</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.9 × 10^9</td>
<td>7.6 × 10^9</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.8 × 10^9</td>
<td>3.4 × 10^10</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean volume of accumulated fluid in ml/cm in ileal loops observed in two rabbits.

Table 2. Multiplication of *A. media* in RIL

**Enterotoxicity of *Aeromonas media***

687
Table 3. Effect of temperature on fluid accumulation in RIL caused by culture filtrates of *A. media*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Fluid accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-88</td>
<td>Untreated</td>
<td></td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>10</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>20</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>10</td>
<td>0.00</td>
</tr>
<tr>
<td>W-20</td>
<td>Untreated</td>
<td></td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>10</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>20</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>10</td>
<td>0.00</td>
</tr>
<tr>
<td>CF-7</td>
<td>Untreated</td>
<td></td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>10</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>20</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>10</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*Each test was done in two rabbits.

Table 4. Effect of pH on enterotoxic activity of *A. media*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Fluid accumulation (ml/cm in RIL) of CF treated with HCl and ammonia solution at pH value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td>D-88</td>
<td>0.34</td>
</tr>
<tr>
<td>W-20</td>
<td>0.21</td>
</tr>
<tr>
<td>CF-7</td>
<td>0.41</td>
</tr>
</tbody>
</table>

*Mean amount of fluid accumulated in two rabbits.

which caused little or no fluid accumulation in the initial experiments, showed enhancement of their enterotoxic activity after serial passages through the gut of a susceptible host. Similar observations on the switching to toxin production by an apparently non-toxic strain on consecutive passage through the gut of a susceptible host were made in earlier studies with *Aeromonas* species [3–7, 12–16], and organisms such as *V. cholerae* non-O1 [24], *Plesiomonas shigelloides* [25], *V. fluvialis* [26], *V. mimicus* [27], Klebsiella pneumoniae [28] and CT gene-negative *V. cholerae* O1 [22]. Such a change may result from the existence of a repression-depression phenomenon that controls the expression of a toxin gene which is dependent on a micro-environment [29]. There is every likelihood that toxigenicity of fresh isolates used in this study may have decreased during one-to-three subcultures in vitro before the ileal loop tests.

Small amounts of fluid accumulated in RILs with inocula of *c. 1 × 10^3* cfu and maximum fluid outpouring was observed with inocula of *c. 1 × 10^4* cfu after 8 h, but no appreciable change in fluid accumulation was observed after 12 or 18 h. No fluid accumulation was observed with smaller inocula, although bacterial multiplication by a factor of *c. 10^4* took place. Moreover, the bacteria multiplied by a factor of 10^3–10^5 in all the loops, but accumulation of fluid took place only when the inocula were *c. 1 × 10^3* cfu or more. It is known that fluid outpouring in the gut of a susceptible host may be due to the elaboration of enterotoxic substance(s) by the organism during multiplication in the intestine [30]. The majority of *A. media* strains caused fluid accumulation in the initial test when *c. 1 × 10^3* cfu of bacteria adhered to intestinal mucosa (data not shown). This study suggests that an optimal number of bacteria need to adhere to the intestinal mucosa in order to multiply and elaborate sufficient amounts of enterotoxic substance(s) to induce optimal fluid accumulation, as reported earlier in *Aeromonas* [31]. Furthermore, the observation that a gradual increase in the range of fluid accumulation corresponded to the increase in size of the inocula indicates that a minimum number of bacterial cells is necessary to cause fluid accumulation. This was substantiated by the fact that there was no increase in fluid outpouring when the size of the bacterial inoculum was increased further.

The observation that a small inoculum size of 0.25 ml of CF caused an optimal secretory response in RIL suggests that *A. media* may secrete potent enterotoxic substance(s). The loss of enterotoxic activity of the CF when held at 56°C for 20 min or 65°C for 10 min, and the retention of biological activity over a wide range of pH (5.0–9.0) suggest that the enterotoxic substance(s) produced by the *A. media* strains was heat-labile and pH-stable.

The present study demonstrates that, regardless of their
source, strains of *A. media* are potentially enterotoxi-
genic and the toxic substances produced are heat-labile and
pH-stable, as are other known heat-labile and pH-
stable enterotoxins. The fact that strains of *A. media*
produced quantitatively more enterotoxin after each
passage suggests that this species, being ubiquitous in
the aquatic environment, may have easy access to the
human intestine, by consumption of either contami-
nated water or under-cooked fish. In this way, increased
enterotoxicity may develop.

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for the ATCC 19350 strain of *A. media* and Dr B. N. Shukla for the
remaining strains.

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ENTEROTOXICITY OF *AEROMONAS MEDIA* 689