INTRODUCTION

*Aeromonas* spp. cause extraintestinal infections and diarrhoea in man (1,2). Production of heat-labile enterotoxin by clinical and environmental isolates of *Aeromonas* was first demonstrated in the ligated rabbit ileal loop model (3-5) and subsequently in various other animal and tissue culture assays (6-11). Among the recently-described species, *Aeromonas hydrophila*, *A. veronii* bv *sobria*, *A. caviae*, *A. trota*, *A. jandaei*, *A. eucrenophila*, and *A. media* have been reported to produce enterotoxin (12-17).

*A. veronii* bv *veronii*, like other aeromonads, commonly isolated from the aquatic environment, is associated with intestinal (18) and extraintestinal infections (19) in human. Attempt was made to examine if these strains produce any diarrhoea in the ileal loops of experimental adult rabbits with or without causing any damage to the intestinal mucosa.
METHODS AND MATERIALS

Organisms

Three isolates of A. veronii bv veronii (two environmental and one blood infection, kindly provided by Dr. JM Janda, Microbial Diseases Laboratory, Department of Health Services, Berkeley, CA, USA) were studied. The isolates were re-identified as A. veronii bv veronii due to their ability to hydrolyze aesculin; ferment salicin, sucrose, and cellobiose; produce gas from glucose, acetoin, and H2S from cysteine; decarboxylate ornithine, and resistant to ampicillin and carbenicillin, following the criteria of Hickmann-Brenner et al. (18) and Joseph et al. (19) and using the standard bacteriological techniques (Table 1). The strains were maintained in peptone agar stab culture at room temperature, and did not undergo more than three subcultures prior to testing in this study.

Ileal loop test and passage through rabbit ileal loops

Cultures and culture filtrates of all the A. veronii bv veronii strains were tested in the ligated ileal loops of adult rabbits (Belgian strain), following the method of De and Chatterji (20). In brief, bacteria, grown in Brain Heart Infusion Broth (BHIB, Difco) for 3 hours, were diluted 10-fold in the same medium, and at that time 1 mL (containing 10^5-10^6 cfu/mL) was inoculated into a rabbit ileal loop. BHIB culture of toxigenic Vibrio cholerae strain 569B and unseeded BHIB served as positive and negative controls respectively. One mL of culture filtrate was also tested in the same way described above. Every test was done in two rabbits, 8-10 loops being ligated in each. The rabbits were sacrificed after 8 hours.

Strains of A. veronii bv veronii that caused little or no accumulation of fluid in the initial tests were passed through the rabbit gut, following the method of Sanyal et al. (21). Briefly, each strain was cultured aseptically from a rabbit ileal loop on nutrient agar. After overnight incubation, 5 or 6 colonies were inoculated into BHIB, incubated for 3 hours and 1 mL of the 10-fold diluted culture was inoculated again into a rabbit ileal loop. The process continued until a positive response was obtained.

Multiplication of the organism in ileal loops

To determine the multiplication of the organism in the rabbit ileal loop, the method described by Sanyal et al. was used (22). Briefly, a 3-4-hour culture in BHIB was diluted 10-fold in the same medium, and 1 mL from each dilution was inoculated into the rabbit ileal loop. After laparotomy, the fluid was collected aseptically, and a viable count was made in duplicate by the pour plate method. Colonies were tested biochemically for identification. Where there was no fluid accumulation in the rabbit ileal loops, fluid for viable count was obtained by removing the loop and washing the lumen twice with 10 mL of sterile isotonic saline.

Preparation of culture filtrate

Culture filtrate of all the A. veronii bv veronii strains, which gave positive ileal loop reaction, was prepared by the method of Annapurna and Sanyal (5). Briefly, 10 mL of BHIB in a 50-mL conical flask was inoculated with 5 or 6 smooth colonies grown overnight on nutrient agar. The flasks were incubated at 37 °C in a water bath by shaking at 80-120 oscillations per minute for 16-18 hours. Culture was centrifuged at 22,000xg for 20 minutes at 4 °C, and the supernatant was filtered through membrane filters (Millipore, 0.22 µm) and stored at 4 °C in small aliquots.

Determination of minimum reacting dose of culture filtrate in ileal loops

Culture filtrate of all the A. veronii bv veronii strains, prepared in BHIB, was inoculated in amounts ranging from 0.1 to 1 mL into different loops of each rabbit. The animals were sacrificed after 8 hours, and the accumulation of fluid in mL per cm of gut was measured. The minimal amount of culture filtrate causing maximum fluid accumulation was regarded as the minimal reacting dose. All experiments were done in duplicate.
Effect of temperature on enterotoxic activity of culture filtrate

Culture filtrate of each strain was heated at different temperatures (56–65 °C) for various time periods (10-20 minutes) in a water bath, and 1 mL was inoculated into a loop. The animals were sacrificed after 8 hours, and the reaction was noted. Each culture filtrate was tested in two rabbits.

Effect of pH on enterotoxic activity of culture filtrate

To examine the effect of pH on enterotoxic activity, the pH of culture filtrate of each strain was adjusted to 4.0, 5.0, and 6.0 by adding 0.1N-HCl solution and to 8.0, 9.0 and 10.0 by adding 1 M of ammonia solution. Culture filtrate was kept overnight at 4 °C, readjusted the pH to 7.2, and 1 mL was tested for enterotoxic activity as described above.

Histopathology of ileal loops

The ileal loop test was done with live cells and culture filtrate of each A. veronii bv veronii strain and V. cholerae 569B, while BHIB-inoculated loops served as negative control. The rabbits were sacrificed after 8 hours, and the reaction was noted. Each loop was immediately removed, cut through its entire length, and completely immersed in 10% formal saline in a petridish. A piece of normal gut measuring a few cm was also removed and treated similarly. The tests were done in duplicate. The section embedded in paraffin blocks was stained with haematoxylineosin, mounted in Canada balsam and examined under the microscope for histological changes.
Results

The Aeromonas isolates, provided by Dr. JM Janda, were re-identified in our laboratory as A. veronii bv veronii, using 26 standard biochemical tests (Table 1) performed by the conventional methods, following the criteria of Hickmann-Brenner et al. (18) and Joseph et al. (19). All the three isolates, when examined at 37°C, were resistant to vibriostatic agent (O/129; 50 µg and 150 µg), and were uniformly positive for oxidase, gas production from glucose, H₂S from cysteine, indole, motility, ornithine decarboxylation, Voges-Praskauer reaction, and haemolysis on 5% sheep blood agar plates. All the strains fermented D-glucose, D-mannitol, D-mannose, salicin, and hydrolyzed aesculin. They were uniformly negative for arginine hydrolysis, lysine decarboxylation, cellobiose, L-arabinose, and inositol fermentation, and were resistant to ampicillin and carbenicillin. These characteristics clearly distinguished the strains from other species of Aeromonas, and were designated as Aeromonas veronii bv veronii.

<table>
<thead>
<tr>
<th>Test</th>
<th>Aeromonas veronii bv veronii strains</th>
<th>Reaction for control strain*</th>
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<tr>
<td>Oxidase</td>
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<td>Motility</td>
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<td>Indole production</td>
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<td>Arginine hydrolysis</td>
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<td>H₂S from triple sugar iron agar</td>
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<td>H₂S from cysteine</td>
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<td>Decarboxylation of Lysine (Moller’s)</td>
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<td>Decarboxylation of Ornithine (Moller’s)</td>
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<td>Susceptibility to</td>
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<td>Ampicillin</td>
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<td>Carbenicillin</td>
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<td>Cephalothin</td>
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<td>Colistin</td>
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<td>O/129 resistance</td>
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<td>Haemolysis on 5% sheep blood agar</td>
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*Criteria of Hickmann-Brenner et al. (18) and Joseph et al. (19) were followed
- : Negative at the end of the appropriate incubation period
+ : Positive after 24 hours or at the end of the incubation period

Live cells of 2 of the 3 A. veronii bv veronii strains caused fluid accumulation in the initial tests. The other isolate that caused no fluid accumulation in the initial test did so after a single passage through the rabbit ileal loop. All the strains also showed gradual enhancement of fluid outpouring after each passage (Table 2). There were strain variations in the volume of fluid accumulated and also variations between individual loops inoculated with the same strain.
All the three strains either from the environment or from blood caused no fluid accumulation with inocula of about $1 \times 10^2 \text{cfu}$, but caused small accumulation of fluid with about $1 \times 10^3 \text{cfu}$, and caused maximal accumulation of fluid with approximately $1 \times 10^4 \text{cfu}$ (Table 3). There was no enhancement of fluid accumulation with the increased inoculum size of $1 \times 10^5 \text{ cfu}$ or more (data not shown). The bacterium multiplied by about $10^3$- to $10^5$-fold in all loops irrespective of the fluid accumulated in the rabbit ileal loop.

Culture filtrates of all the *A. veronii* bv *veronii* strains, prepared in BHIB, also caused accumulation of fluid in volume similar to those caused by the live cells, and were comparable to toxigenic *V. cholerae* 569B.

The minimal loop-reacting dose of the culture filtrate was 0.25 mL. There was no enhancement of the secretory response with increasing doses. Lower doses caused either no or less fluid accumulation.

Culture filtrates prepared with all the strains of *A. veronii* bv *veronii* caused fluid accumulation after holding at 56 °C for 10 minutes (data not shown) and little or no fluid accumulation after the filtrates were held at 56 °C for 20 minutes or 65 °C for 10 minutes.

The optimal pH at which maximal fluid accumulation observed was 7.2. There was proportionally less accumulation of fluid on either side of the pH range (data not shown).

No difference was observed between the ileal loops exposed for 8 hours to live cells and culture filtrates of *A. veronii* bv *veronii* and the control loops. The intestinal mucosa remained intact, and there was mild congestion in blood vessels. Mild mononuclear leukocyte infiltration was noted in the ileal loops exposed to *V. cholerae* 569B and lesser extent in those treated with *A. veronii* bv *veronii*. There was reduction in the number of goblet cells in the intestinal mucosa of loop treated with live cells and culture filtrates.
DISCUSSION

The data of the present study indicate that strains of *A. veronii bv veronii* produce an enterotoxin as judged by fluid accumulation in the rabbit ileal loop, the classical model for detection of enterotoxin production. The differences in fluid accumulation between strains may be due to variation in the amount of toxin released. The observation suggests that *A. veronii bv veronii* is potentially enterotoxigenic, and has the ability to cause diarrhoea in man.

The observation that one of the three isolates of *A. veronii bv veronii* failed to cause fluid accumulation in the initial set of experiments but did so after one passage through the rabbit gut indicates that this strain has also potential to produce enterotoxin. Similar observations on switching over 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 (5,12-17) and other organisms (21,23-27). Such a change may result from the existence of a repression-derepression phenomenon controlling the expression of a toxin gene depending on a microenvironment (28).

No fluid accumulation was observed with smaller inocula, although bacterial multiplication by about a factor of $10^4$ took place. Moreover, the accumulation of fluid took place only when the inocula were approximately $1 \times 10^3$ 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 (29). Furthermore, the observation that the 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 with a further increase in the size of bacterial inocula.

The observation that small inocula of culture filtrate caused an optimal secretory response in the rabbit gut suggests that *A. veronii bv veronii* may secrete a potent enterotoxic substance(s). The loss of enterotoxic activity of the culture filtrate at higher temperature and the retention of biological activity over a wide range of pH suggest that the enterotoxin produced by the *A. veronii bv veronii* strains was heat-labile and pH-stable.

Histopathological examination of the ileal loops exposed to live cells and culture filtrates of *A. veronii bv veronii* up to 8 hours showed no change other than a considerable reduction in the number of goblet cells. This finding indicates a depletion of mucus from these cells (30,31). However, the result that one strain isolated from blood infection caused blenching/haemorrhage of the ileal loops with loss of lustre immediately after inoculation suggests elaboration of additional toxic factors by this organism other than the enterotoxic substance(s). Further, the mild mononuclear infiltration, observed in the ileal loops treated with *A. veronii bv veronii* and *V. cholerae* 569B as well as in the normal ileal loop, might have been due to surgical manipulations (32).

The data, thus, suggest that strains of *A. veronii bv veronii* are able to produce a heat-labile and pH-stable enterotoxic substance(s) without causing any damage to the intestinal mucosa.
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REFERENCES


