BACTERIAL PATHOGENICITY

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

D. V. SINGH

Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India

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 outpouring after each subsequent passage. Inocula of $c. 1 \times 10^4$ viable cells and 0.25 ml of culture filtrate (CF) caused fluid accumulation similar to that of toxigenic *Vibrio cholerae* 569B. The enterotoxic factor(s) were inactivated 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 (HGs) 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 enterotoxic 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 (BHIB; Difco) were diluted 10-fold in the same medium and inoculated into a RIL

Received 28 April 1999; revised version received 23 Oct. 1999; accepted 5 Jan. 2000.

Corresponding author: Dr D. V. Singh. Present address: Rajiv Gandhi Centre for Biotechnology, Jagathy, Thiruvananthapuram 695 014, Kerala, India (e-mail: rgcbt@md2.vsnl.net.in).

in a 1.0-ml dose containing 10^5 – 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 viable 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 were 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 *g* for 20 min at 4°C, and the supernates were filtered through membrane filters (Millipore, 0.22 μ m) and stored at 4°C in small volumes. These CFs were tested for enterotoxigenic 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 enterotoxigenic 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 enterotoxigenic activity of culture filtrates

To examine the effect of pH on enterotoxigenic activity, the pH of each CF was adjusted to 5.0 and 6.0 by the addition of 0.1 N 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 enterotoxigenic 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 μ g and 150 μ g) and uniformly positive for oxidase, indole, motility, aesculin 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 H₂S 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

Table 1. Enhancement of fluid outpouring into RIL after each passage through rabbit gut when inoculated with BHIB culture of *A. media*

Source and strain no.	Fluid accumulation (ml/cm of RIL) and passage number*			
	0	1	2	3
<i>Diarrhoea</i>				
D-12	ND	0.30	0.42	0.59
D-22	0.78	NT	NT	NT
D-58	0.35	0.54	0.61	NT
D-88	0.78	NT	NT	NT
D-Pappu	0.55	0.68	0.67	NT
D-Suchi	0.94	NT	NT	NT
D-Sarita	0.75	NT	NT	NT
<i>Water</i>				
W-4	ND	0.60	0.67	NT
W-13	ND	0.64	0.70	0.77
W-20	0.31	0.60	0.83	NT
W-56	ND	0.53	0.62	NT
<i>Catfish</i>				
CF-7	0.71	NT	NT	NT
CF-40	ND	0.81	0.85	0.91
CF-45	0.44	0.66	NT	NT
CF-75	0.51	0.67	NT	NT
ATCC 35950	ND	ND	0.50	0.63
<i>V. cholerae</i> 569B [†]	0.90	1.10	1.40	NT
BHIB [‡]	0.00	0.00	0.00	0.00

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 diarrhoeal stool, water and infected fish, caused no fluid accumulation when inoculated at a dose of *c.* 1×10^2 cfu, a small accumulation at *c.* 1×10^3 cfu and approached maximal accumulation of fluid at *c.* 1×10^4 cfu. There was no further enhancement of fluid accumulation when the inoculum size was increased to 1×10^5 cfu or more (data not shown). The bacteria multiplied by *c.* 10^3 – 10^5 -fold in all loops, irrespective of the fluid accumulated in RIL.

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 filtrates 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 diarrhoeal 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 haemolysis 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 2. Multiplication of *A. media* in RIL

Strain no.	Source	Viable count of inoculum (cfu/ml)	Viable count after incubation for 8 h (cfu/ml)	Fluid accumulation*
D-88	Diarrhoea	4.0×10^5	3.1×10^9	0.75
		2.1×10^4	5.3×10^8	0.66
		4.3×10^3	2.3×10^7	0.31
		3.7×10^2	1.8×10^6	0
W-20	Water	2.2×10^5	2.7×10^7	0.34
		6.4×10^4	1.9×10^7	0.21
		5.4×10^3	2.2×10^6	0
		4.8×10^2	1.7×10^5	0
CF-7	Catfish	8.1×10^5	6.9×10^9	0.70
		6.6×10^4	4.6×10^9	0.61
		3.9×10^3	7.6×10^7	0.26
		5.8×10^2	3.4×10^6	0

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

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

Mean volume of accumulated fluid (ml/cm in RIL) after heat treatment of culture filtrates*			
Strain no.	Temperature (°C)	Time (min)	Fluid accumulation
D-88	Untreated	—	0.60
	56	10	0.90
	56	20	0.20
	65	10	0.00
W-20	Untreated	—	0.31
	56	10	0.35
	56	20	0.00
	65	10	0.00
CF-7	Untreated	—	0.74
	56	10	0.67
	56	20	0.13
	65	10	0.00

*Each test was done in two rabbits.

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

Fluid accumulation (ml/cm in RIL) of CF treated with HCl and ammonia solution at pH value*					
Strain no.	5	6	7.2	8	9
D-88	0.34	0.57	0.70	0.58	0.41
W-20	0.21	0.30	0.37	0.31	0.25
CF-7	0.41	0.56	0.71	0.63	0.48

*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-derepression 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 enterotoxin 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.* 1×10^5 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 enterotoxin 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 enterotoxin substance(s). The loss of enterotoxin 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 enterotoxin 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 enterotoxigenic 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 contaminated water or under-cooked fish. In this way, increased enterotoxicity may develop.

This study was supported in part by the Council of Scientific and Industrial Research, New Delhi, India. I am grateful to Dr J. M. Janda for the ATCC 35950 strain of *A. media* and Dr B. N. Shukla for the remaining strains.

References

- Janda JM, Duffey PS. Mesophilic aeromonads in human disease: current taxonomy, laboratory identification and infectious disease spectrum. *Rev Infect Dis* 1988; **10**: 980–997.
- Altwegg M, Geiss HK. *Aeromonas* as a human pathogen. *CRC Crit Rev Microbiol* 1989; **16**: 253–286.
- Sanyal SC, Singh SJ, Sen PC. Enteropathogenicity of *Aeromonas hydrophila* and *Plesiomonas shigelloides*. *J Med Microbiol* 1975; **8**: 195–199.
- Annapurna E, Sanyal SC. Studies on enteropathogenicity of *Aeromonas hydrophila* in an experimental animal model. *Indian J Prev Soc Med* 1975; **6**: 234–237.
- Annapurna E, Sanyal SC. Enterotoxigenicity of *Aeromonas hydrophila*. *J Med Microbiol* 1977; **10**: 317–323.
- Dubey RS, Sanyal SC. Enterotoxicity of *Aeromonas hydrophila*: skin responses and *in vitro* neutralisation. *Zentralbl Bakteriol Orig A* 1978; **242**: 487–499.
- Ljungh Å, Poppoff M, Wadström T. *Aeromonas hydrophila* in acute diarrheal disease: detection of enterotoxin and biotyping of strains. *J Clin Microbiol* 1977; **6**: 96–100.
- Burke V, Robinson J, Berry RJ, Gracey M. Detection of enterotoxins of *Aeromonas hydrophila* by a suckling-mouse test. *J Med Microbiol* 1981; **14**: 401–408.
- Chakraborty T, Montenegro MA, Sanyal SC, Helmuth R, Bulling E, Timmis KN. Cloning of enterotoxin gene from *Aeromonas hydrophila* provides conclusive evidence of production of a cytotoxic enterotoxin. *Infect Immun* 1984; **46**: 435–441.
- Turnbull PCB, Lee JV, Miliotis MD *et al.* Enterotoxin production in relation to taxonomic grouping and source of isolation of *Aeromonas* species. *J Clin Microbiol* 1984; **19**: 175–180.
- Kuijper EJ, Steigerwalt AG, Schoenmakers BS, Peeters MF, Zanen HC, Brenner DJ. Phenotypic characterization and DNA relatedness in human fecal isolates of *Aeromonas* spp. *J Clin Microbiol* 1989; **27**: 132–138.
- Singh DV, Sanyal SC. Haemolysin and enterotoxin production by *Aeromonas caviae* isolated from diarrhoeal patients, fish and environment. *J Diarrhoeal Dis Res* 1992; **10**: 16–20.
- Singh DV, Sanyal SC. Enterotoxicity of clinical and environmental isolates of *Aeromonas* spp. *J Med Microbiol* 1992; **36**: 269–272.
- Singh DV, Sanyal SC. Production of haemolysis and its correlation with enterotoxicity in *Aeromonas* spp. *J Med Microbiol* 1992; **37**: 262–267.
- Singh DV, Sanyal SC. Enteropathogenicity of *Aeromonas jandaei* and *A. trota*. *FEMS Immunol Med Microbiol* 1997; **17**: 243–250.
- Singh DV, Sanyal SC. Enterotoxicity, haemolytic activity and antibiotic susceptibility of *Aeromonas eucrenophila* strains isolated from water and infected fish. *Indian J Exp Biol* 1997; **35**: 144–147.
- Rautelin H, Hanninen ML, Sivonen A, Turnunen U, Valtonen V. Chronic diarrhoea due to a single strain of *Aeromonas caviae*. *Eur J Clin Microbiol Infect Dis* 1995; **14**: 51–53.
- Guimaraes MS, Neves MS, Nunes MP. Prevention of suicide phenomenon in aeromonads. *Eur J Clin Microbiol Infect Dis* 1996; **15**: 420–422.
- Allen DA, Austin B, Colwell RR. *Aeromonas media*, a new species isolated from river water. *Int J Syst Bacteriol* 1983; **33**: 599–604.
- Abbott SL, Cheung WKW, Kroske-Bystrom S, Malekzadeh T, Janda JM. Identification of *Aeromonas* strains to the genus-species level in the clinical laboratory. *J Clin Microbiol* 1992; **30**: 1262–1266.
- De SN, Chatterji DN. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. *J Pathol Bacteriol* 1953; **66**: 559–562.
- Sanyal SC, Neogi PKB, Alam K, Huq MI, Al-Mahmud KA. A new enterotoxin produced by *Vibrio cholerae* O1. *J Diarrhoeal Dis Res* 1984; **2**: 3–12.
- Sanyal SC, Huq MI, Neogi PKB, Alam K, Kabir MI, Rahaman ASM. Experimental studies on the pathogenicity of *Vibrio mimicus* strains isolated in Bangladesh. *Aust J Exp Biol Med Sci* 1984; **62**: 515–521.
- Shanker P, Agarwal RK, Sanyal SC. Experimental studies on enteropathogenicity of *Vibrio cholerae* serotypes other than 1. *Zentralbl Bakteriol Mikrobiol Hyg A* 1982; **252**: 514–524.
- Sanyal SC, Saraswathi B, Sharma P. Enteropathogenicity of *Plesiomonas shigelloides*. *J Med Microbiol* 1980; **13**: 401–409.
- Sanyal SC, Agarwal RK, Annapurna E, Lee JV. Enterotoxicity of group F vibrios. *Jpn J Med Sci Biol* 1980; **33**: 217–222.
- Sanyal SC, Huq MI, Neogi PKB, Alam K, Rahman ASM. *Vibrio mimicus*, an aetiological agent of diarrhoea and its pathogenesis. *Indian J Med Microbiol* 1983; **1**: 1–12.
- Raychaudhury A, Agarwal RK, Sanyal SC. Enteropathogenicity of *Klebsiella pneumoniae* strains isolated from stools with diarrhoeal patients and other clinical specimens: an experimental study. *Zentralbl Bakteriol Mikrobiol Hyg A* 1984; **258**: 94–103.
- Singh SJ, Sanyal SC. Enterotoxicity of the so-called NAG vibrios. *Ann Soc Belg Med Trop* 1978; **58**: 133–140.
- Holmes RK, Bramucci HG, Twiddy EM. Genetics of toxinogenesis in *Vibrio cholerae* and *Escherichia coli*. *Contrib Microbiol Immunol* 1979; **6**: 165–177.
- Singh DV, Dubey RS, Sanyal SC. Adherence of haemagglutinating and non-haemagglutinating clinical and environmental isolates of *Aeromonas*. *J Diarrhoeal Dis Res* 1993; **11**: 157–160.