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Biological characterization of *Aeromonas* spp. isolated from the environment

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SUMMARY

Cytotoxic enterotoxin (Act) is a key virulence factor in the pathogenesis of infections caused by *Aeromonas* spp. The cytotoxic enterotoxin gene (*act*) was detected in 32 out of 69 environmental isolates of *Aeromonas* spp. by hybridization with the *act* gene probe. To evaluate the pathogenic potential of the *act* gene probe-positive isolates, 32 *act* gene probe-positive and 31 randomly selected *act* gene probe-negative isolates were tested for enterotoxicity in a suckling mice assay (SMA), for haemolytic activity on sheep blood agar plates, for the presence of CAMP-like factors, and for cytotoxicity in a Vero cell line. The *act* gene probe-positive isolates significantly differed from the toxin gene probe-negative ones with respect to enterotoxicity in the SMA ($P=0{\cdot}009$) and haemolytic activity ($P=0{\cdot}005$). The CAMP–haemolysin phenotype was significantly associated with the rabbit ileal loop assay ($P=0{\cdot}08$), Vero cell assay ($P=0{\cdot}064$), and haemolysin production under the microaerophilic conditions ($P=0{\cdot}056$) of the *act* gene probe-positive isolates of *Aeromonas* spp. These data indicated the role of Act in the pathogenesis of *Aeromonas* infections and that the enterotoxic potential of *Aeromonas* spp. could be assessed by simply performing a CAMP–haemolysin assay.

INTRODUCTION

In humans, *Aeromonas* spp. are associated with life-threatening diseases [1]. Moreover, the *Aeromonas* species is one of the important agents associated with diarrhoea [2]. *Aeromonas* spp.-associated enteric infection is common among young children manifesting diarrhoeal symptoms [3] and is also associated with travellers' diarrhoea [4]. *Aeromonas* spp. multiply in prawns when stored at 4 °C [5] showing that these bacteria may be responsible for spoilage of food stored even at a lower temperature. *Aeromonas* spp. have been identified as one of the important food-borne pathogens [6]. Moreover, *Aeromonas* spp. infestation in ready-to-eat food, such as salad, is a risk factor from the public health point of view [7].

Aeromonas spp. can produce different virulence-associated factors, such as straight [8] and flexible type-IV pili [9]. Two distinct class IV-type pili (bundle forming and Tap) are produced by *Aeromonas* spp. [10, 11] and are associated with virulence. Other virulence-associated factors of *Aeromonas* spp. are collagen-binding protein [12] and haemagglutinin [13]. In addition, *Aeromonas* strains having an S-layer [14] could resist a bactericidal activity of 65% pooled human serum [5]. These strains are more pathogenic to mice compared to S-layer-negative strains [15]. Moreover, *Aeromonas* strains of serotype 0:34 can resist complement-mediated lysis [16]. These properties help the bacteria to cause bacteraemia

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and septicaemia [17, 18]. Invading strains of *Aeromonas* spp. can be disseminated via the bloodstream to other parts of the body to infect vital organs, such as the lung [19] and/or the heart [20].

Various toxins that could lead to tissue damage and enterotoxicity have been reported in *Aeromonas* spp. [21–23]. Chakraborty et al. [24] cloned an aerolysin gene from *A. trota* and conclusively proved its role in systemic infections. Subsequently, Chopra et al. [25] molecularly characterized a cytotoxic enterotoxin (Act) from a diarrhoeal isolate SSU of *A. hydrophila*, that had haemolytic, cytotoxic and enterotoxic activity and was 52 kDa in size. Act was lethal to mice when injected intravenously and the *act* gene isogenic mutant of *A. hydrophila* had a significantly higher 50% lethal dose compared to that of wild-type *Aeromonas* [26]. Detailed structure–function studies were performed on both Act and aerolysin [27] to better understand the regions of the toxins involved in biological function. These two molecules could be differentiated from each other by (i) differential neutralization of Act using Act and aerolysin-specific monoclonal antibodies; (ii) different essential amino-acid residues in Act and aerolysin, which contributed to haemolytic activity, and (iii) the inability of Act to bind to glycophorin, the aerolysin receptor [22, 27]. There have been limited attempts to evaluate the pathogenic potential of *act* gene-positive environmental isolates of *Aeromonas* spp. in animal models and tissue culture. In the present study, 69 environmental isolates of *Aeromonas* spp. were screened for the presence of the cytotoxic enterotoxin (*act*) gene. Furthermore, *act* gene probe-positive and probe-negative isolates were tested for haemolytic activity, enterotoxicity in a suckling mice/rabbit ligated ileal loop assay and cytoxicity in a Vero cell assay to evaluate pathogenicity. The purpose of this study was to correlate those virulence traits of *Aeromonas* spp. that could be associated with human disease.

MATERIALS AND METHODS

Bacterial strains

Various strains of *Aeromonas* spp. were identified by conventional biochemical tests as outlined by Popoff et al. [28]. Initially, strains were screened by a cytochrome oxidase test. Cytochrome oxidase-positive isolates were identified to the species level by their sensitivity to 0/129 (2,4-diamino-6,7-diisopropyl pteridine), dehydrogenation of arginine, decarboxylation of lysine and ornithine, utilization of arabinose, hydrolysis of esculin, the Voges–Proskauer reaction and by growth in potassium cyanide (KCN) and peptone water containing 0, 3 and 6% NaCl. Using these conventional biochemical reactions, 69 isolates were identified as *Aeromonas* spp. (*A. hydrophila*, $n=22$; *A. sobria*, $n=41$; *A. caviae*, $n=6$) and were used in this study. These strains were isolated from water, duckweed (*Lamna minor*) and fish from August 1994 to the end of July 1995. These strains were stored in T1N1 [1% trypticase (Becton Dickinson, La Porte de Claire, France); 1% NaCl (Fisher Scientific, Fair Lawn, NJ, USA); and 1·5% agar (Difco Laboratories, Detroit, MI, USA), pH 7·2)] agar slant until used.

Extraction of DNA

Aeromonas strains were retrieved from stock cultures on Luria–Bertani (LB) agar plates at 37 °C. A portion of one well-isolated aeromonad colony was inoculated into 5 ml LB broth and incubated overnight at 37 °C in a shaking incubator. From 1 ml of overnight culture, chromosomal DNA was extracted and purified using a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). Pelleted DNA was dissolved in 100 μl of of the kit's DNA dissolving solution (Promega). Then the chromosomal DNA was diluted in the proportion of 1:50 and the concentration of DNA measured at $OD_{260\,nm}$ using a UV-1201 spectrophotometer (P/N 206-62409 model, Shimadzu Corporation, Japan). DNA was further diluted in deionized, sterilized distilled water to a final concentration of 0·1 μg/μl.

Dot blotting

An aliquot of DNA solution (1 μg, 10 μl) was taken from the stock DNA (0·1 μg/μl) prepared as described above in a microfuge tube. To this, 90 μl of the final concentration equal to 0·4 M NaOH and 10 mM EDTA was added, heated at 94 °C for 10 min and immediately chilled in ice for 10 min. Subsequently, 100 μl of 2 M ammonium acetate was mixed with denatured chilled DNA for dot blotting.

A piece of Zeta Probe membrane (Bio-Rad, Hercules, CA, USA) was taken from the packet using a pair of forceps and soaked in deionized, sterilized distilled water for 10 min. For dot blotting, a microfiltration apparatus was assembled with a piece of pre-wetted Zeta Probe membrane according to the

manufacturer's instructions and 500 μl deionized distilled water was passed through each well. Then 200 μl of DNA samples (as prepared above) was passed through each well, which was further rinsed with 500 μl of 0·4 M NaOH. The microfiltration apparatus was disassembled to take out the Zeta Probe membrane with immobilized DNA and rinsed in 2× SSC. Finally, UV cross-linking of DNA with the Zeta Probe membrane was performed with the GS Gene Linker UV chamber (Bio-Rad). The membrane was then stored dry between two pieces of filter paper in a plastic bag at room temperature.

Preparation of the *act* gene probe

An internal *Bst*X1 DNA fragment (0·6 kb) of the *act* gene of *A. hydrophila* SSU was used as a probe [25]. For labelling, a non-radioactive ECL direct nucleic acid labelling and detection system (Amersham Life Science, Arlington Height, UK) was used. The detailed procedure was as follows. The initial concentration of probe was 20 μg/μl. An aliquot of the probe (5 μl, 100 μg) was mixed with 5 μl of sterilized, deionized distilled water, boiled for 5 min and chilled immediately in ice. Subsequently, 10 μl of labelling reagent (Amersham) was mixed with the denatured DNA fragment and incubated at 37 °C for 20 min. Following incubation, the mixture was thoroughly mixed, centrifuged briefly, kept on ice, and used immediately.

Pre-hybridization

A Zeta Probe membrane containing immobilized DNA of different *Aeromonas* isolates was taken in 30 ml of Gold hybridization solution (Amersham) (30 ml Gold hybridization buffer, 1·5 g blocking agent, 0·876 g NaCl) in a suitable leak-proof hybridization bag. Air bubbles were completely removed from the hybridization bag which was then sealed and immersed in water for 2 h in a water bath at 42 °C with gentle agitation.

Hybridization

Following incubation, 20 μl of the *act* probe (as prepared above) was added to the hybridization solution-containing blot, mixed thoroughly, and the bubbles completely removed. Finally, the bag was again sealed for overnight incubation at 42 °C in a water bath with gentle agitation.

Post-hybridization washing

Following hybridization, the blot was washed twice in 500 ml of primary wash buffer (0·5× SSC, 0·4% SDS) at 55 °C for 20 min each time with gentle agitation and twice in secondary wash buffer (2× SSC) at room temperature for 5 min each time with gentle agitation. Then the blot was taken on a clean container in a moist condition for the generation of a signal.

Signal generation on X-ray film

Five millilitres each of detection reagents 1 and 2 were added on a moist hybridized blot with the DNA- side up for 1 min. Excess detection reagent was drained from the blot, which was then covered with a piece of plastic wrap and rapidly fixed inside one X-ray film cassette. To generate a prominent signal of hybridization, this blot was exposed to X-ray film in a dark room for at least 5 min at ambient temperature before developing the film.

Preparation of culture filtrate (CF)

Isolates of *Aeromonas* spp. harbouring the *act* gene were retrieved on a gelatin agar (GA) plate. A portion of one well-isolated colony of *Aeromonas* was inoculated into 5 ml of T1N1 broth and incubated overnight at 37 °C in a shaking water bath (120 rpm). Following incubation, 500 μl of overnight culture was inoculated in 10 ml of Richardson's medium [29] in a 50-ml conical flask and incubated overnight at 37 °C in a shaking water bath (120 rpm). Then the culture was centrifuged in a RC5C centrifuge (Sorvall Instrument, Du Pont, New Town, CT, USA) at 10 000 rpm for 15 min at 4 °C. Following centrifugation, the supernatant was decanted and further sterilized by syringe filter (0·22-μm pore, Gelman Sciences, Ann Arbor, MI, USA). Cell-free CF samples were aliquoted in 1·5-ml Eppendorf centrifuge tubes and stored at −20 °C until use. Control CF was also prepared from *Vibrio cholerae* 569B [positive control for the rabbit ileal loop (RIL) assay], *E. coli* 36004 [positive control in the suckling mice assay (SMA)] and *E. coli* 36000 (negative control in the SMA) following the same procedure and stored similarly.

Heat inactivation of CF

CF aliquots were completely defrosted at room temperature. Two aliquots prepared from the same

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strain were then heated separately at 56 °C and 100 °C for 20 min each.

SMA

The SMA was performed following the procedure of Dean et al. [30]. Briefly, a group of 3-day-old mice (Hanover strain, NMRI) were separated from their mothers. An aliquot (0·1 ml) of the CF containing 0·01% (w/v) Evans Blue was directly injected into the milky-white stomach of two of three suckling mice and the third, without injection of CF, was used as a negative control. After 3 h of incubation at room temperature (22 °C), these mice were killed by cervical dislocation. Their intestines were removed, pooled and weighed. Fluid accumulation was expressed as the ratio of weight of the intestine to the remaining body weight. A ratio of $\geqslant 0{\cdot}08$ was considered positive, whereas a ratio of $< 0{\cdot}08$ was considered negative. CFs prepared from *E. coli* 36004 and *E. coli* 36000 were similarly inoculated as positive and negative controls respectively. This experiment was repeated twice.

RIL assay

The enterotoxicity of *Aeromonas* isolates harbouring the *act* gene was tested in the RIL, following the procedure of Sanyal et al. [31]. Briefly, eight samples were tested in eight separate loops each with an interloop between two consecutive loops of an adult rabbit ileum (New Zealand White variety weighing 1·2–1·9 kg). One millilitre of CF was injected into each loop of a rabbit ileum in duplicate rabbits following laparotomy. In the first loop, 1 ml CF of *V. cholerae* 569B was injected as a positive control and 1 ml sterile normal saline was injected in the eighth loop as a negative control. In the remaining loops between positive and negative controls, 1 ml CF of six test isolates was inoculated sequentially. The inoculated rabbits were killed after 16–18 h of incubation to measure the volume of fluid accumulation per cm of gut in each rabbit. Fluid accumulation $\geqslant 0{\cdot}5$ ml/cm of gut was considered a positive response in the RIL. This experiment was repeated twice.

Vero cell assay

The cytotoxic effect of the CF of *Aeromonas* spp. harbouring the *act* gene was tested in a Vero cell line (African Green monkey kidney cells). Vero cells were grown in Eagle's Minimal Essential Medium (EMEM) supplemented with L-glutamate (2·3 mM), heat-inactivated foetal bovine serum (10%) (Gibco–BRL, Grand Island, NY, USA), penicillin (100 000 U/l) and streptomycin (10 mg/l). For adhesion, 200 μl of Vero cells suspended in EMEM (2×10^3/200 μl were placed in the wells of microtitre plates (96-well, 15-cm^2, flat-bottomed microtitre plates) and incubated at 37 °C in a humidified CO_2 incubator for 3 h for adhesion. Serial twofold dilutions of CF were prepared in PBS and 200 μl of crude toxin sample was added to each well of the microtitre plate containing freshly prepared Vero cells. A plate containing Vero cells and toxin was incubated at 37 °C in a humidified CO_2 incubator and change of the cell morphology (cell death or rounding) examined using an inverted microscope after 18–24 h of incubation [32]. This experiment was repeated twice.

Test of haemolytic activity (aerobic)

The haemolytic activity of *act* gene-positive and gene-negative *Aeromonas* isolates was tested on a blood agar plate containing 5% defibrinated sheep blood. Initially, the strains were subcultured onto a gelatin agar (GA) plate and incubated at 37 °C overnight. The lower surface of the blood agar plate was marked into several squares of 0·7 cm each and labelled with the test strain number. A portion of the colony from the overnight *Aeromonas* culture on the GA plate was transferred into one chamber having a corresponding isolate number. The inoculated plates were incubated aerobically at 37 °C overnight. Following incubation, haemolytic strains showed a prominent zone of haemolysis around each colony, and the haemolytic zones were characterized as α, β and γ, depending on the extent of haemolysis around each colony. This experiment was repeated twice.

Test of haemolytic activity under microaerophilic conditions (candle jar)

To study haemolytic activity under microaerophilic conditions, inoculated blood agar plates were placed in a candle jar with a lighted candle on the stack of the plates and the lid of the jar closed tightly before incubation at 37 °C overnight. Following incubation, haemolytic strains showed a prominent zone of haemolysis around each colony, and the haemolytic zones were characterized as α, β and γ, depending on

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Table 1. *Haemolytic activity and toxigenic potential of* act *gene probe-positive and probe-negative isolates of* Aeromonas *spp. in animal model and cell line*

Characteristics of strains	Suckling mice assay with culture filtrate		Vero-cell assay with culture filtrate		Haemolysin assay		CAMP–haemolysin assay (sample positive/tested)	Rabbit ileal loop assay (sample positive/tested)
	Unheated (sample positive/tested)	Heated at 56 °C (sample positive/tested)	Unheated (sample positive/tested)	Heated at 56 °C (sample positive/tested)	Aerobic	Candle jar		
act probe-positive isolates ($n=32$)	10/32	0/30	25/31	0/31	30/32	23/26	14/32	3/27
act probe-negative isolates ($n=31$)	2/31	n.d.	27/30	n.d.	n.d.	n.d.	8/31	n.d.

n.d., Not done.

the extent of haemolysis around each colony. This experiment was repeated twice.

Test of CAMP–haemolysin

Overnight cultures of *Aeromonas* spp. harbouring the *act* gene and *Staphylococcus aureus* (ATCC 25923) were inoculated 8 mm apart on a blood agar plate containing 5% washed sheep blood. Inoculated plates were incubated at 37 °C in a candle jar for 18–24 h [33]. Following incubation, CAMP–haemolysin-positive isolates of *Aeromonas* spp. could only produce a characteristic type of haemolytic zone between the test *Aeromonas* isolate and *S. aureus* ATCC 25923. This experiment was repeated twice.

Data analysis

A χ^2 test was performed using SPSS10 (SPSS Inc., Chicago, IL, USA) to examine the difference between *act* gene-positive and gene-negative isolates of *Aeromonas* spp. with respect to different bioassays. Fisher's exact test was performed to determine the association of the CAMP–haemolysin test with different bioassays.

RESULTS

Probe assay

A prominent hybridization signal with the *act* gene probe was obtained from 32 out of 69 different *Aeromonas* DNA dot-blotted on the Zeta Probe membrane. Some of the strains lost viability during the experiments related to different bioassays. Therefore, all 32 *act* gene probe-positive and randomly selected 31 probe-negative isolates could not be used uniformly to test enterotoxicity in the SMA, Vero cell assay, haemolytic activity measurement under aerobic and microaerophilic conditions and for the CAMP–haemolysin test.

Table 2. *Difference between* act *gene probe-positive and probe-negative isolates of* Aeromonas *spp. with respect to different bioassays*

Bioassay	*act* gene probe-positive isolates (no. positive/no. tested)	*act* gene probe-negative isolates (no. positive/no. tested)	Exact significance (P value)
Suckling mice assay	10/32	2/31	0·011
Vero cell assay	25/31	27/30	0·473
Haemolysin assay	30/32	22/30	0·012
CAMP–haemolysin	14/32	8/30	0·192

Enterotoxicity in the SMA

Ten CFs out of 32 *act* gene probe-positive and 2 out of 31 *act* gene probe-negative *Aeromonas* isolates induced fluid accumulation in the SMA (Table 1). *Aeromonas* isolates harbouring the *act* gene differed from *act* gene-deficient strains with respect to enterotoxicity in the SMA. This difference was significant ($P=0·011$) (Table 2).

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Table 3. *Association of CAMP–haemolysin with different bioassays*

Character	Association of CAMP–haemolysin with				
	Suckling mice assay	Aerobic haemolysin assay	Anaerobic haemolysin assay (candle jar)	Rabbit ileal loop assay	Vero cell assay
act gene probe-positive isolates	$P=0{\cdot}26$	$P=0{\cdot}452$	$P=0{\cdot}085$*	$P=0{\cdot}0648$*	$P=0{\cdot}064$*
act gene probe-negative isolates	$P=1{\cdot}00$	$P=0{\cdot}374$	n.d.	n.d.	$P=0{\cdot}54$

n.d., Not done.
* Significant at 1% level (Fisher's exact test).

Vero cell assay

The CFs of 31 *act* gene probe-positive and 30 gene probe-negative isolates of *Aeromonas* spp. were tested for cytotoxicity in a Vero cell line. Twenty-five out of 31 *act* gene-positive and 27 out of 30 *act* gene-negative isolates of *Aeromonas* spp. showed cytotoxicity in a Vero cell line (Table 1). The cytotoxic effect on Vero cells was lost after the CFs were heated at 56 °C. The cytotoxicity of *act* gene-positive *Aeromonas* isolates could not be differentiated from *act* gene-negative ones with respect to the Vero cell assay. This difference was not significant ($P=0{\cdot}473$) (Table 2).

RIL assay

Twenty-six CFs of *Aeromonas* spp. harbouring the *act* gene were tested in the RIL. Three out of 26 CFs could induce fluid accumulation in the RIL (Table 1). Fluid accumulation ranged between 0·9 and 1·3 ml/cm of the gut.

Haemolytic activity under aerobic conditions of incubation

Haemolytic activity (both α and β) was detected in 30 out of 32 *act* gene probe-positive and 22 out of 30 *act* gene probe-negative *Aeromonas* isolates. Isolates harbouring the *act* gene differed from *act* gene-deficient ones with respect to haemolytic activity on blood agar plates. This difference was significant ($P=0{\cdot}012$) (Table 2).

Haemolytic activity under microaerophilic conditions of incubation

Twenty-six *act* gene-positive isolates were incubated overnight at 37 °C under microaerophilic conditions (candle jar) to study the effect of oxygen on haemolytic activities during incubation. Of these, 23 showed haemolytic activity (Table 1). Three isolates (nos. 97, 155 and 187) produced β-haemolytic activity aerobically but these isolates produced α-haemolytic activity under microaerophilic conditions. Moreover, isolate no. 26 showed α-haemolytic activity under aerobic conditions but this isolate failed to produce haemolytic activity under microaerophilic conditions. From these results, it could be concluded that haemolytic activity of *Aeromonas* spp. might be enhanced due to incubation of isolates under aerobic conditions. Since there was no significant difference between haemolytic activity of *act* gene probe-positive *Aeromonas* isolates under aerobic and microaerophilic conditions, the *act* gene probe-negative isolates were not tested for haemolytic activity under microaerophilic conditions.

CAMP–haemolysin

Production of CAMP–haemolysin was detected in 14 out of 32 *act* gene probe-positive and 8 out of 30 *act* gene probe-negative *Aeromonas* isolates. Some of the isolates produced crescent-shaped CAMP–haemolytic zones, while others produced prominent β-haemolytic zones that extended towards growth of *S. aureus*. Strains of *Aeromonas* spp. harbouring the *act* gene could not be differentiated from *act* gene-deficient isolates with respect to the CAMP-like haemolysin test ($P=0{\cdot}192$) (Table 2). However, a positive association of CAMP-haemolysin phenotype with the RIL assay ($P=0{\cdot}08$), Vero cell assay ($P=0{\cdot}064$) and haemolysin under microaerophilic conditions ($P=0{\cdot}056$) of the *act* gene probe-positive isolates of *Aeromonas* was noted, but in *act* gene probe-negative isolates, association of CAMP– haemolysin with the SMA, aerobic haemolysin and the Vero cell assay was not significant (Table 3).

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DISCUSSION

The SMA was initially devised for evaluating production of heat-stable enterotoxins of *E. coli* [30]. Considering this assay to be relatively cheaper and faster, Burke et al. [34] tested the enterotoxicity of *A. hydrophila* strains collected from different geographical areas [34] and established the SMA as the model for testing enterotoxicity. Subsequently, several investigators successfully used the SMA for testing enterotoxicity of *Aeromonas* spp. [32, 35]. In this study, more *act* gene probe-positive isolates showed enterotoxic activity compared to *act*-gene negative ones. Since 20 CFs of 30 *act* gene probe-positive isolates did not induce fluid secretion in the SMA, it must be determined whether it is related to the amount of Act produced by these isolates or if the presence of other enterotoxin(s) contributed to this effect.

Sanyal et al. [31] first demonstrated the enterotoxicity of live cultures of clinical and environmental isolates of *A. hydrophila* in an RIL model [31]. Using this model, Annapurna and Sanyal [36] of India and Wadstrom et al. [37] and Ljungh et al. [38] of Sweden simultaneously demonstrated the enterotoxicity of cell-free CF of *Aeromonas* isolated from clinical and environmental samples. Thus, the RIL assay is an established assay for testing the enterotoxicity of *Aeromonas*. The RIL assay can be used for detecting *Aeromonas* cytotoxic enterotoxin (Act) in cell-free CF of *A. hydrophila* [39]. It has been demonstrated that 200 ng is the minimum amount of Act required to induce fluid accumulation in the rat ligated ileal loop [39]. In this study, CF of only 3 out of 27 *act* gene probe-positive isolates could induce fluid accumulation. It is possibly due to the fact that *act* gene probe-positive isolates other than these three produced less than 200 ng Act in Richardson's medium [29], which was not detected in the RIL assay. To confirm this observation, it is essential to evaluate various culture media for the better production of Act.

Vero cells have been used to detect Shiga toxin produced by *Shigella dysenteriae* type 1 [40]. Vero toxin-producing *Aeromonas* was reported from meat samples in Australia [13] and from clinical and environmental samples in Japan [32]. The cytotoxic response on Vero cells by the CF of *Aeromonas* isolates in this study could be contributed by Act and possibly by other virulence factors. Whether *Aeromonas* isolates in this study do indeed produce Vero toxin needs to be elucidated in detail using specific DNA probes or by neutralizing the cytotoxic effect in the CF by using antibodies to the Shiga toxin.

Aeromonas spp. isolated from fish [41], clinical specimens and environmental samples [42, 43] could lyse red blood cells (RBC) of different species. On blood agar plate, *Aeromonas* spp. produced two different types of haemolysins, such as α and β [44]. In this study, all *Aeromonas* isolates produced either α- or β-haemolysin. This observation is consistent with past reports from Bangladesh [45]. Moreover, haemolysin is one of the virulence factors of *Aeromonas* [24]. Purified haemolysin showed multiple biological properties, including haemolytic, cytotoxic and enterotoxic activities and lethality in mice [46] like Act of *Aeromonas* [25]. However, this haemolysin was not characterized at the molecular level. Although haemolytic activity is one of the biological properties of Act, molecular cloning and DNA sequence analysis of the *act* gene from *A. hydrophila* [25] revealed that this gene differed significantly from the aerolysin gene of *A. trota* [24, 47]. Due to this type of structural difference, the *act* probe, which was used in this study, possibly failed to hybridize with other haemolytic isolates of *Aeromonas* spp. The ability of the *act* gene probe-negative *Aeromonas* strains to cause haemolysis indicated the presence of other haemolysins that were different from Act.

Isolates of *act* gene probe-positive *Aeromonas* producing β- and α-haemolysin under aerobic conditions respectively, produced α- and γ-haemolysin when incubated under microaerophilic conditions of incubation. This finding indicates that haemolysin production of *Aeromonas* spp. may be enhanced due to the incubation of isolates under aerobic conditions of incubation.

Synergistic CAMP–haemolysin detection was introduced by Christie et al. [48]. This test has been successfully used for differentiating *Vibrio cholerae* O1 El Tor (positive) from classical strains (negative) [33]. In different species of *Aeromonas*, CAMP–haemolysin production differed in different atmospheric conditions of growth, such as aerobic or microaerophilic conditions [49, 50]. Based on a CAMP-like test, Carnahan et al. [51] differentiated *A. hydrophila* and *A. veronii* bv. *sobria* (both positive) from *A. caviae* (negative) [50]. Subsequently, Gubash [52] conducted a CAMP–haemolysin test on a large number of *Aeromonas* isolates and raised questions

about the usefulness of the CAMP–haemolysin assay to differentiate different strains of *Aeromonas* [52]. However, none of these authors made any attempt to correlate the pathogenicity of *Aeromonas* using the CAMP–haemolysin test. In our attempt, we failed to demonstrate significant differences ($P = 0{\cdot}192$) (Table 2) between *act* gene probe-positive and probe-negative isolates with respect to CAMP–haemolysin. However, in *act* gene probe-positive isolates of *Aeromonas* spp., CAMP–haemolysin was associated with some of the pathogenic properties, such as enterotoxicity in the RIL assay, cytoxicity in the Vero cell assay and in the haemolysin assay under microaerophilic conditions (Table 3). Thus, these pathogenic factors could be easily assessed in fresh *Aeromonas* isolates by simply performing the CAMP–haemolysin test. This simple CAMP–haemolysin test could be easily adopted economically in laboratories of developing countries where it is often difficult to set up experiments involving dot–blot hybridization, animal model and tissue culture assays for testing enterotoxicity.

It is evident from this study that environmental isolates of *Aeromonas* spp. tested in this study (32 out of 69) harboured the *act* gene. Strains harbouring the *act* gene significantly differed from *act* gene-deficient strains with respect to enterotoxicity in the SMA and haemolytic activity on blood agar plates. In *act* gene probe-positive isolates of *Aeromonas* spp., the CAMP–haemolysin assay was associated with some of the pathogenic properties, such as enterotoxicity in the RIL assay, cytotoxicity in the Vero cell assay and haemolysin production under microaerophilic conditions. Therefore, enterotoxicity of *Aeromonas* spp. could be easily assessed by simply performing the CAMP–haemolysin test.

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