BACTERIAL PATHOGENICITY

Production of an enterotoxin by a gastro-enteritis-associated *Aeromonas* strain

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The potential of motile *Aeromonas* species to cause human gastrointestinal infections has been recognised recently. Considerable worldwide epidemiological, microbiological and clinical investigations have shown that some strains of the different motile aeromonads are of increasing enteropathogenic significance, especially in children, the elderly and in immunocompromised individuals. Some of the diarrhoeal symptoms of *Aeromonas*-associated gastro-enteritis have been attributed to enterotoxins. In this study, 15 *Aeromonas* isolates from clinical and non-clinical sources, representing the three motile aeromonads commonly associated with gastro-enteritis (*A. caviae*, *A. hydrophila* and *A. veronii* biovar *sobria*), were tested for their ability to cause fluid accumulation in infant mice by the suckling mouse technique. Eight isolates were found to produce enterotoxin. Of these, an *A. veronii* biovar *sobria* strain (AS15), isolated from lamb kidney, was found to produce the highest enterotoxin score. An enterotoxin of c. 40 kDa produced by *A. veronii* biovar *sobria* AS15 was purified by Sephacryl S-100 gel filtration and high-performance liquid chromatography. This enterotoxin caused marked fluid accumulation in infant mice by the suckling mouse technique. The purified enterotoxin cross-reacted with cholera toxin antibodies and was readily inactivated by heating at 56°C for 10 min. The production of a 'cholera-like' enterotoxin by *Aeromonas* isolates from samples of animal origin suggests that these organisms could be of public health significance in food products.

Introduction

The genus *Aeromonas* represents a group of gram-negative, facultatively anaerobic and oxidase-positive organisms that are currently classified, along with the much better known *Vibrio cholerae*, in the family Vibrionaceae. However, molecular evidence has shown that they possess an evolutionary history sufficiently different to warrant a family of their own, the Aeromonadaceae [1, 2]. For systematic, clinical and ecological reasons, aeromonads can be grouped conveniently into the psychrophilic aeromonads of the salmonicida group and the mesophilic aeromonads. The aeromonads of the salmonicida group are non-motile and normally found in surface waters and in bottom sediments whenever certain diseases of fish and other freshwater animals are prevalent. The mesophilic

aeromonads, in contrast, are mostly motile, with the exception of *A. media* [3], and have been the topic of recent renewed interest, principally because of increased awareness of their role in human illnesses. Motile *Aeromonas* species are ubiquitous in nature and have been documented for a long time as autochthonous inhabitants of freshwater and estuarine environments. They can be isolated from many environmental locations, but they are mainly water-borne organisms found in virtually all waters, including chlorinated drinking water [4–11]. However, numerous reports on the incidence of aeromonads in cold-blooded animals, human clinical specimens, food and soil clearly demonstrate that the natural habitats of these organisms are not strictly limited to aquatic environments [12].

Motile aeromonads have been reported to cause a wide range of diseases in both man and animals [10, 13–15]. Human aeromonas infections include skin and soft tissue infections, gastro-enteritis and bacteraemia [16, 17]. Gastro-enteritis is the most common human illness associated with *Aeromonas* spp., mainly affecting the young, the elderly and immunocompromised

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patients [17, 18]. However, illness may also occur in healthy individuals of any age [17, 19, 20].

Aeromonas species produce a wide range of extracellular toxins and enzymes. Other properties such as adherence, invasiveness and possession of certain surface proteins have also been reported for motile *Aeromonas* species. Generally, the multiplicity of extracellular products of motile *Aeromonas* species has led to difficulty in characterising these factors and to disagreement about their properties, as well as about their direct involvement in *aeromonas* enteropathogenicity. This has resulted in a great deal of controversy and confusion. However, such extracellular biologically active factors as enterotoxins, cytotoxins, haemolysins and proteases have been reported to be produced by motile *aeromonads* [21–25]. These properties are found more frequently, or better expressed, in strains isolated from diarrhoeal individuals than in those isolated from healthy control individuals or from the environment [26].

It has been well documented that *Vibrio cholerae* produces enterotoxins and causes profuse watery diarrhoea. The enterotoxins include factors that stimulate cyclic adenosine 3,5-monophosphate (cyclic AMP) production by irreversibly activating the enzyme adenyl cyclase [27, 28]. The abnormally high cellular levels of cyclic AMP produced as a result stimulate active transport of fluid and electrolytes into the lumen of the small bowel, resulting in a watery diarrhoea. It has been reported that in *aeromonas* gastrointestinal infections, profuse watery 'cholera-like' diarrhoea is the most common feature [29, 30]. Furthermore, it has been reported that *Aeromonas* mutants unable to produce or secrete these enterotoxins show reduced pathogenicity [24].

The present study explored the enterotoxigenic prop-

erties of *Aeromonas* isolates from clinical and non-clinical sources and the isolation and partial characterisation of an enterotoxin from an *A. veronii* biovar *sobria* strain are described.

Materials and methods

Aeromonas strains

Fifteen *Aeromonas* strains were included in this study. They represented the three main phenotypes of motile *Aeromonas* species (*A. hydrophila*, *A. caviae* and *A. veronii* biovar *sobria*) and were isolated from different clinical and non-clinical sources (Table 1). All strains were confirmed as *Aeromonas* species by the oxidase test, resistance to vibriostatic agent 0/129, motility and characteristic reaction in *A. hydrophila* (AH) medium [31]. They were further confirmed as motile *Aeromonas* species by the 24E Microbact Test System (Medvet Science, Adelaide, Australia). Microbact test kits were read after 18–24 h at 37°C and re-examined at 48 h if definitive results were not obtained. All strains were stored on frozen beads in a cryopreservative fluid (Technical Services Consultants, The Rope Walk, Schofield Street, Heywood, Lancashire OL10 1DS).

Preparation of cell-free culture filtrates

For the production of crude enterotoxin, the *Aeromonas* strains were propagated in Tryptone Soy Broth (TSB; Oxoid) supplemented with yeast extract (Oxoid) 0.6%. They were incubated at 37°C with agitation at 150 rpm in an environmental incubator shaker (New Brunswick Scientific, Edison, NJ, USA) for 24 h. The cultures were then centrifuged in sterile centrifuge tubes at 10 000 g for 30 min at 4°C. The resulting supernatant fluids were then filtered through sterile 0.45-µm membrane filters (Millipore Medical, Bedford, USA).

Table 1. SMT results of the 15 *Aeromonas* isolates tested

Isolate no.	Species	Source	Enterotoxin score*
[†] AC23 (-ve)	<i>A. caviae</i>	Lamb meat (Qld)	0.073 (-)
AC27	<i>A. caviae</i>	Lamb carcass (Qld)	0.068 (-)
CA182	<i>A. caviae</i>	Faeces, weight loss (Tandy)	0.072 (-)
CA185	<i>A. caviae</i>	Faeces (Tandy)	0.078 (-)
AH06	<i>A. hydrophila</i>	Lamb meat (Qld)	0.090 (+)
AH33	<i>A. hydrophila</i>	Lamb kidney (Qld)	0.065 (-)
CA05	<i>A. hydrophila</i>	Diarrhoeal stool (Tas)	0.066 (-)
CA191.1	<i>A. hydrophila</i>	Faeces (Tandy)	0.087 (+)
P04	<i>A. hydrophila</i>	Non-diarrhoeal stool (Adel)	0.068 (-)
AS02	<i>A. veronii</i> biovar <i>sobria</i>	Lamb meat (Qld)	0.092 (+)
AS05	<i>A. veronii</i> biovar <i>sobria</i>	Lamb faeces (Qld)	0.099 (+)
[‡] AS15	<i>A. veronii</i> biovar <i>sobria</i>	Lamb kidney (Qld)	0.119 (+)
AS16	<i>A. veronii</i> biovar <i>sobria</i>	Lamb kidney (Qld)	0.106 (+)
CA18	<i>A. veronii</i> biovar <i>sobria</i>	Diarrhoeal stool (Tas)	0.116 (+)
[†] CA188.2 (+ve)	<i>A. veronii</i> biovar <i>sobria</i>	Stool, intermittent diarrhoea (Tandy)	0.106 (+)
Purified 40-kDa protein (gel filtration)	<i>A. veronii</i> biovar <i>sobria</i>	Lamb kidney (Qld)	0.108 (+)
Purified 40-kDa protein (HPLC)	<i>A. veronii</i> biovar <i>sobria</i>	Lamb kidney (Qld)	0.088 (+)

Qld, Queensland; Tas, Tasmania; Adel, Adelaide.

*A strain was considered positive (+) if the enterotoxin score was >0.08.

[†]Known positive and negative controls.

[‡]The strain that produced the highest enterotoxin score.

Cell-free filtrates were stored at 4°C for no more than 48 h until their use in the enterotoxin assay.

Enterotoxin assay

The suckling mouse technique (SMT) was performed as described previously [32, 33] to investigate the ability of the isolates to produce enterotoxin. Appropriate approval for these studies was granted by the Monash University Ethics Committee. One hundred μ l of each cell-free filtrate containing 2 μ l of food dye (green SCI44090, McCormack, USA) 2.5% w/v were injected into the stomachs of 2–4-day-old suckling mice. Three mice were used for each culture filtrate and were kept at 28°C for 4 h after injection before they were killed by cervical dislocation. The ratio of combined intestinal weight to combined remaining body weight was determined. A ratio of >0.08 was considered positive for enterotoxin activity. A known enterotoxin-positive strain (CA188, a diarrhoeal isolate kindly provided by S. Kirov, Department of Pathology, University of Tasmania, Australia) and an enterotoxin-negative strain (AC23, isolated from a lamb kidney as described previously) [22] were included in the study as controls.

Isolation of the enterotoxin

The enterotoxin was isolated by a combination of salt precipitation and chromatographic methods [34, 35]. A large volume of TSB supplemented with yeast extract 0.6% was inoculated with a 6-h culture of *A. veronii* biovar *sobria* (AS15) and incubated under the conditions described earlier. The culture was then centrifuged and filtered as described previously. The cell-free supernatant fluid was then stored in sterile bottles at 4°C before further purification.

Ammonium sulphate precipitation

Ammonium sulphate was added to the supernatant fluid at 50% saturation and mixed for up to 4 h. The mixture was then centrifuged at 10 000 g for 40 min at 4°C. The resulting pellet was redissolved in phosphate-buffered saline (PBS), pH 7.4 (Ajax). This was then dialysed against PBS which was changed three times at 2-h intervals to remove any ammonium salts for safe injection into the mice in the SMT.

Gel filtration and SDS-PAGE

A sample of the cell-free supernatant fluid (crude enterotoxin) was loaded on to a Sephacryl S-100 gel filtration column (1.0 $\times$ 120-cm Biorad Econo-column) connected to a protein fractionation system (Pharmacia). The column was packed according to the manufacturer's instructions and equilibrated with PBS until the base line stabilised. Mol. wt standards (Pharmacia) ranging from 13.7 to 67 kDa were run before loading the sample. A volume of 1 ml of PBS,

containing 1 mg of protein of the dialysed 'crude enterotoxin', was loaded on to the column. Elution with PBS was applied over a 24-h period at 4°C. Fractions of 5 ml were collected and the absorbance at 280 nm was measured for all fractions. Fractions with no apparent protein content were pooled and concentrated with the Amicon 50 ml concentrator (Grace). Individual fractions that corresponded with protein peaks were concentrated and examined by SDS-PAGE 12% [36]. They were then tested in suckling mice to identify fractions containing enterotoxin activity.

High-performance liquid chromatography (HPLC)

HPLC (Waters, USA) was employed as another method to further purify the crude enterotoxin. The column used was the Protein Pak 60 (7.8 mm[ID] $\times$ 30 cm) analytical column. For this experiment, a fresh culture was grown as described earlier, and following the dialysis step the crude enterotoxin was concentrated. A sample of 400 μ l was run through the HPLC column. PBS was the mobile phase and the flow rate was 1 ml/min. The detection wavelength used was 280 nm and a retention time of 25 min was observed. Fractions of 1 ml each were collected from the column and analysed by SDS-PAGE in 12% gels. The activity of the protein fractions was determined by the SMT.

Heat stability and activity after freezing

Heat stability was tested by heating the crude enterotoxin preparation in a water bath for 10 min at 56°C [37]. The activity of the protein was subsequently measured in the SMT. The activity of the protein after storage at -20°C for 2 and 3 weeks respectively was also determined by the SMT.

Immunoblotting

After separation on SDS-PAGE, proteins were transferred on to PVDF membranes and then blocked with the chemiluminescence blocking substrate (POD; Boehringer Mannheim). Anti-*V. cholerae* antiserum (O319 'Bengal'; Denka Seikan, Japan) was diluted to 1 in 300 in blocking solution 0.5% w/v (solution 1) and incubated for 1 h. After washing with TBS containing Tween 20 0.1% and solution 1, horseradish peroxidase-conjugated sheep anti-rabbit immunoglobulin (Silenus) diluted to 1 in 1000 in solution 1 was added and incubated for 1 h as before. Reactivity was detected by using the detection solution of chemiluminescence autoradiography according to the manufacturer's instructions (Boehringer Mannheim). Incubation with secondary antibodies was used as a control for non-specificity.

Results

Enterotoxin assay

The results of the enterotoxin assay in suckling mice are shown in Table 1. Of the 15 strains tested, eight (including the positive control) showed positive enterotoxin activity; the other seven lacked this activity. *A. veronii* biovar *sobria* strain AS15 was the strongest enterotoxin producer, eliciting marked fluid accumulation in the infant mice. This isolate was chosen for the isolation of the enterotoxin.

Isolation of the enterotoxin (gel filtration and HPLC)

After separation by Sephacryl S-100 gel filtration and HPLC, fractions were pooled and concentrated. As shown in Fig. 1, after staining with silver stain, a single band with an apparent mol. wt of c. 40 kDa was obtained on SDS-PAGE. Another band of c. 31 kDa was also collected (lane 5). Fractions containing these proteins were tested in the SMT as well as other fractions containing no apparent proteins (applied as controls). The only fractions that showed enterotoxin activity were those with the 40-kDa band (Table 1), demonstrating that the enterotoxin isolated by gel filtration and HPLC corresponds to a protein with a mol. wt of c. 40 kDa.

Stability to heat and freezing

The crude enterotoxin was labile to heating in a water bath at 56°C for 10 min. Storage at -20°C for 2 weeks did not abolish the activity, although the score obtained after testing in the SMT was reduced from the initial value of 0.119 to 0.086. After storage for 3 weeks at -20°C, the toxin had completely lost activity.

Cross-reactivity with cholera toxin

The purified toxin reacted with antibodies to *V. cholerae* (O139 'Bengal') in Western blots to give a single band corresponding to the 40-kDa protein (Fig. 1, lane 6).

Discussion

The work reported here resulted in the isolation of a heat-labile enterotoxin with an apparent mol. wt of c. 40 kDa from a gastro-enteritis-associated *Aeromonas* strain. Enterotoxin production is an important factor in the pathogenesis of *Aeromonas*-associated gastrointestinal disease. The enterotoxin isolated and purified in this study caused marked fluid accumulation in the SMT, consistent with the production of watery diarrhoea in the aetiology of gastro-enteritis.

The SMT was chosen as the appropriate method for the

Fig. 1. SDS-PAGE of the purified enterotoxin and other proteins. Lane 1, mol. wt markers; 2, crude enterotoxin; 3, toxin from Sephacryl S-100 gel filtration; 4, purified toxin from HPLC; 5, fractions containing the 31-kDa protein; 6, autoradiogram of the 40-kDa protein reacting with anti-cholera antibodies.

detection of enterotoxin, as it is a well accepted technique for detecting enterotoxins produced by other enteropathogenic bacteria, including *Escherichia coli* [37]. It is also used frequently for the detection of *aeromonas* enterotoxin [22–25, 37]. The SMT allows for enterotoxin detection on a larger scale than is possible with the rabbit ileal loop test [30, 37, 38]. *Aeromonads* also produce cytotoxins and cytotoxic enterotoxins; therefore, the SMT has the advantage over cell-culture systems which detect only cytotoxicity [30].

The SMT applied to the 15 *Aeromonas* isolates used in this study demonstrated that both clinical and environmental isolates are capable of producing enterotoxins. The enterotoxin itself was isolated from an *A. veronii* biovar *sobria* strain from lamb kidney, illustrating that meat and other foods may be a source of infection [38–41], even if kept refrigerated, as *aeromonads* have the ability to survive and produce exotoxins in this cooler environment [42–44]. In this study, the enterotoxin lost its activity when heated at 56°C for 10 min, demonstrating the importance of cooking foods adequately to inactivate toxins. This study also confirmed that *A. caviae* strains (e.g., isolates CA185 and CA182) could cause enteric infection without the production of enterotoxin (Table 1), indicating that enterotoxin is not the only factor capable of causing gastro-intestinal disease [45–47].

To our knowledge, this is the first report of the isolation of an enterotoxigenic 40-kDa protein from an *A. veronii* biovar *sobria* strain, although a 63-kDa cytotoxic enterotoxin from an *A. sobria* isolate has been described [35]. This study demonstrated that the purified enterotoxin can be stored for 2 weeks at -20°C without losing its activity. Moreover, the crude toxin retained activity if stored below 4°C for <1 week or frozen at -20°C for <2 weeks. The simple and rapid purification processes used in this study also minimised the possible inactivation of the target

protein; gel filtration took 24 h and HPLC took only 25 min, a much shorter time than the classical purification techniques. Immuno-adsorption with anti-cholera toxin antibodies has been employed to purify aeromonad proteins [48]. In this case, three proteins of differing sizes were isolated (27, 29.5 and 43.5 kDa). The drawback of this process is that only those proteins adsorbed to anti-cholera toxin antibodies were studied, thus excluding any possible non-cholera toxin-reactive enterotoxins.

In the present study, the isolation of enterotoxin was achieved with gel filtration columns, which are widely used and readily obtainable. This method produced a pure protein that retained its activity for several days. It will be interesting to apply this method to the isolation of other active proteins from other aeromonad isolates.

In summary, a highly purified protein of c. 40 kDa in size was isolated from an aeromonad strain known to cause gastro-enteritis. This protein isolation procedure used gel filtration and was able to produce a pure protein that was active in the SMT. This result was reproduced with the more sensitive process of HPLC. The toxin itself was heat labile and could not be stored for >3 weeks at -20°C. It was also cross-reactive with *V. cholerae* (O139 'Bengal') antiserum and this may indicate that the enterotoxin causes its diarrhoeal symptoms by a mechanism similar to that of cholera toxin [49]. Further studies are needed to obtain more information about the specificity and other activities related to this enterotoxin. Furthermore, the determination of its amino-acid sequence and three-dimensional structure may provide information on the aeromonad enterotoxins in general, and their specific role in enteric illness.

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