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Characterisation of *Aeromonas hydrophila* Extracellular Products with Reference to Toxicity, Virulence, Protein Profiles and Antigenicity

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Abstract

A comparison of extracellular products (ECP) from a variety of *Aeromonas hydrophila* isolated from fish, with LD_{50} values between 2.3 x10^5 and 3.5 x10^8 CFU ml^{-1}, was conducted using SDS-polyacrylamide gel electrophoresis. Differences were observed in the silver stain profiles of the ECP preparations, while the total amount of protein ob-tained from each isolate were very similar (0.14–0.15 $mgml^{-1}$). Levels of toxicity for the dif-ferent ECP preparations were determined through intraperitoneal injection (0.2 $mgml^{-1}$) of fingerling hybrid catfish (*Clarias gariepinus* x *Clarias macrocephalus*). The ECP prepara-tion from the high virulence isolates produced 100% mortality in all fish within 18 h of in-jection, while the ECP from the low virulence isolates produced 100% mortalities after 96 h. The cytolytic enterotoxin was partially purified by chromatography, and this was then used to immunise a rabbit and a catfish. Antibody titres of 1/1,024 and 1/32,768 were ob-tained through an indirect enzyme linked immunosorbent assay, for the catfish and rabbit sera, respectively. Purified enterotoxin from bacteria with LD_{50} values around 10^5 CFU ml^{-1} contained a strong band at 52 kDa, while one of the avirulent isolates with an LD_{50} value around 10^8 CFUml^{-1} contained a strong band at 45 kDa. The 52 kDa molecule was identified only by the catfish serum in the enterotoxin prepared from virulent isolates of *A. hydrophila*.

Introduction

Aeromonas hydrophila is widely distributed in the aquatic environment and is recognised as a pathogen of animals, including fish and man (Kaper et. al., 1981; Asao et. al., 1984). *A. hydrophila* produces a large variety of extracellular products (ECP) including haemolysins (Allan and Stevenson, 1981; Yadav et. al., 1992; Mateos et. al., 1993), aerolysin (Howard and Buckley,

1985; Chakraborty et. al., 1986), cytotoxin (Boulanger et. al., 1977; Olivier et. al., 1981), enterotoxin (Annapurna and Sanyal, 1977; Olivier et. al., 1981) and cytolytic enterotoxin (Rose et. al., 1989a,b). Such factors are thought to be important in the virulence of *A. hydrophila* infection in fish, which are known either as motile aeromonad septicaemia (McDaniel 1979), haemorrhagic septicaemia (Snieszko et al 1983) or red-sore disease (Reed and Toner 1941,Cahill 1990).

Many of the ECPs produced by *A. hydrophila* have been purified and their characteristics studied. Rose et. al., (1989a,b) described both biochemical and immunological characteristics of a 52 kDa protein, purified from a human *A. hydrophila* isolate (SSU) that not only processed cytotoxic, haemolytic and enterotoxic activities, but also demonstrated immunological cross-reactivity with cholera toxin. The physiochemical properties of the cytolytic enterotoxin from *A. hydrophila* have been shown to be similar to that of its aerolysin (Howard and Buckley, 1985)

The aims of the present study were to examine the protein profiles and toxicity of ECP derived from virulent and avirulent *A. hydrophila* isolated from fish, and to purify cytolytic enterotoxin from the *A. hydrophila* isolates and examine their biological and immunological characteristics. A comparison of these characteristics was then made in an attempt to identify differences between virulent and avirulent isolates of *A. hydrophila.*

Materials and Methods

Bacterial culture

A. hydrophila isolates used in the study were obtained from a survey of diseased fish from various regions within Thailand (Table 1), and their biochemical profiles were compared (Table 2). Bacteria were normally grown for 18 h at 28 °C on tryptic soy agar (TSA, Difco). After isolation, stock-cultures were immediately placed on cryopreservation beads (Technical service consultants Ltd) at -70 °C.

Determination of virulence

Bacteria were grown in tryptic soy broth (TSB, Difco) at 28 °C for 18 h. Cultures were centrifuged at 5000 x g for 10 min, and culture broth were discarded before washing cell pellets with sterile saline [0.85% (w/v) NaCl] by cen-

Table 1. *Aeromonas hydrophila* isolates obtained from diseased hybrid catfish (*Clarias gariepinus* x *Clarias macrocephalus*) cultured in Thailand.

Isolate	Organ	Location	Year
II/1	Kidney	Kampangpeeth	1993
III/22	Liver	Nakhornradchasrima	1993
95023	Lesion	Uthithanee	1995
III/39	Lesion	Nongkhai	1993

Table 2. Biochemical characterisation of *Aeromonas hydrophila* isolates.

Test	*Aeromonas hydrophila* isolate			
	II/1	III/22	95023	III/39
Gram stain	-	-	-	-
Motility	+	+	+	+
Oxidase	+	+	+	+
Catalase	+	-	+	-
O/F test	+/+	+/+	+/+	+/+
O/129	R	R	R	R
Fermentation of sugar				
Glucose	+	+	+	+
Sucrose	+	+	+	+
Lactose	-	-	-	-
Mannitol	+	+	+	+
Maltose	+	+	+	+
Salicin	+	+	+	+
Xylose	-	-	-	-
TSI	K/A	K/A	K/A	K/A
Citrate utilization	+	+	+	+
Esculine hydrolysis	+	+	+	+
Starch hydrolysis	+	-	+	+
Gelatine hydrolysis	+	+	+	+
Growth in KCN broth	+	+	+	+
Nitrate reduction	+	+	+	+
MR-VP	-/+	-/+	+/+	-/+
Indole	+	+	+	+
Decarboxylase reaction				
Arginine	+	+	+	+
Lysine	-	-	-	-
Ornitine	-	-	-	-

+: positive; -: negative; R: resistant to O/129; K/A: uses glucose and sucrose, but not lactose

trifuging at 5000 x g for 10 min. Cell pellets were finally resuspended in sterile saline. Dilutions of the bacterial suspension were prepared in saline and a viable count was established on TSA (Collins and Lyne 1976). Virulence was determined in hybrid catfish (*Clarias gariepinus* x *Clarias macrocephalus*) weighing 30 to 40 g, and twenty fish were used for each bacterial dose tested. The fish were injected intraperitoneally (ip) with 0.1 ml of bacterial suspension. They were maintained in fresh water for four days at approximately 24 °C. Virulence was determined as the lowest bacterial dose, which caused 50% mortalities in the inoculated fish group, as calculated using the method of Reed and Muench (1938).

Preparation of extracellular products

Bacteria cultures were incubated at 28 °C for 24 h in TSB with shaking. Twenty-five ml of each culture was centrifuged at 10,000 x g for 30 min to remove bacterial cells. The supernatant was passed through a 0.45 mm filter and dialysed against distilled water at 4 °C for 24 h. Aliquots (2 ml) of each ECP preparation were stored at -20 °C until required. Protein concentrations of the preparations were determined using a Protein Determination Kit (Bio-Rad), with bovine serum albumin (BSA) (Sigma) as a standard.

Determination of toxicity

The toxicity of the ECPs was tested using hybrid catfish fingerlings weighing 20 to 30 g. Twenty fish were injected with 0.3 ml (0.2 $mgml^{-1}$) for each of the ECP preparation intraperitoneal. Fish were kept at approximately 25 °C in freshwater and mortalities were recorded over a four-day period.

Preparation of cytolytic enterotoxin

A. hydrophila were grown in Casamino Acids and yeast extract (CYE) broth, by adding 10 ml of an overnight culture to 500 ml of CYE broth. The bacteria were then cultured for 18 to 20 h at 28 °C, with shaking at 100 rpm. The bacterial culture was centrifuged at 5000 x g for 10 min, the pellet was discarded, while the supernatant was retained for subsequent studies.

The 20 to 60% ammonium sulphate saturation-range was found to contain the majority of the haemolytic activity, as well as cholera toxin cross-reactivity, assessed by the method of Rose et. al., 1989a. On the basis of these observations, ammonium sulphate (20% saturation) was added to culture supernatants, the pH of the suspension was adjusted to 6.0 and it was stored at 4 °C for 4 h. The first precipitate was removed from the supernatant by centrifugation at 10,400 x g for 30 min and then discarded. Additional ammonium sulphate was added to the supernatant until a final saturation of 60% was achieved. The pH was again adjusted to pH 6.0 and the supernatants were stored at 4 °C for a further 4 h. The precipitate was isolated by centrifugation at 10,400 x g for 30 min, and the resulting pellet was dissolved in phosphate buffered saline [PBS: 0.02 M $NaH_2PO_4.2H_2O$, 0.02 M $Na_2HPO_4.2H_2O$, 0.15 M NaCl, pH 7.2]. The pH of the suspension was adjusted to 7.0.

The dissolved pellets were dialysed against 2.4 M ammonium sulphate in 100 mM sodium phosphate pH 6.8 by applying them to an Econo-Pac HIC Cartridge that had been previously washed in this solution. Bound protein was eluted with a 2.4 M to 0 M gradient of ammonium sulphate.

The biologically active peak from the Econo-Pac HIC Cartridge was applied to a DEAE-Bio-Gel A column, and washed with 0.05 M Tris hydrochloride pH 7.5 containing 1 M urea. Bound material was eluted from the column using a salt gradient (0.05 to 0.35 M NaCl, followed by 0.35 to 1.0 M NaCl in 0.05 M Tris-1 M urea buffer pH 7.5).

Hybrid catfish and a rabbit were immunised with purified cytolytic enterotoxin from virulent *A. hydrophila* isolate II/1 according to Chen et. al., (1997) and the antibody responses of the animals examined by Enzyme Linked Immunosorbent Assay (ELISA) and Western blot analysis.

Electrophoresis

Sodium dodecyl sulphate polyacylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of proteins present in the ECP and in the purified cytolytic enterotoxin using a Mini-Protein II Dual Slab Cell system (Bio-Rad Laboratories). The method of Laemmli (1970) was employed,

using a 12% separating gel. Samples were added to sample buffer (0.5 M Tris-HCl pH 6.8, glycerol, 10% SDS, 0.1% bromophenol blue, 5% b-mercaptoethanol), heated in a boiling water bath for 90 s, and centrifuged briefly in a microcentrifuge at 13,000 rpm before applying to the gels. Broad range molecular weight standards were used as a reference (Bio-Rad). The gel was run at 120 V, until the tracking dye reached the bottom of the gel, and it was then stained with Silver stain (Sigma).

Enzyme Linked Immunosorbent Assay (ELISA)

Microtitre plates (Nunc) were coated with the purified cytolytic enterotoxin, using a concentration of 10 $\mu g ml^{-1}$ in 0.05 M carbonate/bicarbonate buffer, pH 9.6 at 100 $\mu l\ well^{-1}$. The plates were incubated overnight at 4 °C, then washed three times in Low Salt Wash [LSW: 0.02 M Trizma base, 0.38 M NaCl, 0.05% (v/v) Tween-20, pH 7.4]. Non-specific binding sites were blocked with 250 $\mu l\ well^{-1}$ of 1% (w/v) BSA. The plates were incubated at 22 °C for 30 min, then washed three times with LSW. Rabbit or catfish anti-*A. hydrophila* sera (100 $\mu l\ well^{-1}$) was added. Fish sera diluted two fold in PBS containing 1% (w/v) BSA were added to the wells (100 $\mu l\ well^{-1}$). A negative control of PBS was also included. The plates were incubated for 1 h, then washed three times in LSW. Rabbit anti-catfish IgM, diluted 1/1000 in PBS (100 $\mu l\ well^{-1}$) was the added for 1 h at 22 °C, after which the plates were washed with high salt wash buffer [HSWB: 0.02 M Tris, 0.5 M NaCl, 0.1% Tween 20, pH 7.8]. Anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (SAPU: Scottish antibody production unit, Law Hospital, UK), diluted 1/3000 in PBS was added to each well (100 $\mu l\ well^{-1}$). The plates were incubated for 1 h at 22 °C, then washed as previously described. When rabbit anti-*A. hydrophila* serum was used, ten fold dilutions were first made in PBS. These were added to the plate and incubated for 1 h at 22 °C. Plates were washed three times with HSW, and donkey anti-rabbit IgG conjugated with HRP (SAPU) was added at a 1/1000 dilution in PBS. This was incubated for 1 h at 22 °C and plates were again washed three times with HSW. The reaction was developed by adding chromogen [42 mM tetramethyl benzidine dihydrochloride, (Sigma) in 2 M acetic acid diluted 1/100 in substrate buffer (3.33 ml 30% H_2O_2 in 0.1 M citric acid and 20 mM sodium acetate)] to each well (100 $\mu l\ well^{-1}$). The reaction was stopped after 15 min with the addition of 50 $\mu l\ well^{-1}$ 2 M H_2SO_4 and the optical density was determined spectrophotometrically at 450 nm.

Western blot analysis

The proteins, separated by SDS-PAGE, were transferred to a nitrocellulose membrane using a Trans-Blot SD semi-dry transfer cell (Bio-Rad) at 10 V for 20 min. Non-specific binding sites on the membrane were blocked for 1 h with 1% w/v BSA in Tris-buffered saline [TBS: 10 mM Tris, 0.5 M NaCl, pH 7.5] then wash three times with TBS containing 0.1% (v/v) Tween-20 (TBST). The membranes were incubated with 1/10 catfish anti-*A. hydrophila* serum in antibody buffer (1% BSA in TBS) overnight at 4 °C. The blots were washed as

previously described, then incubated in rabbit anti-catfish IgM serum diluted 1/100 in antibody buffer for 1 h at 22 °C. The membranes were again washed, then incubated with anti-rabbit IgG conjugated with HRP, (diluted 1/100 in antibody buffer) for 1 h at 22 °C. After washing, the membrane was placed in 3'3 diaminobenzidine tetrahydrochloride (DAB) in the presence of H_2O_2 to develop the reaction. The reaction was stopped once bands had appeared, by placing the membrane into tap water.

Results and Discussion

The virulence of *A. hydrophila* isolates was determined by challenging hybrid catfish with live bacterial cells injected ip. Mortalities were recorded over four days and LD_{50} values were calculated for each isolate (Table 3). Two isolates had LD_{50} values of around $x10^8$ bacteria ml^{-1} and were classified as avirulent, while the other two isolates were much more virulent with LD_{50} values of $x10^5$ bacteria ml^{-1}. Differences in the ECP composition of the different *A. hydrophila* isolates examined may have been responsible for the variation obtained in their LD_{50} values. Therefore, toxicity levels of the ECP preparations were determined by injecting 0.2 $mgml^{-1}$ of the ECP into fingerling hybrid catfish. The ECP preparation from the high virulence isolates produced 100% mortality in all fish within 18 h of injection, while the ECP from the low virulence isolates produced 100% mortalities after 96 h. The total amount of protein obtained in the ECP of the four *A. hydrophila* isolates examined was very similar, with values of 0.144, 0.148, 0.150 and 0.151 $mgml^{-1}$ for isolates III/39, 95023, III/22 and II/1 respectively.

The protein compositions of the four *A. hydrophila* ECP preparations were examined using SDS-PAGE, and differences were identified in the protein profiles between the various preparations (Figure 1). With the virulent isolates, major bands were evident at 88, and 52 kDa in the ECP of isolate II/1, and at 88, 52 and 45 kDa in the ECP of isolate III/22. There was some staining of low molecular weight material with these two isolates. The silver staining profiles of the ECP from the avirulent isolates had bands at 88, 45, 31 and 24 kDa with isolate 95023 and at 70, 50, 33 and 20 kDa with isolate III/39. Again staining of low molecular weight material was evident. The more virulent isolates of *A. hydrophila* (II/1 and III/22) expressed protein bands at 52 kDa, which were not evident in the ECP of the less virulent isolates (95023

Table 3. Lethal Dose (LD_{50}) values of *Aeromonas hydrophila* isolates determined by intraperitoneal injection of the bacterium into hybrid catfish (*Clarias gariepinus* x *Clarias macrocephalus*)

Isolates of *A. hydrophila*	*LD_{50}
II/1	2.3×10^5
III/22	2.8×10^5
95023	1.4×10^8
III/39	3.5×10^8

*LD_{50} values represent 50% mortalities over 96 h.

and III/39). The 88 kDa protein band, present in the ECP of isolates II/1, III/22 and 95023, has been identified as a haemolysin (Allan and Stevenson, 1981; Boulanger et. al., 1977; Ellis et. al., 1988; Santos et. al., 1992; Yadav et. al., 1992), capable of damaging red blood cells of fish, while the 52 kDa protein possesses haemolytic enterotoxin and cytolytic activities, found to be correlated with the virulence of the bacterium (Rose et. al., 1989a, b).

The enterotoxin from the various *A. hydrophila* isolates was purified using ammonium sulphate precipitation, followed by hydrophobic column chromatography and anion exchange chromatography. The protein composition of the purified *A. hydrophila* enterotoxins were also compared by SDS-PAGE, and silver stain profiles obtained for the preparations varied in the number of bands that were present (Fig. 2). The major component in the purified enterotoxins from the virulent isolates of *A. hydrophila* (LD_{50} around 10^5 $CFUml^{-1}$) was found to be the band located at 52 kDa. Silver staining showed that the less virulent isolates (LD_{50} around 10^8 $CFUml^{-1}$) contained much lower amounts of this protein than the virulent isolates. The primary function of this aerolysin, located at 52 kDa, appears to be that of killing cells so as to protect the bacterium from attack by the immune defences of its host, or as a source of nutrition (Howard et. al., 1996). This protein has been identified at 52 kDa in all known aeromonad species, including *A. hydrophila, A. salmonicida, A. sobria* and *A. trota* (Howard and Buckley 1986).

The antibody titre of serum obtained from hybrid catfish or rabbit, immunised with the enterotoxin purified from the ECP of virulent isolate II/1, was determined using an indirect ELISA. The antibody titres were obtained 1/1,024 and 1/32,768 for the catfish and rabbit sera, respectively. The antigenicity of the enterotoxin was examined by Western blot analysis using the immune catfish serum, which identified a predominant band at 52 kDa in the ECP of the virulent isolates (Fig. 3).

The results of this study indicate that the aerolysin at 52 kDa, a well-characterised toxin of *A. hydrophila*, is expressed in greater amounts in the ECP of virulent isolates of the bacterium than in avirulent isolates. The antibodies of fish immunised with cytolytic enterotoxin, purified from virulent isolate II/1, were able to identify the 52 kDa aerolysin of the two virulent isolates examined, but did not recognise the molecule in the enterotoxin purified from the avirulent isolates.

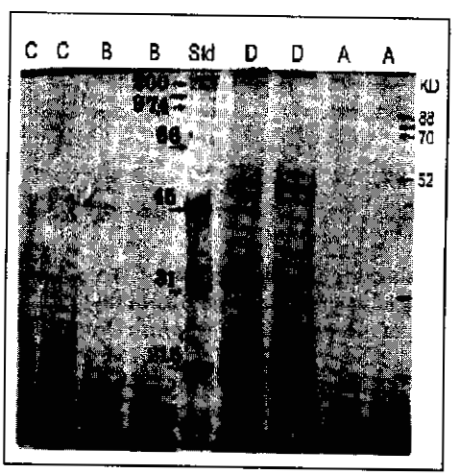

Fig. 1. SDS- polyacrylamide gel electrophoresis (12% polyacrylamide) of extracellular products of *Aeromonas hydrophila*, stained with silver stain. Lanes: (A)-isolate II/1; (B)- isolate III/22; (C)- isolate 95023; (D)-isolate III/39; (Std)-molecular weight standards.

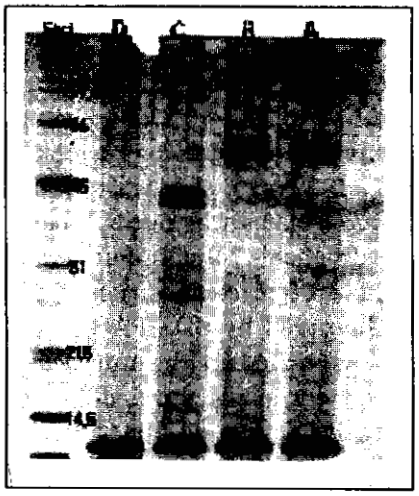

Fig. 2. SDS- polyacrylamide gel electrophoresis (12% polyacrylamide) of purified cytolytic enterotoxin of *Aeromonas hydrophila*, stained with silver stain: Lanes (A)-isolate II/1; (B)-isolate III/22; (C)-isolate 95023; (D)-isolate III/39; (Std)-molecular weight standards.

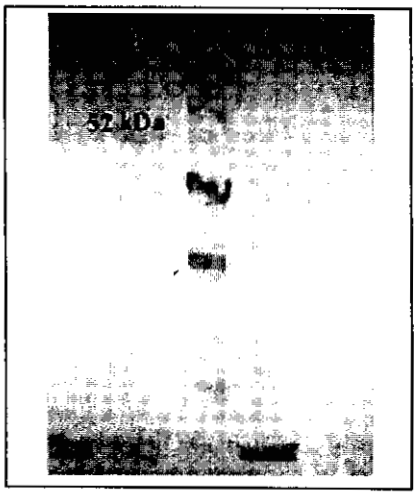

Fig. 3. Response of hybrid catfish (*Clarias gariepinus* x *Clarias macrocephalus*) anti *Aeromonas hydrophila* sera (pooled) against purified cytolytic enterotoxin of *A. hydrophila*, examined by Western blot analysis. Lanes (A)-isolate II/1; (B)-isolate III/22; (C)-isolate 95023; (D)-isolate III/39; (Std)-molecular weight standards.

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