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ORIGINAL RESEARCH

Aeromonas salmonicida isolated from wild and farmed fish and invertebrates in Oman

Aliya Alghabshi · Brian Austin · Margaret Crumlish

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Abstract *Aeromonas salmonicida* was isolated from red spot emperor, king soldier bream, white-spotted rabbit fish and tilapia, and an invertebrate (abalone) in Oman during December 2011–May 2012. The cytotoxic enterotoxin *ast* gene was found widely distributed among the isolates; aerolysin-like protein (*act*) and the flagellin structural gene *lafA* less so; and the nuclease gene (*nuc*) not at all. However, there was not any evidence of pathogenicity among the isolates when tested in laboratory-based experiments using rainbow trout and Nile tilapia. Therefore, the risk of the pathogen to fish in Oman is unclear.

Keywords *Aeromonas salmonicida* · Fish · Invertebrates · Aquaculture · Oman

Introduction

Aeromonas salmonicida is the aetiological agent of furunculosis in salmonids and causes ulcer disease in cyprinids and marine flatfish (Austin and Austin 2016). Traditionally, the organism is regarded as an obligate fish pathogen (Schubert 1974) being only recoverable from clinically diseased fish. In part, the restricted ecology reflected the difficulty of recovering viable and culturable cells from fish in the absence of clinical disease or from environmental samples (Austin and Austin 2016). Pathogenesis of *Aeromonas* infections may be correlated with stress of the susceptible fish and the production of cell-associated and extracellular virulence determinants (Austin and Austin 2016). Although numerous virulence factors, such as surface polysaccharides (capsule, lipopolysaccharide, and glucan), iron-binding systems, exotoxins and extracellular enzymes, secretion systems, fimbriae and flagella, contribute to pathogenesis of fish and human diseases caused by *Aeromonas* spp., none of the factors alone are responsible for all of the clinical signs of disease presented during an infection (Ali et al. 1996). Variations in the distribution of potential virulence genes between *Aeromonas* isolates may well contribute to their degree of pathogenicity (Albert et al. 2000). Sha et al. (2002) reported the presence and expression of three enterotoxin genes (*alt*, *ast* and *act* genes) in *Aeromonas* spp. that led to a 100% reduction in fluid secretion in a mouse model. Conversely, Sen and Rodgers (2004) reported that the mere presence of these toxins may not be sufficient for virulence. Therefore,

A. Alghabshi (✉)
Microbiology Section, Fish Quality Control Centre, Ministry of Agriculture and Fisheries Wealth, Muscat, Sultanate of Oman
e-mail: aliya.7744@gmail.com

B. Austin · M. Crumlish
Institute of Aquaculture, University of Stirling, Stirling, UK

there is a need to continuously assess the presence of several accepted virulence factors in *Aeromonas* isolates for better understanding of the overall pathogenesis of infections (Sen and Rodgers 2004). The present study was undertaken to investigate the pathogenicity of strains of *A. salmonicida* recovered from a range of aquatic animals in Oman.

Materials and methods

Bacterial isolates

In this study, 9 isolates of *A. salmonicida* were recovered from skin, gill and kidney of 417 fish representing 4 wild fish, including red spot emperor (*Lethrinus lentjan*), king soldier bream (*Argyrops spinifer*), white-spotted rabbit fish (*Siganus canaliculatus*), abalone (*Haliotis mariae*) and one farmed tilapia (*Oreochromis niloticus*). The fish were collected from 3 areas (Muscat, Mudhaibi and Salalah) considered suitable for aquaculture in Oman based on the Atlas of suitable sites for aquaculture in Oman. The animals were mostly healthy, as judged visually, except for one tilapia that demonstrated abnormal behaviour. The abalone was from a population that had a history of high mortality albeit without any clinical signs of disease.

From freshly dead fish and muscle from the abalone, samples were taken by means of inoculating loops from skin, gill and kidney, and were directly streaked onto the surface of tryptone soya agar plates (TSA; CM0131, Oxoid) with incubation at 28 °C for 48 h. From each plate with dense growth, colonies representing the most commonly occurring morphological types were aseptically selected and subcultured for purity. Smears were prepared for the Gram-staining reaction and micro-morphology. Catalase production and the determination of motility followed the methods of Frerichs and Millar (1993) and Martin-Carnahan and Joseph (2005), respectively. Oxidase production was determined using oxidase strips (OxiStrips^{TM}, Oxoid). The API 20E rapid identification system (BioMérieux, France) was used following the manufacturer's instructions, except that the inoculated strips were incubated at 28 °C and the results read at 48 h (Crumlish et al. 2002). The demonstration of haemolysin activity was assessed from the presence of complete (β-haemolysis) or incomplete (α-haemolysin) zones of clearing around colonies on 5% sheep blood agar following incubation at 28 °C for 48 h. The susceptibility to the vibriostatic agent (O/129) (150- and 10-μg discs; Oxoid) was achieved by the disc method of Kirby and Bauer on TSA agar plates (Buller 2004). After 24-h incubation at 28 °C, zones of clearing of < 7 and > 7 mm were recorded as indicative of resistance and sensitivity, respectively (Whitman 2004). Serology was determined by indirect agglutination reactions using the MONO-As kit (BIONOR AS, Skien, Norway) for *A. salmonicida*, following the manufacturer's instructions. This kit consists of antibody-coated latex beads, and is designed for specific identification of all *A. salmonicida*. Growth at 5, 20, 22, 28, 30 and 37 °C and the ability to produce brown diffusible pigment were evaluated on TSA (Austin et al. 1998). Here, inoculated media were examined every 24 h for up to 5 days to determine the presence of colony growth, and up to 14 days to assess for brown diffusible pigment production. For each test performed, a positive control, i.e. *A. salmonicida* subsp. *salmonicida* (NCIMB 1102^{T}), was included.

Molecular identification

Genotyping of the suspected *A. salmonicida* used 16S rRNA sequencing (Borrell et al. 1997) and rDNA-RFLP (Borrell et al. 1997; Figueras et al. 2000). Species identification was confirmed by comparing the DNA sequence obtained in this study with the NCBI GenBank database using BLAST [basic local alignment search tool, standard nucleotide comparison (http://www.ncbi.nlm.nih.gov/BLAST/)] (Altschul et al. 1990). The sequences were aligned using ClustalW for multiple sequence alignment with the DNASTAR computer program and phylogenetic analyses (maximum likelihood method) were conducted by the MEGA software version 6.0 (Tamura et al. 2013) to provide a phylogenetic tree. Standard errors were obtained with 1000 bootstrap replicates.

Preparation of extracellular products (ECP)

ECP was prepared using the cellophane overlay method of Gudmundsdóttir (1996). The bacteria were grown in tryptone soya broth (TSB; Oxoid) for 24 h at 28 °C, and centrifuged at 10,000×*g* at 4 °C for 30 min. The supernatant containing the ECP fractions was sterilized by filtration through 0.22-µm filters (Millipore). To confirm the absence of bacterial colonies, 0.2 ml volume of the filtered supernatants was streaked over TSA plates and incubated for 48 h at 28 °C. The protein concentration of the ECP was determined by the method of Bradford (1976) using a protein determination kit (Bio-Rad, USA) according to the manufacturer's instructions with bovine serum albumin (BSA; Sigma-Aldrich, UK) as the standard. The ECP extractions were then subsequently stored at − 20 °C until required.

Determination of putative virulence characteristics

Bacteria from 24-h-old cultures on TSA and filtered supernatants obtained from the ECP preparations were used to assess protease activity (Gudmundsdóttir 1996), haemolytic activity (Brender and Janda 1987), DNase activity (Buller 2004), Congo red dye uptake (Crump and Kay 2008) and the production of A-layer as visualized on Coomassie brilliant blue agar (CBB; Cipriano and Bertolini 1988). Positivity for A-layer was indicated by the presence of dark-red colonies on Congo red agar and dark-blue colonies on CBB agar, whereas negativity was indicated by the presence of pale colonies on these media.

Detection of virulence genes

The presence of genes encoding the virulence factors aerolysin (*aer*) (Pollard et al. 1990), aerolysin-like proteins (*act*) (Sen and Rodgers 2004), cytotoxic enterotoxins (*ast, alt*) (Aguilera-Arreola et al. 2005), glycerophospholipid cholesterol acyltransferase (*gcat*) (In-Young and Kiseong 2007), structural gene flagellin (*lafA, lafB*) (Aguilera-Arreola et al. 2005) (In-Young and Kiseong 2007) and serine protease (In-Young and Kiseong 2007) was determined by use of the polymerase chain reaction (PCR) using primers and conditions already published.

Determination of pathogenicity

Cells from 24-h cultures in TSB were harvested by centrifugation, washed in 0.85% (w/v) saline and the optical density (OD_{600}) was adjusted to 1. Viable colony counts were performed following the Miles and Misra (1938) method and tenfold serial dilutions prepared for challenge studies in tilapia and rainbow trout. The first challenge was performed in the Fishery Quality Control Centre in Oman using farmed tilapia stocks of 30 g average weight. The fish were fed with commercial pelleted fish diet for the 5-day period of each experiment with quarantine for 14 days prior to administration of 0.1 ml volume of bacterial suspension containing 10^8 cells $fish^{-1}$ by intraperitoneal (i.p) and intramuscular (i.m) injections. Control fish received 0.1 ml volume of sterile 0.85% (w/v) saline by i.p. and i.m. injections. A second experiment was performed at the University of Stirling using the same concentration of bacterial cells in an in-house tilapia population of 30 g average weight and farmed rainbow trout of 50 g average weight. The fish at Stirling were health checked prior to use, where tissue samples were aseptically taken from a subsamples of the group and fixed in 10% neutral buffered formalin before processing into wax-embedded tissue sections. These were trimmed and 5-µm sections cut, stained with haemotoxylin and eosin and viewed for health check prior to any experimentation. Additional fish experiments sought to determine the effect of ECPs, which were injected i.m. and i.p. in 0.1 ml volume (0.1 mg of protein ml^{-1}). Control fish received 0.1 ml volume of sterile 0.85% (w/v) saline. All fish were maintained in freshwater at 26 ± 2 and 15 ± 1 °C for tilapia and rainbow trout, respectively, and examined daily for 5 days. Any dead or clinically diseased fish were sampled microbiologically in which loopfuls of samples from kidney and spleen were streaked onto TSA plates with incubation at 28 °C for 24 h, and the resulting colonies identified as previously described. Also, spleen and kidney tissues were fixed in 10% (v/v) neutral buffered formalin for 24 h before embedding in paraffin following routine tissue processing. Thus, 5-µm-thick sections were cut and stained with haematoxylin and eosin (H&E) (Oliveira Ribeiro et al. 1981) and Gram stained for histological examination. All slides were examined at a magnification of 400× using a Carl Zeiss microscope.

Results and discussion

Nine isolates of *A. salmonicida* were recovered and identified from skin, gill and kidney from four fish species, i.e. red spot emperor, king soldier bream, white-spotted rabbit fish, tilapia and one of abalone, all of which are considered as commercially important for aquaculture development in Oman.

These isolates were all tentatively equated with *A. salmonicida* (Martin-Carnahan and Joseph 2005), insofar as they produced smooth convex colonies on TSA, and comprised Gram-negative rod-shaped bacteria that grew at 5–30 °C, produced catalase, β-galactosidase, gelatinase, indole and oxidase, but not tryptophan deaminase or urease, fermented glucose, mannose, rhamnose, melibiose and sucrose but not inositol, they did not utilize sodium citrate and were positive for the Voges–Proskauer reaction. All biochemical test results using the API 20E rapid identification kit were in agreement with those of the NCIMB reference strain (Table 1). Unlike descriptions of virulent *A. salmonicida* subsp. *salmonicida* as recovered from furunculosis in salmonids (McCarthy and Roberts 1980), the Omani strains did not produce brown diffusible pigment around the colonies on TSA, and so the isolates correspond to the description of 'atypical' *A. salmonicida* (Austin et al. 1998; Martin-Carnahan and Joseph 2005). Sequencing of the 16S rRNA gene revealed that three isolates, 291MS, 293MS and 295MS, had the highest (99%) similarity with *A. salmonicida* spp., sequences deposited in GenBank, whereas isolates 373MG and 388MS demonstrated 98% homology, and 395M, 340M, 26 MS2 and 16MG revealed 95–92% identity levels, respectively (Fig. 1). It is emphasized that reliance only on sequence analysis of the 16S rRNA gene to identify *A. salmonicida* has limited value, although the technique is useful for confirmation of membership of the genus *Aeromonas* (Figueras et al. 2011; Han et al. 2011).

Extracellular nuclease and haemolysin activities are considered to be virulence-associated factors belonging to many *Aeromonas* species (González-Rodríguez et al. 2002; Rahman et al. 2002). Haemolytic activity was present in 67% of the isolates, whereas extracellular protease occurred in 44% of the cultures from Oman. Only a minority of the isolates demonstrated uptake of Congo red (22%) and CBB (33%).

Table 1 Differential phenotypic characteristics of the isolates

Phenotypic characteristics	Isolate no.								
	A. salmonicida[a]	16MG	26MS2	291MS	293MS	295MS	340M	373MG	388MS	395M
Colonial colour on TSA	N	B	B	B	B	B	A	B	B	B
Motility	V	−	+	+	+	+	−	+	+	+
Pigment production	V	−	−	−	−	−	−	−	−	−
Autoagglutination in saline	V	−	−	+	−	−	−	−	−	−
Growth at:										
5 °C	V	−	−	+	+	+	−	−	+	−
37 °C	V	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	V	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	V	+	+	+	−	−	+	+	+	+
Ornithine decarboxylase	V	−	+	−	−	−	−	+	−	−
H_2S production	V	−	−	−	−	−	−	−	−	−
Fermentation of sorbitol	V	+	−	+	−	−	+	+	−−	−
Fermentation of rhamnose	_	+	−	−	−	−	+	−	−	−
Fermentation of melibiose	_	+	−	−	−	−	+	−	−	−
Fermentation of amygdalin	V	+	−	+	+	−	+	+	+	+
Fermentation of arabinose	V	+	−	+	+	+	+	+	−	−
Susceptibility to the vibriostatic agent O/129:150 μg/10 μg	R/R	R/R	S/S	R/R	R/R	R/R	S/R	S/R	S/S	R/R

A, light creamy colonies; B, yellowish colonies; +, positive; −, negative; V, variable result; N, no data; R, resistant; S, sensitive

[a]Data are from the following references: Abbott et al. (1992), Austin et al. (1989), Carnahan and Altwegg (1996), Huys et al. (1996), Griffiths et al. (1953), Schubert (1974), McCarthy and Roberts (1980), Pavan et al. (2000) and Yamada et al. (2000)

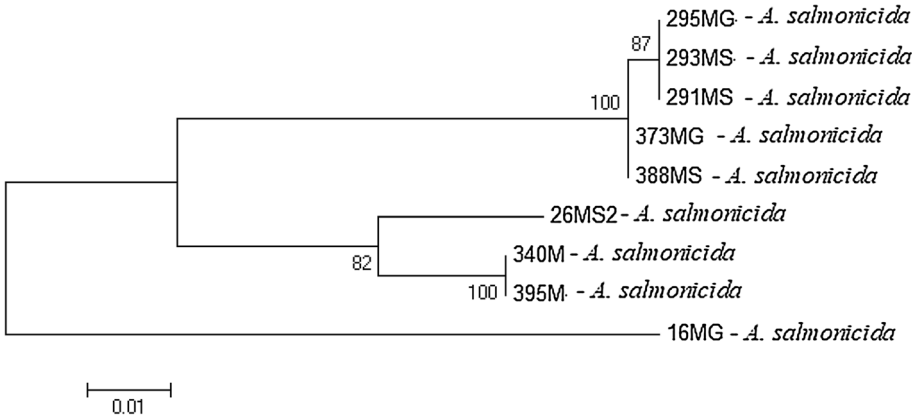

Fig. 1 The phylogenetic tree based on 16S rRNA fragment sequences, showing relationship of the *A. salmonicida* cultures (constructed by maximum likelihood method using MEGA6 software); scale bar 0.01 represents sequence divergence

Haemolytic and proteolytic activities were not seen in any of the ECPs. The distribution of the putative virulence genes has been presented in Table 2. Thus, the *ast* gene was distributed among 67% of the isolates; slightly less (56%) contained aerolysin-like proteins (*act*). The genes for *lafA* (33% of isolates) were less common. Also, *aerA*, *alt*, *gcat* and *ser* were present in only 22% of the cultures, *lafB* occurred in a single isolate; and *nuc* not at all.

In this study, mortalities were not recorded in any of the challenge experiments. Indeed, pathological changes were not observed in any of the tissues examined by histology (Fig. 2), although i.p. injection of isolate 340M in rainbow trout led to the development of pale liver and darkened kidneys. Moreover, only fish injected with 16MG and 340M contained culturable cells at the end of the experiments.

The discrepancy in the presence of aerolysin (*aerA*) and aerolysin-like protein (*act*) genes among the Omani cultures suggested that the isolates may possess but not express these genes (Wang et al. 2003) under the situations described. Enterotoxin genes *alt*, *ast* and *act* were not expressed in any of the isolates. Two cultures harboured both *act* and *ast* genes, which is unusual as this combination has been only rarely reported among environmental isolates (Albert et al. 2000; Chang et al. 2008). Yet, pathogenicity was not recorded among these isolates. Some studies reported a correlation between the higher number of virulence genes harboured in *Aeromonas* spp. and their potential for causing disease (Albert et al. 2000; Chang et al. 2008). These workers mentioned that the number of isolates positive for both the *alt* and *ast* genes was significantly higher in children with diarrhoea than for healthy controls. In this study, there was not any such correlation. An explanation could be that the experimental conditions used in this study influenced the expression of the genes involved in pathogenicity. Also, the level of virulence has inevitably been correlated with the amount of enzymes and toxins produced (Kozińska 1996). Another possibility is that their presence in *A. salmonicida* does not infer that disease is inevitable reflecting the susceptibility of the host, immune state and actual number of bacterial cells in and around the host (Ottaviani et al. 2011). Notwithstanding, some isolates did lead to the development of small haemorrhages in/on the internal organs, as reported previously (Austin and Adams 1996). In this respect, it is worthwhile to consider the comments of Austin and Austin (1993) and Austin (2011), who considered that loss of virulence might well reflect the effects of storage, i.e. the transition to what are effectively laboratory cultures, and the inability to replicate conditions of the initial disease, which led to the recovery of the cultures.

The recovery of *A. salmonicida* from Omani fish and abalone in the absence of clinical signs of disease contradicts the commonly held view that the organism is an obligate fish pathogen. However, this may reflect that scientists have focused on recovery only from diseased fish, namely salmonids, cyprinids and marine flatfish, rather than other groups of aquatic animals and environmental samples. All the isolates recovered in this study had similar morphologies and lacked diffusible brown pigment production. Also, there was not any direct relationship found between pathogenicity and the presence of putative virulence factors. Again, it is questionable whether this reflects the loss of activity during storage. Clearly, further research is needed to extend the knowledge of this group of organisms, particularly in an emerging aquaculture industry.

Table 2 Results of infectivity studies, and presence of different virulence factors expression among the isolates

Isolates no.	Recovered from	Location	Clinical signs	Genetic method									Phenotypic method			
				Areo-gene	ACT	AST	ALT	GCAT	SER	NUC	*LafB*	*LafA*	Haemolysin	Congo red/CBB	Protease	DNase
16MG	Tilapia	Mudhaibi	Weakness, swimming on one side	−	−	+	−	−	−	−	−	−	γ	−	+	−
26MS2	Tilapia	Mudhaibi	NC	−	+	+	−	−	−	−	−	−	+ β	−	+	+
291MS	White-spotted rabbit fish	Muscat	NC	−	+	−	−	+	+	−	+	+	+ β	−	−	+
293MS	White-spotted rabbit fish	Muscat	NC	−	−	+	−	−	−	−	−	−	γ	−	−	+
295MG	King soldier bream	Muscat	NC	+	+	+	−	+	+	−	−	+	α	−	−	+
340M	Abalone	Salalah	History of high mortality with no clear clinical sign	−	−	+	−	−	−	−	−	+	γ	−	−	−
373MG	Red spot emperor	Muscat	NC	+	−	+	−	−	−	−	−	−	+ β	−	−	−
388MS	Tilapia	Mudhaibi	NC	−	+	−	+	−	−	−	−	−	+ β	−	+	+
395M	Abalone	Salalah	NC	−	+	−	+	−	−	−	−	−	+ β	−	+	+

M, muscle; MG, mucus of gill; NC, no clinical signs of disease; α, α-haemolysis; β, β-haemolysis; γ, γ-haemolysis; +, presence; −, absence

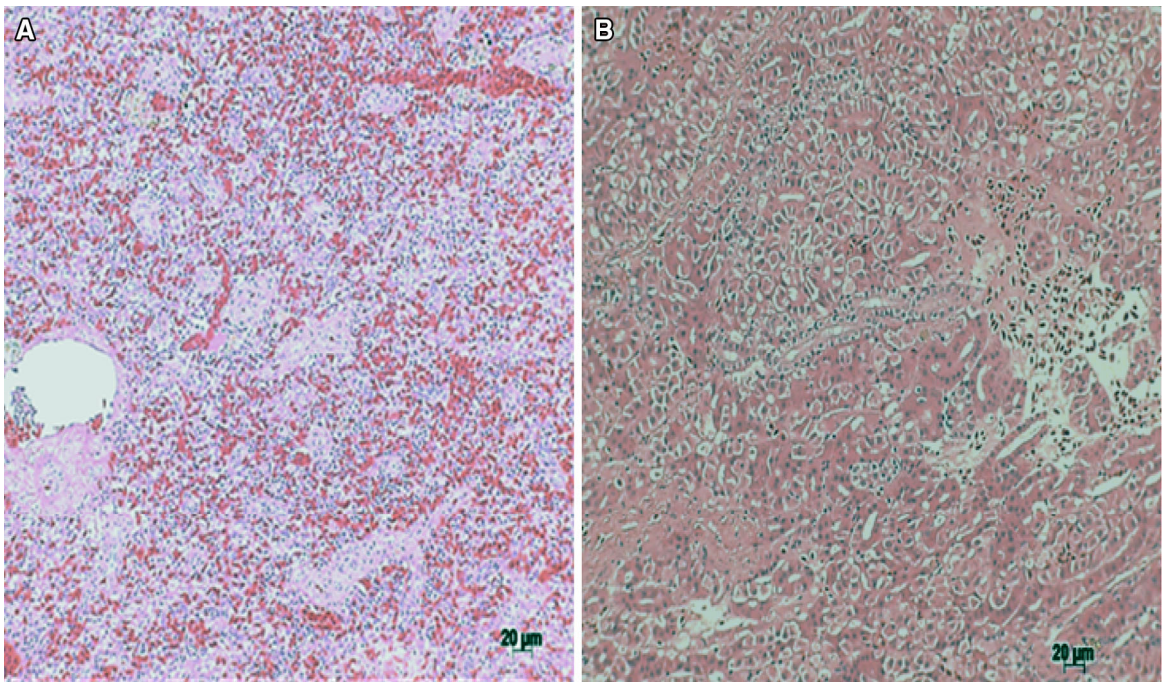

Fig. 2 Light microscopic appearance of the (**a**) spleen and (**b**) kidney of tilapia injected with extracellular products (ECP) of *A. salmonicida* isolates (H&E, scale bar 20 µm)

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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