Vol. 94: 29–39, 2011
doi: 10.3354/dao02304

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published March 16

Isolation and characterization of virulent *Aeromonas veronii* from ascitic fluid of oscar *Astronotus ocellatus* showing signs of infectious dropsy

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ABSTRACT: The cichlid oscar *Astronotus ocellatus* has worldwide commercial value in the pet fish industry because of its early maturation, relatively high fecundity, ability to identify its caretaker and also to alter colouration amongst conspecifics. Pathogenic strains of *Aeromonas veronii* resistant to multiple antibiotics were isolated from *A. ocellatus* individuals showing signs of infectious abdominal dropsy. The moribund fish showed haemorrhage in all internal organs, and pure cultures could be obtained from the abdominal fluid. The isolates recovered were biochemically identified as *A. veronii* biovar *sobria* and genetically confirmed as *A. veronii* based on 16S rRNA gene sequence analysis (GenBank accession no. FJ573179). The RAPD profile using 3 primers (OPA-3, OPA-4 and OPD-20) generated similar banding patterns for all isolates. They displayed cytotoxic and haemolytic activity and produced several exoenzymes which were responsible for the pathogenic potential of the isolates. In the representative isolate MCCB 137, virulence genes such as enterotoxin *act*, haemolytic toxin *aerA*, type 3 secretion genes such as *aexT*, *ascV* and *ascF-ascG*, and *gcat* (glycerophospholipid-cholesterol acyltransferase) could be amplified. MCCB 137 exhibited a 50% lethal dose (LD_{50}) of $10^{5.071}$ colony-forming units ml^{-1} in goldfish and could be subsequently recovered from lesions as well as from the internal organs. This is the first description of a virulent *A. veronii* from oscar.

KEY WORDS: *Aeromonas veronii* · Oscar · *Astronotus ocellatus* · Virulence · Ornamental fishes

INTRODUCTION

Aeromonas spp. are primary pathogens of freshwater fish or secondary opportunistic pathogens of compromised or stressed hosts (Jeney & Jeney 1995). Of the *Aeromonas* spp., *A. hydrophila*, *A. bestiarum*, *A. salmonicida, A. veronii* biovar *sobria*, *A. caviae*, *A. veronii* and *A. jandaei* have been reported as pathogens of various fish species (Kozinska et al. 2002, Rahman et al. 2002, Shome et al. 2005, Wahli et al. 2005, Hossain 2008, Martínez-Murcia et al. 2008, Sreedharan 2008). Motile aeromonads are associated with more than one disease, manifested through several clinical signs such as fin rot/tail rot, ulceration, exophthalmia and abdominal distention (dropsy). They are responsible for motile aeromonad septicaemia (MAS) and bacterial haemorrhagic septicaemia (BHS) and are associated with epizootic ulcerative syndrome in numerous freshwater (Rahman et al. 2004, Shao et al. 2004) and marine species (Lilley et al. 1997).

Among the aeromonads, *Aeromonas veronii* has the greatest range in virulence (Janda & Kokka 1991). *A. veronii* was originally described by Hickman-Brenner et al. (1987) as a novel species in the genus *Aeromonas* that had previously been referred to by the Center for Disease Control as Enteric Group 77. Genetic studies have indicated that this species consists of 2 biovars, *A. veronii* biovar *sobria*, which is negative for esculin hydrolysis and ornithine decarboxy-

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lase, and *A. veronii* biovar *veronii*, which is positive for these reactions (Altwegg et al. 1990, Carnahan & Altwegg 1996). The incidence of *A. veronii* from diseased fishes has been reported (Hossain 2008, Ma et al. 2009), but not from oscar *Astronotus ocellatus*.

The oscar is a cichlid that has worldwide commercial value as an ornamental fish (Staeck & Linke 1995) because of its early maturation and relatively high fecundity (Paiva & Nepomuceno 1989). These fish are regarded as being among the most intelligent tropical fish species, as they identify their caretakers and sense their surroundings (Von 2010). The species is able to rapidly alter its colouration, a trait which facilitates ritualized territorial and combat behaviours amongst conspecifics (Beeching 1995).

While carrying out a survey on diseases of ornamental fishes across the state of Kerala, India, characteristic abdominal dropsy was detected among oscar, from which *Aeromonas* in pure culture was isolated especially from the ascitic fluid. This paper describes characterization of the isolates and their pathogenicity, giving insight into their role in the development of dropsy in fishes.

MATERIALS AND METHODS

Diseased fish. A few (4) moribund fishes from among a population of oscar were bought from an ornamental fish breeding unit. They showed anorexia and lethargy, and exhibited clinical signs of dropsy such as distended abdomen, scale protrusion and petechial haemorrhage. On postmortem examination, the ascitic fluid appeared straw yellow. A water sample was collected from the rearing facility and major parameters (pH, dissolved oxygen, ammonia, alkalinity and hardness) were measured (APHA 1995).

Isolation of the associated bacteria. The abdominal fluid was serially diluted in physiological saline and spread-plated onto nutrient agar (peptone, 5.0 g l^{-1}; beef extract, 5.0 g l^{-1}; NaCl, 5.0 g l^{-1}; agar, 20.0 g l^{-1}; pH 7.5 ± 0.3). The plates were incubated at 28°C for 24 to 48 h. Colonies were isolated at random from all plates to nutrient agar slants and their purity was confirmed by repeated streaking on plates of the same medium and by subsequent Gram staining and observation.

Phenotypic characterization. The isolates were examined for Kovac's cytochrome oxidase, O/129 sensitivity (Oxoid), catalase, production of hydrogen sulphide in triple sugar iron (TSI), arginine dihydrolase, lysine and ornithine decarboxylase, indole production, methyl red test, Voges-Proskauer reaction (acetoin production), citrate utilization, urease production, phenylalanine deaminase, gluconate oxidation, nitrate reduction, ortho-nitrophenyl-galactoside oxidation (β-galactosidase production), and acid production from sugars as described by Collee et al. (1996). The isolates were tested for hydrolysis of esculin (Wilcox et al. 1992), production of alkylsulphatase (Janda et al. 1996), pyrazinamidase (Carnahan et al. 1990), and utilization of DL-lactate (Janda et al. 1996), malonate and acetate (Ewing 1986). The identification was accomplished following Aerokey II (Carnahan et al. 1991).

Random amplified polymorphic DNA (RAPD). RAPD-PCR was carried out using a set of 3 primers (OPA-3: AGT CAG CCA C; OPA-4: AAT CGG GCT C; and OPD-20: ACC CGG TCA C) as previously described (Sudheesh et al. 2002). The reaction mixture consisted of 2.5 µl of 10× buffer, 1.5 µl of 25 mM $MgCl_2$, 1.5 µl of 10 pmol of oligonucleotide primer, 1.0 µl of DNA template, 2 µl of 2.5 mM of each deoxynucleoside triphosphate and 1.0 µl of *Taq* polymerase in a total volume of 25 µl. The reaction was performed in a DNA thermal cycler (Eppendorf), and the amplification conditions consisted of initial denaturation at 95°C for 4 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and primer extension at 72°C for 2 min, with a final extension at 72°C for 10 min. The amplified products were electrophoresed on 1.2% agarose gels in Tris-acetate-EDTA (TAE) buffer at a constant current of 60 mA.

Molecular identification by 16S rRNA gene sequence analysis. As all isolates were phenotypically identical, one representative culture (MCCB 137) was chosen for 16S rRNA gene sequence analysis.

***Extraction of total DNA*:** Cell suspension (1 ml) grown in Luria Bertani (LB) medium was centrifuged at 10 000 × *g* (10 min, 4°C), and the pellet was resuspended in 500 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and centrifuged at 10 000 × *g* (10 min, 4°C). The pellet was resuspended in 500 µl lysis buffer (Tris-HCl 0.05 mM, pH 8.0, EDTA 0.05 mM, NaCl, 0.1 mM, SDS 2%, PVP 0.2% and 0.1% mercaptoethanol; Lee et al. 2003) and 10 µl of Proteinase K were added and incubated initially for 1 h at 37°C and then for 2 h at 55°C. Further extraction was carried out by phenol–chloroform extraction method as described by Sambrook & Russell (2001).

Amplification of the 16S rRNA gene from extracted DNA: 16S rRNA gene amplification was accomplished following Reddy et al. (2000) and employing the universal primers 16S1 (GAG TTT GAT CCT GGC TCA) and 16S2 (ACG GCT ACC TTG TTA CGA CTT).

The reaction was performed in a DNA thermal cycler (Eppendorf) with reaction mixtures (final volume 25 µl) containing 2.5 µl of 10× buffer, 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of 10 pmol of each oligonucleotide primer, 1.0 µl of DNA template, 2 µl of 2.5 mM of each deoxynucleoside triphosphate and 1 µl of *Taq* DNA polymerase.

The amplification profile consisted of initial denaturation at 95°C for 5 min, followed by 34 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 30 s and extension at 68°C for 2 min, with a final extension of 68°C for 10 min.

The PCR products were analyzed by electrophoresis on a 1% agarose gel prepared in 1× TAE buffer. The gels were stained with ethidium bromide (0.5 µg ml^{-1}), visualised on a UV light transilluminator and documented.

***Cloning onto pGEM-T Easy vector*:** The PCR product was cloned into pGEM-T Easy vector (Promega) as per the manufacturer's instructions and used for transforming *Escherichia coli* JM 109. Plasmids from the positive clones were extracted using the GenElute HP plasmid miniprep kit (Sigma-Aldrich). Nucleotide sequencing was performed using an ABI PRISM 3700 Big Dye Sequencer at Microsynth AG, Switzerland. The primers used were T7 (TAA TAC GAC TCA CTA TAG GG) and SP6 (GAT TTA GGT GAC ACT ATA G).

***16S rRNA gene sequence similarity and phylogenetic analysis*:** Sequenced DNA data were compiled and analysed, and matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al. 1990) from the NCBI website (www.ncbi.nlm.nih.gov). The sequences were multiply aligned using the programme Clustal W (Thompson et al. 1994). Genetic distances were obtained using Kimura's 2-parameter model (Kimura 1980), and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei 1987) using the software MEGA4 (Tamura et al. 2007).

Phenotypic expression of virulence: *in vitro* assays. All isolates were tested for the production of DNase (Jeffries et al. 1957), caseinase, chitinase, phospholipase (lecithinase), gelatinase and degradation of tributyrin (for lipase; Collee et al. 1996). Elastase activity on solid medium was detected by spot inoculating the organisms on LB medium supplemented with 0.2% elastin-Congo red (Sigma-Aldrich) with a clear zone around the growth and diffusion of Congo red into the clear zone, and haemolytic activity on LB agar containing 5% (vol/vol) human blood (Swift et al. 1999). The other virulence traits tested were slime formation (Freeman et al. 1989) and surface hydrophobicity (Rosenberg et al. 1980, Scoaris et al. 2008). Cytotoxicity of culture supernatant was examined on Hep-2 cells by incubating exponentially growing cells with the supernatant following the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983, Greenman et al. 1997).

Detection of virulence genes. The representative culture, MCCB 137, was subjected to PCR to detect virulence genes such as enterotoxins (*act, alt* and *ast*), haemolytic toxins (*hlyA* and *aerA*), genes involved in the type 3 secretion system (TTSS: *ascV*, *aexT*, *aopP*, *aopO*, *ascF–ascG* and *aopH*), and glycerophospholipid-cholesterol acyltransferase (*gcat*). The previously described primers and PCR conditions were used for the specific amplification of virulence genes. Characteristics of primers used for the PCR amplification of virulence genes are summarized in Table 1. The PCR products were visualised in a 1.5% gel stained with ethidium bromide.

Antibiotic susceptibility test. Susceptibility to selected antibiotics was tested on nutrient agar plates by the disc diffusion method of Bauer et al. (1966). Briefly, the nutrient agar plates were swabbed with cultures of the isolates grown overnight. Ready-made antibiotic discs from HiMedia Laboratories were aseptically placed on the swabbed plates. The plates were incubated at 28 ± 1°C for 18 h, and the clearing zone formed around the discs was recorded using Hi Antibiotic Zone Scale (HiMedia). The multiple antibiotic resistance (MAR) index (number of antibiotics to which the isolate was resistant/total number of antibiotics tested) was determined for each isolate (Krumperman 1985).

Pathogenicity *in vivo*. The pathogenicity of *Aeromonas* MCCB 137 *in vivo* was tested on goldfish *Carassius carassius* as the challenge model. All experiments were conducted in a bioassay system. The fish (weighing about 10 to 15 g) were anaesthetised using clove oil (80 ppm). For 50% lethal dose (LD_{50}) determination, 5 groups of 7 fish each were intramuscularly injected with 0.1 ml of a saline suspension of the pathogen at 10^4, 10^5, 10^6, 10^7 and 10^8 colony-forming units (CFU) ml^{-1}. One group that served as a control was injected with 0.1 ml of saline. Morbidity and death of the fishes were monitored daily for 7 d, and the moribund specimens were subjected to routine bacteriological examination for re-isolation of the organism. The LD_{50} was calculated following Reed & Muench (1938).

RESULTS

Phenotypic and molecular characterization

All cultures (n = 20) were of uniform colony morphology (circular, entire, convex and opaque light brown), Gram-negative short rods, motile, fermentative with gas production from glucose, Kovac's oxidase positive and with uniformity in all other characteristics examined (Table 2). On the basis of phenotypic characteristics, the isolates were identified as *Aeromonas veronii* biovar *sobria*.

The PCR product (1506 bp) of the 16S rRNA gene of *Aeromonas* MCCB 137 (1.5 kb) was subjected to sequencing after purification and cloning. The BLAST

Table 1. Primers (F: forward; R: reverse) used for the amplification of virulence genes

Virulence gene	Primer	DNA sequence (5′–3′)	Product size (bp)	Source
Enterotoxins				
act	F	AGAAGGTGACCACCAAGAACA	232	Kingombe et al. (1999)
	R	AACTGACATCGGCCTTGAACTC		
ast	F	TCTCCATGCTTCCCTTCCACT	331	Kingombe et al. (1999)
	R	GTGTAGGGATTGAAGAAGCCG		
alt	F	TGACCCAGTCCTGGCACGGC	442	Kingombe et al. (1999)
	R	GGTGATCGATCACCACCAGC		
Haemolytic toxins				
aerA	F	CCCGCCGATCTGCAACCGGG	489	Ormen & Ostensvik (2001)
	R	CTGGTCTGGATAGACGGGCTCTGCC		
hlyA	F	GGCCGGTGGCCCGAAGATACGGG	597	Heuzenroeder et al. (1999)
	R	GGCGGCGCCGGACGAGACGGG		
Type 3 secretion genes				
aexT	F	GGCGCTTGGGCTCTACAC	535	Burr & Frey (2007)
	R	GAGCCCGCGCATCTTCAG		
ascV	F	GCCCGTTTTGCCTATCAA	807	Burr & Frey (2007)
	R	GCGCCGATATCGGTACCC		
aopP	F	GAGAGTTGGCTAGCGGTGAG	490	Burr & Frey (2007)
	R	TCCTCATGGAGCGCATCCAG		
aopO	F	CGAGACAGACAAGTTTGC	401	Burr & Frey (2007)
	R	TGTCGTTGTGGACTATCC		
aopH	F	TCAATCAGGACGATGTCG	518	Burr & Frey (2007)
	R	GTTGGCATTGAGATCTGC		
ascF–ascG	F	ATGAGGTCATCTGCTCGCGC	789	Wu et al. (2007)
	R	GGAGCACAACCATGGCTGAT		
GCAT	F	CTCCTGGAATCCCAAGTATCAG	237	Nerland (1996)
	R	GGCAGGTTGAACAGCAGTATCT		

results of the sequence obtained matched 99.7% with *A.veronii*. A phylogenetic tree could be constructed with its most similar matches in the GenBank database (Fig. 1), and the sequence was submitted under accession no. FJ573179.

RAPD profile

The RAPD patterns of all isolates were identical and were therefore considered genetically related. The fingerprints of the isolates consisted of 6 to 12 bands ranging from 250 to 2450 bp (Fig. 2). The maximum number of bands was exhibited by the primer OPD-20 (12 bands at positions 1650, 1550, 1150, 1050, 900, 750, 650, 600, 550, 450, 400 and 300), followed by OPA-3 (8 bands at positions 2450, 1150, 900, 850, 750, 700, 600 and 250) and OPA-4 (6 bands at positions 1850, 1650, 1500, 850, 600 and 450).

Phenotypic expression of virulence and detection of virulence genes

All isolates produced amylase, lipase, lecithinase, DNase, caseinase, chitinase and gelatinase, but not elastase. All were haemolytic on human blood agar and produced slime. They were hydrophobic and cytotoxic to the Hep-2 cell line. Major cytopathic effects included rounding, granulation and cell burst (Fig. 3). In the representative strain, *Aeromonas* MCCB 137, the virulence genes such as enterotoxin *act* and haemolytic toxin *aerA*, and type 3 secretion genes such as *aexT*, *ascV* and *ascF-ascG*, and *gcat* could be amplified (Fig. 4).

Antibiotic susceptibility and expression of pathogenicity *in vivo*

All 20 strains isolated in this study exhibited the same antibiotic susceptibility pattern (Table 3) and had a MAR index of 0.3.

Virulence of the representative strain *Aeromonas* MCCB 137 was assessed *in vivo* based on the LD_{50} values in goldfish as the test model, and it was found to be $10^{5.071}$ CFU ml^{-1}. External signs such as reddening at the site of injection appeared in both experimental and control groups as early as 1 h post infection. However, further signs of haemorrhagic scale pockets and loss of scales at the site of injection were only observed in test fish.

Table 2. Phenotypic characterization of the bacterial isolates from oscar (n = 20). MOF: marine oxidation fermentation; F: fermentative; TSI: triple sugar iron; ONPG: ortho-nitrophenyl-β-galactoside

Phenotypic characteristic	Result
Gram stain	–
Motility	+
MOF	F
Kovac's oxidase	+
Catalase	+
O/129 sensitivity	–
Nitrate reduction	+
Indole production	+
Voges-Proskauer reaction	–
Methyl red test	+
Utilization of:	
citrate	–
malonate	–
acetate	–
DL-lactate	–
Reaction on TSI	Alkaline slant/acid butt
Oxidation of ONPG	+
Gluconate oxidation	–
Production of:	
urease	–
alkyl sulphatase	+
pyrazinamidase	+
Hydrolysis of esculin	–
Arginine dihydrolase	+
Lysine decarboxylase	+
Ornithine decarboxylase	–
Acid production from:	
sucrose	+
D-fructose	+
D-mannose	+
D-mannitol	+
D-maltose	+
trehalose	+
dextrin	+
starch	+
D-galactose	+
D-ribose	+
glycerol	+
salicin	–
D-sorbitol	–
L-rhamnose	–
D-melibiose	–
m-inositol	–
raffinose	–
D-lactose	–
adonitol	–
D-cellobiose	+
inulin	–
L-arabinose	–

DISCUSSION

Husbandry of ornamental fish is gaining popularity worldwide, leading to the opening of prospective markets with increased employment opportunities. In accordance with the global scenario, the ornamental fish trade in India has also been showing signs of improvement. Most of the ornamental fishes cultured and marketed in India are exotic species; based on FAO export data, India was ranked 26th in the world in 2004, accounting for 0.5% of world exports (FAO 2006). In India, several governmental agencies such as the Marine Products Development Authority (MPEDA) and Kerala Aqua Ventures International Ltd (KAVIL) have been involved to boost ornamental fish production. As in any other aquaculture practice, the intensification of ornamental fish culture has led to the emergence of diseases and mortality with varied manifestations. In our study, we found that *Aeromonas* spp. were the associated bacterial flora in the majority of disease outbreaks.

Aeromonas spp. are the most frequently encountered bacterial agents associated with fish diseases in freshwater tropical environments (Karunasagar et al. 2003). The presence of virulence and antibiotic-resistance genes in *Aeromonas* isolates from fresh water is probably also responsible for human infections (Henriques et al. 2006, Scoaris et al. 2008). Moreover, there have been reports on zoonoses of infectious *Aeromonas* from fishes following injuries during handling, working in aquaculture systems, or pet fish keeping (Filler et al. 2000, Lehane & Rawlin 2000). In view of these reports, a comprehensive study was undertaken to look into the characteristics, virulence potential and antibiotic susceptibility pattern of *Aeromonas* spp. recovered from diseased ornamental fishes.

Aeromonas spp. is considered to be an opportunistic agent that only provokes clinical signs in stressed fish or fish affected by concurrent infections (van der Marel et al. 2008). The major predisposing stress factors include temperature shock, low oxygen level, high ammonia and other adverse water quality problems (Plumb & Hanson 2010). In our study, the pH in the rearing facility was low (5.8), as were dissolved oxygen (3 mg l^{-1}) and hardness (25 mg l^{-1}). These sub-optimal husbandry conditions in the rearing facility may have made the fish susceptible to the infection, suggesting that in order to avoid an infection from *Aeromonas* in aquaculture, the fish should be maintained free from stressors.

Information on diseases of oscar is fairly sparse, with the exception of several reports on bacterial infections (McCormick et al. 1995, Soltani et al. 1998, Tukmechi et al. 2009) and parasitic infestations (Kim et al. 2002, Toksen 2006). In the present study, the organisms recovered from oscar could be identified as *Aeromonas veronii* biovar *sobria* based on their phenotypic characteristics. However, 16S rRNA gene sequence analysis and multiple alignment with sequences from the GenBank database revealed that the representative isolate *Aeromonas* MCCB 137 exhibited 99.7% similarity with *A. veronii*, and only 98.7% with *A. veronii*

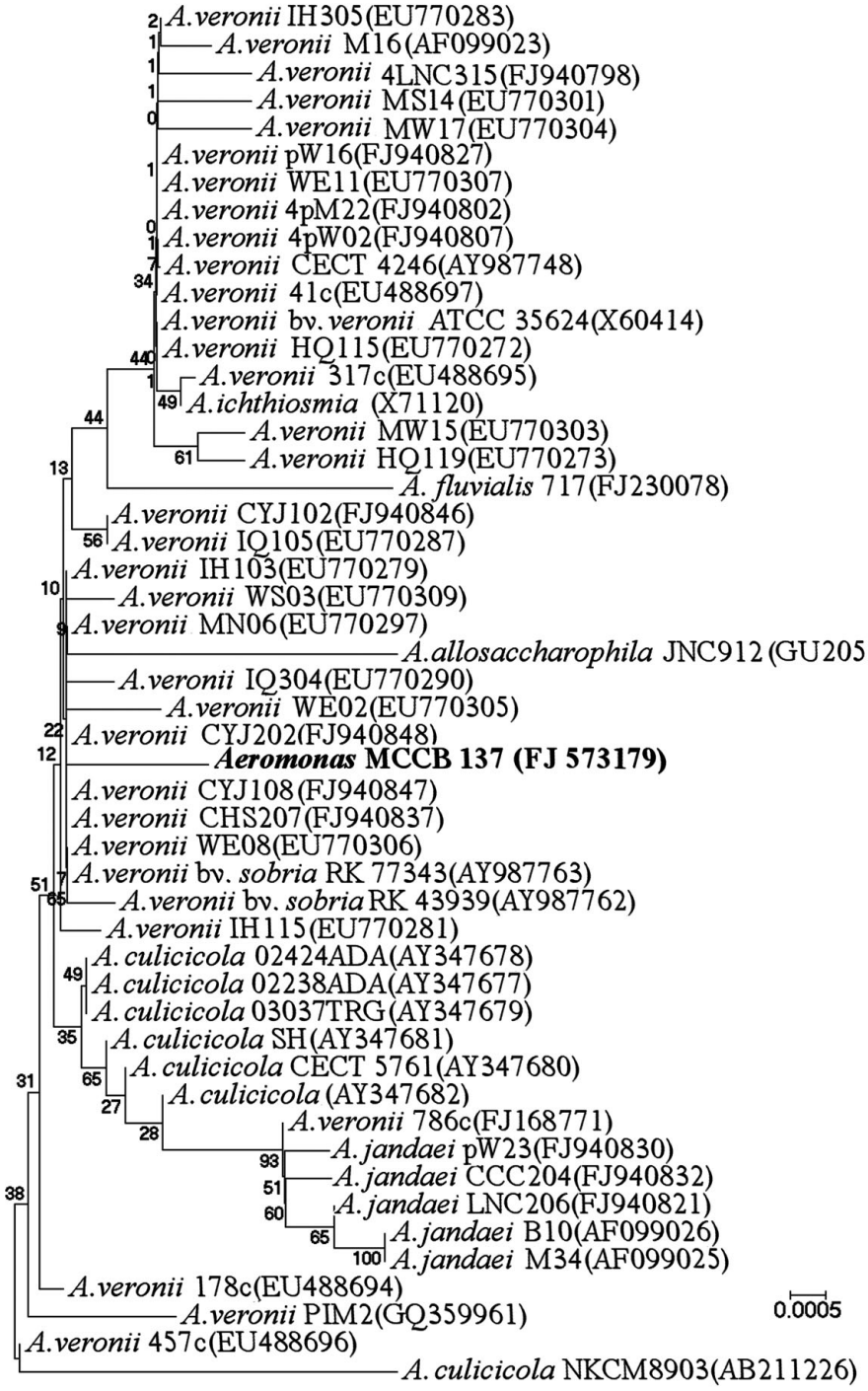

Fig. 1. *Aeromonas* spp. Neighbour-joining phylogenetic tree constructed with the 16S rRNA gene sequences of *A. veronii* MCCB 137 (in bold) with its most similar matches in GenBank using MEGA4. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are shown as the number of base substitutions per site

biovar *sobria*. Nevertheless, all isolates were negative for esculin hydrolysis and ornithine decarboxylase, which is characteristic of *A. veronii* biovar *sobria*. The phylogenetic tree that we constructed indicated the closeness of the isolate *Aeromonas* MCCB 137 with the *Aeromonas* spp. available in GenBank.

In our study, all isolates produced hydrolytic enzymes such as gelatinase, lecithinase, caseinase, chitinase, amylase, lipase and DNase, but not elastase. The cultures exhibited β-haemolysis, slime formation, hydrophobicity and cytotoxicity on the Hep-2 cell line. Extracellular proteases aid the organism in overcoming the initial host defence mechanism such as resistance to serum killing (Leung & Stevenson 1988) and are needed for the maturation of exotoxins such as aerolysin (Howard & Buckley 1985). Lipases play an

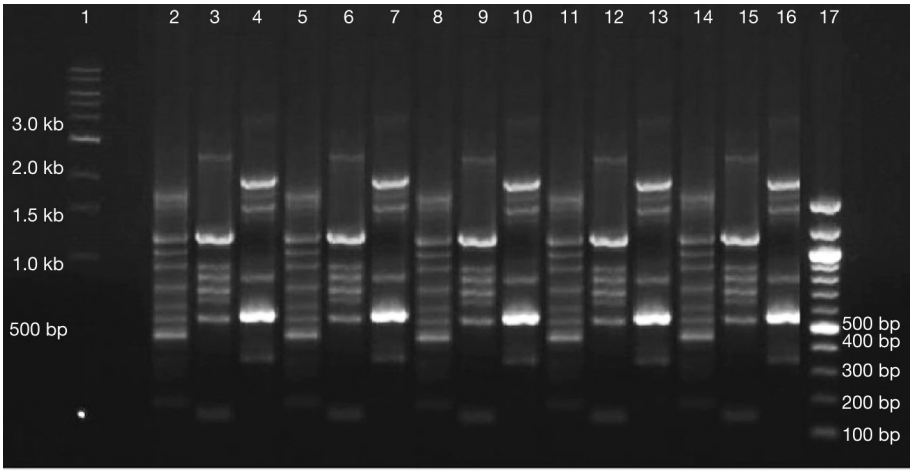

Fig. 2. *Aeromonas veronii.* RAPD profile of isolates from oscar (profiles of AOB-1, AOB-5, AOB-8, AOB-12 and AOB-15 are shown). The profiles were generated by using 3 primers (OPA-3, OPA-4 and OPD-20). Lane 1: 1 kb molecular mass marker; Lanes 2, 5, 8, 11 and 14: RAPD profile using OPD-20; Lanes 3, 6, 9, 12 and 15: RAPD profile using OPA-3; Lanes 4, 7, 10, 13 and 16: RAPD profile using OPA-4; Lane 17: 100 bp molecular mass marker

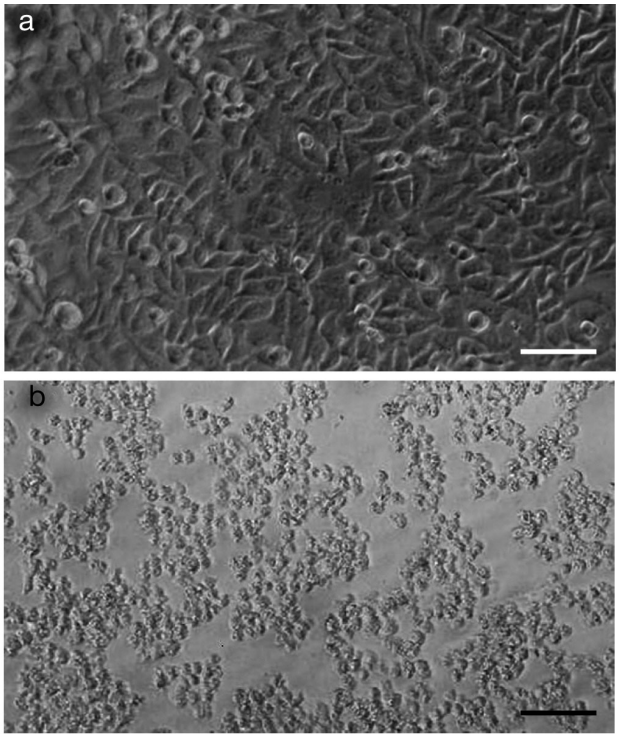

Fig. 3. (a) Normal Hep-2 cell line (Control). Scale bar = 250 µm. (b) Hep-2 cell line subsequent to the addition of the culture supernatant of *Aeromonas* MCCB 113 (125th dilution). Rounding and dislodgement of cells can be seen. Scale bar = 250 µm

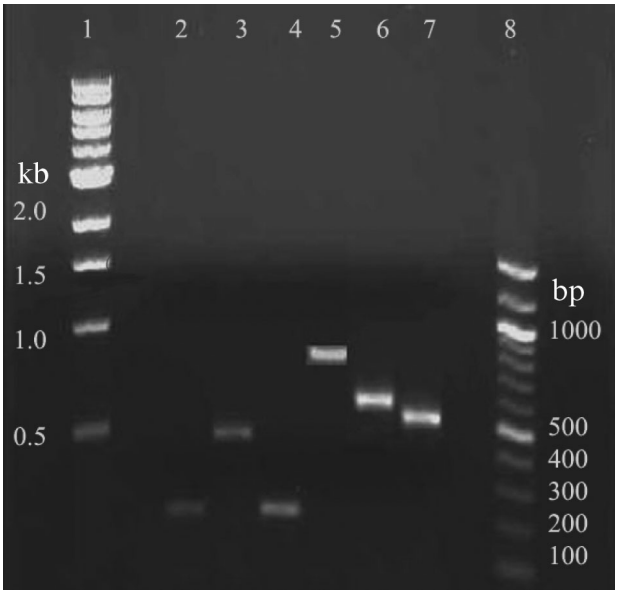

Fig. 4. *Aeromonas veronii.* PCR amplification of virulence genes from the genomic DNA of strain MCCB 137. Lane 1: 1 kb molecular mass marker; Lane 2: *act* (232 bp) gene; Lane 3: *aerA* gene (489 bp); Lane 4: *gcat* gene (237 bp); Lane 5: *ascV* gene (807 bp); Lane 6: *ascF–ascG* gene (789 bp); Lane 7: *aexT* gene (535 bp); Lane 8: 100 bp molecular mass marker

important role in invasiveness and establishment of infections (Timpe et al. 2003). Members of the family *Aeromonadaceae* produce secreted phospholipases, some of which act as haemolysins and some as glycerophospholipid-cholesterol acyl-transferases (Scoaris et al. 2008).

The production of haemolytic toxins has been regarded as strong evidence for the pathogenic potential of aeromonads (Santos et al. 1999). All isolates were β-haemolytic to human red blood cells, strongly supporting virulence as suggested by Hazen et al. (1978).

Table 3. *Aeromonas veronii.* Antibiotic susceptibility pattern of strains (n = 20) isolated from oscar. Disc content is in µg unless given as U. R: resistant; S: susceptible

Antibiotic group	Antibiotic	Disc content	Result
Beta lactams	Amoxicillin	10	R
	Ampicillin	10	R
	Methicillin	5	R
	Oxacillin	1	R
	Piperacillin	100	S
	Penicillin G	10 U	R
	Carbenicillin	100	S
	Cefaclor	30	S
	Cephadroxil	30	S
	Cephalothin	30	S
	Cefoperazone	75	S
	Cloxacillin	1	R
	Cefazolin	30	S
	Cephalexin	30	S
	Cephradine	25	S
	Cephoxitin	30	S
	Ceftizoxime	30	S
	Ceftazidime	30	S
	Cephalodrine	10	S
	Cefamandole	30	R
	Ceftriaxone	10	S
	Ticarcillin	75	R
	Imipenam	10	S
Glycopeptides	Vancomycin	5	R
Polypeptides	Bacitracin	10 U	R
	Polymixin B	50 U	R
	Colistin	10	R
Aminoglycosides	Amikacin	10	S
	Gentamycin	10	S
	Kanamycin	30	S
	Neomycin	30	S
	Netillin	10	S
	Streptomycin	10	S
	Tobramycin	10	S
Tetracyclines	Doxyxycline HCl	10	R
	Chlortetracycline	30	S
	Minocycline	30	S
	Oxytetracycline	30	R
	Tetracycline	10	R
Chloramphenicols	Chloramphenicol	10	S
Rifamycins	Rifampicin	2	S
Lincosamides	Clindamycin	2	R
	Lincomycin	2	R
Steroids	Fusidic acid	10	R
Nitrofurans	Nitrofurazone	100	S
	Furazolidone	50	S
	Furaxone	100	S
Sulphonamides	Trimethoprim	5	S
	Sulphadiazine	100	S
	Sulphafurazole	300	S
	Sulphaphenazole	200	S
Quinolones/ Fluoroquinolones	Ciprofloxacin	5	S
	Enrofloxacin	5	S
	Floxidine	20	S
	Pipemidic acid	20	S
	Nalidixic acid	30	R
	Nitroxoline	30	S
	Norfloxacin	10	S
	Ofloxacin	2	S
	Pefloxacin	5	S
	Sparfloxacin	5	S
Aminocoumarins	Novobiocin	30	S
Nitrofurantoin	Nitrofurantoin	100	S
Fosfomycin	Fosfomycin	50	S
Macrolides	Azithromycin	15	S
	Clarithromycin	15	S
	Erythromycin	10	R
	Tylosine	15	S
	Oleandomycin	15	R
	Spiramycin	30	S

High cell surface hydrophobicity is considered an advantage in the colonization of mucosal surfaces by bacteria and formation of biofilm and adhesion to epithelial cells (Scoaris et al. 2008). All isolates of *Aeromonas* studied here were strongly hydrophobic, with the percentage of adhesion being 56.9 ± 0.311% (Lee & Yii 1996). Slime production reflects a microorganism's capacity to adhere to specific host tissues and thereby to produce invasive micro colonies (Lilenbaum et al. 1998) and diverse illnesses (Tenover et al. 1988). All isolates of *Aeromonas* produced black colonies in Congo red agar with dry crystalline consistency and were regarded as slime positive.

The isolates were cytotoxic to the Hep-2 cell line. One of the most important virulence properties of haemolysins and enterotoxin of aeromonads involves exhibition of cytotoxicity *in vitro* (Ghatak et al. 2006). Microscopic examination of the cell line following exposure to and incubation with cell-free supernatant revealed cytopathic effects including rounding, shrinkage of cytoplasm and dislodgement of cells (Fig. 3).

In our study, all isolates showed uniformity in phenotypic characteristics, hydrolytic potential and RAPD pattern. Therefore, we selected 1 representative strain (*Aeromonas veronii* MCCB 137) for screening virulence genes and for the infection trial. *Aeromonas* MCCB 137 has the enterotoxin gene *act*, the haemolytic toxin genes *aerA* and *gcat*, and the type 3 secretion genes *aexT*, *ascF–ascG* and *ascV*, confirming its potential pathogenicity. All of these virulence genes encode for secreted enzymes and toxins that contribute to the pathogenicity of the organism. Earlier studies suggested that *act* possessed multiple biological activities, including haemolysis, cytotoxicity and lethality when injected into mice (Chopra et al. 2000, Sha et al. 2005). Aerolysin is an extracellular, soluble and hydrophilic protein exhibiting both haemolytic and cytolytic properties and is considered a putative virulence factor related to the pathogenicity of several *Aeromonas* strains (Biscardi et al. 2002, Epple et al. 2004). GCAT has lipase or phospholipase activity and could cause erythrocyte lysis by digesting their plasma membrane (Pemberton et al. 1997).

The genes encoding effector proteins, translocated by the TTSS, disrupt the cytoskeleton or interfere with cell signalling cascades of eukaryotic host cells (Galan & Wolf-Watz 2006). *aexT* is a bifunctional protein with ADP-ribosylating activity and guanosine triphosphatase-activating protein (GAP) activity (Fehr et al. 2007). *aexT* mediates ADP-ribosylation of both muscular and non-muscular actin *in vitro* (Fehr et al. 2007), thereby preventing polymerization (Masignani et al. 2006). It also induces morphological changes in fish cells, including cell rounding and subsequent lysis

(Fehr et al. 2007, Vilches et al. 2008). *ascV* is a gene that serves as an indicator for the presence of the TTSS machinery (Stuber et al. 2003), which encodes an inner membrane component of the TTSS apparatus, and *ascF–ascG* encode the needle complex and a chaperone, respectively (Ghosh 2004). Several previous studies have examined the prevalence of the TTSS among *Aeromonas veronii* isolates (Chacon et al. 2004, Burr et al. 2005). In our study, *ascV*, *ascF–ascG* and *aexT* were detected, as was also reported by Silver & Graf (2009).

The LD_{50} value of $10^{5.071}$ CFU ml^{-1} confirms the virulence of *Aeromonas* MCCB 137 according to Lallier & Daigneault (1984). At the site of injection of the pathogen, ulceration commenced as sloughing off of the scales, followed by the occurrence of a haemorrhagic spot which progressed to form an epidermal lesion as observed earlier by Harikrishnan et al. (2003).

As *Aeromonas* spp. are zoonotic, it is imperative to combat the organism in the rearing facility, and for this purpose, we performed antimicrobial susceptibility tests on the organisms. As antibiotic susceptibility varies among *Aeromonas* species, it is better to identify the organism to species level prior to administration of the drugs (Trakhna et al. 2009). The multiple-antibiotic resistances of the isolates suggest the potential loss of fish including brood stock, as antibiotics might not protect a population from infectious death. The MAR index >0.2 suggests that the isolates originated from a high-risk source of antibiotic contamination. The widespread antibiotic use in the ornamental fish culture facilities might have resulted in the rapid spread of multidrug-resistant pathogens across the systems.

Here we characterized *Aeromonas veronii* associated with abdominal dropsy of oscar. The isolates possessed several virulence factors that could potentially play an important role in the development of dropsy in fishes. To our knowledge, this is the first report of virulent and multiple antibiotic resistant *A. veronii* associated with dropsy in oscar.

Acknowledgements. We thank the Marine Products Export Development Authority (MPEDA), Ministry of Commerce and Industry, Government of India (project code: 3/3/OFD/HO/2003 dated 25-02-2004), and the Department of Biotechnology (DBT), Government of India (project code: BT/PR4012/AAQ/03/204/2003), for financial support. K.S. thanks MPEDA for a fellowship.

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Editorial responsibility: Roland Anderson, Seattle, Washington, USA

Submitted: August 2, 2010; Accepted: October 18, 2010
Proofs received from author(s): February 15, 2011