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Characterization and virulence potential of phenotypically diverse *Aeromonas veronii* isolates recovered from moribund freshwater ornamental fishes of Kerala, India

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Abstract In the present study, we investigated the involvement of *Aeromonas* spp. in eliciting disease outbreaks in freshwater ornamental fishes across the state of Kerala, India. We investigated three incidences of disease, in which the moribund fishes exhibited clinical signs such as haemorrhagic septicemia (in gouramy, *Trichogaster* sp.), dropsy (in Oscar, *Astronotus ocellatus*) and tail rot/fin rot (in gold fish, *Carassius carassius*). Pure cultures ($n = 20$ from each fish; 60 in total) of *Aeromonas* spp. were recovered from the abdominal fluid as well as from internal organs of affected fishes, although they could not be identified to species level because of the variations in their phenotypic characters. The molecular fingerprinting of the isolates using Enterobacterial Repetitive Intergenic Consensus PCR proved the genetic diversity of the isolates from the three sites. The phylogenetic trees constructed using concatenated sequences (using 16S rRNA, *gyrA*, *gyrB* and *rpoD* genes) indicated that they were related to *Aeromonas veronii*. They exhibited marked cytotoxic and haemolytic activity, which were responsible for the pathogenic potential of the isolates. The isolates possessed multiple virulence genes such as enterotoxins (*act* and *alt*), haemolytic toxins (*aerA* and *hlyA*), genes involved in type III secretion system (*ascV*, *aexT* and *ascF–ascG*), glycerophospholipid-cholesterol acyltransferase (*gcat*) and a type IV pilus (*tapA*) gene, as determined by PCR. Virulence of representative isolates to goldfish was also tested, and we found LD_{50} values of $10^{4.07}$–$10^{5.35}$ cfu/fish. Furthermore, the organisms could be recovered as pure cultures from the lesions as well as from the internal organs.

Keywords *Aeromonas veronii* · Ornamental fishes · Phenotypic diversity · Virulence potential

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Introduction

Diseases are the primary constraints to the culture of many aquatic species, impeding both economic and social developments and a significant constraint on aquaculture production and trade (Smith 2006). It is recognized that bacteria are one of the important causative agents of fish diseases (Yesmin et al. 2004) and motile aeromonads represent the most frequently

encountered bacterial agents associated with fish diseases in freshwater tropical environments (Karunasagar et al. 2003).

The genus *Aeromonas* (family *Aeromonadaceae*, class Gammaproteobacteria) includes bacteria that are considered autochthonous to aquatic environments and have recently been considered emerging pathogens, causing a multitude of diseases in many species of animals, including fishes and humans (Janda and Abbott 2010; Parker and Shaw 2011). Currently there are 25 described and accepted *Aeromonas* species (Figueras et al. 2011). The pathogenicity of *Aeromonas* spp. is complex and multifactorial, and is attributed to multiple potential virulence factors. They possess a variety of biologically active substances such as cell structural lipopolysaccharides, outer-membrane proteins, pili and flagella, and extracellular factors such as enzymes and toxins (Von Gravenitz 2007; Janda and Abbott 2010).

Of the *Aeromonas* species, *Aeromonas veronii* appears to have the greatest host range in virulence and also has been reported to cause wound infections, diarrhea and septicemia in humans (Janda and Abbott 2010; Silver et al. 2011). In fish, *A. veronii* has been reported to be an opportunistic pathogen and also a digestive-tract symbiont of zebra fish and medicinal leech (Rahman et al. 2002; Bates et al. 2006; Graf et al. 2006; Hossain 2008; Ma et al. 2009). *A. veronii* has been divided into two biovars, *A. veronii* bv. *veronii* and *A. veronii* bv. *sobria*, with the latter being considered more virulent (Janda and Abbott 2010). It has been reported that *A. veronii* bv. *sobria* is negative for esculin hydrolysis and ornithine decarboxylase, whereas *A. veronii* bv. *veronii* is positive for these reactions (Altwegg et al. 1990, Carnahan and Altwegg 1996). Moreover, *A. veronii* bv. *veronii* is negative for arginine dihydrolase, produces acid from salicin and utilizes tartrate (Abbott et al. 2003).

The aquarium fish industry constitutes a large proportion of the pet animal industry having global marketing network. However, diseases in different stages of production, transportation and marketing have been a major stumbling block in its progress. The objective of the present study was to characterize phenotypically and genotypically *Aeromonas* strains associated with specific diseases commonly observed in freshwater ornamental fishes and to determine the presence of genes encoding virulence factors and virulence potential through in vivo assays.

Materials and methods

Diseased fish

While implementing a disease surveillance programme in fresh water ornamental fishes across the state of Kerala, we came across several incidents of disease outbreaks, the majority of which were with clinical signs such as haemorrhagic septicemia, dropsy and tail and fin rot, at three locations. The gouramy with haemorrhagic septicemia was collected from an ornamental fish farm (earthen pond) located in Trichur District, Kerala, India. In this facility, mortality of fishes commenced subsequent to their transfer to cement tanks for sale. The fishes were found swimming around nearer to water surface with petechial haemorrhage. The moribund Oscar with dropsy was collected from M/S Marvel Aquarium Systems, Cochin, Kerala, which displayed distended abdomen and hemorrhages on the body surface. On postmortem examination, ascitic fluid was found amber coloured. Tail and fin rot in goldfish were noticed in a home aquarium in Cochin. The affected fishes showed gradual and progressive erosion of fin rays.

Measurement of water quality parameters

To identify the relationships between the water quality parameters and the incidence of disease outbreaks, temperature, pH, dissolved oxygen, hardness, ammonia, and nitrite of three affected ornamental fish culture systems were measured. Water samples (100 mL) were collected according to standard methods for examination of water and wastewater (APHA 1995) in plastic bottles, kept in ice and examined within 24 h.

Isolation of the associated bacteria and phenotypic characterization

Moribund animals were surface disinfected with 100 mg/L available chlorine in the form of sodium hypochlorite. Samples were aseptically taken from the body fluid (in the case of Oscar) and the internal organs (in the case of gouramy and goldfish) such as kidney, liver and spleen and plated onto nutrient agar (g/L peptone, 5.0; beef extract, 5.0; NaCl, 5.0; agar, 20.0; pH 7.5 ± 0.3) after serial dilution in

Table 1 Comparison of phenotypic characteristics of three groups of *Aeromonas* strains isolated from moribund ornamental fishes with the type strains of *A. veronii* bv. *sobria* CECT 4246^T and *A. veronii* bv. *veronii* CECT 4257^R

Phenotypic characteristics	Fish isolates			Type strains[b]	
	$N = 20$ represented by MCCB 137	$N = 20$ represented by MCCB 140	$N = 20$ represented by MCCB 142	*A. veronii* bv. *sobria*	*A. veronii* bv. *veronii*
Gas from glucose	–	–	+	+	V
Triple sugar iron agar[a]	Acid/acid	Acid/acid	Alk/acid	NA	NA
Citrate utilization	–	–	+	V	+
Utilization of acetate	–	+	+	+	V
Utilization of tartrate	+	+	+	–	+
Gluconate oxidation	–	+	+	V	V
Voges-proskauer	–	+	+	+	V
Methyl red test	+	–	–	NA	NA
Arginine dihydrolase	+	+	+	+	–
Hydrolysis of esculin	–	–	+	–	+
Acid from					
L-Arabinose	–	–	+	V	–
D-Cellobiose	+	–	+	V	V
Salicin	–	–	+	–	+
D-Raffinose	–	–	+	V	–
Melibiose	–	–	+	V	–

Positive reactions shown by the fish isolates: production of kovac's oxidase, indole and catalase, nitrate reduction, oxidation of ONPG, utilization of tartrate, production of alkyl sulfatase and pyridaminase, production of arginine dihydrolase and lysine decarboxylase, growth in 0 and 3 % NaCl, production of DNase, gelatinase, and lipase, acid production from sucrose, fructose, D-mannose, D-mannitol, D-maltose, trehalose, dextrin, starch, D-galactose, D-ribose and glycerol. *Negative reactions shown by the isolates*: utilization of malonate and DL-lactate, production of urease, elastase and ornithine decarboxylase, O/129 sensitivity, growth in 6, 8 and 10 % NaCl, acid production from sorbitol, L-rhamnose, m-inositol, D-lactose, D-arabinose, adonitol, arabitol, dulcitol, erythritol and inulin

NA not available, *V* variable

[a] Slant/Butt

[b] Data taken from Abbott et al. (2003)

physiological saline (0.85 % NaCl in distilled water). The plates were incubated at 28 °C for 24–48 h.

After incubation, colonies were isolated at random from the plates onto nutrient agar slants and their purity was examined by repeated streaking on nutrient agar plates, both prepared in distilled water. Subsequent to confirming purity, the isolates were characterized based on their morphological, physiological and biochemical traits as described by Abbott et al. (2003) (Table 1).

Molecular characterization

Identification of the isolates

As all isolates were phenotypically and genotypically identical (as judged by ERIC PCR as shown below), one representative culture from each disease incident (*Aeromonas* sp. MCCB 137 from Oscar, *Aeromonas* sp. MCCB 140 from gourami, *Aeromonas* sp. MCCB 142 from gold fish) was chosen for molecular

Table 2 Primers used for the amplification of 16S rRNA and house keeping genes

Gene	Primer Sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference
16S rRNA	16S1: GAGTTTGATCCTGGCTCA	1,500	58	Reddy et al. (2000)
	16S2: ACGGCTACCTTGTTACGACTT			
gyrA	F: TCCTATCTTGATTACGCCATG	441	55	Goni-Urriza et al. (2002)
	R: CATGCCATACCTACCGCGAT			
gyrB	gyrB3F: TCCGGCGGTCTGCACGGCGT	1,100	57	Yanez et al. (2003)
	gyrB14R :TTGTCCGGGTTGTACTCGTC			
rpoD	70Fs: ACGACTGACCCGGTACGCATGTA	820	57	Yamamoto et al. (2000)
	70Rs: ATAGAAATAACCAGACGTAAGTT			

characterization. A polyphasic approach using the 16S rRNA gene and the house keeping genes *gyrA*, *gyrB* and *rpoD* was used. Genomic DNA of the isolates was extracted by the phenol–chloroform method (Sambrook and Russell 2001). Previously described primers and PCR conditions were used as shown in the Table 2.

The PCR reaction was performed in a DNA thermal cycler (Eppendorf AG, Hamburg, Germany) with the reaction mixture (final volume 25 µL) containing 2.5 µL 10× buffer, 1.5 µL of 25 mM $MgCl_2$, 1.0 µL of 10 pmol of forward and reverse oligonucleotide primers, 1.0 µL of DNA template, 2 µL of 2.5 mM each deoxynucleoside triphosphate and 1 µL of *Taq* DNA polymerase (New England Bio Labs).

The PCR products were analyzed by electrophoresis on 1.5 % agarose gel prepared in 1× TAE buffer and stained with ethidium bromide (0.5 µg/mL), visualized under UV and digitalized with a Gel Doc™ XR + Image System (Bio-Rad, USA).

Nucleotide sequencing was performed using an ABI PRISM 3700 Big Dye Sequencer using the specific primers. The sequences obtained were aligned with the sequences of type and references strains of all members of the genus *Aeromonas* that were available in GenBank (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore) by using the CLUSTAL W program, version 1.83 (Thompson et al. 1994).

The multi-locus phylogenetic analysis (MLPA) of the house keeping genes was performed and their alignments were concatenated using the DAMBE program (Xia and Xie 2001). The evolutionary tree was constructed using the maximum likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei 1993) implemented in MEGA 5 (Tamura et al. 2011). Stability of relationships was assessed by bootstrapping (500 replicates).

Phylogenetic trees of 16S rRNA gene, *gyrA*, *gyrB* and *rpoD* were also constructed by the ML method using the MEGA 5 (Tamura et al. 2011). Stability of relationships was assessed by bootstrapping (500 replicates).

Molecular fingerprinting

The molecular typing of all 60 isolates was performed by ERIC PCR, using the primers ERIC 1 (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC 2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) (Versalovic et al. 1991). The fingerprinting profiles were visualized on 1.2 % agarose gels in TAE buffer at a constant current of 60 mA.

Nucleotide accession numbers

16S rRNA, *gyrB*, *rpoD* and *gyrA* gene sequences of representative isolates were deposited in the Genbank nucleotide sequence databases with the following accession numbers: FJ573179, JN602735, JQ403281 and JQ319807 respectively for *Aeromonas* sp. MCCB 137; JN559374, JN602734, JQ403282 and JQ319808 respectively for *Aeromonas* sp. MCCB 140; JN559373, JN602733, JQ403283 and JQ319809, respectively for *Aeromonas* sp. MCCB 142.

Evaluation of the isolates' virulence

In vitro assays for the phenotypic expression of virulence

Haemolytic activity of the isolates was performed on Luria–Bertani (LB) agar containing 5 % (vol/vol) human blood (Swift et al. 1999). The cytotoxicity of the culture supernatant was examined on an exponentially growing Hep-2 (Human laryngeal epithelial) cell line by incubating with the bacterial cell free supernatant followed by a methyl thiazolyl diphenyl-tetrazolium bromide (MTT) assay (Mosmann 1983; Greenman et al. 1997). The relative hydrophobicity of bacterial cells was determined by microbial adhesion to hydrocarbons (MATH), with xylene as the hydrocarbon choice (Rosenberg et al. 1980; Scoaris et al. 2008). The other virulent traits tested include slime formation using the congo red agar (Freeman et al. 1989), agglutination in 0.2 % acriflavine, auto-agglutination and precipitation after boiling (Janda 1987), crystal violet binding (Paniagua et al. 1990) and suicidal activity using nutrient broth containing 0.5 % glucose (Namdari and Bottone 1988). Quantitative determination of biofilm production was carried out using a crystal violet adherence assay on 96-well tissue culture plates as described previously (Peeters et al. 2008; Zmantar et al. 2010). Statistical data were expressed as mean values ± standard deviation (SD).

Screening for virulence genes

The representative isolates, *Aeromonas* sp. MCCB 137, MCCB 140 and *Aeromonas* sp. MCCB 142 were subjected to PCR to detect virulence genes such as enterotoxins [(cytotoxic enterotoxin gene (*act*), heat labile cytotonic enterotoxin gene (*alt*) and heat-stable cytotonic enterotoxin gene (*ast*)], haemolytic toxins [*hlyA* and *aerA*], genes involved in TTSS [*ascV*, *aexT*, *aopP*, *aopO*, *ascF–ascG*, and *aopH*], *gcat* and *tapA* gene (Kingombe et al. 1999; Ormen and Ostensvik 2001; Heuzenroeder et al. 1999; Burr and Frey 2007; Wu et al. 2007; Nerland 1996; Barnett et al. 1997). Previously described primers and PCR conditions were used for the specific amplification of virulence genes as summarized in Table 3. The PCR products were visualized in 1.5 % agarose gel stained with ethidium bromide.

Pathogenicity test in vivo

The in vivo pathogenicity of the *Aeromonas* isolates MCCB 137, MCCB 140 and MCCB 142 was tested in gold fish. The fishes (weighing about 10–15 g) were anaesthetized using clove oil (80 mg/L). LD_{50} tests with batches of seven fish per dose were conducted by intramuscular injection of 0.1 mL of a physiological saline suspension of the pathogen (10^4–10^8 cfu/fish). One group served as control, which were injected with 0.1 mL saline. Morbidity and death of the fishes were monitored daily for seven days and the moribund specimens were subjected for routine bacteriological examination for the re-isolation of the microorganism. The LD_{50} was calculated following the Reed and Muench (1938) methodology.

Results

Measurement of water quality parameters

The deterioration of water quality parameters often contributes to the development of diseases in aquaculture systems. In our study, it was noticed that the pH in the Oscar fish rearing facility was low (5.8) in combination with low dissolved oxygen (3 mg/L) and hardness (25 mg/L). In the case of the site containing infected goldfish with tail and fin rot, the pH was alkaline (8.3) with subsequent increase in ammonia level (3 mg/L). However, the water quality parameters in the gouramy rearing facility appeared normal. In this system mortality of gouramy started immediately after they were harvested using hand nets.

Phenotypic characterization

Pure cultures of bacteria could be isolated from the diseased fishes (Oscar, gouramy and goldfish) analyzed in each disease incidence. A total of 60 colonies were randomly selected (20 from each disease incidence). All the cultures were of uniform colony morphology (circular, entire, convex, and opaque light brown) and were motile Gram-negative short rods. Of the 58 biochemical characteristics evaluated for all 60 isolates of the putative *Aeromonas*, 45 tests (77 %) yielded uniform results. The remaining 13 tests produced variable results dividing the isolates into 3 distinct phenogroups, consistent with their source of

Table 3 Primers used for the amplification of virulence factor genes

Gene	DNA sequences (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference
act	AGAAGGTGACCACCAAGAACA AACTGACATCGGCCTTGAACTC	232	55	Kingombe et al. (1999)
ast	TCTCCATGCTTCCCTTCCACT GTGTAGGGATTGAAGAAGCCG	331	55	Kingombe et al. (1999)
alt	TGACCCAGTCCTGGCACGGC GGTGATCGATCACCACCAGC	442	55	Kingombe et al. (1999)
aerA	CCCGCCGATCTGCAACCGGG CTGGTCTGGATAGACGGGCTCTGCC	489	68	Ormen and Ostensvik (2001)
hlyA	GGCCGGTGGCCCGAAGATACGGG GGCGGCGCCGGACGAGACGGG	597	62	Heuzenroeder et al. (1999)
aexT	GGCGCTTGGGCTCTACAC GAGCCCGCGCATCTTCAG	535	55	Burr and Frey (2007)
ascV	GCCCGTTTTGCCTATCAA GCGCCGATATCGGTACCC	807	55	Burr and Frey (2007)
*aop*P	GAGAGTTGGCTAGCGGTGAG TCCTCATGGAGCGCATCCAG	490	58	Burr and Frey (2007)
*aop*O	CGAGACAGACAAGTTTGC TGTCGTTGTGGACTATCC	401	55	Burr and Frey (2007)
*aop*H	TCAATCAGGACGATGTCG GTTGGCATTGAGATCTGC	518	55	Burr and Frey (2007)
*asc*F–*asc*G	ATGAGGTCATCTGCTCGCGC GGAGCACAACCATGGCTGAT	789	55	Wu et al. (2007)
gcat	CTCCTGGAATCCCAAGTATCAG GGCAGGTTGAACAGCAGTATCT	237	55	Nerland (1996)
tapA	ATGACCTCTAGCCCCAATA ACCCGATTGATTTCTGCC	550	55	Barnett et al. (1997)

isolation (Table 1). However, the putative *Aeromonas* isolates could not be identified to species level because of the diversity in their phenotypic characters. An isolate was chosen as representative of the cultures obtained from each of the diseased fish type. Accordingly, the isolate MCCB 137 represented strains obtained from Oscar; MCCB 140 represented strains from gouramy and MCCB 142 represented strains from goldfish. Uniformity of these isolates was confirmed by ERIC PCR (as shown below).

Molecular characterization

Identification of the isolates

The 16S rRNA gene nucleotide sequences of *Aeromonas* isolates MCCB 137, 140 and 142 shared 99.9 % similarity with each other. The overall nucleotide sequence similarity of the selected housekeeping genes of these representative isolates ranged between 97.9 and 99.2 %. The MLPA tree (Fig. 1) revealed that the three strains *Aeromonas* sp. MCCB 137, 140 and 142 recovered from the moribund ornamental fishes were phylogenetically related to *A. veronii*. However, the phenotypic characteristics were not sufficient to differentiate which of the biovars of *A. veronii* (*sobria* or *veronii*) were present.

The phylogenetic trees of the 16S rRNA, *gyrA*, *gyrB* and *rpoD* gene sequences were constructed with their most similar matches in the GenBank database (Supplementary Fig. 1a–d). These trees also showed the same pattern as MPLA tree.

Molecular finger printing

Recently, molecular techniques have been used as typing procedures for epidemiological studies of clinical and environmental *Aeromonas* strains, and in our study ERIC PCR was employed. The ERIC PCR profiles of all the isolates of *Aeromonas* spp. resulted

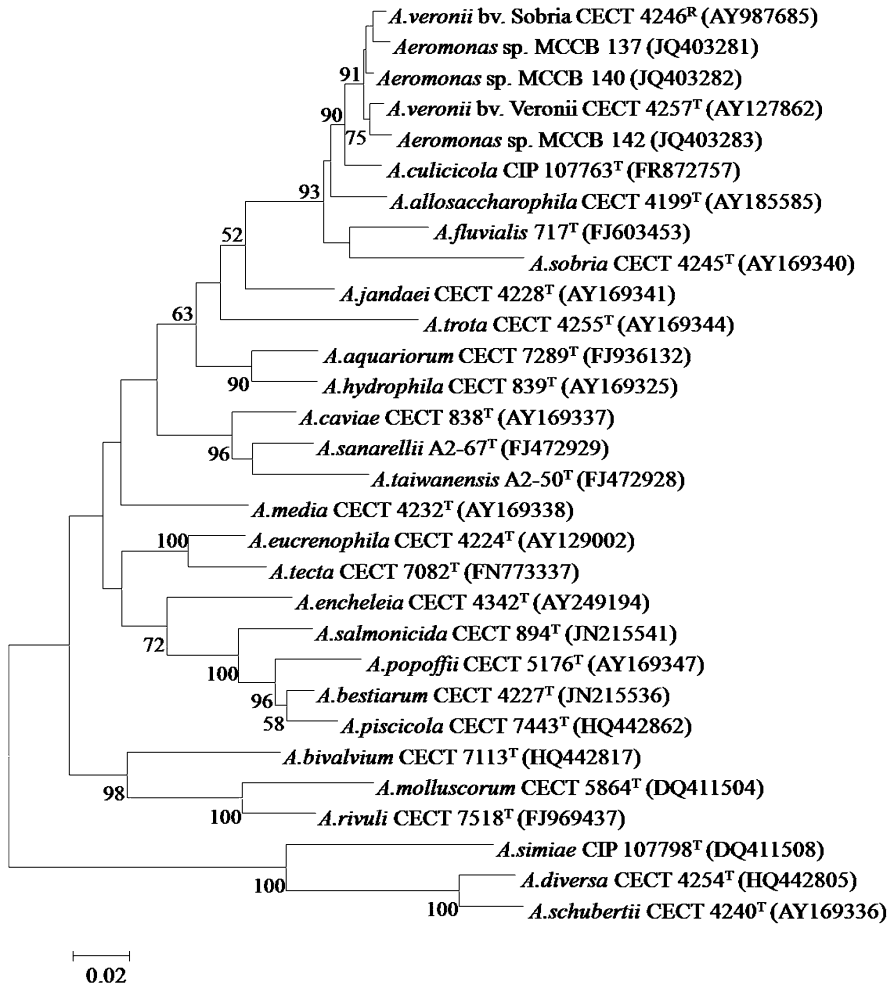

Fig. 1 Unrooted ML phylogenetic tree derived from MLPA (*gyrA*, *gyrB* and *rpoD*) showing the relationships of strains *Aeromonas* sp. MCCB 137, MCCB 140 and MCCB 142 to currently known species of *Aeromonas*. The phylogenetic tree was constructed with a concatenated sequence of 2472 nt using MEGA 5. Bootstrap values (>50 %) based on 500 replicates are shown at nodes. *Numbers in the parenthesis* represents accession numbers. *Bar* 0.02 substitutions per nucleotide position

in 5–11 bands ranging from 100 to 2,500 bp (Supplementary Fig. 2a–c). The ERIC PCR confirms that each isolate chosen to represent the 20 isolates from each fish source was indeed representative of the strains isolated. Moreover, the profiles confirmed that each three sets of isolates was genetically distinct.

Phenotypic expression of virulence characteristics—In vitro assays

It is important in pathology to differentiate pathogenic strains from non-pathogens and in the present study certain in vitro assays were performed, which are indirectly correlated with the virulence characteristics of *Aeromonas* isolates. All strains were haemolytic on human blood agar and produced slime. They were non-suicidal, cytotoxic on Hep-2 cell line and exhibited auto-agglutination. Microscopic examination of a human cell line following exposure to and incubation with cell free supernatant revealed cytopathic effects including rounding, shrinkage of cytoplasm and dislodgement of cells (Fig. 2). The isolates did not agglutinate in 0.2 % acriflavine and displayed precipitation after boiling and crystal violet binding in varying proportions. Furthermore, they showed varying degree of biofilm formation and hydrophobicity indices (Table 4).

Detection of virulence genes

A prerequisite to understand the pathogenicity mechanisms of an organism is the identification and

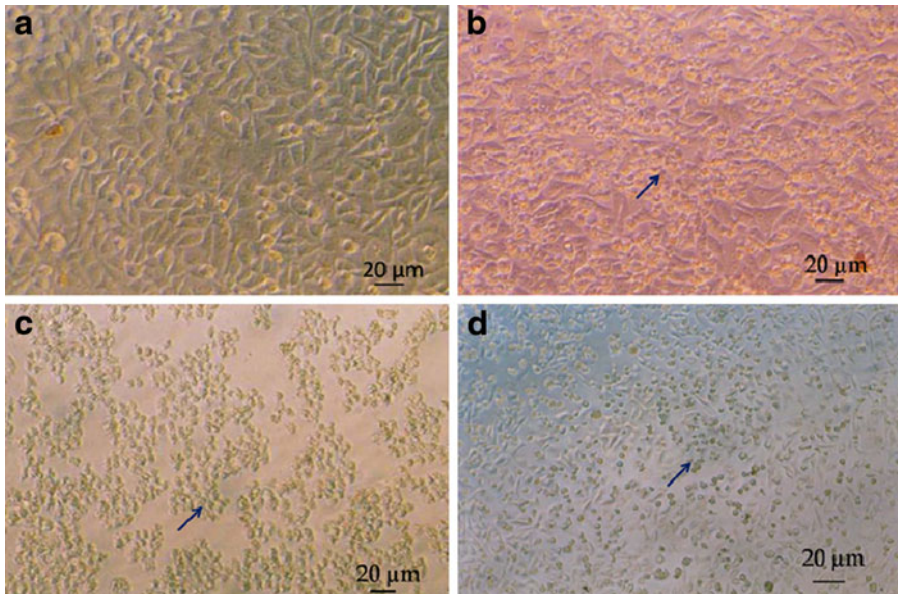

Fig. 2 Cytotoxicity of bacterial culture supernatant on Hep-2 cell line. **a** Normal Hep-2 cell line (Control); **b–d** Hep-2 cell line subsequent to the addition of the culture supernatant of *Aeromonas* sp. MCCB 137 (3,125th dilution), *Aeromonas* sp. MCCB 140 (625th dilution) and *Aeromonas* sp. MCCB 142 (1,000th dilution) respectively. Rounding, dislodgement of cells and granulation can be seen (*arrows*)

Table 4 In vitro assays for the phenotypic expression of virulence-related traits

Assay	Isolates		
	MCCB 137	MCCB 140	MCCB 142
Precipitation after boiling (PAB)	+	–	–
Relative degree of precipitation (RDP)	0.683 ± 0.07	–	–
CSH index[a]	56.9 ± 0.311	53.87 ± 0.269	37.08 ± 0.544
Biofilm index[b]	0.10 ± 0.02	0.27 ± 0.01	0.40 ± 0.01
Crystal violet binding	–	+	–

The data were expressed as mean values ± SD

[a] The values of strong, moderate and negative hydrophobicity degree were >50 %, 20–50 % and <20 % respectively (Lee and Yii 1996)

[b] Biofilm formation was categorized as strong ($OD_{570} \geq 0.5$), medium ($OD_{570} \geq 0.2$ to <0.5) and weak (OD_{570} 0 to <0.2) (Ando et al. 2004)

examination of its virulence genes (Strauss and Falkow 1997). In the present study, PCR amplification of the genes for virulence factors from the *Aeromonas* sp. MCCB 137, 140 and 142 was attempted. They shared the enterotoxin gene *act*, haemolytic toxin genes *aerA* and *hlyA*, TTSS genes such as *aexT*, *ascV* and *ascF-ascG*, and *gcat.* However, the enterotoxin gene *alt* could be amplified from *Aeromonas* sp. MCCB 140 and MCCB 142 only and the *tapA* gene from *Aeromonas* sp. MCCB 137 and MCCB 140 only. None of the isolates showed the presence of the enterotoxin gene *ast* and TTSS genes such as *aopP*, *aopO* and *aopH*.

Expression of pathogenicity in vivo

Virulence of *Aeromonas* strains MCCB 137, 138 and 142 was assessed in vivo based on the LD_{50} values in

Table 5 In vivo pathogenicity of *Aeromonas* strains MCCB 137, MCCB 140 and MCCB 142 in goldfish

Bacterial dose (cfu/fish)	Mortality (%)		
	MCCB 137	MCCB 140	MCCB 142
10E8	100	100	100
10E7	100	100	95.6
10E6	100	94.1	88.9
10E5	84.6	78.6	76.9
10E4	58.3	53.8	50
Control (0.85 % saline)	0	0	0
LD_{50} (cfu/fish)	10E4–10E5 ($10^{4.07}$)	10E4–10E5 ($10^{4.14}$)	10E5–10E6 ($10^{5.35}$)

gold fish as the test model. LD_{50} values were found to be $10^{4.07}$, $10^{4.14}$ and $10^{5.35}$ cfu/fish respectively (Table 5). External signs such as reddening at the site of injection appeared in both experimental and control groups as early as one hour post infection. However, further signs of haemorrhagic scale pockets and loss of scales at the site of injection were observed in test fishes alone. The injected isolates could be recovered from the lesions as well as from the internal organs thus satisfying Koch's postulates.

Discussion

Aeromonas spp. are opportunistic pathogens that produce diseases in a variety of fresh water fishes (van der Marel et al. 2008). In our study, we noticed that the involvement of *A. veronii* in causing disease outbreaks across freshwater ornamental fish culture systems coincided with adverse environmental conditions. The major predisposing stress factors include improper handling, temperature shock, low oxygen level, high ammonia and other adverse water quality problems (Plumb and Hanson 2010). In the present study, each disease incidence was found to be associated with some kind of stress. From the results, it can be speculated the defective husbandry conditions of the rearing facilities might have made the fishes susceptible to infection.

Identification of *Aeromonas* strains to the species level is complicated by the lack of clear-cut phenotypic tests for distinguishing between these groups (Abbott et al. 2003). In our study, the isolates exhibited marked variations in their phenotypic characters, making the species identification difficult. Three phenogroups were obtained among the 60 isolates recovered from three independent disease incidences. Interestingly, the isolates represented by *Aeromonas* sp. MCCB 142 were able to produce acid from raffinose and melibiose, traits which were described by Abbott et al. (2003) as atypical phenotypic characteristics of the genus. They also showed many biochemical properties (such as hydrolysis of esculin, acid production from L-arabinose and salicin, and citrate utilization), which were not shown by the two groups of isolates represented by *Aeromonas* sp. MCCB 137 and 140 (Table 1).

In the present study, sequencing of the 16S rRNA gene, *gyrA*, *gyrB* and *rpoD* were used for molecular identification. However, speciation of the aeromonads based on 16S rRNA gene sequences alone is controversial because the 16S rRNA gene sequence has a high similarity (98–100 %) between closely related species such as *Aeromonas bestiarum*, *Aeromonas salmonicida* and *Aeromonas piscicola* (Beaz-Hidalgo et al. 2010). Analysis based on the sequences of one or two housekeeping genes has proven to be a useful tool for species differentiation of the genus *Aeromonas* (Alperi et al. 2010; Figueras et al. 2011). In our work, when the phylogenetic tree was constructed using the concatenated nucleotide sequences of housekeeping gene sequences, the representative strains *Aeromonas* sp. MCCB 137, MCCB 140 and MCCB 142 were found to clustered with *A. veronii* bv. *sobria* and *A. veronii* bv. *veronii* (Fig. 1). The phylogenetic tree constructed using 16S rRNA, *gyrA*, *gyrB*, and *rpoD* genes alone also showed the same pattern (Supplementary Fig. 1a–d). These data indicate that the three isolates characterized here belong to *A. veronii*. However, the phenotypic characters were not sufficient to allow the assignation of these isolates to one of the two biovars of *A. veronii* due to their diversity (Table 1).

The ERIC sequences are genetically stable and differ only in their copy number and chromosomal locations, making them a good target for strain differentiation (Hulton et al. 1991). The intra-specific diversity of *A. veronii* strains generated by ERIC PCR in our work indicated diversity within this species. Clearly, this genetic diversity may reflect the different sources of *A. veronii* infection in the ornamental fishes under study. The molecular finger printing indicated

that the different fish types were not infected with clonally related strains.

A few in vitro assays were employed in the present study, which measure parameters indirectly correlated with the virulence of *Aeromonas* isolates. High cell surface hydrophobicity (CSH) 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). In our study, isolates *Aeromonas* sp. MCCB 137 and 140 were strongly hydrophobic, whereas MCCB 142 was moderately hydrophobic (Lee and Yii 1996). Hydrophobic strains of *A. salmonicida* are typically more virulent than hydrophilic strains (Sakai, 1986). It has been reported that hydrophobic cells of *A.salmonicida* adhere to fish surfaces and macrophages and subsequently invade and proliferate (Graham et al. 1988; Bar-Or 1990). Slime production, which reflects the microorganism's capacity to adhere to specific host tissues and to produce invasive micro colonies (Lilenbaum et al. 1998) and diverse illness (Tenover et al. 1988), was displayed by all the isolates. Isolates which produced black colonies with dry crystalline consistency were regarded as slime positive, whereas those which showed pink colonies were slime negative. It has been reported that bacteria in biofilms resist antibiotics, biocides, and disinfectants and that biofilm formation protects against host defense mechanisms and facilitates bacterial communication leading to the expression of virulence determinants (Lavender et al. 2004). It may also provide an appropriate environment for microorganisms to initiate outbreaks of disease or the recurrence of infection in aquaculture environments (Coquet et al. 2002). It has been postulated that biofilm formation in *Aeromonas* is a multifactorial process involving both pili and flagella, as well as other determinants (Kirov et al. 2004). All isolates in the present study produced biofilms in varying proportions. Mittal et al. (1980) concluded that highly virulent strains of *Aeromonas* could be differentiated by cell surface characteristics, and they did not agglutinate in acriflavine and precipitated after boiling. Lallier and Daigneault (1984) also reported that lack of agglutination in acriflavine was highly correlated with fish pathogenicity. It has been stated that auto-agglutinating (AA^+) *Aeromonas* strains shared several additional features, including enhanced virulence for animals (LD_{50} in the range of $10^{4.50}$–$10^{7.43}$) and the presence of S-layer peripheral to the cell wall (Dooley and Trust 1988; Paula et al. 1988). According to Esteve et al. (2004), precipitation after boiling (PAB^+) is a suitable marker to search for virulent and S-layer positive motile *Aeromonas* strains from epizootic and clinical samples. Crystal violet binding has been associated with pilins and/or outer membrane proteins that have been demonstrated to be critical for pathogenesis (Golden and Acheson 2002; Yucel et al. 2005). Furthermore, the lack of suicidal activity in these cultures indicate their role in virulence whereby suicidal strains, which spontaneously auto-agglutinate, are inevitably avirulent (Namdari and Bottone 1988; Ballal et al. 2001). In our study, all strains displayed auto-agglutination and non-suicidal activity; however, they failed to agglutinate in acriflavine. The isolates from Oscar showed precipitation after boiling, whereas the one from gouramy was positive to crystal violet binding.

The production of haemolytic toxins has been regarded as strong evidence of pathogenic potential in aeromonads (Santos et al. 1999). In the present study we observed profound haemolytic activities of the isolates. They also harboured genes, which mediate the haemolysis of red blood cells. The enterotoxin gene *act*, one of the most potent cytotoxic genes, encodes haemolytic, cytotoxic and cytolytic activities and induces apoptosis of epithelial cells and macrophages (Krzyminska et al. 2012). Mutagenesis studies indicated that the hemolytic activity of *Aeromonas* sp. is related to both the hemolysin and the aerolysin genes, and they both act by inducing pore formation in the membranes of affected cells (Heuzenroeder et al. 1999; Wong et al. 1998). Moreover, beta-hemolytic isolates of *Aeromonas* were found to cause significantly more fluid accumulation in the ileal loops of experimentally infected rabbits than the alpha-hemolytic and non-hemolytic isolates, regardless of their species designation (Singh and Sanyal 1992). Wang et al. (2003) postulated a correlation between the pathogenic potential and the hemolytic activity of *Aeromonas* sp. In their study, the majority of *A. veronii* bv. *sobria* and *A. veronii* bv. *veronii* strains were highly haemolytic, consistent with our data. Testing clinical and environmental *Aeromonas* sp., Heuzenroeder et al. (1999) found that $aerA^+$ $hlyA^-$ phenotype was dominant in *A. veronii* bv. *sobria*. In contrast, our results indicate that all the *A. veronii* isolates recovered from the moribund fishes harbored both *aerA* and *hlyA* genes in accordance with the results of

Senderovich et al. (2012). The presence of the *gcat* gene indicates potential lipase or phospholipase activity of isolates that mediate erythrocyte lysis by digesting their plasma membrane (Pemberton et al. 1997). It has been reported that the *gcat* gene is present in all *Aeromonas* strains tested, including the representatives of all species (Puthucheary et al. 2012). Therefore, the role of *gcat* in pathogenesis has been questioned. All three of the isolates tested contained the *gcat* gene.

It has been reported that *A. veronii* produced significantly more enterotoxins than other *Aeromonas* sp. (Trower et al. 2000). Sha et al. (2002) noticed an association of *Aeromonas* with diarrhea in humans and showed that enterotoxins were involved in *Aeromonas*-associated gastroenteritis. Their studies also indicated that bloody diarrhea was most commonly associated with the production of the cytotoxic enterotoxin Act, while non-bloody diarrhea was correlated with the elaboration of the cytotonic enterotoxins Alt and Ast. Chopra et al. (2000) demonstrated that Act and Alt promoted fluid accumulation in ligated ileal loops in animal models. However, the role of enterotoxins in fishes has not been extensively studied. It has been reported that the cytotoxins produced by *Aeromonas* strains capable of adhesion to fish mucus may cause damage on the skin after penetration through the mucus layer (Krovacek et al. 1987). Although the presence of *ast* gene in *A. veronii* strains of clinical and environmental origin has been reported previously (Puthucheary et al. 2012; Aguilera-Arreola et al. 2007), in the present study, the strains lacked the *ast* gene. It has been postulated that different prevalence of the toxin genes may be related to geographical distribution (Albert et al. 2000).

The presence of type III secretion systems (TTSS), which delivers toxins directly to the cytosol of eukaryotic host through needle like structures, can be used as a general indicator of virulence (Stuber et al. 2003). It has been reported that inactivation of the TTSS system through defined mutations in the genes was found to attenuate virulence of *A. salmonicida* (Dacanay et al. 2006; Burr et al. 2005). In our study, the isolates possessed the machinery for TTSS such as *ascV*, which encodes a highly conserved inner membrane component of the TTSS apparatus, and *ascF-ascG* encoding the needle complex and a chaperone, respectively (Ghosh 2004). It has been also reported that a combination of the *ascF-ascG* and *alt* genes played a synergistic role in quorum sensing (Sha et al. 2005). Surprisingly, the isolates MCCB 140 and MCCB 142 possessed this combination. The TTSS also disrupts the cytoskeletal structures of the host. For instance, AexT, a bifunctional protein with ADP-ribosylating activity and GAP (GTPase-activating protein) activity, mediates ADP-ribosylation of both muscular and non-muscular actin in vitro (Fehr et al. 2007), thereby preventing polymerization (Masignani et al. 2006). TTSS-containing pathogens could modify the levels of phosphoinositides that anchor the actin cytoskeleton to the plasma membrane that facilitated bacterial invasion (Krzyminska et al. 2012). Several previous studies examined the prevalence of the TTSS among *A veronii* isolates (Puthucheary et al. 2012; Senderovich et al. 2012; Burr et al. 2005). In the present study, *ascV*, *ascF-ascG* and *aexT* were detected as reported by Silver and Graf (2009). However, Senderovich et al. (2012) noticed the absence of TTSS genes in one strain of *A. veronii* (H65AT3) isolated from diarrheal patients.

The interaction of enteropathogens with epithelial cells is the first stage of successive bacterial invasion of the host (Shames and Finlay 2010). In our study, the isolates displayed cytotoxicity towards the Hep-2 cell line (Fig. 2). Sha et al. (2005) suggested that the Act produced by *Aeromonas* strains was the only cytotoxic factor present in bacterial culture supernatants which was responsible for host cell damage and death, once secreted in sufficient quantities. It has been demonstrated that the interaction of the Act from *A. veronii* bv. *sobria* with epithelial cells resulted in extensive generation of reactive oxygen species (ROS) and nitric oxide radicals (NO^{-}) and caused cytotoxicity (Krzyminska et al. 2012). It has also been reported that the presence of the TTSS mediated cell-contact cytotoxicity, destruction of host cells and contributed to tissue damage (Krzyminska et al. 2012).

In the present study, the *tapA* gene encoding the type IV pilus was detected in *Aeromonas* sp. MCCB 137 and MCCB 140. In several pathogenic bacteria, type IV pili are highly associated with expression of adhesins and are responsible for biofilm formation and auto-agglutination (Mattick 2002; Misawa and Blaser 2000). Earlier studies (Masada et al. 2002) in *A. salmonicida* indicated that a Tap pili mutant was slightly less pathogenic for rainbow trout compared to the wild-type strain. However, recent studies showed that the Tap pili of *A. salmonicida* made moderate

contributions to bacterial virulence in Atlantic salmon (Boyd et al. 2008).

The virulence potential of the isolates was also assessed by determining the LD_{50} value in a challenge test, which confirmed the virulence of the representative isolates *Aeromonas* sp. MCCB 137, MCCB 140 and 142. In general bacterial isolates are grouped as virulent, weakly virulent and avirulent according to the LD_{50} values $10^{4.5-5.5}$, $10^{5.5-6.5}$, and $>10^7$ cfu/mL respectively (Lallier and Daigneault 1984). In our study, the LD_{50} of these representative strains ranged between $10^{4.07}$ and $10^{5.35}$ cfu/fish. Han et al. (2008) reported that *A. veroni* bv. *veronii* strain RY001 was virulent to goldfish with an LD_{50} value of 10^7 cfu/fish. However, Cai et al. (2012) reported an LD_{50} of 10^5 cfu/fish in Chinese long snout catfish (*Leiocassis longirostris* Günther) when *A. veronii* bv. *veronii* strain PY50 was injected intraperitoneally.

In the present study, a comprehensive investigation of the occurrence of phenotypically diverse, yet potentially virulent isolates belonging to *A. veronii* from different moribund ornamental fish species (Oscar, gold fish and gouramy) has been accomplished. It is presumed that the isolates in the present study likely have a wide host range and there is the possibility for each pathogenic clone to be proliferative in the ornamental fish culture systems during moments of stress. This implies that to avoid aeromonad disease outbreaks, the ornamental fish culture facility should be maintained free from stressors. The maintenance of good water quality is essential for both survival and optimum growth of cultured organisms as well.

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