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Aeromonas hydrophila associated with mass mortality of adult goldfish *Carassius auratus* (Linnaeus 1758) in ornamental farms in India

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ABSTRACT

Aeromonas hydrophila was identified the causative agent of a disease outbreak in goldfish, *Carassius auratus* from four ornamental fish farms in Kerala, India. Seven bacterial isolates *viz., Plesiomonas shigelloides* (NPPS-1), *Aeromonas hydrophila* (NPAH-1, 2, 3 and 4), *Citrobacter freundii* (NPCF-1) and *Acinetobacter* spp. (NPA-1) were isolated from the affected fish. Further, all four *A. hydrophila* (NPAH-1, 2, 3 and 4) isolates were identified by amplification of gyrB and rpoD genes. The RAPD profile using 3 primers confirmed that all four *A. hydrophila* were genetically similar. No cytopathic effect was observed on goldfish fin (GFF) cell line after inoculation of the tissue homogenate from the affected fish and affected tissues were found negative for koi herpesvirus (KHV), cyprinid herpesvirus-2 (CyHV-2) and spring viraemia of carp virus (SVCV). Experimental challenge resulted in mortality of fish injected with *A. hydrophila* only. *A. hydrophila* was observed to be cytotoxic on GFF cell line and exhibited haemolytic activity on 5% sheep blood agar. *A. hydrophila* possessed multiple virulence genes *viz.,* enterotoxins, haemolytic toxins and outer membrane protein as determined by PCR. *A. hydrophila* was sensitive to Cefixime, Chloramphenicol, Nitrofurantoin, Kanamycin, Ciprofloxacin, Furazolidone and Cefixime/Clavulanic acid. After treatment with the suggested antibiotics, the fish were recovered from the disease.

Keywords: *Aeromonas hydrophila,* Antimicrobial resistance, Cytotoxicity, Goldfish, Phylogenetic identification, Virulence genes

Introduction

Ornamental fish are gaining popularity in India and their farming is becoming popular in many places in the country. The ornamental fish industry in India is rapidly growing at the rate of 20% per annum (Ayyappan *et al.,* 2006). Noga (2010) has reported that the successful production in fish culture has been hampered by factors like diseases. High stocking levels of ornamental fishes in culture ponds has resulted in various disease outbreaks leading to huge economic losses. Fishes are always exposed to bacterial pathogens in the aquatic environment, which has led to many bacterial infections in aquaculture systems affecting fish production (Omprakasam and Manohar, 1991). A range of pathogenic bacteria such as *Aeromonas* spp., *Bacillus* spp., *Citrobacter* spp., *Edwardsiella* spp., *Flavobacterium* spp., *Serratia* spp., *Proteus* spp., *Vibrio* spp., *Providencia* spp. and *Klebsiella* spp. have been reported to cause diseases in ornamental fish (Austin and Austin, 2007). Many reports on the bacterial diseases of Indian freshwater fishes have been published earlier (Das *et al.,* 1999; Vivekanandhan *et al.,* 2002; Mohanty and Sahoo, 2007). Kumar *et al*., (2015) reported mass mortality in ornamental koi carp due to a bacterial pathogen, *Proteus hauseri* in India.

Goldfish, *Carassius auratus* (Linnaeus 1758) is a common domesticated ornamental fish in garden ponds and aquaria. A disease outbreak in goldfish during the rainy months of June and July 2015, was detected in an ornamental fish farm in Ernakulam District, Kerala during the routine aquatic animal disease surveillance of ornamental fish farms, under the National Surveillance Programme of Aquatic Animal Diseases (NSPAAD). The disease spread was rapid affecting the goldfish population in the 3 nearby farms also, with a cumulative mortality of 70-90% over 10 days time in all 4 farms. Clinical signs which were clearly evident in the moribund fish were haemorrhages on the fin, tail and dropsy. The present study investigated the cause of the disease outbreak in goldfish.

Materials and methods

Sampling

Moribund goldfish specimens (n=45, body weight, 18-23 g) were transported alive in aerated plastic bags containing water from the pond to the laboratory, from a disease outbreak in four ornamental fish farms sharing water from a common stream in Kerala. and screened for external parasites and other microbial pathogens. Tissue

samples from gills, fin, heart, kidney and spleen were collected aseptically from 10-12 fish per farm and pooled farm-wise for bacterial isolation. Tissue samples (gills, fin, heart, kidney and spleen) were also collected in tissue culture medium, as well as in 95% ethanol for screening for viruses.

Screening for viral pathogens from fish samples

Viral isolation was attempted on goldfish fin (GFF) cell line by inoculating with fish tissue homogenates from affected fish. The internal organs *viz.,* heart, kidney, liver and spleen as well as gills from each of the infected fish was collected aseptically, pooled and homogenised in Leibovitz's 15 (L-15) medium containing antibiotic and antimycotic solution (Gibco, Carlsbad, CA, USA) at a final dilution of 1/10. The tissue homogenate was centrifuged at 3000 *g*, 10 min at 10°C and then the supernatant was filtered using 0.22 µm syringe membrane filter (Millipore). The filtered homogenate was inoculated to 80% confluent GFF cell line in a 25 cm^2 flask. The cells were then incubated for 1 h at room temperature and then supernatant was discarded and replaced with L-15 medium supplemented with 5% FBS and incubated at 28°C. The cells were observed for any cytopathic effect (CPE) and 5 blind passages were done. GFF cells and cell culture supernatant were used for extraction of the viral nucleic acids for virus screening. Extraction of RNA/DNA from the inoculated GFF cell line and the affected goldfish tissues was carried out using Gene JET RNA purification kit (Thermo Scientific, Lithuania) and DNAeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) respectively and the manufacturer's instructions were followed for production of quality templates in PCR reactions. Amplification of nucleic acids were carried out using published primers to screen for koi herpesvirus (KHV), goldfish herpesvirus haematopoietic necrosis virus (GHHNV) or cyprinid herpesvirus-2 (CyHV-2) and spring viraemia of carp virus (SVCV). Primer details are listed in Table 1.

Bacterial isolation and analyses

The organs collected from moribund fish from each farm were pooled separately and homogenised for bacterial isolation. The samples were inoculated into sterile nutrient broth and incubated at 28°C for 24 h. The overnight grown culture was then streaked in Trypticase soya agar (HiMedia) plates and was incubated at 28°C for 48 h. Different types of bacterial colonies were isolated from all the samples. Bacterial identification was done as per Barrow and Feltham (2004). The results of biochemical tests for different bacteria were compared with corresponding reference strains as described by various authors (Brenner *et al.,* 1993; Krovacek *et al.,* 2000; Constantiniu *et al.,* 2004; Chen *et al.,* 2012). The identified bacterial isolates were stored at -80°C in 20% (v/v) glycerol for further use. Extraction of DNA from bacterial isolates were done as described by Miller *et al.* (1988). The extracted DNA quality and quantity were checked using UV spectrophotometer (Beckman, USA), measuring the optical density (OD) at 260 and 280 nm. All the bacterial strains were identified and confirmed 16S rRNA sequencing using the 16S rRNA bacterial universal primers and PCR conditions as per Weisburg *et al.* (1991). Briefly, amplifications were performed in a total volume of 25 µl reaction mixture containing 1X reaction buffer (50 mm Potassium Chloride, 10 mm Tris, 0.01% gelatin, pH 9.0) with 2.0 mm magnesium chloride (Fermentas), 5 p mol each of forward and reverse primers, 200 mm dNTPs (Fermentas), 1.5 U Taq DNA polymerase (Fermentas) and template DNA (25 - 50 ng). The reaction mixture was pre-heated at 94°C for 3 min followed by 30 cycles of 94°C for 1.3 min, annealing at 52°C for 40 s and extension at 72°C for 1 min. After completion of the reaction. The PCR products were detected by running a sample from each reaction tube to 1.5% agarose gel electrophoresis, stained with ethidium bromide and the product size yield was approximately 1450 bp. Further, the species-level confirmation of four isolates of *A. hydrophila* was carried out by phylogenetic analysis using gyrB and rpoD genes. PCR amplification and sequencing for both genes were performed using primer sequences, as described by Martınez-Murcia *et al.* (2011). PCR amplicons for each primer set were sequenced by Sanger's method at the automated sequencing facility (Scigenom Pvt. Ltd., India). Clustal W in MEGA 5 software (Tamura *et al.,* 2011) was employed for alignment of the partial gene sequences with respective bacterial reference strains. NCBI Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/) and Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu) were used for molecular identification and homology comparison of 16S rRNA sequences as well as the gyrB and rpoD sequences of the four *A. hydrophila* isolates. The nucleotide sequences of the bacterial isolates obtained were deposited in NCBI GenBank database.

Random amplified polymorphic DNA (RAPD)-PCR analysis

Genomic DNA was isolated from broth culture of the four *A. hydrophila* isolates at log phase and approximately 30-35 µg of DNA was yielded from 2 ml of each bacterial culture. Out of the 15 decamer random primers (M/S Operon Technologies) screened, OPA-2, OPA-3 and OPA-4

Table 1. Details of the primers used in the study

Target	Primer sequence	Size (bp)	PCR conditions	Reference
A. hydrophila hem	hemF TCG GGG AAG ATA TGG ATG TT hemR ATA CAT TGC CAA ACC CTT CG	963	35 cycles of: 30 s, 94°C 30 s, 54°C 1min, 72°C 10 min, 72°C	Designed in the present study (AF410466)
*A. hydrophila aer*A	aerF: TGTCGGGGATGACATGAACGTG aerR: CCAGTTCCAGTCCCACCACTTCA	720	35 cycles of: 30 s, 94°C 30 s, 54°C 60 s, 72°C 10 min, 72°C	Wang *et al.* (2012)
A. hydrophila alt	altF: ATGACCCAGTCCTGGCACGG altR: GCCGCTCAGGCCGAAGCCGC	480	35 cycles of: 30 s, 94°C 30 s, 54°C 60 s, 72°C 10 min, 72°C	Wang *et al.* (2012)
*A. hydrophila aha*1	ahalF: GCCGCTAACGCTGCAGTGGTTTACGAC ahalR: CGCAGAGGCTAGATTAGAAGTTGTATTG	1087	35 cycles of: 30 s, 94°C 30 s, 54°C 60 s, 72°C 10 min, 72°C	Wang *et al.* (2012)
*A. hydrophila omp*TS	OmpTSF: GCAGTGGTATATGACAAAGAC OmpTSR: TTAGAAGTTGTATTGCAGGGC	1002	35 cycles of: 1 min, 95°C 1 min, 60°C 1min, 72°C 10 min, 72°C	Khushiramani *et al.* (2007)
Koi herpes virus (KHV)	TKF- GGGTTACCTGTACGAG TKR- CACCCAGTAGATTATGC	409	40 cycles of: 1 min, 95°C 1 min, 52°C 1 min, 72°C 10 min, 72°C	Bercovier *et al.* (2005)
Spring viraemia of carp virus (SUCV)	SVCV F1 -TCTTGGAGCCAAATAGCTCARRTC SVCVR2- AGATGGTATGGACCCCAATACATHACNCAY	714	35 cycles of: 1 min, 95°C 1 min, 55°C 1 min, 72°C 10 min, 72°C	Stone *et al.* (2003)
Cyprinid herpesvirus (2CyHv-2)	CyHVpolF CCCAGCAACATGTGCGACGG CyHVpolR CCGTARTGAGAGTTGGCGCA	362	40 cycles of 1 min, 95°C 1 min, 55°C 1 min, 72°C 10 min, 72°C	Jeffery *et al.* (2007)

were selected for RAPD analyses. The genomic DNA of all four *A. hydrophila* isolates was amplified using the three primers and their repeatability was ascertained. The PCR cocktail consisted of Taq reaction buffer 2.5 µl (100 mm Tris-HCl pH 8.3, 500 mm KCl and 0.001% gelatin), dNTPs - 0.2 mm, Taq DNA polymerase - 1 unit, $MgCl_2$ - 2 mm, each primer - 7.5 pM and DNA template - 30 ng in a total reaction volume of 25 µl. RAPD was performed with the following parameters: an initial denaturation cycle of 95°C, 5 min; followed by 45 cycles of denaturation at 94°C, 1 min; primer annealing at 36°C, 1 min and primers extension at 72°C, 4 min with final extension at 72°C 12 min.

Screening for virulence genes in A. hydrophila NPAH-1

A. hydrophila (NPAH-1) isolate was screened for presence of virulence genes *viz.,* enterotoxins (*alt*), aerolysin (*aer*A), outer membrane proteins (*aha*1 and *omp* TS) and also heamolysin (*hem*). The primer details and PCR conditions used for screening the five virulence genes of *A. hydrophila* are given in the Table 1.

Phenotypic expression of virulence of A. hydrophila (NPAH-1)

Luria-Bertani (LB) agar containing 5% (v/v) sheep blood was used to determine the haemolytic activity of *A. hydrophila* (NPAH-1). Further, bacterial extracellular products (ECP, prepared following the cellophane overlay technique as per Liu, 1957) was exposed to GFF cell line, to determine the cytotoxicity of the bacteria. Morphological changes of infected GFF cells were observed at regular intervals for 4 days post-inoculation (dpi).

Experimental challenge trials

A total of 50 healthy goldfishes (mean weight 20.6 g ±1.65) were used for infection experiments. Fish were maintained in 500 l capacity glass aquaria containing 300 l UV treated water. Continued aeration was provided with 50% of water exchange daily. Water temperature in the tanks was recorded twice daily which ranged from 26 to 29ºC. The fish were acclimatised for 15 days in the laboratory, before challenge studies. Fifty fishes were divided into five experimental groups. The experimental animals were anaesthetised using MS-222 (Sigma), at 75 to 150 µg l^{-1} of water for 2-5 min. Each group was injected intramuscularly (i/m) with 100 µl of one of the 4 bacterial strains of *P. shigelloides* (NPPS-1), *A. hydrophila* (NPAH-1), *C. freundii* (NPCF -1) and *Acinetobacter* spp. (NPA-1) isolated from diseased goldfish and the fifth group which served as control were injected with 100 µl sterile phosphate buffered saline (PBS). The inocula of all the seven bacterial isolates were prepared as follows: A single colony of each bacterial isolate was inoculated in 5 ml of TSB and incubated at 28°C overnight and the bacterial cell density was determined by plate count method prior to challenging. The bacterial broth was centrifuged at 300 *g* for 10 min. The cell pellets were suspended in sterile PBS and serially diluted upto 10^{-7} and cell counts were determined on plate count agar. Fishes were monitored for 15 days post-challenge and mortalities were recorded. Mortalities were considered to be due to the bacterial inoculation, only when the bacterial strain was reisolated in pure culture from internal organs (kidney, spleen, liver).

Calculation of LD_{50} in vivo

The virulence of the *A. hydrophila* (NPAH-1) was further explored *in vivo* based on 50% lethal dose (LD_{50}) in gold fish. For determination of 50% lethal dose (LD_{50}), intramuscular injection of 0.1 ml bacterial suspension each of 10^4, 10^5, 10^6 and 10^7 CFU per fish was employed, in groups of 10 fishes each. Control group comprising 10 fish were injected with 0.1 ml each of normal saline. Morbidity and mortality of the injected fishes were observed daily for a week and the affected animals were subjected for routine bacteriological examination. Reed and Muench (1938) methodology was followed to calculate LD_{50} value of *A. hydrophila* NPAH-1.

Antibiotic susceptibility

Standard procedure of disc diffusion method as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009) was employed for determining the antibiotic susceptibility of *A. hydrophila* (NPAH-1). Antibiotic discs used in this study were procured from HiMedia Laboratories, India. A total of 16 antimicrobials agents *viz.,* Cefixime, Chloramphenicol, Bacitracin, Nitrofurantoin, Azithromycin, Erythromycin, Gentamicin, Kanamycin, Cefalexin, Oxytetracycline, Ciprofloxacin, Amoxycillin, Enrofloxacin, Furazolidone, Ampicillin and Cifixime/Clavulanic acid were tested. By measuring the diameter of the clearance zones (mm) around the disc, antibiotics were interpreted as sensitive, resistant and intermediate using CLSI criteria (CLSI, 2009) and criteria set by the manufacturer.

Results and discussion

Viral pathogens screening

No cytopathic effect in GFF cell line was observed upto 2 weeks after inoculating tissue homogenates and also even after 5 blind passages. No amplifications in PCR and RT-PCR for KHV, CyHV-2 and SVCV were observed from the GFF cell line supernatant and all the pooled samples were also found negative for KHV, CyHV-2 and SVCV through PCR and RT PCR. The respective positive control for the KHV, CyHV-2 and SVCV successfully produced amplification for the target gene fragments.

Bacterial isolation and identification

It has been known for decades that the motile aeromonads cause diseases among freshwater fishes. The interactions of fishes with *Aeromonas* spp. in aquatic environments are continual and enable the bacteria to be an opportunistic pathogen (Ottaviani *et al*., 2011). *A. hydrophila* have been a major causative agent for fish bacterial disease outbreaks around the world, resulting in enormous economic losses (Janda and Abbott, 2010). Virulent *A. hydrophila* were more frequently isolated from diseased fish than from healthy fish (Nielsen *et al*., 2001). In the present study, four isolates of *A. hydrophila* (NPAH-1, 2, 3, 4) along with three more bacteria *viz., P. shigelloides* (NPPS-1), *C. freundii* (NPCF-1) and *Acinetobacter* spp. (NPA-1) were recovered from gills, fin, heart, kidney and spleen of diseased goldfish from four ornamental fish farms in Kerala and confirmed by both traditional biochemical methods and 16S rRNA gene PCR amplification. The details of the farms and bacteria isolated from different farms are given in Table 2. The biochemical

Table 2. Details of sample collection and bacteria isolated from the affected farms

Farm	GPS co-ordinates	Date of collection	No. of fishes collected	Bacteria isolated
1	10°04'06.5"N 76°31'04.2"E	02.11.2016	15	*Aeromonas hydrophila* (NPAH-1)
2	10°04'14.1"N 76°31'02.8"E	08.11.2016	8	*Plesiomonas shigelloides* (NPPS-1) and *Aeromonas hydrophila* (NPAH- 2)
3	10°04'02"N 76°31'21.2"E	08.11.2016	10	*Acinetobacter* (NPA-1) and *Aeromonas hydrophila* (NPAH-3)
4	10°03'58.4"N 76°29'15.3"E	11.11.2016	12	*Citrobacter freundii* (NPCF-1) and *Aeromonas hydrophila* (NPAH-4)

tests for seven different bacterial isolates from diseased goldfish were compared with respective type strains (Table 3). The 16S rRNA 1450 bp products of all 4 isolates were sequenced and they were aligned with BLAST and RDP. The RDP results showed 98.6% homology with *A. hydrophila,* 98.8% homology with *Acinetobacter* spp., 99.3% homology with *C. freundii* and 99.1% similarity with *P. shigelloides* of the respective bacteria isolated in this study. The bacterial isolates were confirmed as *P. shigelloides, A. hydrophila, C. freundii* and *Acinetobacter* spp. and the sequences were submitted to GenBank, NCBI under the accession numbers KT361192, KT361193, KT361194 and KT361195 respectively.

Sequencing of gyrB and rpoD genes for molecular identification of *A. hydrophila* was also carried out. Even though, sequence analysis of 16S rRNA is the recommended criterion for the delineation of a bacterial species (Moore *et al.,* 1996; Bennasar *et al.,* 1998) and for bacterial identification, the 16S rRNA gene sequence projects high similarity among *Aeromonas* spp. (Soler *et al.,* 2004). For example, *A. aquariorum* has been often mistakenly identified as *A. hydrophila* or *A. caviae* due to the matching results obtained by 16S rRNA sequences and biochemical profiles among these three species (Figueras *et al.,* 2011) and so 16S rRNA gene sequences alone for speciation of the aeromonads is controversial. The

Table 3. Biochemical characteristics of the four bacterial strains isolated from diseased goldfish in the present study, along with characteristics of reference strains

Biochemical tests	*Plesiomonas shigelloides*		*Aeromonas hydrophila*		*Citrobacter freundii*		*Acinetobacter* sp	
	NPPS-1	Krovaceka *et al.* (2000)	NPAH-1	Chen *et al.* (2012)	NPCF-1	Brenner *et al.* (1993)	NPA-1	Constantiniu *et al.* (2004)
Colony morphology	Round, mucoid, milky white and elevated		Pin head sized, round, semi translucent, yellowish and flattened		Smooth, opaque and moist		Smooth and pale	
Gram's staining	-	-	-	-	-	-	-	-
Catalase	+	+	+	+		+	+	+
Oxidase	+	+	+	+	-	-	-	-
Motility	+	+	+	+	+	+		-
Indole		-	+	+		-		
Urease	-	-	-	-	-	-		
Simmon's citrate		-	+	+	+	+	+	+
Methyl red	+	+	+	+	+	+		-
Voges Proskauer		-	+	+	-	-	-	-
Lysine decarboxylase	+	+	+	+		-		
Ornithine decarboxylase	+	+	-	-	-	-		
Glucose		+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+		
Inositol	+	+	-	-	-	-		
Lactose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+			+	+
Raffinose	-	-	-	-	+	+		
Rhamnose	-	-	-	-				
Sorbitol	-	-	-	-	+	+		
Xylose	-	-	-	-	+	+		

benefits of housekeeping genes have been well studied in recent times (*e.g.,* gyrB and rpoD) in the identification of *Aeromonas* spp. (Soler *et al.,* 2004). Among all *Aeromonas* spp., the sequencing of the house keeping genes has revealed a higher sequence divergence than that of the 16S rRNA sequence (Kupfer *et al.,* 2006). In our work, when the phylogenetic tree was constructed using the nucleotide sequences of 16S rRNA gene and housekeeping genes sequences, the four strains of *A. hydrophila* (NPAH-1, 2, 3 and 4) recovered from the moribund ornamental fishes were found phylogenetically related to *A. hydrophila* (Fig. 1a). The overall nucleotide sequence similarity of gyrB and rpoD genes of all the four *A. hydrophila* isolates ranged between 98.9 and 99.2%. GyrB and rpoD gene sequences of four strains of *A. hydrophila* were used for the phylogenetic tree construction, with their most similar matches in the GenBank database and the sequences were submitted to NCBI GenBank (Fig. 1b and c).

RAPD profile

Amplification of the DNA from each of 4 *A. hydrophila* isolates with the three random primers OPA-2, OPA-3 and OPA-4 produced amplicons, which were consistent and appeared as distinct bands on agarose gel after electrophoresis. The RAPD patterns of all 4 isolates of *A. hydrophila* were similar and therefore referred to as genetically related. The fingerprints pattern generated by 3 random primers *viz.,* OPA-2, OPA-3 and OPA-4 of

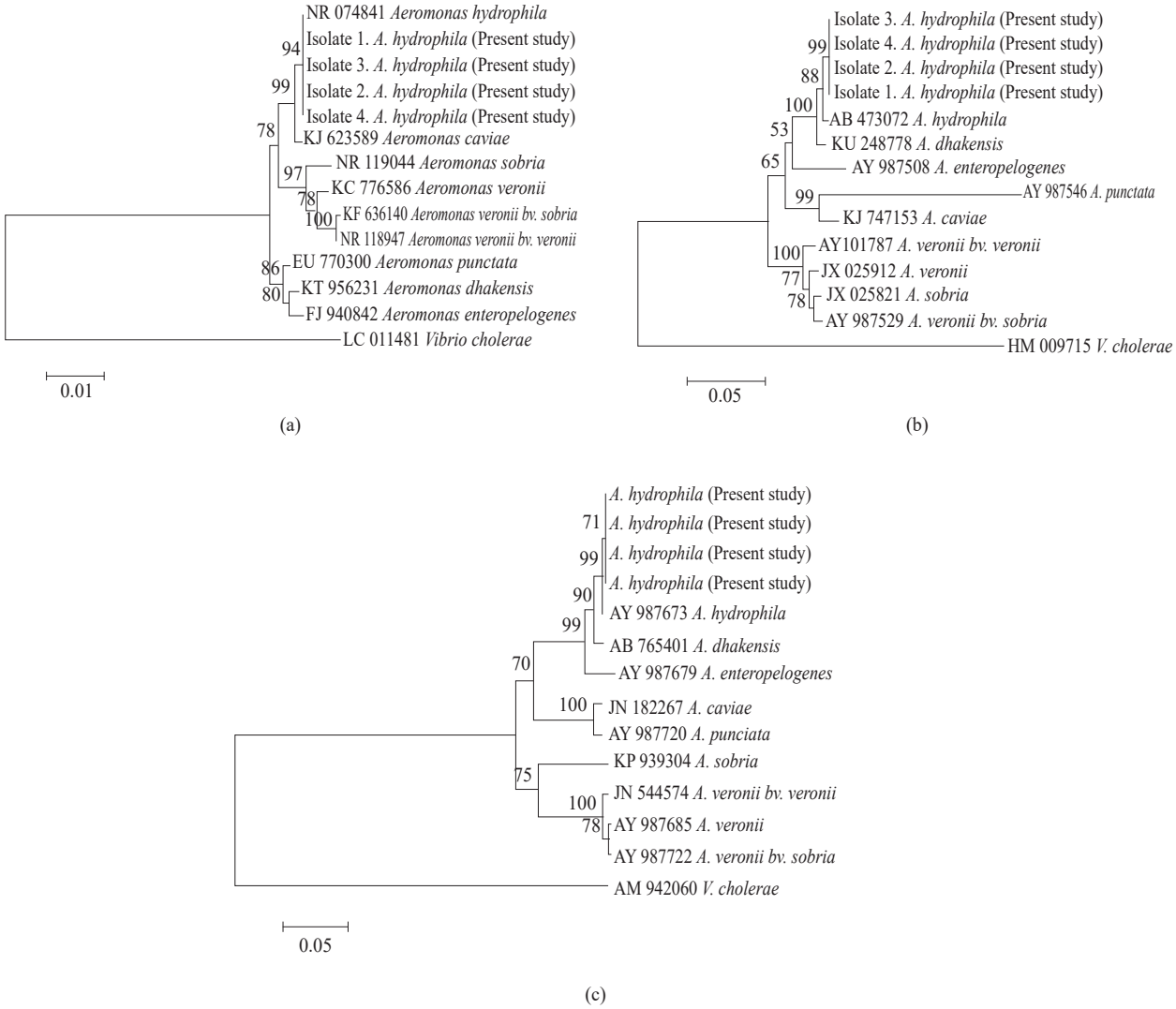


Fig. 1. Phylogenetic tree based on the nucleotide sequences of 16S rRNA (Fig. 1a), gyrB (Fig 1b) and rpoD (Fig 1c) of four isolates of *A. hydrophila* (NPAH-1, 2, 3 and 4) and related *Aeromonas* spp. The tree was constructed by neighbour-joining algorithms with genetic distance computed by Kimura's 2-parameter method. Bootstrap values of 1000 simulations are indicated at the branches. The bar indicates percentage difference.

the isolates contained 5 to 12 bands ranging from 400 to 3530 bp (Fig. 2). In our study, all isolates showed uniformity in biochemical characteristics and RAPD pattern. Therefore, we randomly selected one representative strain (*A. hydrophila,* NPAH-1) for screening virulence genes, cytotoxicity analysis, antibiotic sensitivity assay and for the experimental challenge trial.

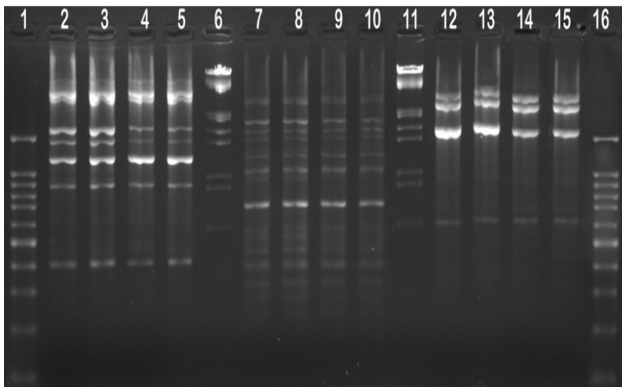


Fig. 2. RAPD profiles of *A. hydrophila* (NPAH-1, 2, 3 and 4) isolated from goldfish.
The profiles were generated using 3 primers (OPA-2, OPA-3 and OPD-4). Lane 1: 100 bp to 1500 bp ladder (Origin, India); Lane 2-5: RAPD profile of NPAH-1, 2, 3 and 4 of using OPD-2; Lane 6: Lamda DNA/EcoRI Hind III double digest (GeNei); Lane 7-10: RAPD profile of NPAH-1, 2, 3 and 4 using OPD-3; Lane 11: Lamda DNA/EcoRI Hind III double digest (GeNei); Lane 12-15: RAPD profile of NPAH-1, 2, 3 and 4 using OPD-4; Lane 16: 100 bp to 1500 bp ladder (Origin, India)

Virulence genes

Pathogenesis of *Aeromonas* spp. has been believed to be caused by multi-factorial toxins, such as enterotoxin and extracellular products (Sha *et al.,* 2002; Janda and Abbott, 2010). Therefore, these virulence factor encoding genes have been applied for evaluating the pathogenicity of *Aeromonas* spp. isolated from different sources *viz.,* environment, foodstuffs, fish, human and aquatic environments (Ottaviani *et al.,* 2011; Puthucheary *et al.,* 2012). The identification and examination of virulence genes is a prerequisite to understand the pathogenicity mechanisms of an organism (Strauss and Falkow, 1997). The *Aeromonas* strains isolated either from diseased fish or healthy fish and water samples often had number of virulence genes in different combinations (Santos *et al.,* 1999; Sreedharan *et al.,* 2012). A variety of toxins are secreted by aeromonads that boosts the severity of many infections (Cahill, 1990; Vadivelu *et al.,* 1995; Chopra and Houston, 1999; Sen and Rodgers, 2004). However, not all species of aeromonads produce all the toxins (Chopra and Houston 1999). PCR amplification and sequencing of the genes for virulence factors *viz.,* hemolysin gene, aerolysin gene (*aer*A), cytotonic enterotoxin gene (*alt*), adhesion gene (*aha*1) and outer membrane protein (*omp* TS) from the *A. hydrophila* (NPAH-1) isolate was attempted and the expected PCR products of size 963 bp, 720 bp, 480 bp, 1087 bp and 1002 bp respectively were obtained (Fig. 3) and the sequences were submitted to NCBI GenBank (Accession nos.: KU527552, KU527544, KU527550, KU527545 and KU527551 respectively). In agreement with previous studies (Hu *et al.,* 2012; Yi *et al.,* 2013), we found expression of 5 different virulence genes in the tested isolate.

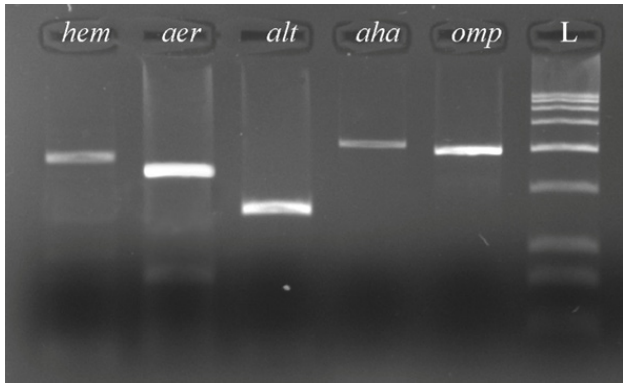


Fig. 3. Detection and identification of *A. hydrophila* (NPAH-1) virulence genes by amplification of fragments in the PCR assay.
Lane 1: *hem*; Lane 2: *aer*A; Lane 3: *alt*; Lane 4: *aha*1; Lane 5: *omp* TS; Lane 6: Low range DNA ruler 100 bp to 3 kbp (GeNei)

Phenotypic expression of virulence by invitro assay of A. hydrophila (NPAH-1)

In the present study, *A. hydrophila* (NPAH-1) was found haemolytic on goat blood agar and produced β haemolysis (Fig 4). When ECP from *A. hydrophila* (NPAH-1) was tested on GFF cell line (Fig. 5a) signs of cytotoxicity including dislodgement of cells, rounding and shrinkage of cytoplasm were observed, which directly correlates with the virulence of *A. hydrophila* isolate. The changes indicating cytotoxicity started in GFF cells by 1 dpi and death of the cells appeared on 3 dpi (Fig. 5b).

Experimental challenge trials

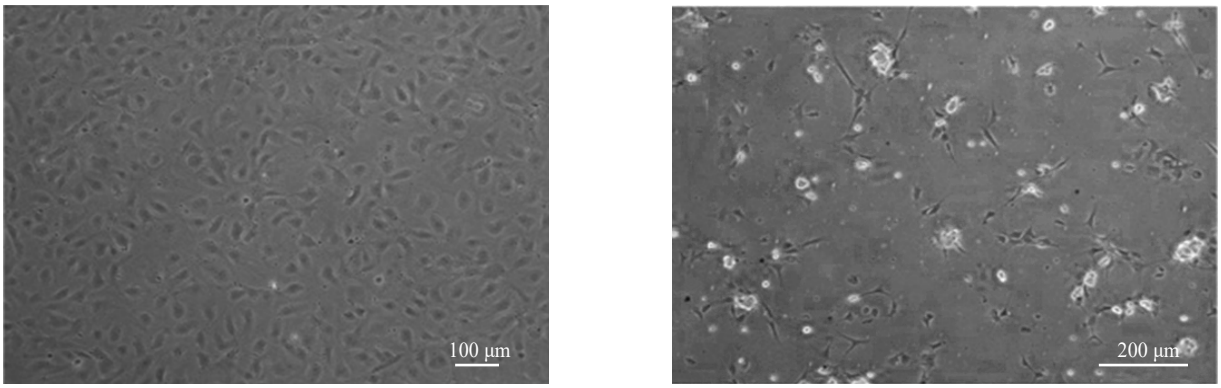
The differentiation of pathogenic strains from non-pathogens is inevitable in pathological investigations. In the experimental challenge, cumulative mortality rate of 100% was reached in goldfish injected with overnight grown *A. hydrophila* (NPAH-1). The experimentally infected goldfish displayed clinical signs of scale protrusion, distended abdomen, tail rot and haemorrhages

Fig. 4. β haemolytic colonies of *A. hydrophila* (NPAH-1) on 5% goat blood agar

results showed that mortality of the fishes in the farm were mainly due to *A. hydrophila*. The other three bacteria *viz.,* NPPS-1, NPCF-1, NPA-1 were found to be secondary bacterial pathogens.

LD_{50} in vivo

In general bacterial isolates are grouped as virulent, weakly virulent and avirulent according to the LD_{50} values $10^{4.5\text{-}5.5}$, $10^{5.5\text{-}6.5}$ and $>10^{7}$ cfu ml^{-1} respectively (Lallier and Daigneault, 1984). While calculating LD_{50} values of *A. hydrophila* (NPAH-1), the cumulative mortality rates of 12-21%, 34-48%, 62-90% and 100% were recorded in goldfish injected at doses of $1x10^{4}$, $1x10^{5}$, $1x10^{6}$ and $1x\ 10^{7}$ CFU per fish, respectively. LD_{50} value was estimated as



(a) (b)

Fig. 5. Cytotoxicity of ECP of *A. hydrophila* (NPAH-1) on GFF cell line. (a) Uninoculated GFF cell line (control); (b) GFF cell line subsequent to addition of ECP from *A. hydrophila* (NPAH-1) isolated from goldfish

on tail as reported in the naturally affected goldfish collected from the farms (Fig. 6a, b). No mortality was observed in the group receiving bacterial isolates NPPS-1, NPCF-1, NPA-1 and control group up to 15 days post-challenge. Pure bacterial colonies recovered from the liver and kidney of all freshly dead fish exhibited homologous biochemical characteristics with the original isolate. The $10^{5.35}$ CFU per fish. In most animals, distended abdomen, scale protrusion, tail rot, haemorrhages on tail and scale loss were also noticed finally leading to fish death in 1 week.

Antibiotic sensitivity

Various studies have reported antimicrobial resistance in bacteria isolated from ornamental

(a) (b)

Fig. 6. Clinical signs *viz*., distended abdomen, scale protrusion, tail rot and haemorrhages on body of affected goldfish. (a) Infected goldfish collected from the affected farm; (b) Goldfish experimentally challenged with A. hydrophila (NPAH-1)

fish and their environment (Verner-Jeffreys *et al.,* 2009; Cizek *et al.,* 2010; Dias *et al.,* 2012). *A. hydrophila* (NPAH-1) strain was screened for antibiotic susceptibility using the agar diffusion method and the results are shown in Table 4. *A. hydrophila* (NPAH-1) was found resistant (R) to six antibiotics *viz.,* Bacitracin, Azithromycin, Cefalexin, Amoxycillin, Enrofloxacin and Ampicillin. The strain was intermediate (I) sensitive to Erythromycin, Gentamicin and Oxytetracycline while susceptible to Cefixime, Chloramphenicol, Nitrofurantoin, Kanamycin, Ciprofloxacin, Furazolidone and Cifixime/Clavulanic acid. Resistance to these antibiotics has likely resulted from their indiscriminate use in the aquarium fish industry to treat diseases, while resistance can also result from gene mutations or by acquisition of transferable genetic elements (Jacobs and Chenia, 2007).

Table 4. Antibiotic sensitivity pattern of *Aeromonas hydrophila* (NPAH-1) isolate

Antibiotics	Concentration (µg disc^{-1})	Inhibition zone (mm)	Inference
Cefixime	5	25	S
Chloramphenicol	25	22	S
Bacitracin	10	No zone	R
Nitrofurantoin	100	19	S
Azithromycin	30	12	R
Erythromycin	10	11	I
Gentamicin	120	13	I
Kanamycin	30	20	S
Cefalexin	30	No zone	R
Oxytetracycline	30	17	I
Ciprofloxacin	30	21	S
Amoxycillin	25	No zone	R
Enrofloxacin	10	13	R
Furazolidone	100	18	S
Ampicillin	25	11	R
Cifixime / Clavulanic acid	5/10	21	S

R: Resistant; S: Sensitive; I: Intermediate sensitive

Intramuscular injection of Ciprofloxacin at a dose of 10 mg kg^{-1} body weight for five days was advised to all the fish farms. After the antibiotic treatment, the fish recovered from haemorrhages on the tail, fin, dropsy and no further mortality of fishes was noticed in the farms.

The present study highlights *A. hydrophila* could potentially be associated with bacterial infection in goldfish. *A. hydrophila* NPAH-1 isolates harbour multiple virulence genes with multiple antibiotic resistance. These might be a possible reason for treatment failures in fish disease outbreaks. The *A. hydrophila* isolated in the present study could be proliferative in the fish during stress conditions. Therefore to avoid bacterial disease outbreaks, ornamental fish farms should be maintained with minimum stress conditions. Maintenance of good water quality and minimum stress are essential for the survival as well for the optimum growth of cultured organisms. Overall, the results obtained highlight the need to promote responsible ornamental fish farming, good husbandry practices and prudent use of antimicrobials in the ornamental fish industry.

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