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 antymotic 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 cm² 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 pmol 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 Martinez-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

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Aeromonas hydrophila associated with mass mortality of Carassius auratus 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, MgCl₂ - 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-I

A. hydrophila (NPAH-1) isolate was screened for presence of virulence genes viz., enterotoxins (alt), aerolysin (aerA), outer membrane proteins (aha1 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.

| Target                  | Primer sequence          | Size (bp) | PCR conditions                              | Reference                  |
|-------------------------|--------------------------|-----------|---------------------------------------------|----------------------------|
| A. hydrophila hem       | hemF: TCG GGG AAG ATA TGG ATG TT  
                         |            | 963                                            | 35 cycles of:  
                         | hemR: ATA CAT TGC CAA ACC CTT CG              | Designed in the present study (AF410466)   |
| A. hydrophila aerA      | aerF: TGTCGGGATGACATGAACGTG  
                         |            | 720                                            | 35 cycles of:  
                         | aerR: CCAAGCTCGAACCACACTCAGG                 | Wang et al. (2012)                         |
| A. hydrophila alt       | altF: ATGACCCAGTCTGGCAGCAGG  
                         |            | 480                                            | 35 cycles of:  
                         | altR: CCCGCTACGGCGAGCGCGCGCGG                | Wang et al. (2012)                         |
| A. hydrophila aha1      | ahlF: GCCGCTAACGCTCGATGTTTACGAC  
                         |            | 1087                                           | 35 cycles of:  
                         | ahlR: GCAGAGGCTAGATAGAAGTGTTATTG             | Wang et al. (2012)                         |
| A. hydrophila ompTS     | OmpTSF: GCAGTGGTATATGCAAAAGAC  
                         |            | 1002                                           | 35 cycles of:  
                         | OmpTSR: TTAGAAGTGTATTGCAAGGGCG               | Khishiraman et al. (2007)                  |
| Koi herpes virus (KHV)  | TKF- GGTTAACCTGTACGAG      
                         |            | 409                                            | 40 cycles of:  
                         | TKR- CACCCAGTAGATTATGC                       | Bercovier et al. (2005)                    |
| Spring viraemia of     | SVCV F1 -TCTTGGAGCCCAAATAGCTCARRTC  
                         |            | 714                                            | 35 cycles of:  
                         | SVCVR2- AGATGGTATGGACCCCAAATACATHACNCA       | Stone et al. (2003)                        |
| Cyprinid herpesvirus (2CyHv-2) | CyHVpolF CCCAGCAACATGGTGCGACGG  
                        |            | 362                                            | 40 cycles of:  
                         | CyHVpolR CCCAGTGAGTGTTGGCGCA                 | Jeffery et al. (2007)                      |
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 which ranged from 26 to 29°C. 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 up to 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 LD₅₀ in vivo

The virulence of the A. hydrophila (NPAH-1) was further explored in vivo based on 50% lethal dose (LD₅₀) in goldfish. For determination of 50% lethal dose (LD₅₀), intramuscular injection of 0.1 ml bacterial suspension each of 10⁴, 10⁵, 10⁶ and 10⁷ 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 LD₅₀ 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, Amoxicillin, 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) |

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                   | NPAH-1                | NPCF-1               | NPA-1            |
| 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           | +                        | +                    | +                    | +                |
| Malose            | +                        | +                    | +                    | +                |
| 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 housekeeping 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 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 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

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**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.

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), cytotoxic 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.

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

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 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<sub>50</sub> in vivo**

In general bacterial isolates are grouped as virulent, weakly virulent and avirulent according to the LD<sub>50</sub> values 10<sup>4.5-5.5</sup>, 10<sup>5.5-6.5</sup> and >10<sup>7</sup> cfu ml<sup>-1</sup> respectively (Lallier and Daigneault, 1984). While calculating LD<sub>50</sub> 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<sup>4</sup>, 1x10<sup>5</sup>, 1x10<sup>6</sup> and 1x 10<sup>7</sup> CFU per fish, respectively. LD<sub>50</sub> value was estimated as 10<sup>5.35</sup> 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
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         |
| Cefixime / Clavulanic acid   | 5/10                      | 21                   | S         |

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

The strain was intermediate (I) sensitive to Erythromycin, Gentamicin and Oxytetracycline while susceptible to Cefixime, Chloramphenicol, Nitrofurantoin, Kanamycin, Ciprofloxacin, Furazolidone and Cefixime/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).

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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References

Austin, B. and Austin, D. 2007. Bacterial fish pathogens: Diseases of farmed and wild fish, 4th edn. Praxis Publishing Ltd., Chichester, UK.

Ayyappan, S., Jena, J. K., Gopalakrishnan, A. and Pandey, A. K. 2006. Handbook of fisheries and aquaculture. Directorate of Information and Publications of Agriculture, Indian Council of Agricultural Research, New Delhi, 22, 354 pp.

Barrow, G. I. and Feltham, R. K. A. 2004. Cowen and Steel’s manual for the identification of medical bacteria. Cambridge University Press, UK.

Bennasar, A., Guasp, J. and Lalucat, J. 1998. Molecular methods for the detection and identification of Pseudomonas stutzeri in pure culture and environmental samples. Microb. Ecol., 35(1): 22-53. DOI:10.1007/s002489900057.
Aeromonas hydrophila associated with mass mortality of Carassius auratus

Moore, E. R. B., Mau, M., Arnseidt, A., Böttger, E. C., Hutson, R. A., Collins, M. D., Van de, P eer Y., De Wachter, R. and Timmis, K. N. 1996. The determination and comparison of the 16S rRNA gene sequence of species of the genus Pseudomonas (sensu stricto) and estimation of the natural intrageneric relationships. Syst. Appl. Microbiol., 19(4): 478-492. doi:10.1016/S0723-2200(96)80021-X.

Nielsen, M. E., Hoi, L., Schmidt, A. S., Qian, D., Shimada, T., Shen, J. Y. and Larsen, J. L. 2001. Is Aeromonas hydrophila the dominant motile Aeromonas species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China. Dis. Aquat. Organ., 46(1): 23-29. DOI: 10.3354/daa046023.

Noga, E. J. 2010. Fish disease: Diagnosis and treatment, 2nd edn. John Wiley & Sons, Inc., Iowa, USA.

Omparkasam, M. and Manohar, L. 1991. Experimental infection of some bacterial fish pathogens in the cichlid fish, Oreochromis mossambicus. Ind. J. Fish., 38: 106-110.

Ottaviani, D., Pariani, C., Citterio, B., Masini, L., Leoni, F., Canonico, C., Sabatini, L. and Bruscolini, F. 2011. Putative virulence properties of Aeromonas strains isolated from food, environmental and clinical sources in Italy: a comparative study. Int. J. Food Microbiol., 144(3): 538-545. doi: 10.1016/j.ijfoodmicro.2010.11.020.

Puthucheary, S. D., Puah, S. M. and Chua, K. H. 2012. Molecular characterisation of clinical isolates of Aeromonas species from Malaysia. PLoS ONE, 7(2): e30205.

Reed, M. J. and Muench, M. 1938. A simple method for estimating fifty percent endpoints. Am. J. Hyg., 27(3): 493-497.

Santos, J. A., Gonzalez, C. J., Otero, A. and Garcia-Lopez, M. L. 1999. Haemolytic activity and siderophore production in different Aeromonas species isolated from fish. Appl. Environ. Microbiol., 65(12): 5612-5614.

Sen, K. and Rodgers, M. 2004. Distribution of six virulence factors in Aeromonas species isolated from US drinking water utilites: a PCR identification. J. Appl. Microbiol., 97(5): 1077-1086.

Sha, J., Kozlova, E. V. and Chopra, A. K. 2002. Role of various enterotoxins in Aeromonas hydrophila-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. Infect Immun., 70(4): 1924-1935. doi: 10.1128/IAI.70.4.1924-1935.2002.

Soler, L., Yanez, M. A., Chacon, M. R., Aguilar-Areola, M. G., Catalan, V., Figueras, M. J. and Martinez-Murcia, A. J. 2004. Phylogenetic analysis of the genus Aeromonas based on two housekeeping genes. Int. J. Syst. Evol. Microbiol., 54(5): 1511-1519. DOI:10.1099/ijs.0.03048-0.

Sreedharan, K., Philip, R. and Singh, I. S. B. 2012. Virulence potential and antibiotic susceptibility pattern of motile aeromonads associated with freshwater ornamental fish culture systems: a possible threat to public health. Braz. J. Microbiol., 43(2): 754-765. doi: 10.1590/S1517-83822012000200040.

Stone, D. M., Ahne, W., Denham, K. D., Dixon, P. F., Liu, C. T. Y., Sheppard, A. M., Taylor, G. R. and Way, K. 2003. Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhadovirus isolates reveals four genogroups. Dis. Aquat. Org., 53(3): 203-210. DOI:10.3354/dao053203.

Strauss, E. J. and Falkow, S. 1997. Microbial pathogenesis: genomic and beyond. Science, 276(5313): 707-712. DOI: 10.1126/science.276.5313.707.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. Mol. Biol. Evol., 28(10): 2731-2739. doi: 10.1093/molbev/msr121.

Vadivelu, J., Puthucheary, S. D., Phillips, M. and Chee, Y. W. 1995. Possible virulence factors involved in bacteremia caused by Aeromonas hydrophila. J. Med. Microbiol., 42(3): 171-174. doi: 10.1099/00222615-42-3-171.

Verner-Jeffreys, D. W., Welch, T. J., Schwarzand, T., Pond, M. J., Woodward, M. J., Haig, S. J., Rimmer, G. S. E., Roberts, E., Morrison, V. and Austin, C. B. 2009. High prevalence of multidrug-tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. PLoS ONE, 4: e8388.

Vivekanandhan, G., Savithamani, K., Hathaand, A. A. and Lakshmanaperumalsamy, P. 2002. Antibiotic resistance of Aeromonas hydrophila isolated from marketed fish and prawn of South India. Int. J. Food Microbiol., 76(1): 165-168. DOI: 10.1016/S0168-1605(02)00009-0.

Wang, L., Wei, Y., Yuan, G., Dai, M. and Chen, X. 2012. Molecular characterisation and virulence genes of Aeromonas hydrophila isolated from the Chinese giant salamander (Andrias davidianus). Asian Herpetol. Res., 3(4): 303-309.

Weisburg, W. G., Barns, S. M., Pelletierand, D. A. and Lane, D. J. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol., 173(2): 697-703. DOI: 10.1128/jb.173.2.697-703.1991.

Yi, S. W., You, M. J., Cho, H. S., Lee, C. S., Kwonand, J. K. and Shin, G. W. 2013. Molecular characterisation of Aeromonas species isolated from farmed eels (Anguilla japonica). Vet. Microbiol., 164(1): 195-200. doi: 10.1016/j.vetmic.2013.02.006.

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