Journal of Fisheries
Volume 10 Issue 1 April 2022
Peer Reviewed | Open Access

eISSN 2311-3111
pISSN 2311-729X

Original Article

Phenotypic characterization, genetic analysis and antibiotic sensitivity of *Aeromonas hydrophila* isolates causing dropsy in cultured *Labeo rohita* from Punjab, Pakistan

Fatima Sughra[1] • Muhammad Hafeez-ur-Rehman[1] • Farzana Abbas[1] • Imran Altaf[2] • Saira Razzaq[1] • Sidra Kanwal[1] • Muhammad Inayat[1] • Iqra Anwer[1] • Muhammad Akram[1]

[1] *Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[2] *Quality Operations and Research Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan*

Correspondence
Fatima Sughra; Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore, Pakistan
2017-phd-1034@uvas.edu.pk, mhafeezurehman@uvas.edu.pk

Manuscript history
Received 19 October 2021 | Accepted 31 January 2022 | Published online 25 March 2022

Citation
Sughra F, Hafeez-ur-Rehman M, Abbas F, Altaf I, Razzaq S, Kanwal S, Inayat M, Anwer I, Akram M (2022) Phenotypic characterization, genetic analysis and antibiotic sensitivity of *Aeromonas hydrophila* isolates causing dropsy in cultured *Labeo rohita* from Punjab, Pakistan. Journal of Fisheries 10(1): 101207.

Abstract
Motile Aeromonas septicemia (MAS) is a common freshwater fish disease and major threat to the aquaculture in Pakistan. The present study was carried out on suspected fish samples to isolate and characterize local strains of *Aeromonas hydrophila*, a key pathogen responsible for the said disease in aquacultured fishes. A total of ninety suspected fish specimens were collected from fish farms in Kasur, Okara and Gujranwala districts of Punjab, Pakistan from June 2018 to April 2019. The specimens were processed and *A. hydrophila* strains were isolated. The primary identification of sixty seven isolates were verified by colony morphology, microscopy and phenotypic characterization with ten biochemical reactions. The *A. hydrophila* strains of test samples were molecularly characterized by polymerase chain reaction (PCR) using 16S rRNA at desired size of 356bp. The PCR amplified product was subjected to DNA sequencing and phylogenetic analysis showed homology with related strains of *Aeromonas* spp. By antibiotic sensitivity test, the isolates were checked for nine antibiotics in which the pathogen was sensitive to four and resistant to five drugs. Results of genetic analysis confirmed strains as *A. hydrophila* which are useful to take preventive measures against the said disease.

Keywords: 16S rRNA; *Aeromonas hydrophila*; antibiotic sensitivity; dropsy disease; *Labeo rohita*; phylogenetic analysis; polymerase chain reaction

1 | INTRODUCTION

The carps are important source of fish meat in Pakistan and neighboring countries. Indian major carps including Rohu (*Labeo rohita*), Mori (*Cirrhinus mrigala*) and Thaila (*Catla catla*) are considered potential source of protein in Pakistan. These carps are considered high-value commercial fish species and being cultured on priority by local farmers (Sheikh *et al.* 2017). On the other hand, Pakistan aquaculture industry is facing threats from several sources, with disease being the most critical hurdle to more semi-intensive, intensive carp farming and feral systems (Iqbal 2016). This bacteria especially belonging to the genus *Aeromonas* (family Aeromonadaceae) are widespread in freshwater environment, and have been implicated as fish pathogens (Pridgeon and Klesius 2011; Mishra *et al.* 2017). It is an opportunistic pathogen most likely responsible for infectious abdominal dropsy, ulcer disease, Motile Aeromonas Septicemia (MAS), hemor-

rhagic septicemia and red-sore disease at different growth stages leading to high mortality rates in aquaculture (Toranzo *et al.* 2005; Igbinosa *et al.* 2012). This bacterium can be found in fresh, salt, marine, estuarine, chlorinated, and un-chlorinated waters and can survive in both aerobic and anaerobic environments (Bartlett *et al.* 2011).

Aeromonas hydrophila is rod shaped heterotrophic, gram-negative bacterium ubiquitously present in freshwater environment. It is normally inhabited in gastrointestinal tract of fish and water bodies and has ability to grow at low temperature (Abbott *et al.* 2003). *Aeromonas hydrophila* is a diet tolerated pathogen that is accountable for severe zoonotic diseases (Harikrishnan and Balasundaram 2005; Rey *et al.* 2009; AlYahya *et al.* 2018). Seasonal incidents also cause more stress in fish farms. In particular during monsoon period when fish breeding season is at peak in Punjab region, the high temperature, high levels of ammonia and nitrites, less amount of dissolved oxygen (DO), presence of high amount of carbon dioxide (CO_2), organic pollution and malnutrition enable the *A. hydrophila* to develop in aquacultures quite rapidly. Moreover, heavy infestation with parasites, presence of injured fish and spawning activity creates a stressed environment for fish while favorable environment for *A. hydrophila* to grow (Iqbal 2016; Kotob *et al.* 2016).

In a variety of freshwater species, the existence and pathogenicity of *A. hydrophila* has previously been reported in different fish species particularly *Carassius auratus* (Citarasu *et al.* 2011), *Cyprinus carpio* (Citarasu *et al.* 2011), *Anabas testudineus* (Hossain *et al.* 2011; Sarder *et al.* 2016), *Clarias gariepinus* (Laith and Najiah 2013), *Oncorhynchus mykiss* (Cagatay and Sen 2014), *Oreochromis mossambicus* (Sarder *et al.* 2016) and *Potamotrygon motoro* (Yun *et al.* 2017). It has been recently reported that *A. hydrophila* is the key bacterial pathogen that causes diseases in freshwater fish species (Stratev and Odeyemi 2016). The pathogen has recently emerged in United States where it has been responsible for extensive farm losses in catfish aquaculture (Peatman *et al.* 2018).

The fish diseases caused by *A. hydrophila* are responsible for major economical losses of local fish farmers in Pakistan (Iqbal 2016). Diseases in inland aquaculture are currently being treated by antibiotic products. To our knowledge, disease prevention mechanisms in terms of local or imported vaccine products have not been explored for fish culture systems in the country to date. Under these circumstances, it will be worthwhile to characterize the local bacterial strains that may be considered potential threat to fish farming industry and pave the way forward for its preventive measures in terms of autogenous vaccines.

The premier objective of present work is to perform phenotypic identification, molecular characterization and phylogenetic analysis of local strains of *A. hydrophila* in aquacultured carps of Pakistan. The resulting bacterial isolates are to be used for subsequent remedial actions against *A. hydrophila* strains for prevention of disease in farmed fish species of Pakistan.

2 | METHODOLOGY

2.1 Specimens collection

A total of ninety specimens of naturally infected *Labeo rohita* were carefully collected from eight fish farms and barrages in Kasur (31°05′N 74°30′E), Okara (30°50′N 73°31′E) and Gujranwala (32°10′N 74°12°E) districts of Punjab, Pakistan. The live and dead samples weighing 175 ± 25 g or length 10 ± 1 cm, were immediately transported in a cleaning bag in containers with cooled ice bags to Quality Operations Laboratory (QOL), University of Veterinary and Animal Sciences (UVAS), Lahore for further processing within 3 h. Morphological examination of samples showed symptoms of abdominal dropsy, exophthalmia, skin discoloration, shedding of the scales, hemorrhages on body surface, distended vent, ulceration on skin assorted from deep of necrotizing skin ulcers, fin erosions, sero-hemorrhagic and discharge of fluid from vents.

2.2 Isolation of bacteria

For isolation of bacteria nutrient agar, nutrient broth and MacConkey agar media were used with standard compositions. Sterilization was done by autoclaving media at 121°C under 15 lbs and glassware in hot air oven at 180°C for 10 minutes. Pouring of autoclaved media was done for preparation of MacConkey agar plates. For the sterilization check, the plates were incubated at 37°C for 24 h. Body surfaces of fish samples were first cleaned with 70% alcohol and then dissected under sterile laboratory conditions. Smears were taken aseptically using sterile loop from kidney, liver and spleen and streaked to MacConkey agar plates. After streaking the plates were incubated at 37°C for 24 h following the procedure described by Al-Fatlawy and Al-Hadrawy (2014) and Cagatay and Sen (2014).

2.3 Phenotypic characterization of isolates

The identifications of bacterial isolates were verified by colony morphology, microscopy and biochemical characterization. The isolated strains were tested using ten different phenotypic tests. These tests were performed in a conventional format as previously described, and appropriate positive and negative controls were included for each test (Jayavignesh *et al.* 2011). Morphological characterization of isolates included size and shape of colony and Gram's reaction along with motility test (Shahzad *et al.* 2016). Catalase test, urease test, Voges-Proskauer (VP) test and five different carbohydrate fermentation tests were also conducted for bio-chemical characterization of *A. hydrophila* (Samal *et al.* 2014). Observations on carbohydrate metabolism reaction included production of acid

and gas in sucrose and glycerol tests; and production of acid in L-arabinose, D-mannitol, and salicin tests. For biochemical tests, bioMérieux® API-20E microbiological kit (reference No. 20160, United States) was used (Abbot *et al.* 2003).

2.4 Genetic analysis of isolates

Gnomic DNA extraction: The DNA extraction was done from a representative isolate of the biochemical results using GeneAll® ExGene™ DNA purification kit (model: Clinic SV, Korea) following manufacturer's protocol (Byers *et al.* 2002). The cells were harvested by centrifugation. The supernatant was discarded and then re-suspended to 20 µl of proteinase K solution (20 mg ml^{-1}) and 200 µl of CL buffer. The mixture was heated in water bath at 56°C for 15 min. Then concisely spin down the tube to eliminate drops from the lid. The tube was filled with 200 µl BL buffer. Then placed in the water bath for 10 min at 70°C and the mixture was concisely spin down the tube to eliminate drops from the lid. Absolute ethanol (200 µl) was added in tube, mixed by vortex and was spin to eliminate the drops. The mixture was carefully moved to SV column then centrifuged at 8000 rpm for 1 minute. After that 600 µl BW buffer was added and centrifuged at 8000 rpm for 1 minute. Mixture was transferred into new SV column. After adding 700 µl TW buffer mixture was centrifuged at 8000 rpm for 1 minute and supernatant was removed. Then SV column was transferred into collection tube. The residual wash buffer was eliminated by centrifugation for 1 minute at 13000 rpm. The SV column was placed in 1.5 ml of micro centrifuge tube and 200 µl of AE buffer was added. As a last step, tubes were incubated and centrifuged for 1 minute at 13000 rpm.

PCR amplification: The genomic DNA of *A. hydrophila* was amplified by using universal primer purchased from Gene Link™ (NY, USA). For 16S ribotyping, universal primer with sense 5'GGG AGT GCC TTC GGG AAT CAG A'3 and antisense 5'TCA CCG CAA CAT TCT GAT TTG'3 with product size of 356 bp was used. For PCR, reaction mixture of 25 µl was comprised of followings: Master mix (12.5 µl), DNA sample (2 µl), forward primer (1 µl), reverse primer (1 µl) and nucleus free water (8.5 µl). The composition of the master mixture was 0.05 U μL^{-1} Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$ and 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). PCR amplification was done by using BIO-RAD® T100 Thermal Cycler™ (model: T100, United States) for 50 cycles with each cycle's conditions as follows: 5 minutes for initial denaturation at 95°C, 0.5 minute for denaturation at 95°C, 0.5 minute for annealing at 56.9°C, 0.5 minutes for elongation at 72°C and finally 7 minutes for elongation at 72°C. After the completion of 50 cycles the yield was observed by 1% agarose gel electrophoresis. The amplification primers listed in Table 1 were used for the forward and reverse sequencing. The target genes amplified in this study along with primer pairs, sequences, amplified product size and references are also provided in Table 1.

TABLE 2 Primers used for PCR amplification.

Target gene	Primer pair	Sequence (5'–3')	Amplified product size	References
16S rRNA	Ah16SF Ah16SR	GGG AGT GCC TTC GGG AAT CAG A TCA CCG CAA CAT TCT GAT TTG	356	Wang *et al.* (2003); Furmanek-Blaszk (2014)
16S rRNA Universal PCR primer	27F 1492R	AGA GTT TGA TCM TGG CTC AG TAC GGY TAC CTT GTT ACG ACT T	1538	Ludwig *et al.* 1995 (Acc # NR_119190)
16S rRNA Universal sequencing primer	785F 907R	GGA TTA GAT ACC CTG GTA CCG TCA ATT CMT TTR AGT TT	1538	Ludwig *et al.* 1995 (Acc # NR_119190)

Gel electrophoresis: For electrophoresis, the buffer was prepared as per manufacturer's instruction. One percent agarose gel was prepared by adding 100 ml of TAE buffer in 1 g of agarose. The solution was heated in microwave oven until it became transparent and 5 µl ethidium bromide was added. Agarose solution was then poured carefully in electrophoresis tray with suitable comb. After 30 to 40 minutes the gel solidified the comb was removed and tray was placed in electrophoresis tank filled with electrophoresis buffer. The 2 µl of loading dye was mixed with 5 µl of DNA sample. By using micropipette the samples were loaded on gel. After that 2 µl of DNA ladder was loaded in first well at the right and last well at left side of the gel. After power on, the gel was run and DNA moved towards the anode. The voltage was applied at 120 V cm^{-1} for 35 minutes. The gel tray was further removed and placed on a transilluminator. The DNA bands were visualized under Bio-Rad® UV transilluminator (United States). The amplified PCR products were sequenced directly from MacroGen® (South Korea).

Analysis of nucleotide sequence: The initial and final portions of the sequences were manually trimmed in BioEdit Sequence Alignment Editing software (version 7.2.5). The resulting high-quality fragments of the sequences were exported in FASTA format for comparison with GenBank database using online BLAST (by NCBI, USA) optimized for highly similar sequences (megablast). The

query coverage and percent identity values ≥98% were considered for specific identification. The three sequences obtained in the present study were deposited at NCBI GenBank under nucleotide accession numbers MT249820, MT249821 and MT249822. The supplementary information in terms of fish species, place of origin, year and season of collection, and size of amplified PCR products was also provided.

The consensus nucleotide sequences were aligned with the help of ClustalW bioinformatics software (Tamura *et al.* 2013). The genetic distance of aligned sequences was obtained using "Kimura's Two-parameter Model" (Kimura 1980). Phylogenetic analysis was performed for isolated strains with reference strains using neighbor-joining method, and bootstrap values were calculated with 1000 replicates. Evolutionary trees were constructed with the help of MEGA software (version 6.06) by Kumar *et al.* (2001).

Phylogenetic analysis: The phylogenetic diagrams were constructed for verification of sequencing data using highly similar reference sequences and out groups from NCBI Genebank along with three sequences of present study. All the sequences were taken in FASTA format for subsequent pair-wise and multiple alignment using the ClustalW alignment tool integrated within MEGA software v. 6.06 (Thompson *et al.* 1997). The aligned sequences were then subject to phylogenetic analysis. As a result of the preliminary analysis, a neighbor-joining phylogenetic diagram was constructed using the Kimura 2-parameter model, a gamma-shape parameter with 5 categories and nearest-neighbor-interchange tree inference options. Bootstrap analysis with 1000 replications was used for assessment of stability in internal nodes.

2.5 Antibiotic sensitivity analysis

The antibiogram of isolates against antibiotics was studied by standard disc diffusion assay method (Vivekanandhan *et al.* 2002; Ramalivhana *et al.* 2009). The sterilized media was poured into petri plates. By using sterile swabs the isolates were spread on the plates. Antibiotic disc was placed on the surface of the nutrient agar for the sensitivity test. The plates were incubated at 37°C for 24 h. Nine antibiotics were used is this study as follows: penicillin, colistin sulphate, oxytetracycline, novobiocin, ciprofloxacin, gentamicin, trimethoprim, tetracycline, nitrofurantoin. After 24 h, the zones of inhibition were measured (Odeyemi and Ahmad 2017).

3 | RESULTS

3.1 Clinical and post mortem findings

The fish was analyzed in terms of total length, fork length and weight. The naturally infected *L. rohita* showed symptoms like abdominal swelling, scales extrusions, hemorrhagic septicemia, necrotizing ulcer on body, exophthalmia and sero-hemorrhagic fluids from the vent (Figure 1).

The post-mortem examination of diseased fish revealed septicemia represented by gill and kidney congestion. The lesions appeared on infected fish as hemorrhage at base of fins or on the skin (Figure 1a), distended abdomens (Figures 1b and 1d), and protruded eyes (Figure 1c).

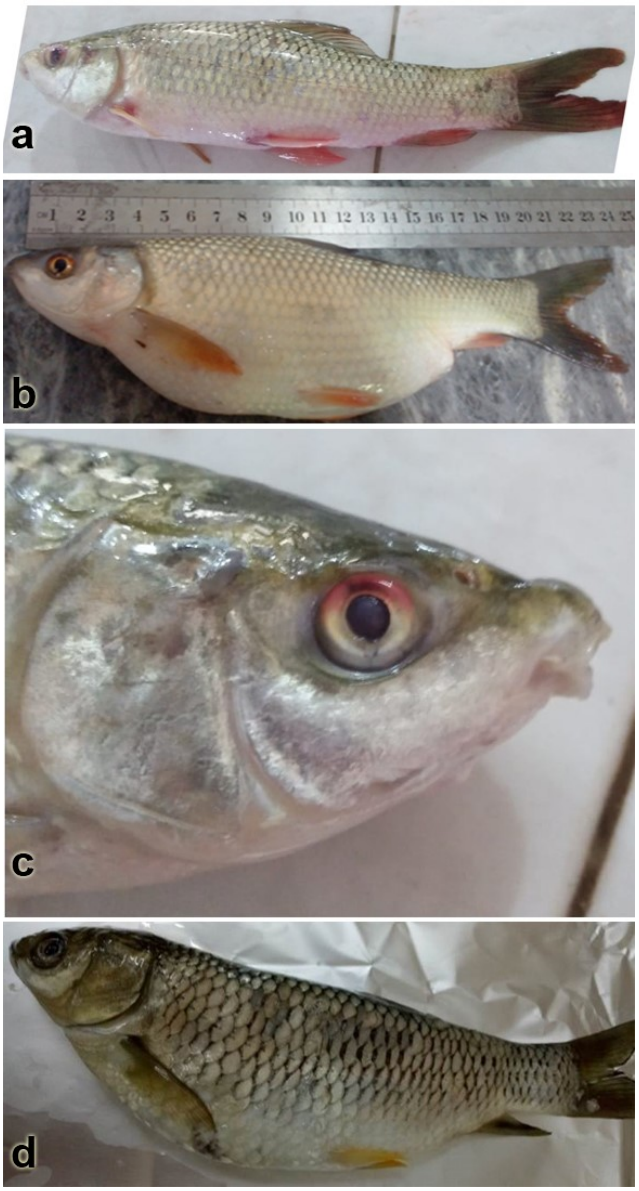

FIGURE 1 Naturally infected *Labeo rohita*. a, skin ulceration; b, abdominal dropsy; c, infected eye; d, abdominal dropsy.

3.2 Phenotypic characterization

Out of ninety specimens, 67 (74%) were identified as *A. hydrophila* through phenotypic characterization. *Aeromonas hydrophila* colonies appeared in pale yellowish color, round shaped with rounded end on MacConkey agar medium when incubated at 37°C for 24 h (Figure 2).

For the microscopic study, the bacterial cultures were examined by Gram's staining method. After staining they were observed as rod shape, single or paired and infrequently as short chains non-spore forming gram-

negative bacillus (Figure 2). All the isolates were motile in distilled water as well as peptone water on slide, which indicates the positive result in distilled water motility test.

Biochemical test results for three isolates are provided in Table 2, which show that bacterial isolates are uniformly positive for catalase and voges proskauer (VP) tests whereas negative reaction was observed for urease test. Out of ten biochemical tests, five glucose fermentation tests were performed in which isolates utilized sucrose, D-mannitol, glycerol and salicin. However L-arabinose did not ferment. On the basis of biochemical reactions and glucose fermentation, the isolates were identified as *A. hydrophila*.

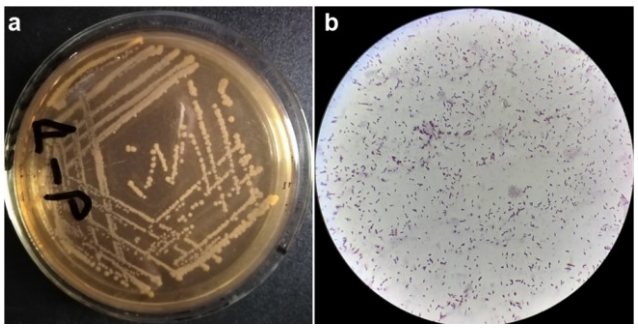

FIGURE 2 (a) Phenotypic characters of *Aeromonas hydrophila* strain on MacConkey agar medium; (b) Gram's staining appearance of *A. hydrophila*.

TABLE 2 Reaction results of *Aeromonas hydrophila* isolates.

Test	QOL787	QOL788	QOL789
Gram's reaction	Negative	Negative	Negative
Motility test	Positive	Positive	Positive
Catalase test	Positive	Positive	Positive
Voges proskauer test	Positive	Positive	Positive
Urease test	Negative	Negative	Negative
Sucrose test	Positive	Positive	Positive
D-mannitol test	Positive	Positive	Positive
Glycerol test	Positive	Positive	Positive
Salicin test	Positive	Positive	Positive
L-arabinose test	Negative	Negative	Negative

3.3 Genetic analysis

Molecular characterization revealed that all the isolates were *A. hydrophila* based on amplification by PCR technique. The genomic DNA of bacterial isolates was extracted as per protocols described by GeneAll® for ExGene™ DNA purification kit. Universal 16S rRNA gene has been amplified from genomic DNA of all *A. hydrophila* isolates.

The DNA were examined by electrophoresis on 1% agarose gels with 100 bp ladder for estimating the bands. The DNA bands were documented and pictured in gel documentation system (Biorad® Gel Doc XR system, United States). The samples provided were confirmed through PCR as *A. hydrophila*. PCR was run using universal primer for 16S rRNA gene and resulting band having amplicon size 356 bp (Figure 3).

To validate the *Aeromonas* characterization, the chosen strains were identified by 16S rRNA PCR pattern analysis on 1% agarose gel electrophoresis. The 16S rRNA sequences of the bacterial strains acquired in this study were aligned with bacterial nucleotide sequence data available at GenBank database using online BLAST program (Basic Local Alignment Search Tool) by National Center for Biotechnology Information (NCBI), USA. The obtained sequences highly resembled with nucleotide of the same bacterial species as listed in Table 3.

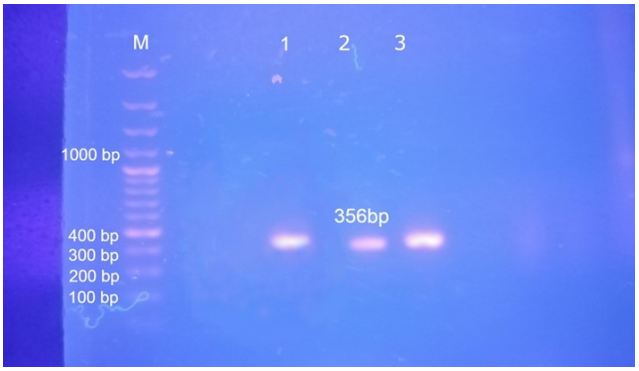

FIGURE 3 Agarose gel electrophoresis (1%) of PCR Amplify of 16S rRNA gene of *Aeromonas hydrophila* isolates.

The phylogenetic tree of Figure 4 shows close resemblance of our isolate QOL788 to *A. hydrophila* strains on GenBank database after performing BLAST. The phylogenetic tree of Figure 5 depicts a consolidated genetic relationship of isolated strains among each other as well as with reference strains. The trees also show genetic heterogeneity and distance within the species due to different sources of collection. However all three isolates were confirmed as *A. hydrophila*.

3.4 Antibiotic sensitivity

The results of the antibiotic resistance tests of each isolate on nine antibiotics are presented in Table 4. Resistance test results of *A. hydrophila* against some antibiotics were marked by the formation of clear zones around the antibiotic dishes. *Aeromonas hydrophila* isolates were resistant to penicillin and novobiocin and sensitive to colistin sulphate, oxytetracycline, ciprofloxacin, gentamicin, trimethoprim, tetracycline and nitrofurantoin. Figure 6 shows the measurement of inhibition zones of different antibiotics for QOL787 sample.

4 | DISCUSSION

Fish is a significant source of protein in daily food and plays a vital role in agricultural economy. Farmed fish species especially *Labeo rohita*, is suffering from infectious diseases caused by microbial pathogens (Iqbal 2016). Predominantly these pathogens have bacterial origin and among those, *Aeromonas* species like *A. hydrophila* causes majority of the diseases in different fish

species. The gram-negative *A. hydrophila* is pervasive in freshwater ponds causing fish mortality and economic losses to farmers mainly in semi-intensive culture system in Pakistan (Sheikh *et al.* 2017).

TABLE 3 Selected Consensus sequences showing significant resemblance with *Aeromonas hydrophila* isolates (MT249820 - MT249822).

NCBI Description	Max score	Query coverage	Max. identity	Accession No.	Origin
Aeromonas dhakensis strain SA	1256	100%	100%	MT193203	Tamilnadu, India; 2019
Aeromonas caviae strain ACDMC1235	1256	100%	100%	MK598335	Tamilnadu, India; 2019
Aeromonas hydrophila strain RP1	1256	100%	100%	MG607374	Chandigarh, India; 2017
Aeromonas dhakensis strain VITSMBJ1	1709	100%	99.68%	MN854047	Tamilnadu, India; 2019
Aeromonas hydrophila gene for 16S ribosomal RNA, partial sequence, strain: BR	1703	100%	99.57%	AB901365	Tamilnadu, India; 2014
Aeromonas hydrophila strain S2-112	1227	98%	99.26%	MF111726	Beijng, China; 2017

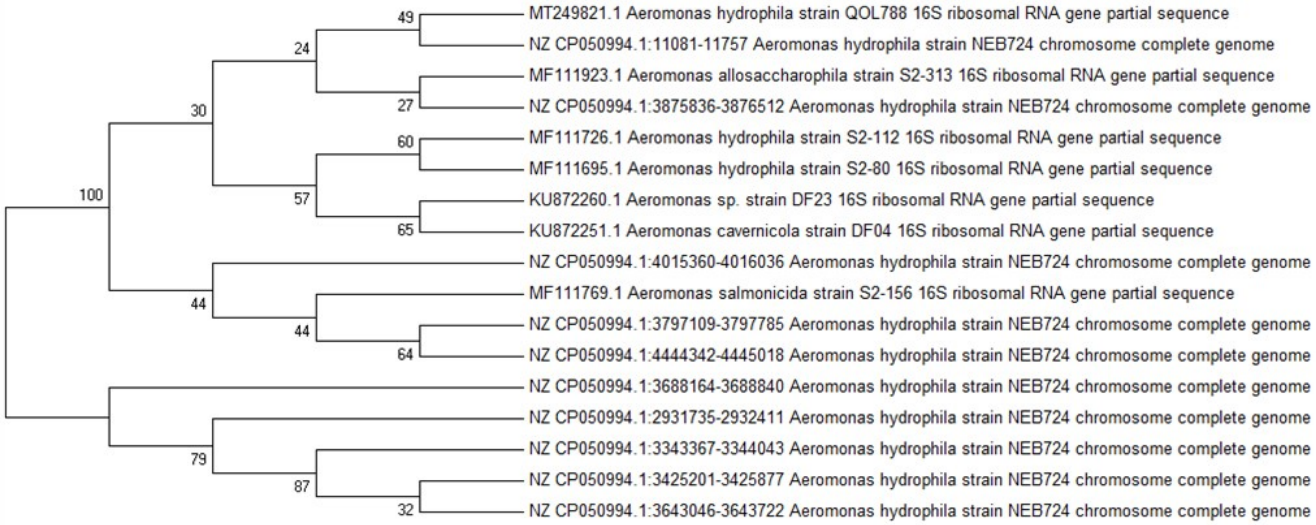

FIGURE 4 Phylogenetic topology of QOL788 isolate showing genetic relationship with reference strains of *Aeromonas* spp.

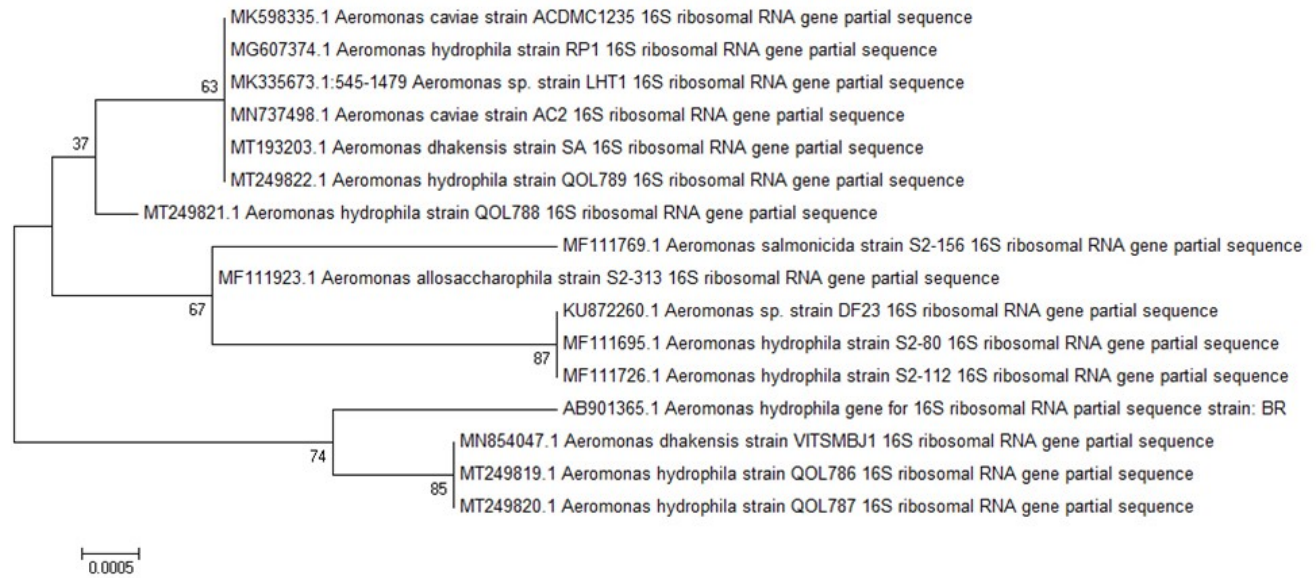

FIGURE 5 Phylogenetic tree of *Aeromonas hydrophila* isolates (MT249820 – MT249822) was computed using neighbor-joining method with MEGA 6.06 showing close similarity to other species of *Aeromonas* spp.

TABLE 4 Antibiotic sensitivity tests of *Aeromonas hydrophila* strains (QOL787 – QOL789). S, sensitive; R, resistant

Antibiotic drugs with symbols	Disk potency (µg disk^{-1})	Isolate Codes					
		QOL787		QOL788		QOL789	
		Inhib. zone (cm)	Result	Inhib. zone (cm)	Result	Inhib. zone (cm)	Result
Penicillin (GP)	10 U	0	R	0	R	0	R
Colistin sulphate (CT)	10	1.5	S	1.7	S	1.4	S
Oxytetracycline (OT)	30	3.0	S	3.0	S	3.0	S
Novobiocin (NV)	30	1.4	R	1.7	R	1.3	R
Ciprofloxacin (CIP)	5	4.3	S	4.7	S	2.8	S
Gentamicin (CN)	10	3.1	S	2.4	S	2.7	S
Trimethoprim (W)	5	2.0	S	1.9	S	2.0	S
Tetracycline (TE)	10	2.3	S	2.4	S	2.7	S
Nitrofurantoin (F)	300	2.1	S	2.3	S	2.2	S

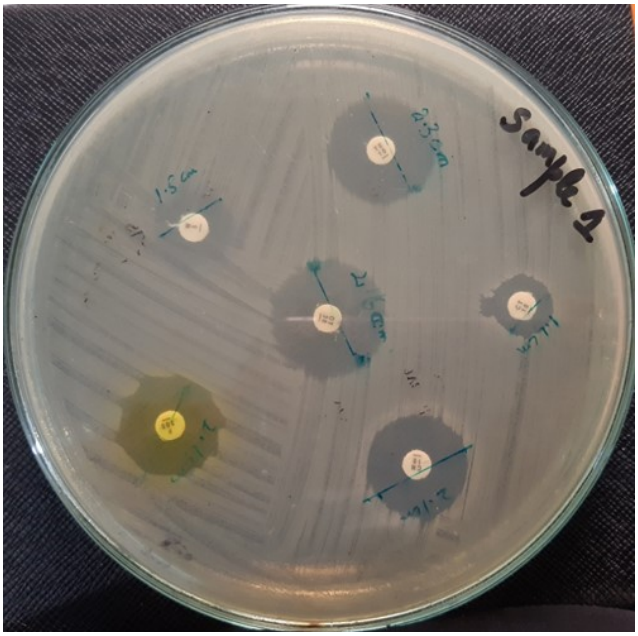

FIGURE 6 Antibiotic sensitivity analysis for sample 1 (QOL787) with colistin sulphate, tetracycline, novobiocin, nitrofurantoin and trimethoprim antibiotic drugs.

In present study, three isolates of *A. hydrophila* were recovered from infected *L. rohita* specimens collected from different fish farms in Punjab, Pakistan. Post mortem findings of the infected *L. rohita* were hemorrhages at the base of fin and edge of head, ulcerative skin lesions on body and tail erosion which are more or less similar with the findings of Rahman *et al.* (2002), Mathur *et al.* (2005), Hassan *et al.* (2017), and Saharia *et al.* (2018). Congested liver and internal organs were also observed in the infected fishes by Dahdouh *et al.* (2016) and Matter *et al.* (2018). Minor variations might be due to different fish species and seasonal variations that influence the disease incidence.

For bacterial identification, biochemical reactions are very important. These reactions help bacteria to provide energy by oxidation of organic substances or by fermentation. Based on ten bio-chemical reactions conducted in this study, the bacteria were identified as *A. hydrophila* which conform to previous biochemical studies in India (Jayvignaish *et al.* 2011; Samal *et al.* 2014), , United States (Nawaz *et al.* 2006), and Egypt (Wassif 2018). Our study extends the work of Shahzad *et al.* (2016) towards molecular characterization using 16S rRNA universal gene. The 16S rRNA gene sequencing is an accurate and objective method for identification of microorganisms in the clinical laboratory (Bisen *et al.* 2012).

Species of the genus *Aeromonas* exhibit very high levels of overall 16S rRNA gene sequence similarity in nucleotides. Species such as *A. hydrophila*, *A. dhakensis* and *A. caviae* exhibit very few differences. Through phylogenetic analysis, it is found that our local isolate MT249820 is most closely related to *A. hydrophila* strains (Accession No. AB901365) which was reported earlier in 2014 from Tamilnadu, India. Our strains are very closely related to *Aeromonas dhakensis* strain (Accession No. MT193203) which is more recently discovered strain from Tamilnadu, India. The results of present study confirm that *A. hydrophila* strains are geographically more related to those in India (Sarkar *et al.* 2012; Rani *et al.* 2016), Bangladesh (Sarder *et al.* 2016; Monir *et al.* 2017), China (Nielsen *et al.* 2001; Hu *et al.* 2012) and South Korea (Yun *et al.* 2017).

The results of sensitivity tests to a wide number of antibiotics could be used for diagnosis of MAS and other infectious diseases in different outbreak and epidemiological conditions in aquaculture. Current results showed that all *A. hydrophila* isolates were sensitive to the selected antibiotics of colistin sulphate, oxytetracycline, ciprofloxacin, gentamicin, trimethoprim, tetracycline and nitrofurantoin. Shahzad *et al.* (2016) from Pakistan and Kusdarwati *et al.* (2018) from Indonesia obtained similar results on the chosen antibiotics. Resistance of *A. hydrophila* isolates against amoxicillin and penicillin has previously been reported from fish affected with bacterial diseases in India (Saha and Pal 2002), United Arab Emirates (Awan *et al.* 2009), Bangladesh (Nahar *et al.* 2016), Egypt (Hafez *et al.* 2018) and South Korea (Yun *et al.*

2017).

From this study, fish farmers will be benefited for controlling abdominal dropsy and other infectious diseases caused by *A. hydrophila* by the administration of specific theraputents. In particular, the *A. hydrophila* isolates of present study will be used for future research towards disease prevention against local strains of *A. hydrophila* in aquacultures of Pakistan.

ACKNOWLEDGEMENTS

The work presented in this paper is part of a project sponsored by Pakistan Science Foundation (PSF; grant No. PSF/NSLP/P-UVAS(701)). We express our gratitude to Quality Operation Lab and Parasitology Lab, UVAS for providing the assistance for conducting microbiological research work in this project.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Conceptualization, **FS** and **MH**; methodology, **FS** and **IA**; software, **FS** and **FA**; validation, **FS**, **MH** and **FA**; formal analysis, **IA**; investigation, **FS**, **SR** and **MA**; resources, **MA**; data curation, **IA**, **SR**, **SK**, **MI** and **FA**; writing—original draft preparation, **FS**; writing—review and editing, **MH**, **IA** and **FA**; visualization, **IA**, **MI**, **SR** and **IA**; supervision, **MH** and **IA**; project administration, **MH**; funding acquisition, **MH**. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The datasets have been deposited in the NCBI database under accession numbers MT249820, MT249821, MT249822.

REFERENCES

Abbott SL, Cheung WKW, Janda JM (2003) The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. Journal of Clinical Microbiology 41: 2348–2357.

Al-Fatlawy HNK, Al-Hadrawy HA (2014) Isolation and characterization of *A. hydrophila* from the Al-Jadryia River in Baghdad (Iraq). American Journal of Educational Research 2: 658–662.

AlYahya SA, Ameen F, Al-Niaeem KS, Al-Sa'adi BA, Hadi S, Mostafa AA (2018) Histopathological studies of experimental *Aeromonas hydrophila* infection in blue tilapia, *Oreochromis aureus*. Saudi Journal of Biological Sciences 25: 182–185.

Awan MB, Maqbool A, Bari A, Krovacek K (2009) Antibiotic susceptibility profile of *Aeromonas* spp. isolates from food in Abu Dhabi, United Arab Emirates. The New Microbiologica 32: 17.

Bartlett JG, Auwaerter PG, Pham PA (2011) Johns Hopkins ABX Guide 2012. Jones & Bartlett Publishers, Burlington, Massachusetts, United States.

Bisen PS, Debnath M, Prasad G (2012) Microbes: concepts and applications. John Wiley & Sons, NJ, United States.

Byers HK, Gudkovs N, Crane MSJ (2002) PCR-based assays for the fish pathogen *Aeromonas salmonicida*. I. Evaluation of three PCR primer sets for detection and identification. Diseases of Aquatic Organisms 49: 129–138.

Cagatay IT, Sen EB (2014) Detection of pathogenic Aeromonas hydrophila from rainbow trout (*Oncorhynchus mykiss*) farms in Turkey. International Journal of Agriculture and Biology 16(2): 435–438.

Citarasu T, Dhas A, Velmurugan S, Viji T, Kumaran T, ... Selvaraj T (2011) Isolation of *Aeromonas hydrophila* from infected ornamental fish hatchery during massive disease outbreak. International Journal of Current Research 2: 37–41.

Dahdouh B, Basha O, Khalil S, Tanekhy M (2016) Molecular characterization, antimicrobial susceptibility and salt tolerance of *Aeromonas hydrophila* from fresh, brackish and marine fishes. Alexandria Journal of Veterinary Sciences 48: 46–53.

Furmanek-Blaszk B (2014) Phenotypic and molecular characteristics of an *Aeromonas hydrophila* strain isolated from the river Nile. Microbiological Research 169: 547–552.

Hafez A-EE, Darwish WS, Elbayomi RM, Hussein MA, El Nahal SM (2018) Prevalence, antibiogram and molecular characterization of *Aeromonas hydrophila* isolated from frozen fish marketed in Egypt. Slovenian Veterinary Research 55: 445–454

Harikrishnan R, Balasundaram C (2005) Modern trends in *Aeromonas hydrophila* disease management with fish. Reviews in Fisheries Science 13: 281–320.

Hassan MA, Noureldin E, Mahmoud MA, Fita NA (2017) Molecular identification and epizootiology of *Aeromonas veronii* infection among farmed *Oreochromis niloticus* in Eastern Province, KSA. The Egyptian Journal of Aquatic Research 43: 161–167.

Hossain MF, Rahman MM, Sayed M (2011) Experimental infection of indigenous climbing perch *Anabas testudineus* with *Aeromonas hydrophila* bacteria. Progressive Agriculture 22: 105–114.

Hu M, Wang N, Pan Z, Lu C, Liu Y (2012) Identity and virulence properties of *Aeromonas* isolates from diseased fish, healthy controls and water environment in China. Letters in Applied Microbiology 55: 224–233.

Igbinosa IH, Igumbor EU, Aghdasi F, Tom M, Okoh AI (2012) Emerging *Aeromonas* species infections and their significance in public health. The Scientific World Journal 2012: 625023.

Iqbal Z (2016) An overview of diseases in commercial

fishes in Punjab, Pakistan. Fish Pathology 51: S30–S35.

Jayavignesh V, Sendesh Kannan K, Bhat AD (2011) Biochemical characterization and cytotoxicity of the *Aeromonas hydrophila* isolated from Catfish. Archives of Applied Science Research 3: 85–93.

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16: 111–120.

Kotob MH, Menanteau-Ledouble S, Kumar G, Abdelzaher M, El-Matbouli M (2016) The impact of co-infections on fish: a review. Veterinary Research 47: 98.

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17: 1244–1245.

Kusdarwati R, Rozi, Dinda ND, Nurjanah I (2018) Antimicrobial resistance prevalence of *Aeromonas hydrophila* isolates from motile aeromonas septicemia disease. IOP Conference Series: Earth and Environmental Science 137: 012076.

Laith AR, Najiah M (2013) *Aeromonas hydrophila*: antimicrobial susceptibility and histopathology of isolates from diseased catfish, *Clarias gariepinus* (Burchell). Journal of Aquaculture Research & Development 5: 215.

Ludwig W, Rossellö-Mora R, Aznar R, Klugbauer S, Spring S, ... Dorn S (1995) Comparative sequence analysis of 23S rRNA from proteobacteria. Systematic and Applied Microbiology 18: 164–88.

Mathur AK, Kumar P, Mehrotra S (2005) Abdominal dropsy disease in major carps of Meghalaya: isolation and characterization of *Aeromonas hydrophila*. Current Science India 88: 1897–1900.

Matter AF, El Asely AM, Shaheen AA, El-Gawad EAA, El-Abd H, Abbass AA (2018) Phenotypic and molecular characterization of bacterial pathogens isolated from diseased freshwater fishes. International Journal of Fisheries Aquatic Studies 6: 34–41.

Mishra SS, Rakesh D, Dhiman M, Choudhary P, Debbarma J, ... Ananda K (2017) Present status of fish disease management in freshwater aquaculture in India: state-of-the-art-review. Journal of Aquaculture & Fisheries 1: 003.

Monir M, Bagum N, Kabir S, Borty S, Ud-Doulah M (2017) Isolation, molecular identification and characterization of *Aeromonas hydrophila* from infected air-breathing catfish magur (*Clarias batrachus*) cultured in Mymensingh, Bangladesh. Asian-Australasian Journal of Food Safety and Security 1: 17–24.

Nahar S, Rahman MM, Ahmed GU, Faruk MAR (2016) Isolation, identification, and characterization of *Aeromonas hydrophila* from juvenile farmed pangasius (*Pangasianodon hypophthalmus*). International Journal of Fisheries and Aquatic Studies 4: 52–60.

Nawaz M, Sung K, Khan SA, Khan AA, Steele R (2006) Biochemical and molecular characterization of tetracycline-resistant *Aeromonas veronii* isolates from catfish. Applied Environmental Microbiology 72: 6461–6466.

Nielsen ME, Høi L, Schmidt A, Qian D, Shimada T, ... Larsen J (2001) Is *Aeromonas hydrophila* the dominant motile *Aeromonas* species that causes disease outbreaks in aquaculture production in the Zhejiang Province of China? Diseases of Aquatic Orgnanisms 46: 23–29.

Odeyemi OA, Ahmad A (2017) Antibiotic resistance profiling and phenotyping of *Aeromonas* species isolated from aquatic sources. Saudi Journal of Biological Sciences 24: 65–70.

Peatman E, Mohammed H, Kirby A, Shoemaker CA, Yildirim-Aksoy M, Beck BH (2018) Mechanisms of pathogen virulence and host susceptibility in virulent *Aeromonas hydrophila* infections of channel catfish (*Ictalurus punctatus*). Aquaculture 482: 1–8.

Pridgeon JW, Klesius PH (2011) Molecular identification and virulence of three *Aeromonas hydrophila* isolates cultured from infected channel catfish during a disease outbreak in west Alabama (USA) in 2009. Diseases of Aquatic Organisms 94: 249–253.

Rahman M, Colque-Navarro P, Kuhn I, Huys G, Swings J, Mollby R (2002) Identification and characterization of pathogenic *Aeromonas veronii biovar sobria* associated with epizootic ulcerative syndrome in fish in Bangladesh. Applied Environmental Microbiology 68: 650–655.

Ramalivhana J, Obi C, Moyo S (2009) Antimicrobial susceptibility testing of *Aeromonas hydrophila* isolated from Limpopo Province, South Africa using VITEK 2 system, Micro Scan Walk Away, disk diffusion and E-test method. African Journal of Microbiology Research 3: 903–913.

Rani MK, Chelladurai G, Jayanthi G (2016) Isolation and identification of bacteria from marine market fish *Scomberomorus guttatus* (Bloch and Schneider, 1801) from Madurai district, Tamil Nadu, India. Journal of Parasitic Diseases 40: 1062–1065.

Rey G, Fouillet A, Bessemoulin P, Frayssinet P, Dufour A, ... Hémon D (2009) Heat exposure and socio-economic vulnerability as synergistic factors in heat-wave-related mortality. European Journal of Epidemiology 24: 495–502.

Saha D, Pal J (2002) In vitro antibiotic susceptibility of bacteria isolated from EUS-affected fishes in India. Letters in Applied Microbiology 34: 311–316.

Saharia P, Pokhrel H, Kalita B, Hussain IA, Islam S (2018) Histopathological changes in Indian major carp, *Labeo rohita* (Hamilton), experimentally infected with *Aeromonas hydrophila* associated with hemorrhagic septicemia of Central Brahmaputra valley of Assam,

India. Journal of Entomology and Zoology Studies 6: 6–11.

Samal SK, Das BK, Pal BB (2014) Isolation, biochemical characterization, antibiotic susceptibility study of *Aeromonas hydrophila* isolated from freshwater fish. International Journal of Current Microbiology and Applied Sciences 3: 259–267.

Sarder H, Punom NJ, Khan T, Saha ML, Mandal SC, Rahman MS (2016) Prevalence and antibiotic susceptibility of *Aeromonas hydrophila* isolated from freshwater fishes. Journal of Fisheries 4(3): 411–419.

Sarkar A, Saha M, Roy P (2012) Identification and typing of *Aeromonas hydrophila* through 16S rDNA-PCR fingerprinting. Journal of Aquaculture Research & Development 3: 142.

Shahzad A, Akhter S, Ali M, Khan I, Khan WA, ... Qurban A (2016) Identification, characterization and antibiotic sensitivity of *Aeromonas hydrophila*, a causative agent of epizootic ulcerative syndrome in wild and farmed fish from Potohar, Pakistan. Pakistan Journal of Zoology 48(3): 899–901.

Sheikh M, Laghari M, Lashari P, Khooharo A, Narejo N (2017) Current status of three major carps (*Labeo rohita*, *Cirrhinus mrigala* and *Catla catla*) in the downstream Indus River, Sindh. Fisheries and Aquaculture Journal 8: 222.

Stratev D, Odeyemi OA (2016) Antimicrobial resistance of *Aeromonas hydrophila* isolated from different food sources: a mini-review. Journal of Infection and Public Health 9: 535–544.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30: 2725–2729.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876–4882.

Toranzo AE, Magariños B, Romalde JL (2005) A review of the main bacterial fish diseases in mariculture systems. Aquaculture 246: 37–61.

Vivekanandhan G, Savithamani K, Hatha A, Lakshmanaperumalsamy P (2002) Antibiotic resistance of *Aeromonas hydrophila* isolated from marketed fish and prawn of South India. International Journal of Food Microbiology 76: 165–168.

Wang G, Clark CG, Liu C, Pucknell C, Munro CK, ... Rodgers FG (2003) Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. Journal of Clinical Microbiology 41: 1048–1054.

Wassif IM (2018) Biochemical and molecular characterization of *Aeromonas* species isolated from fish. Alexandria Journal of Veterinary Sciences 57(1): 32–39.

Yun S, Lee Y-R, Giri SS, Kim HJ, Chi C, ... Park SC (2017) Isolation of a zoonotic pathogen *Aeromonas hydrophila* from freshwater stingray (*Potamotrygon motoro*) kept in a Korean aquarium with ricefish (*Oryzias latipes*). Korean Journal of Veterinary Research 57: 67–69.

F Sughra 0000-0002-4470-8196
M Hafeez-ur-Rehman 0000-0002-6609-4244
F Abbas 0000-0002-9036-2050
I Altaf 0000-0002-0157-4239
S Razzaq 0000-0002-0157-4239
S Kanwal 0000-0002-0157-4239
M Inayat 0000-0002-0157-4239
I Anwer 0000-0002-0157-4239
M Akram 0000-0002-0157-4239