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

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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).
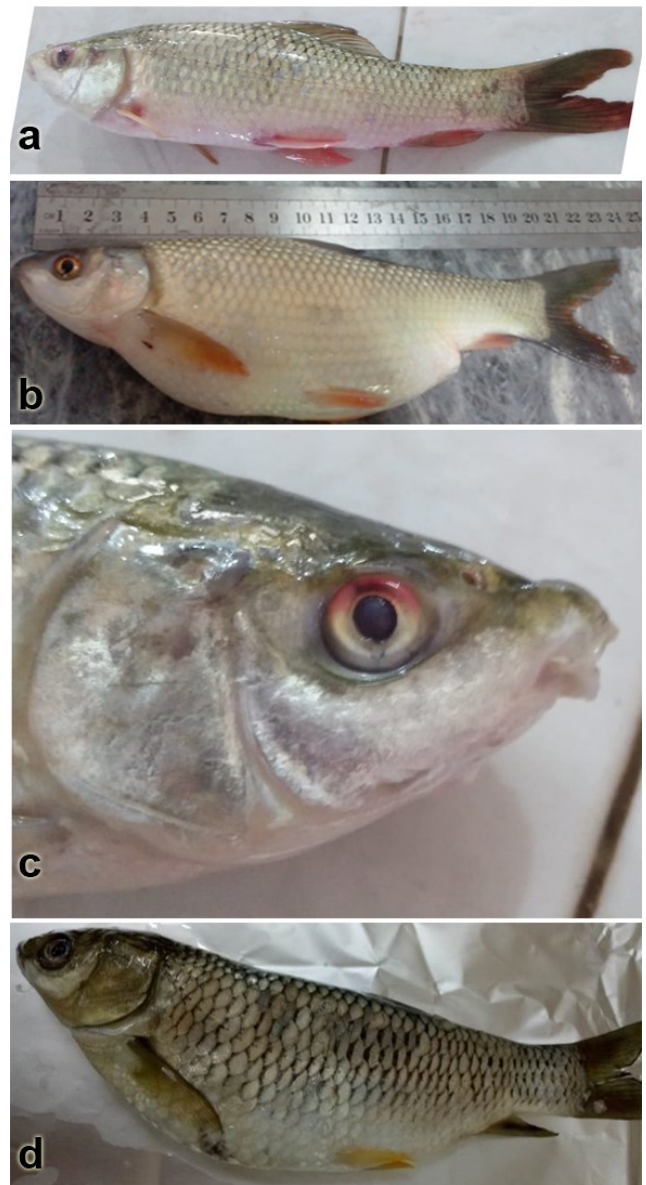

FIGURE 1 Naturally infected *Labeo rohita*. a, skin ulceration; b, abdominal dropsy; c, infected eye; d, abdominal dropsy.

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*.
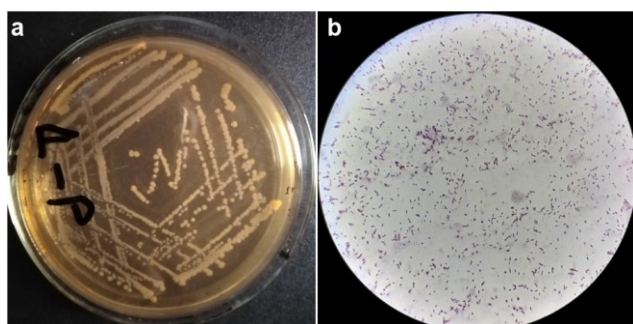

FIGURE 2 (a) Phenotypic characters of *Aeromonas hydrophila* strain on MacConkey agar medium; (b) Gram's staining appearance of *A. hydrophila*.

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.
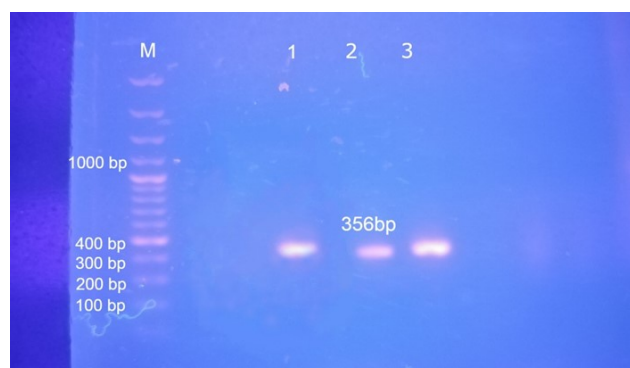

FIGURE 3 Agarose gel electrophoresis (1%) of PCR Amplify of 16S rRNA gene of *Aeromonas hydrophila* isolates.

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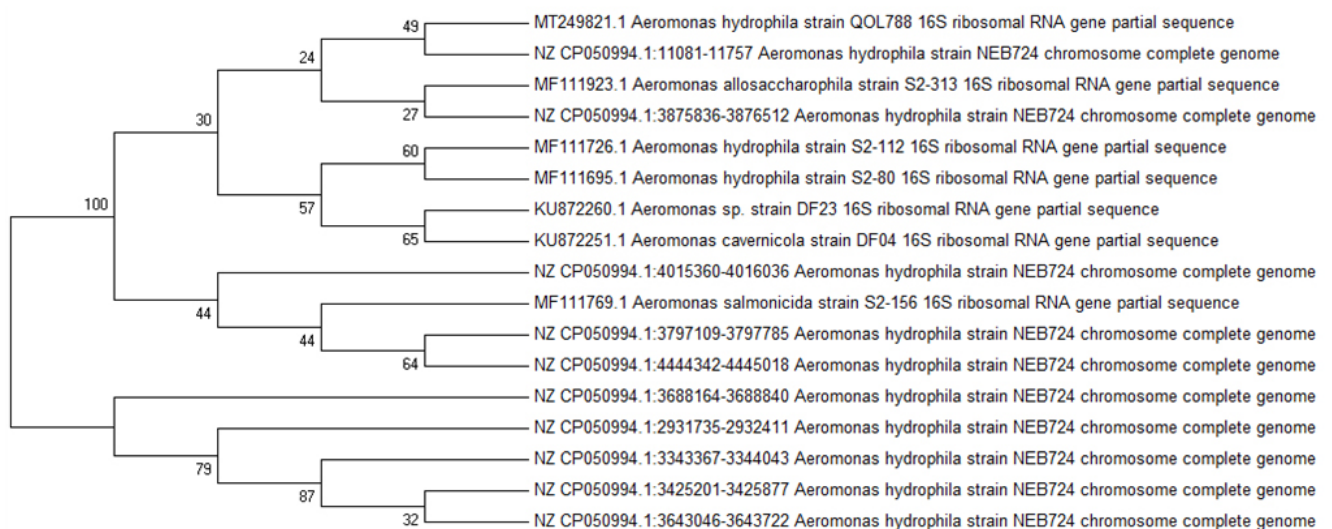
FIGURE 4 Phylogenetic topology of QOL788 isolate showing genetic relationship with reference strains of *Aeromonas* spp.
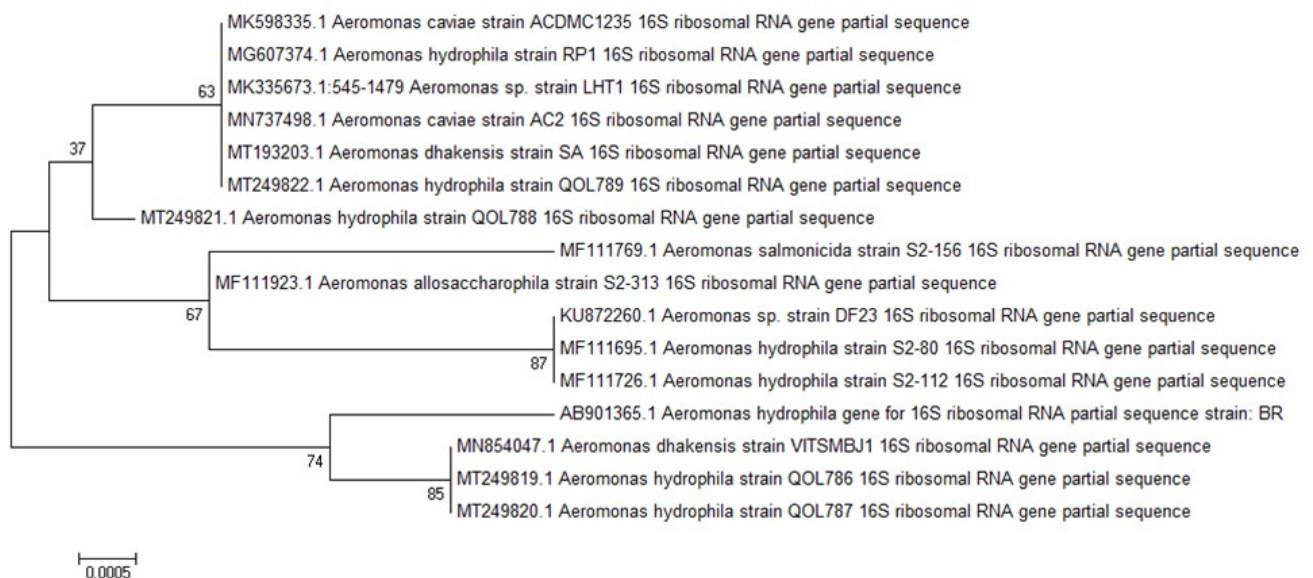

FIGURE 5 Phylogenetic tree of *Aeromonas hydrophila* isolates (MT249820 – MT249822) was computed using neighbor-joining method with MEGA 6.06 showing close similarity to other species of *Aeromonas* spp.

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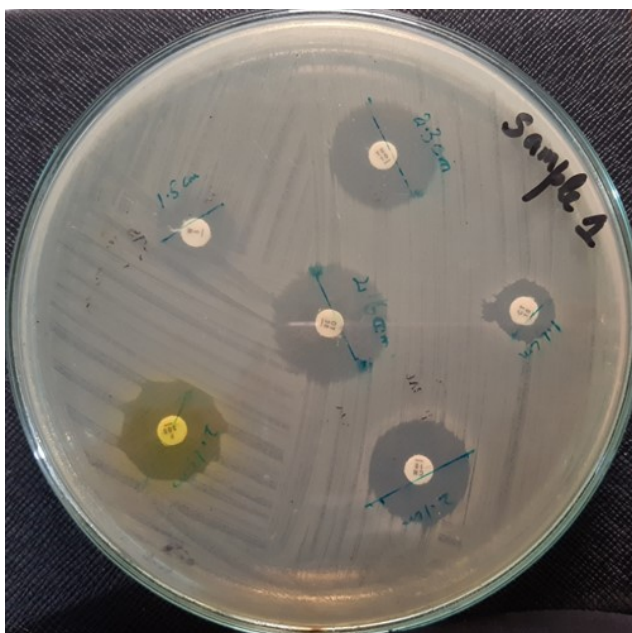
FIGURE 6 Antibiotic sensitivity analysis for sample 1 (QOL787) with colistin sulphate, tetracycline, novobiocin, nitrofurantoin and trimethoprim antibiotic drugs.

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.

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.

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