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Phenotypic Characterization, Genetic Analysis and Antibiotic Sensitivity of *Aeromonas hydrophila* Isolates causing Abdominal Dropsy in *Labeo rohita* collected from Punjab, Pakistan

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ABSTRACT

Motile *Aeromonas* Septicaemia (MAS) is a common fresh water fish disease and major threat to the aquaculture in Pakistan. The present study was carried out on suspected cases of Rohu (*Labeo rohita*) to characterize and genetically analyze local strains of *Aeromonas (A.) hydrophila*, a key pathogen responsible for the said disease in farmed fish of Pakistan. A total of ninety suspected samples were collected from fish farms in various districts of Punjab from June 2018 to April 2019. The samples were processed and *A. hydrophila* strains were isolated. The primary identification of sixty seven isolates was verified by colony morphology, microscopy and phenotypic characterization with ten biochemical reactions. The *A. hydrophila* strains of test samples were characterized by polymerase chain reaction (PCR) using 16S rRNA at desired size of 356 bp. 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 pathogen was sensitive to four and resistant to five drugs. Results of molecular characterization confirmed strains as *A. hydrophila* which are useful to take preventive measures against the said disease.

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 as potential source of protein in Pakistan. These carps are considered as high-value commercial fish species and being cultured on priority by local farmers[1]. 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. This bacteria especially belonging to the genus *Aeromonas* (family *Aeromonadaceae*) are widespread in freshwater environment, and have been implicated as fish pathogens[2,3]. It is an opportunistic pathogen most likely responsible for Infectious Abdominal Dropsy, Ulcer Disease, Motile *Aeromonas* Septicemia (MAS), Hemorrhagic Septicemia and Red-Sore Disease at different growth stages leading to high mortality rates in aquaculture[4,5]. This bacterium can be found in fresh, salt, marine, estuarine, chlorinated, and un-chlorinated waters and can survive in both aerobic and anaerobic environments[6].

A. hydrophila is rod shaped heterotrophic, gram-negative bacterium ubiquitously present in freshwater environment[7]. It is normally inhabited in gastrointestinal tract of fish and water bodies and has ability to grow at low temperature. *A. hydrophila* is a diet tolerated pathogen that is accountable for severe zoonotic diseases[8–10]. 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 aquaculture 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[11,12].

In a variety of freshwater species, the existence and pathogenicity of *A. hydrophila* has previously been reported in different fish species particularly *Carassius auratus*[13], *Cyprinus carpio*[13], *Anabas testudineus*[14], *Clarias gariepinus*[15], *Oncorhynchus mykiss*[16]and *Potamotrygon motoro*[17]. It has been recently reported that *A. hydrophila* is the key bacterial pathogen that causes diseases in freshwater fish species[18]. The pathogen has recently emerged in United States where it has been responsible for

extensive farm losses in Catfish aquaculture[19].

The fish diseases caused by *A. hydrophila* are responsible for major economical losses of local fish farmers in Pakistan. 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 as 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 cultured 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.

Methods

Ethics declarations

- All methods in this work were carried out in accordance with "National Biosafety Guidelines" of Pakistan Environmental Protection Agency, Ministry of Environment, Government of Pakistan (http://www.environment.gov.pk/images/guidelines/BiosftyGlines2005.pdf).
- All experimental protocols were approved by Ethical Review Committee, University of Veterinary and Animal Sciences (UVAS), Pakistan

  (http://www.uvas.edu.pk/doc/directorates/2014/Research/Guidelines-Protocols.pdf).
- This study was carried out in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines https://arriveguidelines.org/arrive-guidelines.

Sample collection

A total of 90 samples of naturally infected *Labeo rohita* were carefully collected from eight (08) different 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 between 150-200 grams and average length of 9-11 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 hours. 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.

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 121ºC under 15 lbs and glassware in hot air oven at 180ºC for 10 minutes. Pouring of autoclaved media was done for preparation of MacConkey agar plates. For the sterilization check, the plates were incubated at 37ºC for 24h. 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 hours following the procedure described in[20] and[16].

Phenotypic characterization of isolates

The identifications of bacterial isolates were verified by colony morphology, microscopy and biochemical characterization. The isolated strains were tested for 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[21]. Morphological characterization of isolates included size and shape of colony and Gram's reaction along with motility test[22]. Catalase test, Urease test, Voges-Proskauer (VP) test and five (5) different carbohydrate fermentation tests were also conducted for bio-chemical characterization of *A. hydrophila*[23]. 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 was used[24].

Genetic analysis of isolates

The DNA extraction was done from a representative isolate of the biochemical results using GeneAll® ExGene™ DNA purification kit following manufacturer's protocol[25]. The cells were harvested by centrifugation. The supernatant was discarded

and then re-suspended to 20 μl of proteinase K solution (20 mg/ml) and 200 μl of CL buffer. The mixture was heated in water bath at 56ºC for 15 minutes. Then concisely spun 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 minutes at 70ºC 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 centrifuging for 1 minute at 13000 rpm. 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.

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 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™ for 50 cycles with each cycle's conditions as follows: 5 minutes for initial denaturation at 95ºC, 0.5 minute for denaturation at 95ºC, 0.5 minute for annealing at 56.9ºC, 0.5 minutes for elongation at 72ºC and finally 7 minutes for elongation at 72ºC. After the completion of 50 cycles the yield was observed by gel electrophoresis.

For electrophoresis, the buffer was prepared as per manufacturer's instruction. Gel was prepared by adding 100 ml of TAE buffer in 1 gram 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 for 35 minutes. The gel tray was further removed and placed on a Tran's illuminator. The DNA bands were visualized under Bio-Rad® UV transilluminator. The amplified PCR products were sequenced directly from MacroGen®, South Korea.

The initial and final portions of the sequences were manually trimmed in BioEdit sequence alignment editor software. 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 $\geq 98\%$ were considered for specific identification. The three (03) 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 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[26]. 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.

Antibiotic sensitivity

Antibiogram of the of isolates against antibiotics was studied by standard disc diffusion assay method[27–29]. 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 37ºC for 24 hours. Nine antibiotics were used is this study are: Penicillin, Colistin sulphate, Oxytetracycline, Novobiocin, Ciprofloxacin, Gentamicin, Trimethoprim, Tetracycline and Nitrofurantoin. After 24 hours the zones of inhibition were measured[30].

Results

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

(a) (b) (c) (d)

Figure 1. Naturally infected Rohu (*Labeo rohita*) samples

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 1(a)), distended abdomens (Figure 1(b), 1(d)), protruded eyes (Figure 1(c)) which are the same lesions of with hemorrhagic septicemia disease caused by *Aeromonas* spp.[31,32].

Phenotypic characterization

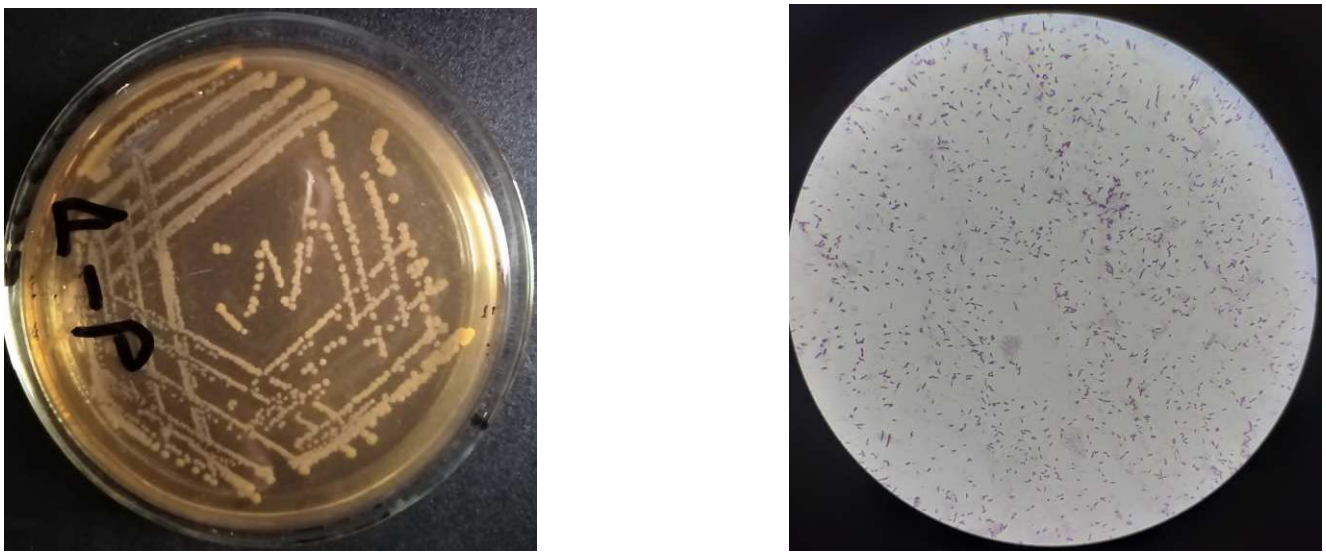
Out of 90 samples, 67 (74%) were identified as *A. hydrophila* through phenotypic characterization. *A. hydrophila* colonies appeared in pale yellowish color, round shaped with rounded end on MacConkey agar medium when incubated at 37°C for 24 hours (Figure 2(a)).

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(b)). 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 (03) isolates are provided in Table 1, 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 (10) biochemical tests, five (05) 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*. The results are in close correspondence with work of Halima *et al.*[33], Furmanek-Blaszk[34] and Nawaz *et al.*[35].

Genetic analysis

Molecular characterization revealed that all the isolates were *A. hydrophila* based on amplification by real-time polymerase chain reaction (PCR) technique. The genomic DNA of bacterial isolates were 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 amplification primers listed in Table 2 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 2.

The DNA were examined by electrophoresis on 1% agarose gels with 100bp ladder for estimating the bands. The DNA bands were documented and pictured in Gel documentation system (Biorad® Gel Doc XR system). The samples provided were



(a) (b)

Figure 2. (a) Cultural characters of *A. hydrophila* strain on MacConkey agar medium; (b) Gram staining appearance of *A. hydrophila*

Test	QOL786	QOL787	QOL788
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

Table 1. Reaction results of *A. hydrophila* isolates

confirmed through polymerase chain reaction (PCR) as *A. hydrophila*. PCR was run using universal primer for 16S rRNA gene and resulting band having amplicon size 356bp was observed in Bio-Rad® UV transilluminator as shown in Figure 3.

To validate the *Aeromonas* characterization, the chosen strains were identified by 16S rRNA PCR pattern analysis on 1% agarose gel electrophoresis. The product was selected for subsequent sequencing from MacroGen®, South Korea. 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.

The consensus nucleotide sequences were aligned with the help of ClustalW bioinformatics software[38]. The genetic distance of aligned sequences were obtained using "Kimura's Two-parameter Model"[39]. Phylogenetic analysis was performed for isolated strains with reference strains using neighbour-joining method, and bootstrap values were calculated with 1000 replicates. Evolutionary trees were constructed with the help of MEGA v. 6.06 software by Kumar *et al.*[40]. 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*.

Target gene	Primer pair	Sequence (5'–3')	Amplified product size	References
16S rRNA	Ah16SF	GGG AGT GCC TTC GGG AAT CAG A	356	Furmanek-Blaszk (2014)[34], Wang *et al.* (2003)[36]
	Ah16SR	TCA CCG CAA CAT TCT GAT TTG		
16S rRNA Universal PCR primer	27F	AGA GTT TGA TCM TGG CTC AG	1538	Ludwig *et al.* (1995)[37] (Acc # NR_119190)
	1492R	TAC GGY TAC CTT GTT ACG ACT T		
16S rRNA Universal sequencing primer	785F	GGA TTA GAT ACC CTG GTA	1538	
	907R	CCG TCA ATT CMT TTR AGT TT		

Table 2. Primers used for PCR amplification

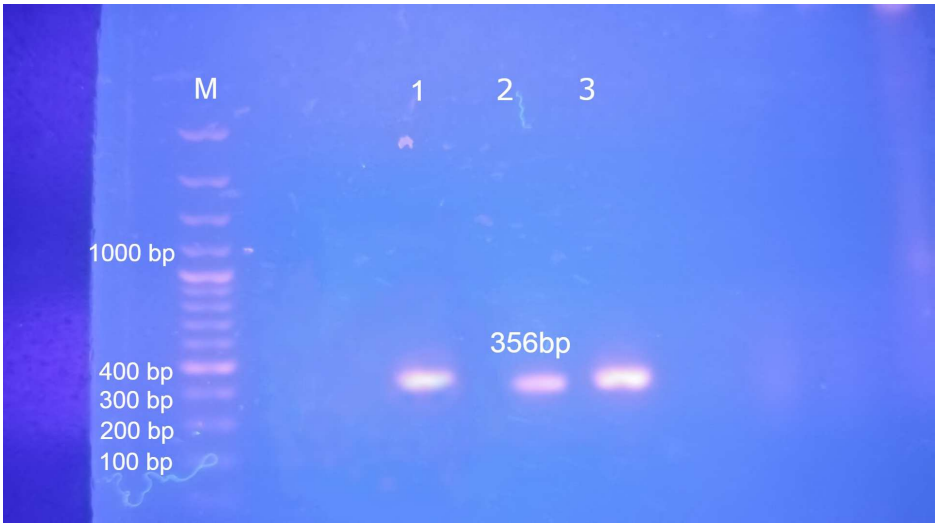


Figure 3. Agarose gel electrophoresis (1%) of PCR amplification of 16S rRNA gene of *A. hydrophila* isolates

Antibiotic sensitivity

The results of the antibiotic resistance tests of each isolate on nine (09) 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. *A. 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 (Sample 1).

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[12]. 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[1].

In present study three (03) isolates of *A. hydrophila* were recovered from infected *Labeo rohita* samples collected from different fish farms in Punjab, Pakistan. Post mortem findings of the infected fish 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 Mathur *et al.*[41] and Rahman *et al.*[42]. Congested liver and internal organs were also observed in the infected fishes by Dahdouh *et al.*[43] and Matter

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

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

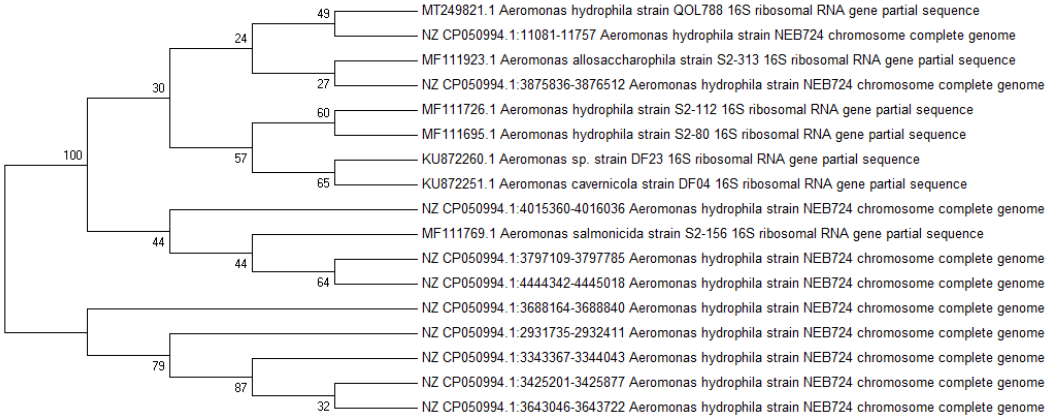


Figure 4. Phylogenetic topology of QOL788 (MT249821) isolate showing genetic relationship with reference strains of *Aeromonas* spp.

et al.[44]. 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[21,23] and Egypt[45]. Our study extends the work of Shahzad *et al.*[22] 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[46,47].

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 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[48,49], Bangladesh[33,50], China[51,52] and South Korea[17].

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.*[22] from Pakistan and Kusdarwati *et al.*[53] 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[54], United Arab Emirates[55], Bangladesh[56], Egypt[57] and South Korea[17].

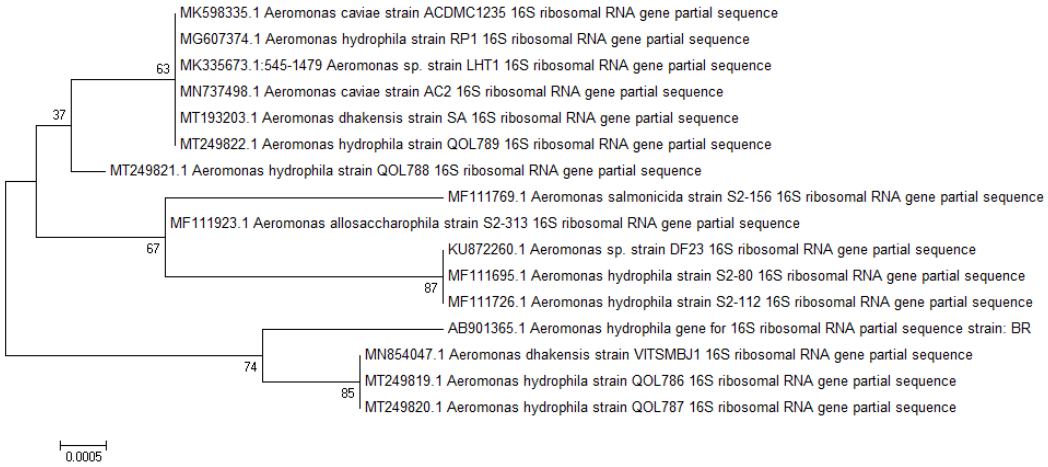


Figure 5. Phylogenetic tree of *A. hydrophila* isolates (MT249820-MT249822) was computed using neighbor-joining method with MEGA 6.06 showing close similarity to other species of *Aeromonas* spp.

Antibiotic drugs with symbols	Disk potency (µg / disk)	Isolates					
		QOL786		QOL787		QOL788	
		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

Table 4. Antibiotic sensitivity tests of *A. hydrophila* strains (QOL787 - QOL789) S = sensitive, R = resistant

Subsequently 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 therapeutants. 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 aquaculture of Pakistan.

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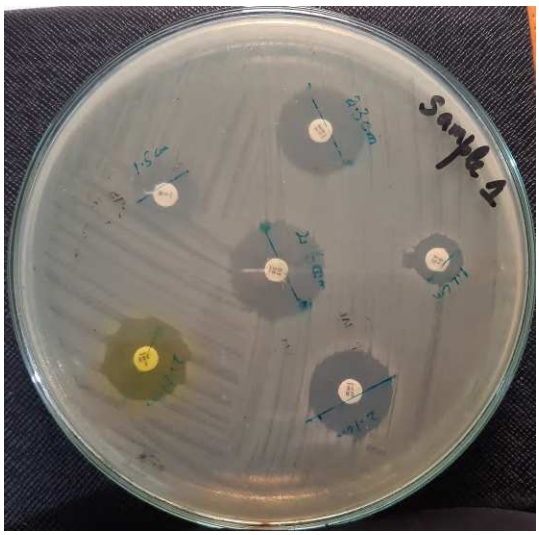

Figure 6. Antibiotic sensitivity analysis for sample 1 (QOL787) with CT, TE, NV F and W antibiotic drugs

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Author contributions statement

F.S. and M.H. conceptualized research; F.S. and I.A. devised methodology; F.S., M.H. and F.A. validated data; I.A. performed formal analysis; F.S. and M.A. carried investigations; M.A. provided resources; I.A. and F.A. did data curation; F.S. prepared original draft; M.H., I.A. and F.A. reviewed and edited manuscript; M.A. provided visualization; M.H. and I.A. supervised research; M.H. administered project; M.H. acquired funding; All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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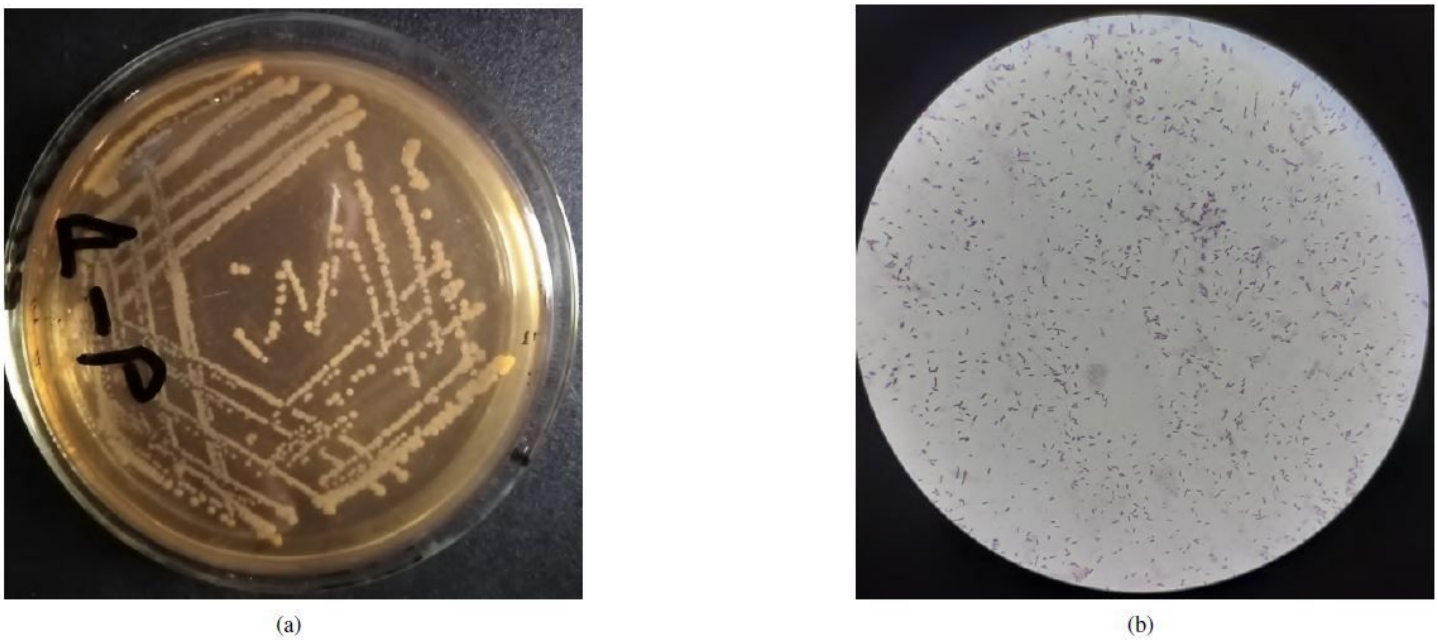
Figures

(a) (b) (c) (d)

Figure 1

Naturally infected Rohu (Labeo rohita) samples



(a) (b)

Figure 2

(a) Cultural characters of A. hydrophila strain on MacConkey agar medium; (b) Gram staining appearance of A. hydrophila

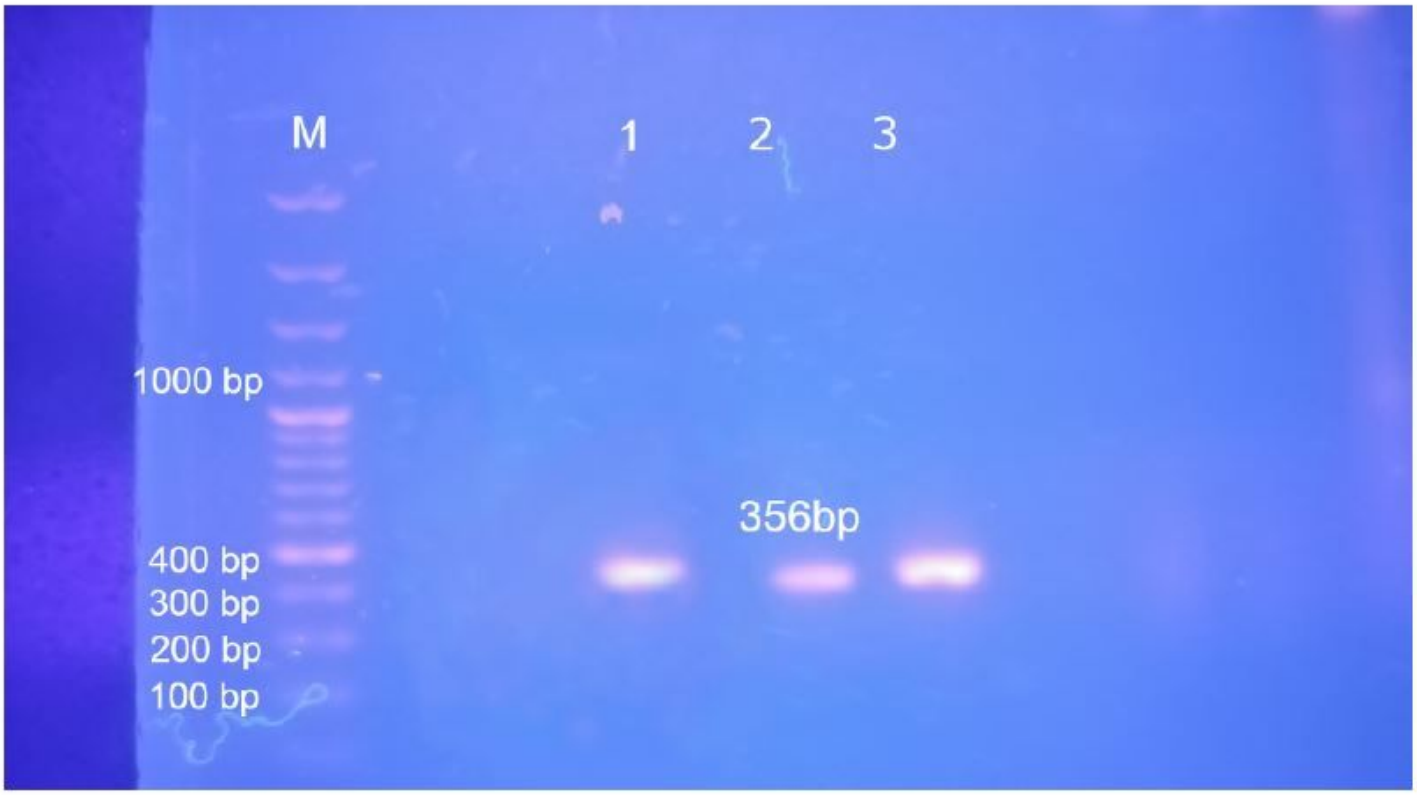


Figure 3

Agarose gel electrophoresis (1%) of PCR amplification of 16S rRNA gene of A. hydrophila isolates

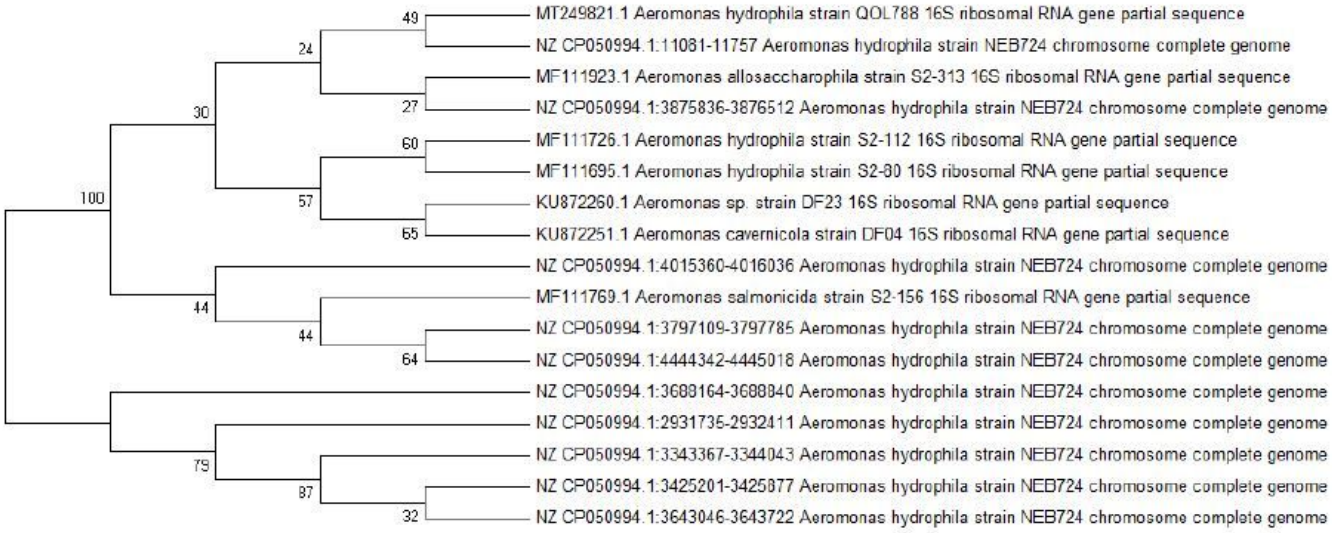


Figure 4

Phylogenetic topology of QOL788 (MT249821) isolate showing genetic relationship with reference strains of Aeromonas spp.

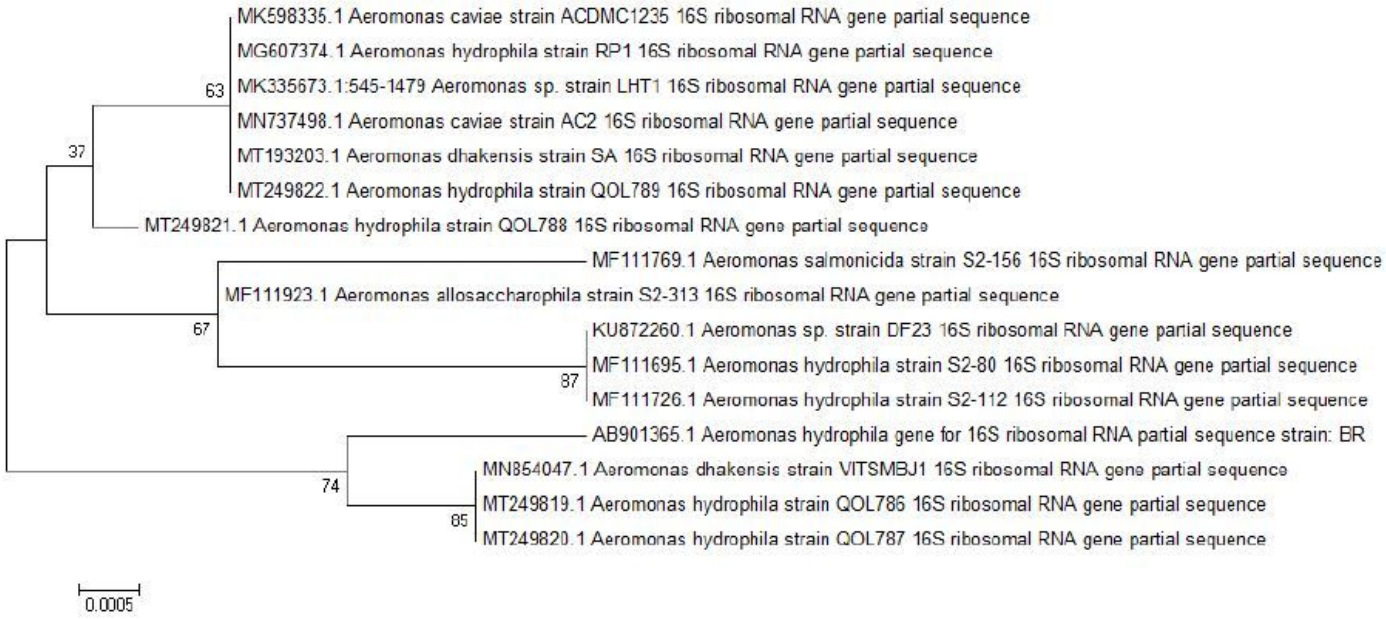


Figure 5

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

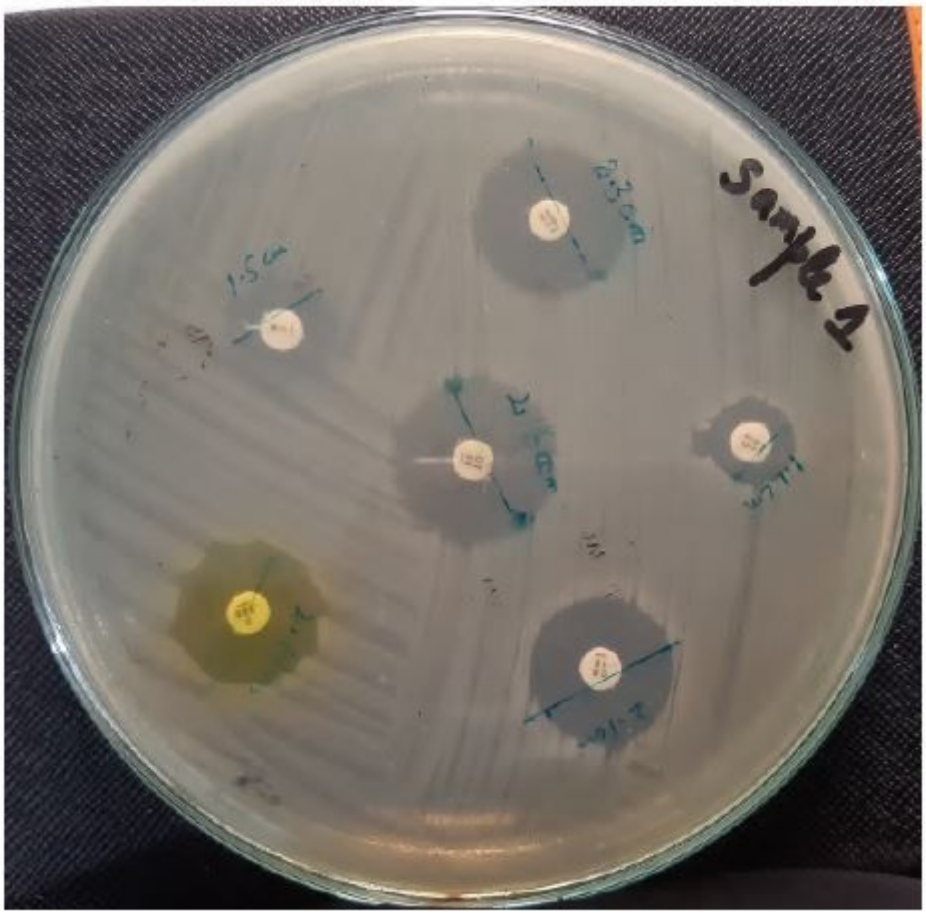


Figure 6

Antibiotic sensitivity analysis for sample 1 (QOL787) with CT, TE, NV F and W antibiotic drugs