Journal of Fisheries
Volume 10 Issue 3 December 2022
Peer Reviewed | Open Access

eISSN 2311-3111
pISSN 2311-729X

DOI: https://doi.org/10.17017/j.fish.466

Short Communication

Molecular characterisation and genetic analysis of aerolysin and haemolysin in *Aeromonas hydrophila* isolated from diseased *Labeo rohita* by polymerase chain reaction

Fatima Sughra[1] • Muhammad Hafeez-ur-Rehman[2] • Farzana Abbas[2] • Imran Altaf[3] • Zohal Hassan[3] • Ayesha Bhatti[2] • Kashif Ali[4]

[1] *Department of Zoology, Division of Science and Technology, University of Education, Lahore, Pakistan*
[2] *Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[3] *Quality Operations and Research Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[4] *Department of Wildlife & Ecology, University of Veterinary and Animal Sciences, Lahore, Pakistan*

Correspondence
Fatima Sughra; Department of Zoology, Division of Science and Technology, University of Education, Lahore, Pakistan.
fatima.sughra@ue.edu.pk

Manuscript history
Received 6 July 2022 | Accepted 30 July 2022 | Published online 13 December 2022

Citation
Sughra F, Hafeez-ur-Rehman M, Abbas F, Altaf I, Hassan Z, Bhatti A, Ali K (2022) Molecular characterisation and genetic analysis of aerolysin and haemolysin in *Aeromonas hydrophila* isolated from diseased *Labeo rohita* by polymerase chain reaction. Journal of Fisheries 10(3): 103402. DOI: 10.17017/j.fish.466

Abstract
The present study investigates pathogenicity of local *Aeromonas hydrophila* strains by molecular characterisation of two virulence factor genes: aerolysin (aerA) and haemolysin (Ahh1) using polymerase chain reaction (PCR) technique. Phenotypically identified presumptive *Aeromonas* isolates recovered from diseased *Labeo rohita* were genetically analysed using type-specific primers by amplifying 309 bp and 130 bp conserved regions of aerolysin and haemolysin genes respectively. The partial nucleotide sequences of aerA and Ahh1 were determined from representative strains in which aerA was confirmed in 75% isolates, whereas Ahh1 was confirmed in 50% isolates. The nucleotide blast results of the representative strains revealed close homology of 95% (aerolysin) and 97% (haemolysin) with published sequences.

Keywords: aerolysin; *Aeromonas hydrophila*; DNA; haemolysin; polymerase chain reaction; sequencing

1 | INTRODUCTION

Aeromonas hydrophila is a bacterial pathogen associated with infectious diseases of freshwater fish cultured in South Asia. *Aeromonas hydrophila* carries many virulence factors such as aerolysin, haemolysin, elastase, lipase and hidrolipase genes. Extracellular enzymes such as haemolysis, lipases, proteases, β-lactamases, amylases, chitinases and nucleases produced by *Aeromonas* have been involved in their ecology, survival and pathogenicity (Stratev *et al.* 2015) which contribute to their ability to attach with host cells and finally promote disease development.

The fish diseases caused by *A. hydrophila* are responsible for major economic losses of local fish farmers in Pakistan. Diseases in inland aquaculture are currently being treated by antibiotic products. Several studies briefly elucidate the incidence of *A. hydrophila* among different fish species in Pakistan such as Shahzad *et al.* (2016) and Iqbal (2016). Studies on the pathogenicity, molecular typing and biodiversity of *A. hydrophila* infection, particularly those are based on carp aquaculture of Pakistan has only been recently reported (Tooba *et al.* 2022). The current study aimed to emphasize the prevalence and pathogenicity of *A. hydrophila* in cultured fish. The genetic typing of all retrieved strains and their heterogeneity were evaluated using polymerase chain reaction (PCR).

The resulting bacterial isolates are to be used for subsequent remedial actions for prevention of disease in farmed fish species of Pakistan and elsewhere.

2 | METHODOLOGY

Ninety specimens of naturally infected and moribund individuals of *Labeo rohita* weighing 175 ± 25 g and average length of 10 ± 1 cm, were collected from private and government fish farms situated 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. For isolation of bacteria nutrient agar, nutrient broth and MacConkey agar media were used with standard compositions (Al-Fatlawy and AL-Hadrawy 2014; Cagatay and Sen 2014). The isolates were phenotypically characterised using ten biochemical tests (Fatima *et al.* 2022).

The DNA extraction was done from representative isolates 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 one minute. After that 600 µl BW buffer was added and centrifuged at 8000 rpm for one minute. Mixture was transferred into new SV column. After adding 700 µl TW buffer mixture was centrifuged at 8000rpm for one minute and supernatant was removed. Then SV column was transferred into collection tube. The residual wash buffer was eliminated by centrifugation for one 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 one minute at 13000 rpm. The supernatant containing bacterial template DNA was used directly in the specific-PCR for the detection of aerolysin (aerA) and haemolysin (Ahh1) genes.

The virulent gene aerolysin was first targeted for confirmation of pathogenicity described as AH-aerAR (5′ACG AAG GTG TGG TTC CAG T′3) and AH-aerAF (5′CAA GAA CAA GTT CAA GTG GCC A′3) with amplicon size 309 bp (Wang *et al.* 2003; Furmanek-Blaszk 2014). The virulent gene haemolysin was targeted for confirmation of pathogenicity described as Ahh1F (5′GCC GAG CGC CCA GAA GGT GAG TT′3) and Ahh1R (5′GAG CGG CTG GAT GCG GTT GT′3) with amplicon size 130 bp (Wang *et al.* 2003; Furmanek-Blaszk 2014). For both PCRs, reaction mixture of 25 µl was comprised of followings: master mix (12.5 µl), DNA sample (2 µl), forward primer (1 µl for aerolysin: 0.6 µl for haemolysin), reverse primer (1 µl for aerolysin: 0.6 µl for haemolysin) and nucleus free water (8.5 µl for aerolysin: 8.1 µl for haemolysin). The composition of the master mixture for both reactions 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). Colony PCR method was used for PCR amplification of both genes using BIO-RAD® T100 Thermal CyclerTM. The thermal conditions for both PCR reactions are provided in Table 1.

TABLE 1 Thermal conditions for PCR to detect aerolysin and haemolysin. Temperature (Temp) and time values represent °C and minute respectively.

Steps	Aerolysin			Hemolysin		
	Temp	Time	Cycle	Temp	Time	Cycle
Initial denaturation	95	5	1	95	5	1
Denaturation	95	0.5	50	95	1	35
Annealing	56.9	0.5	50	58	1	35
Elongation	72	0.5	50	72	1	35
Final extension	72	7	1	72	10	1
Storage	4	∞	1	4	∞	1

The genomic DNA of representative isolates was extracted as per protocols described by GeneAll® for ExGeneTM DNA purification kit. The aerolysin gene was amplified from genomic DNA of isolates. For this purpose, PCR was run for type-specific gene (aerolysin) by using specific primer pair (AH-aerA). The DNA bands were documented and pictured in Gel documentation system (Bio-rad® Gel Doc XR system, United States).

3 | RESULTS AND DISCUSSION

The PCR product of representative isolates was examined by electrophoresis on 1% agarose gel with 100 bp ladder for estimating the bands. Resulting bands having amplicon size 309 bp were observed in gel electrophoresis as shown in Figure 1 which confirms the identity of pathogenic gene aerolysin of *A. hydrophila* in three isolates.

The haemolysin gene was also amplified from genomic DNA of isolates. For this purpose, another PCR was run for type-specific gene (haemolysin) by using specific primer pair (Ahh1). The resulting amplified product size was 130 bp which confirms the identity of pathogenic gene haemolysin of *A. hydrophila* in two isolates. The results of gel electrophoresis are depicted in Figure 2(b) in which bands for test samples are visible in Lane 3 and Lane 6 respectively. The two haemolysin sequences obtained in present study are formally submitted at NCBI database with accession numbers MZ223859 and MW972019.

The sequences of the *A. hydrophila* strains acquired in this study were checked for similarity with bacterial

nucleotide sequence data available at GenBank database using online BLASTN program (Basic Local Alignment Search Tool) by National Center for Biotechnology Information (NCBI), USA. The phylogenetic tree of aerolysin sequence presented close similarity with *A. hydrophila* aerolysin gene partial sequences isolated from intestine of *Carassius carassius* in Wuhuan, China with accession no. JQ003204 and JQ003205 (Figure 2). The phylogenetic tree of haemolysin sequence showed close genetic relationship with *Aeromonas* sp. ASNIH3 (Accession no. CP026222), *Aeromonas* media strain T5-8 (Accession no. CP038444) and *Aeromonas caviae* DNA (Accession no. AP022254) as shown in Figure 3.

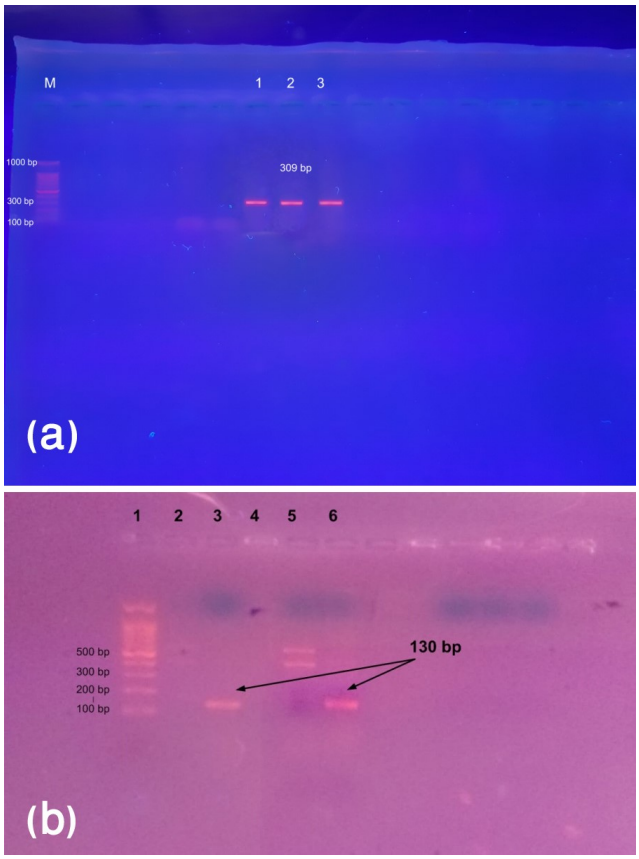

FIGURE 1 (a) Electrophoresis of PCR amplification products on 1% agarose gel, Lane 1: marker (100-1000) bp; Lane 2: positive control; Lane 3: negative control; Lane 4: negative control; Lane 5: Other strain; Lane 6: Test-1; Lane 7: Test-2; Lane 8: Test-3; Lane 9: Test-4 representative aerolysin gene in *Aeromonas hydrophila*; **(b)** Electrophoresis of PCR amplification products on 1% agarose gel, Lane 1: marker 100bp; Lane 2: negative control; Lane 3: Test-1; Lane 4: Test-2; Lane-5: Test-3; Lane 6: Test-4 representative haemolysin gene in *A. hydrophila.*

FIGURE 2 Phylogenetic tree of aerolysin sequence (R_UVAS) of *Aeromonas hydrophila* isolate was computed using maximum likelihood method showing similarity to aerolysin (aerA) sequences.

In present study, the selection of isolates from different fish farms allowed the observation of different virulence factor combinations, confirming the multifactorial virulence profile in *Aeromonas* spp. Among the tested virulence genes, aerolysin was more common gene and prevalent in 71.8% of the isolates. These genes play an important role in the pathogenicity observed in dropsy disease in fresh water cultured systems (Rahman *et al.* 2002; Bhowmik *et al.* 2009; Pandey *et al.* 2010; Tomás 2012) as well as diarrheal diseases in humans (Citterio

and Biavasco 2015; Pessoa *et al.* 2019).

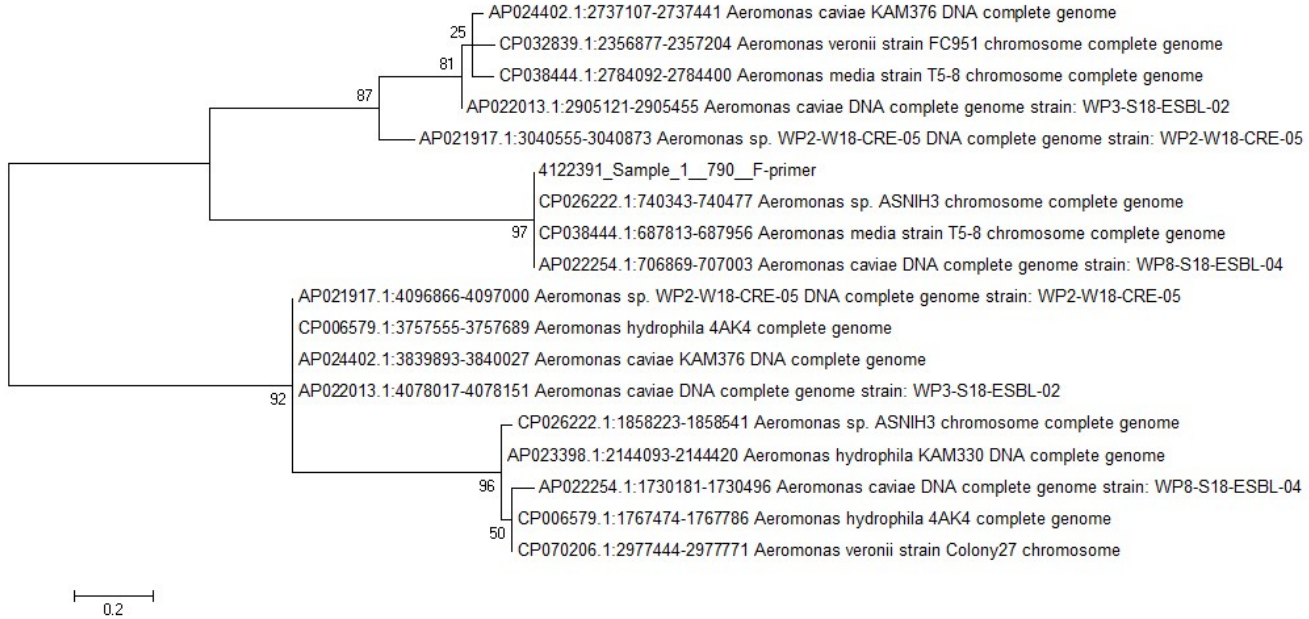

FIGURE 3 Phylogenetic tree of haemolysin sequence (Sample1_790) of *Aeromonas hydrophila* isolate was computed using maximum likelihood method showing similarity to *Aeromonas* spp.

Aerolysin and haemolysin are major contributors to the virulence of pathogenic *Aeromonas* isolates (Iacovache *et al.* 2016). Aerolysin is a pore forming toxin that binds to the receptors on target cell membrane. After proteolytic activation, it induces formation of pore or channel, which ultimately leads to destruction of membrane permeability, cytolysis, and cell death (Cirauqui *et al.* 2017). The evaluation of the haemolysin gene is the most prevalent gene in the studied marine animals. Our work is identical to the findings of previous reports (Oliveira *et al.* 2012; Amsaveni *et al.* 2014). In our study, 75% *A. hydrophila* isolates harboured this gene causing cytotoxic effects. Similar findings were reported by others (Degiacomi *et al.* 2013; Podobnik *et al.* 2017).

The *A. hydrophila* isolates of present study will be used for future research towards disease prevention against local strains of *A. hydrophila* in Pakistan's aquaculture. Based on this experiment, fish farmers may be benefited for controlling abdominal dropsy and other infectious diseases caused by *A. hydrophila* by the administration of specific therapeutant.

ACKNOWLEDGEMENTS
The work presented in this paper is part of a project sponsored by Pakistan Science Foundation (PSF) under 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 author declares 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, ZH and KA; resources: AB; data curation: IA, AB, KA, ZH and FS; writing—original draft preparation: FS; writing—review and editing: MH, IA and FA; visualization: IA, KA, ZH, AB; 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 MZ223859 and MW972019.

REFERENCES
Al-Fatlawy HNK, AL-Hadrawy H (2014) Isolation and Characterization of *Aeromonas hydrophila* from the Al-Jadryia River in Baghdad (Iraq). American Journal of Educational Research 2: 658–662.

Amsaveni R, Muthusamy S, Vivekanandhan G (2014) Screening of pathogenic *Aeromonas* species from marketed fish samples. International Journal of Pharmacy and Pharmaceutical Sciences 6(8): 148–150.

Bhowmik P, Bag PK, Hajra TK, De R, Sarkar P, Ramamurthy T (2009) Pathogenic potential of *Aeromonas hydrophila* isolated from surface waters in Kolkata, In-

dia. Journal of Medical Microbiology 58: 1549–1558.

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 Agriculture Biology 16: 435–438.

Cirauqui N, Abriata LA, van Der Goot FG, Dal Peraro M (2017) Structural, physicochemical and dynamic features conserved within the aerolysin pore-forming toxin family. Scientific Reports 7: 13932.

Citterio B, Biavasco F (2015). *Aeromonas hydrophila* virulence. Virulence 6: 417–418.

Degiacomi MT, Iacovache I, Pernot L, Chami M, Kudryashev M, ... Dal Peraro M (2013) Molecular assembly of the aerolysin pore reveals a swirling membrane-insertion mechanism. Nature Chemical Biology 9: 623–629.

Fatima S, Muhammad H-u-R, Farzana A, Imran A, Saira R, ... Muhammad A (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: 101207.

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

Iacovache I, De Carlo S, Cirauqui N, Dal Peraro M, van Der Goot FG, Zuber B (2016) Cryo-EM structure of aerolysin variants reveals a novel protein fold and the pore-formation process. Nature Communications 7: 12062.

Iqbal Z (2016) An overview of diseases in commercial fishes in Punjab, Pakistan. Fish Pathology 51: S30–S35.

Oliveira STL, Veneroni-Gouveia G, Costa MM (2012) Molecular characterization of virulence factors in *Aeromonas hydrophila* obtained from fish. Pesquisa Veterinaria Brasileira 32: 701–706.

Pandey A, Naik M, Dubey SK (2010) Hemolysin, protease, and EPS producing pathogenic *Aeromonas hydrophila* strain An4 shows antibacterial activity against marine bacterial fish pathogens. Journal of the Marine Biological Association of the United Kingdom 2010: 563205.

Pessoa RBG, de Oliveira WF, Marques DSC, dos Santos Correia MT, de Carvalho EVM, Coelho LCBB (2019) The genus *Aeromonas*: a general approach. Microbial Pathogenesis 130: 81–94.

Podobnik M, Kisovec M, Anderluh G (2017) Molecular mechanism of pore formation by aerolysin-like proteins. Philosophical Transactions of the Royal Society B 372: 20160209.

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 and Environmental Microbiology 68: 650–655.

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: 899–901.

Stratev D, Daskalov H, Vashin I (2015) Characterisation and determination of antimicrobial resistance of β-haemolytic *Aeromonas* spp. isolated from common carp (*Cyprinus carpio* L.). Revue de Médecine Vétérinaire 166: 54–61.

Tomás JM (2012) The main *Aeromonas* pathogenic factors. ISRN Microbiology 2012: 256261.

Tooba L, Shahzad A, Zahid M, Muhammad R, Anam I, ... Mater HM (2022). Molecular characterization of *Aeromonas hydrophila* isolates from diseased fishes in district Kasur, Punjab, Pakistan. Brazilian Journal of Biology = Revista Brasleira de Biologia 84: e254816.

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.