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Characterization of *Aeromonas hydrophila* Strains Isolated from Carp Culture Pond Water: Virulence, Antibiotic Resistance, and Pathogenicity Evaluation

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Abstract

Aeromonas hydrophila, a notorious pathogen in aquaculture, poses significant threats to fish health and industry sustainability, necessitating detailed investigations into its identity, virulence mechanisms, and antibiotic resistance. This study evaluated the identity, virulence properties, and antibiotic susceptibility of four *A. hydrophila* strains (COFCAU_AH1, COFCAU_AH2, COFCAU_AH3, COFCAU_AH4) isolated from carp culture pond water in Tripura, India. The strains were confirmed as *A. hydrophila* through a combination of morphological, physiological, biochemical analyses, and 16S rRNA gene sequencing. All strains were susceptible to several antibiotics but some showed resistance to kanamycin, tobramycin, and polymyxin B. Virulence genes such as hlyA, alt, ast, ela, and ascC were found in COFCAU_AH3, and COFCAU_AH4, while the lipase gene (lip) was present in all four strains. The *in vivo* pathogenicity test determined the LD_{50} for COFCAU_AH1, COFCAU_AH3 and COFCAU_AH4 as $10^{9.5}$, $10^{4.4}$, and $10^{4.5}$ cells/fish, respectively, with no mortality in fish exposed to COFCAU_AH2. Infected fish displayed clinical signs like exophthalmia, scale erosion, fin and tail rot, hemorrhages, and abdominal dropsy. These findings highlight the risk of virulent *A. hydrophila* strains in aquaculture, emphasizing the need for effective monitoring and management.

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Introduction

Fish diseases have become a primary constraint hindering the development and sustainability of aquaculture practices worldwide (Smith, 2006). These disease issues are driven by various global factors, including the rise in commerce and market globalization, the intensification of fish farming practices to boost productivity, and the introduction of new species for aquaculture development (Bondad-Reantaso et al., 2005). Various infectious agents have been reported to cause significant losses, among which bacterial pathogens are more prevalent, causing high mortalities in various fishes at different stages of their growth (Swain, Nayak, Sahu, Mohapatra, & Meher, 2002; Yesmin et al., 2004).

Aeromonas species are Gram-negative, rod-shaped, opportunistic, and zoonotically important bacterial fish pathogens, widespread worldwide, predominantly found in freshwater habitats such as lakes, rivers, and domestic sewage. According to several studies, members of the genus *Aeromonas* are among the most significant pathogens that have been linked to the pathogenesis of several systemic and localized diseases in humans, as well as fish and other aquatic animals (Janda & Abbott, 2010; Beaz-Hidalgo & Figueras, 2013; Kumar et al., 2022; Devi, Khan, Choudhury, Pradhan, & Kamilya, 2024). Among *Aeromonas* spp., *Aeromonas hydrophila* is a major etiologic agent in infections in fresh- and warm-water fish farming, causing significant economic

problems worldwide (Semwal, Kumar, & Kumar, 2023). *A. hydrophila* cause 'Motile Aeromonad Septicaemia' in fish, which is characterized by swollen abdomen, red mouth, and haemorrhage in the external surface and surrounding the anus (Alain, 2009). Additionally, it has been reported to induce liver and kidney necrosis, tissue degradation, and haemorrhages throughout the body (Johnson, 1993).

A. hydrophila possess multiple virulence genes, along with other virulence factors and mechanisms, that aid in their pathogenicity and disease development. Several researchers have described various virulence factors in *A. hydrophila,* including aerolysin, haemolysin, and enterotoxins (Yogananth, Bhakyaraj, Chanthuru, Anbalagan, & Nila, 2009; Chakraborty, Huhle, Bergbauer, & Goebel, 1986), exoenzymes such as amylase, protease, hydrolase, elastase, and lipase (Leung & Stevenson, 1988; Pemberton, Kidd, & Schmidt, 1997; Semwal et al., 2023), type III secretion system (Vilches, Jimenez, Tomás, & Merino, 2009), S-layer (Dooley & Trust, 1988), antigen-O, and the presence of capsules (Zhang, Arakawa, & Leung, 2002). These factors are reported to act in a multifunctional and multifactorial manner (Citterio & Biavasco, 2015).

Environmental isolates of *A. hydrophila,* carrying various virulence factors, pose a significant threat to aquaculture. Controlling the spread of these environmental isolates is crucial for maintaining the sustainability and profitability of aquaculture, necessitating stringent biosecurity measures and effective management strategies to mitigate the risks associated with *A. hydrophila*. Understanding the pathogenicity and virulence pathways of environmental isolates of *A. hydrophila* will be essential in controlling these diseases and limiting their spread. In this study, four *A. hydrophila* strains were recovered from water samples of different polyculture ponds in Tripura, India. These strains were identified using physiological, biochemical, and molecular methods, and were screened for their virulent potential through phenotypic and genotypic analysis. The *in vivo* pathogenicity and antibiotic susceptibility profile are also reported.

Materials and Methods

Bacteria were isolated from water samples collected from fish polyculture ponds in Tripura, India (Fig. 1), where many fish exhibited petechial hemorrhages, following the method described by Pradhan et al. (2023). Sterile glass bottles were used to collect 500 mL of water from a depth of approximately 20 cm below the water surface. The samples were then preserved in ice bags during transport to the laboratory. Bacteriological analyses were conducted within 6h of sample collection. A culture-dependent approach was employed to isolate bacteria, utilizing the spread plating technique. Diluted water samples (10^{-1} to 10^{-3}) in physiological saline (0.85% NaCl) were inoculated onto an *Aeromonas* isolation medium (HiMedia, India) and incubated at 30°C for 24-48h (Devi et al., 2024). Presumptive *Aeromonas* spp. were identified and preserved at -80°C in the glycerol stock.

The identification of the isolates involved a comprehensive assessment encompassing morphological, physiological, and biochemical parameters, following the guidelines outlined by MacFaddin (1980) and the work of Austin and Austin (2007). Furthermore, the distinctive characteristics outlined in *Bergey's Manual of Systematic Bacteriology* (Martin-Carnahan & Joseph, 2005) were used as reference points for identification.

A. hydrophila genomic DNA extracted employing the CTAB method (Wilson, 2001) was utilized in the PCR amplification of 16S rRNA gene with the universal primers 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′) (Weisburg, Barns, Pelletier, & Lane, 1991). The amplification reaction was performed using a thermal cycler (Applied Biosystems, USA). The final reaction mixture (25 µL) contained 1.0 µL of bacterial genomic DNA, 1.0 unit of Taq DNA polymerase, 5 µL of 10X PCR

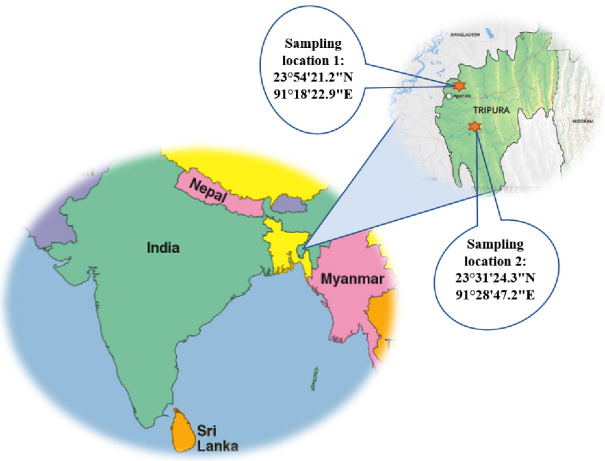

Fig. 1. Location of sampling

amplification buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 200 μM deoxynucleotide triphosphate (dNTP), and 10 pmoles of each primer. The PCR amplification process included an initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min. Subsequently, a final extension step at 72°C for 7 min was incorporated. The PCR products were then analyzed on a 2% agarose gel stained with ethidium bromide and visualized using a UV transilluminator (Bio-rad, USA). The PCR products were purified (Thermo Fisher Scientific, USA) and sequenced (Bioserve Biotechnologies, India). Sequences were analyzed with Chromas (Technelysium, Australia) and matched to GenBank via BLAST. A phylogenetic tree was constructed with MEGA 11 using the neighbor-joining method from the 16S rRNA sequence.

The antibiotic susceptibility of isolates was evaluated using the disc diffusion method on Mueller-Hinton agar plates (Bauer, Kirby, Sherris, & Turck, 1966). Overnight cultures were swabbed onto the plates, and antibiotic-impregnated discs (HiMedia) were placed aseptically, followed by incubation at 30°C for 24h.The results were interpreted according to the Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2016) for 13 antibiotics, including gentamicin (10 μg), kanamycin (300 μg), azithromycin (15 μg), polymyxin B (300 μg), tetracycline (30 μg), cephalexin (30 μg), amoxyclav (30 μg), erythromycin (15 μg), vancomycin (30 μg), and oxacillin (10 μg).

The isolates were evaluated for their potential to produce extracellular substances capable of causing pathological effects in the host. The ability to produce haemolysins was assessed by examining

Table 1. Primers for PCR analysis

Sl. No.	Gene name	Encoded genes	Primer Sequence (5′-3′)	Product size	References
1.	*aerA*	Aerolysin	F- CCCGCCGATCTGCAACCGGGR-CTGGTCTGGATAGACGGGCTCTGCC	489 bp	Ørmen and Østensvik (2001)
2.	*act*	Cytotoxic enterotoxin	F- AGAAGGTGACCACCAAGAACAR-AACTGACATCGGCCTTGAACTC	232 bp	Kingombe et al. (1999)
3.	*ast*	Cytotonic enterotoxins	F- TCTCCATGCTTCCCTTCCACTR-GTGTAGGGATTGAAGAAGCCG	331 bp al. (1999)	Kingombe et
4.	*alt*	Cytotonic enterotoxins	F- TGACCCAGTCCTGGCACGGCR – GGTGATCGATCACCACCAGC	442 bp	Kingombe et al. (1999)
5.	*hlyA*	Hemolysin	F- GGCCGGTGGCCCGAAGATACGGGR – GGCGGCGCCGGACGAGACGGG	597 bp	Heuzenroeder, Wong, and Flower (1999)
6.	*lip*	Lipase	F- ATCTTCTCCGACTGGTTCGGR-CCGTGCCAGGACTGGGTCTT	382 bp	Sen and Rodgers (2004)
7.	*exsA*	T3SS transcriptional regulator	F -TACCACAGAGAAGGGCGATA R- GCGAGCAGAAACAGCAACT	435 bp	Lim and Hong (2020)
8.	*ascV*	Outer membrane ring of T3SS	F-ATGGACGGCGCCATGAAGTT R- TATTCGCCTTCACCCATCCC	710 bp	Chacón, Soler, Groisman, Guarro, and Figueras (2004)
9.	*ascC*	Inner membrane ring of T3SS	F -GCATTGGAGCAACAGTCCCA R- CCTTCAATCCCCTTGCGAT	476 bp	Lim and Hong (2020)
10.	*ela*	Elastase	F ACACGGTCAAGGAGATCAAC R CGCTGGTGTTGGCCAGCAGG	513 bp	Sen and Rodgers (2004)

the isolates on blood agar (HiMedia) containing 5% fish blood (Pradhan et al., 2023). Amylase activity was evaluated on starch ampicillin agar (HiMedia), where clear zones around the colonies indicated starch hydrolysis, as confirmed by flooding the plates with Lugol's iodine solution (Shameena, Kumar, Kumar, Kumar, & Rathore, 2020). The ability of the isolates to hydrolyze lipids by producing lipase enzymes was tested on tributyrin agar (HiMedia) containing tributyrin as the lipid substrate (Harley & Prescott, 2002). Gelatinase activity was identified by clear zones around the colonies, upon flooding the gelatin agar plates with a mercuric chloride solution (dela Cruz & Torres, 2012). Caseinase and DNase activity was carried out on skim milk agar plates containing casein and DNase agar plates containing DNA and toluidine blue (Huys, Kesters, Coopman, Janssen, & Kersters, 1996), respectively.

Putative virulence genes, including aerolysin (*aerA*), cytotoxic (*act*), cytotonic enterotoxin (*ast* and *alt*), lipase (*lip*), hemolysin (*hlyA*), elastase (*ela*), transcriptional regulator (*exsA*) of type-three secretion system (T3SS), and outer and inner membrane ring of T3SS (*ascV* and *ascC*) were detected using PCR with specific primers (Table 1). The PCR was conducted as described in the preceding section. The PCR conditions for *aerA*, *act*, *ast*, and *alt* were similar with an annealing temperature of 58°C. Similarly, amplification of the other six genes, viz., *lip*, *hlyA*, *ela*, *exsA*, *ascV,* and *ascC,* was carried out under similar PCR conditions with an annealing temperature of 55°C.

The *in vivo* pathogenicity of the isolates was assessed by determining the fifty percent lethal dosage (LD_{50}) through probit analysis (Finney, 1952). For each *A. hydrophila* isolate, a separate experiment was conducted at 30±2°C with *Labeo rohita* fingerlings, weighing 14±5 g and measuring 12±2 cm in length, obtained from a local fish farm. Ten fish were placed in each tank with a volume of 200 L and continuous aeration for two weeks before being challenged. The fish were injected intraperitoneally with 100 ìL of bacterial suspension at six different concentrations of *A. hydrophila* strain (10^4-10^9 cells m L^{-1}), while the control received 100 ìL phosphate buffered saline in a completely randomized design with three replications. Mortality rates and clinical symptoms of each group were documented daily for 14 days following infection. Fish showing signs of morbidity were further subjected to regular bacteriological analysis to enable the re-isolation and re-identification of the microorganism.

Results and Discussion

The presumptive *Aeromonas* isolates recovered from the water samples were gram-negative, motile, and rod-shaped. The isolates tested positive for oxidase, catalase, indole, Voges-Proskauer, and lysine decarboxylation, but negative for methyl red, ornithine decarboxylase, citrate utilization, and urea hydrolysis. The isolates exhibited a fermentative metabolism, producing acid from glucose, sucrose, and arabinose, but not from sorbitol or inositol. These biochemical findings indicated that these strains are classified within the *Aeromonas* genus by the guidelines provided in Bergey's Manual of Systematic Bacteriology (Martin-Carnahan & Joseph, 2005). Similar biochemical properties of *A. hydrophila* strains were also reported by Borty et al. (2016), & Monir, Bagum, Kabir, Borty, and Ud Doulah (2017).

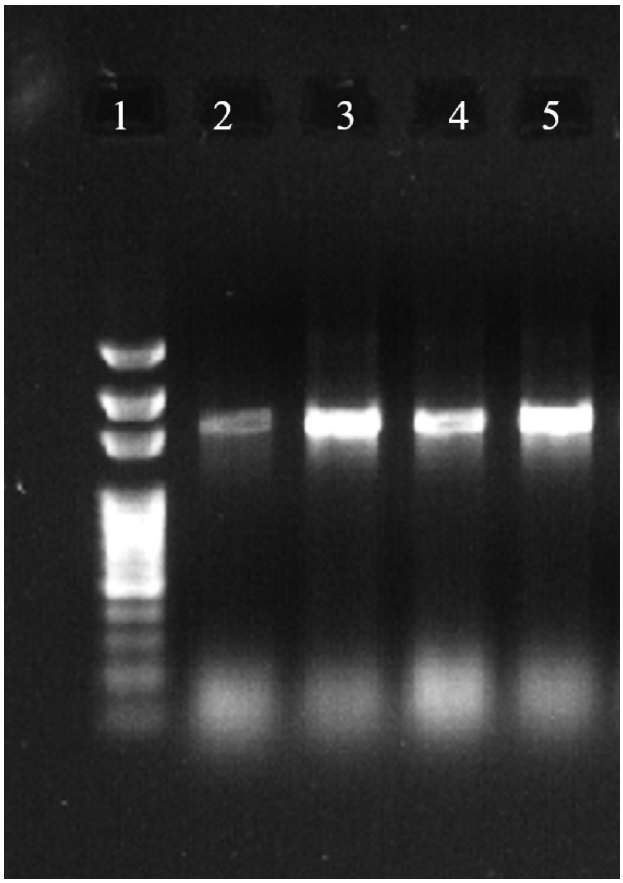

Fig. 2. PCR amplicon of 16s rRNA of *Aeromonas hydrophila* [Lane 1: 100 bp DNA ladder; Lane 2 to 5: A. hydrophila COFCAU_AH1, COFCAU_AH2, COFCAU_AH3, COFCAU_AH4, respectively]

In addition to biochemical characterization, the isolates were subjected to PCR amplification of the 16S rRNA gene followed by sequencing for molecular identification (Fig. 2). The results of molecular analyses confirmed the strains as *A. hydrophila.* The 16S rRNA gene sequences of the strains were submitted to the National Centre for Biotechnology Information (NCBI) database, obtaining GenBank accession numbers MK907589 (842 bp), MK907590 (726 bp), MK907591 (790 bp), and MK907595 (886 bp), respectively with all sequences being 100% identical to *A. hydrophila*. The phylogenetic tree further confirmed the similarity of the strains to other *A. hydrophila* isolates (Fig. 3).

The antibiotic susceptibility test results are summarized in Fig. 4. All tested isolates were susceptible to amoxyclav, cephalexin, gentamicin, oxacillin, and tetracycline. However, COFCAU_AH1 exhibited resistance to kanamycin and tobramycin, while COFCAU_AH2 showed resistance to polymyxin B, ticarcillin, and tobramycin. Additionally, COFCAU_AH3, and COFCAU_AH4 were both resistant to erythromycin. The observed antibiotic resistance patterns in the four *Aeromonas* strains isolated from Tripura fish ponds likely stem from a combination of factors, including intrinsic resistance mechanisms, acquired resistance genes, and selective pressure from antibiotic use in aquaculture (Muziasari et al., 2016; Stratev & Odeyemi, 2016). The emergence of multiple antibiotic resistance in *A. hydrophila* isolated from aquaculture systems poses a significant challenge (Hatha, Vivekanandhan, Joice, & Christol, 2005). Kumar and Rathore (2024) further emphasized the prevalence and geographical distribution of antimicrobial resistance (AMR) in freshwater fish farms in India, highlighting the need for spatial assessments and mitigation strategies to combat AMR in aquaculture.

The results of the phenotypic determinants of virulence and virulence gene detection are presented in Table 2. Determining the production of exoenzymes and toxins by a bacterium is a direct method to demonstrate its pathogenic potential (Sreedharan, Philip, & Singh, 2012). Three strains of *A. hydrophila* (COFCAU_AH1, COFCAU_AH3, and COFCAU_AH4) produced hydrolytic enzymes,

Table 2. Phenotypic virulent determinants and distribution of major virulence genes in *A. hydrophila* isolates

Sl. No.	Phenotypes activity	*A. hydrophila* COFCAU			
		AH1	AH2	AH3	AH4
1.	Haemolytic activity	-	-	+	+
2.	Lipase activity	+	+	+	+
3.	Gelatinase activity	+	-	+	+
4.	Amylase activity	-	-	+	+
5.	DNase activity	-	-	+	+
6.	Caseinase activity	+	-	+	+
	Genes	AH1	AH2	AH3	AH4
1.	*aerA*	-	-	+	-
2.	*act*	-	-	-	-
3.	*ast*	-	-	+	+
4.	*alt*	-	-	+	+
5.	*hlyA*	-	-	+	+
6.	*Lip*	+	+	+	+
7.	*Ela*	-	-	+	+
8.	*exsA*	-	-	-	-
9.	*ascV*	-	-	-	-
10.	*ascC*	-	-	+	+

Note. + : Positive; - : Negative

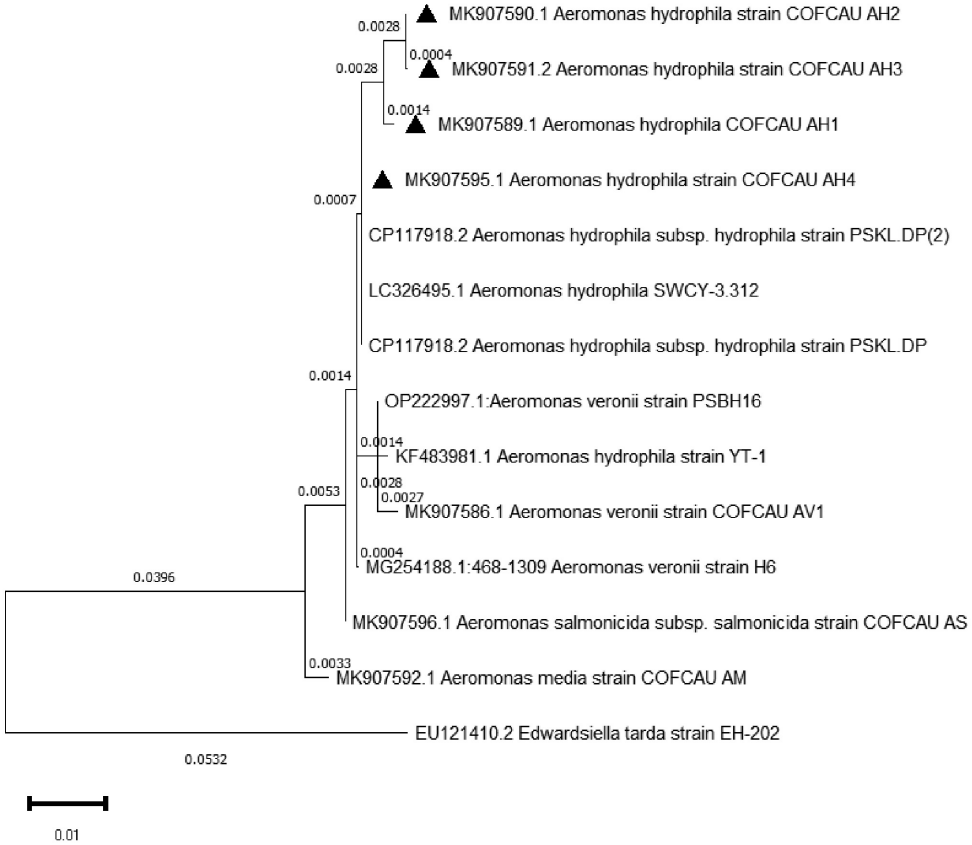

Fig. 3. The phylogenetic tree based on partial 16s rRNA gene sequence of *Aeromonas hydrophila* strains (▲). The tree was constructed using Maximum Likelihood method with genetic distance calculated according to Kimura's 2-parameter model of MEGA11 software.

notably gelatinase and caseinase. All four strains produced lipase. The isolates COFCAU_AH3 and COFCAU_AH4 exhibited high levels of amylase, DNase, and hemolytic activity. The pathogenicity of *Aeromonas* spp. is attributed to the involvement and phenotypic expressions of various secreted enzymes (Citterio & Biavasco, 2015). The phenotypic virulence characteristics of *A. hydrophila* isolates have been described by several authors as being positive for generating amylase and decomposing starch, as well as demonstrating positive reactivity to lipase, gelatinase, caseinase, and DNase activities (Kerigano et al., 2023), which is consistent with current findings. In zebrafish, the phenotypic manifestation of virulence in *A. hydrophila* have been attributed to enzyme-mediated activities via DNase, gelatinase, lipase, caseinase, and haemolysin (Chandrarathna et al., 2018; Hossain, De Silva, Dahanayake, & Heo, 2018).

The virulence gene content of the studied strains of *A. hydrophila* was directly correlated with the pathogenicity of the strains, and the pathogenicity was also associated with the number of genes harboured by the isolates. Virulence genes such as *hylA* (597 bp), *ast* (331 bp), *ela* (513 bp), *ascC* (476) bp, and *alt* (476 bp) were detected in isolates COFCAU_AH3 and COFCAU_AH4 which indicates their pathogenic character. The lipase gene (*lip* - 382 bp) was detected in all four isolates, which aligns with past studies showing that *A. hydrophila* commonly harbors haemolytic genes, which may be used to assess virulence (Hamdan et al., 2015; Roges et al., 2020). However, Park, Kim, Choi, and Rhee (2021) reported that *ast* (heat-stable enterotoxin) and *alt* (heat-labile enterotoxin) were the most frequently detected genetic patterns across all isolates in their study with *A. hydrophila*. Samayanpaulraj et al. (2020) noted that elastase and lipase are essential for

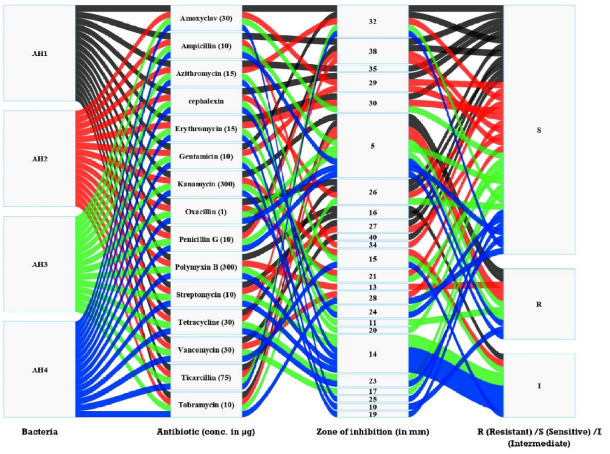

Fig. 4. Antibiotic sensitivity testing of *A. hydrophila* isolates by disc diffusion based on CLSI guidelines (CLSI, 2016)

virulent *A. hydrophila* to invade the intestinal epithelium and contributed to disease development in the host. The presence of the *ascC* virulence gene in *A. hydrophila* strains was also observed by Vilches et al. (2009) and Sha et al. (2005). Additionally, only COFCAU_AH3 contained the aerolysin *aer* (498 bp) gene. Saleh, Elkenany, and Younis (2021) revealed that, out of 187 *A. hydrophila* isolates from diseased fish, 40% possess the aerolysin. None of the examined *A. hydrophila* strains harbored the cytotoxic heat-stable enterotoxin (*act*), *ascV*, or *exsA* genes. A previous study by Li et al. (2021) found a higher frequency of virulent genes in isolates from diseased animals compared to those from healthy fish or water environments.

The first mortality occured 12h after the intraperitoneal injection. It was observed that the mortality rate increased with increasing concentrations. Different concentrations of *A. hydrophila* (10^4-10^9 cells mL^{-1}) resulted in mortality rates of 10-97% for COFCAU_AH1, 0% for COFCAU_AH2, 43-100% for COFCAU_AH3, and 40-100% for COFCAU_AH4. The days post-mortality pattern for fish challenged with different strains and bacterial concentrations are shown in Kaplan-Meier survival curves (Fig. 5). Some of the external clinical signs that were observed included dropsy, a reddish vent, scale erosion, tail rot, erythema at the bases of the fins, bilateral exophthalmia, haemorrhage in the abdomen area, and excessive mucus secretion (Fig. 6). Pale liver and gills, a deposit of bloody fluid in the abdominal cavity, hemorrhage in the kidney, muscle, gastrointestinal tract, and air bladder were some internal signs. The LD_{50} of *A. hydrophila*

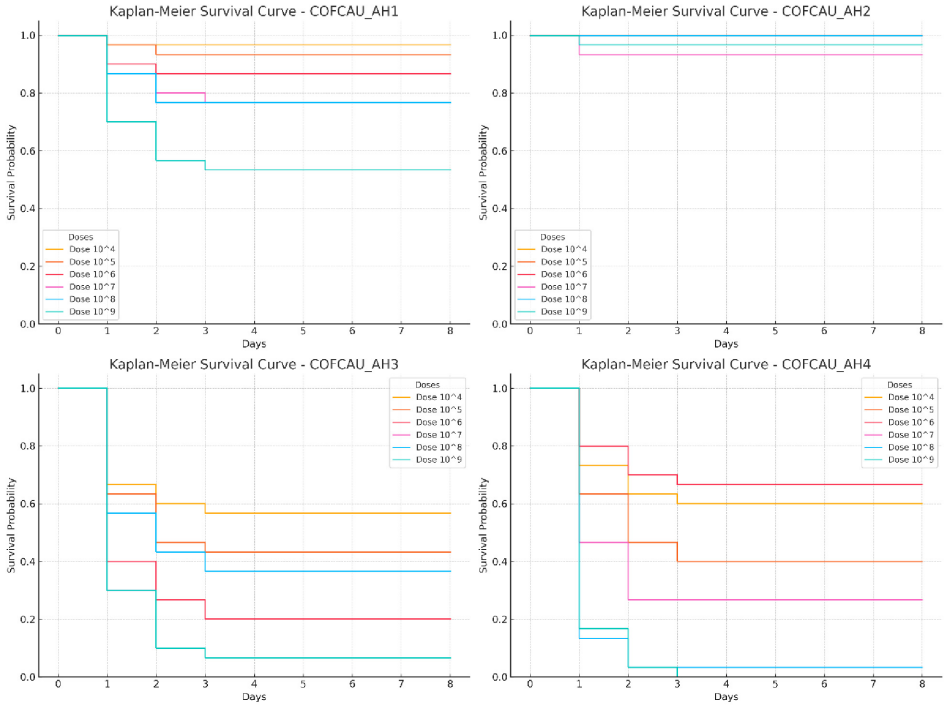

Fig. 5. Kaplan-Meier survival curves for *Aeromonas hydrophila* strains (COFCAU_AH1, COFCAU_AH2, COFCAU_AH3, COFCAU_AH4) across different doses in *Labeo rohita* fingerlings

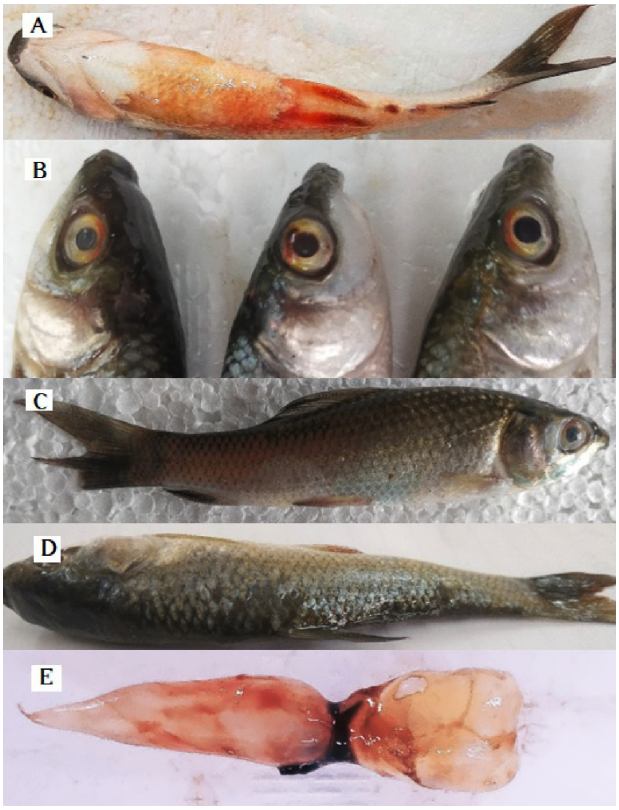

Fig. 6. Hemorrhage in the abdominal region (A), hemorrhage around the eye (B), tail rot (C), erosion of scales (D), and hemorrhage and hyperemia in the air bladder of *L. rohita* challenged with *A. hydrophila*

COFCAU_AH1, COFCAU_AH3, and COFCAU_AH4 for *L. rohita* was estimated to be $10^{9.5}$, $10^{4.4}$, and $10^{4.5}$ cells fish^{-1}, respectively. No mortality was observed for fish infected with COFCAU_AH2.

The current results revealed that COFCAU_AH3 had highest virulence characteristics, followed by COFCAU_AH4 and COFCAU_AH1 in experimental studies, which substantiate the *in vitro* virulence characterization. Generally, isolates from diseased fish show higher virulence than environmental isolates, as observed in zebrafish by Li et al. (2021). However, this study demonstrates that environmental isolates, which carry various virulence factors can also cause significant damage to fish.

The pathogenic species *A. hydrophila* is ubiquitous in aquatic environments and is associated with diseases affecting both humans and various fish species (Daskalov et al., 2006). While environmental isolates of *A. hydrophila* are typically less virulent than the disease-causing isolates found in affected fish, they still pose risks to aquaculture (Abdella, Abozahra, Shokrak, Mohamed, & El-Helow, 2023). Environmental isolates may not cause acute disease in fish under normal circumstances, but virulent isolates can become pathogenic under certain circumstances. These isolates can compromise fish health when they are stressed, or their immune systems are compromised due to poor water quality, overcrowding, or other environmental stressors. Therefore, they can contribute to the overall disease burden and negatively impact the overall health and productivity of the aquaculture systems.

All the isolates exhibited varying levels of phenotypic expression of virulence factors. COFCAU_AH3 and COFCAU_AH4 carried multiple virulence genes, and the LD_{50} dose also substantiates these. Based on the results of the study of virulent determinants and the pathogenicity test, it can be concluded that the environmental isolates of *A. hydrophila* carrying various virulent factors may pose a significant threat to the aquaculture industry.

Acknowledgments

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Ethics Statement

All experiments involving fish were conducted in accordance with the standard guidelines and policies suggested by the Institutional Animal Ethics Committee (IAEC), College of Fisheries, Central Agricultural University, Imphal, Tripura, India (CAU-CF/48/1AEC/2018/03 dated 07/02/2022).

References

Abdella, B., Abozahra, N. A., Shokrak, N. M., Mohamed, R. A., & El-Helow, E. R. (2023). Whole spectrum of *Aeromonas hydrophila* virulence determinants and the identification of novel SNPs using comparative pathogenomics. *Scientific Reports, 13*(1), Article 7712. https://doi.org/10.1038/s41598-023-34887-1.

Alain, K. (2009). Isolation of *Aeromonas hydrophila* from naturally diseased Thai pangas *Pangasius hypophthalmus* (M.Sc. Thesis). Department of Aquaculture, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Austin, B., & Austin, D. A. (2007). *Bacterial fish pathogens: disease of farmed and wild fish* (6th ed.). Springer International Publishing, Switzerland.

Bauer, A. W., Kirby, W. M., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology, 45:* 493-496

Beaz-Hidalgo, R., & Figueras, M. J. (2013). *Aeromonas* spp. whole genomes and virulence factors implicated in fish disease. *Journal of Fish Diseases*, *36*(4), 371-388. https://doi.org/10.1111/jfd.12025.

Bondad-Reantaso, M. G., Subasinghe, R. P., Arthur, J. R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z., & Shariff, M. (2005). Disease and health management in Asian aquaculture. *Veterinary Parasitology*, *132*(3-4), 249-272. https://doi.org/10.1016/j.vetpar.2005.07.005.

Borty, S. C., Rahman, F., Reza, A. A., Khatun, M. S., Kabir, M. L., Rahman, M. H., & Monir, M. S. (2016). Isolation, molecular identification, and antibiotic susceptibility profile of *Aeromonas hydrophila* from cultured indigenous Koi (*Anabus testudineus*) of Bangladesh. *Asian Journal of Medical and Biological Research*, *2*(2), 332-340. https://doi.org/10.3329/ajmbr.v2i2.29078.

Chacón, M. R., Soler, L., Groisman, E. A., Guarro, J., & Figueras, M. J. (2004). Type III secretion system genes in clinical *Aeromonas* isolates. *Journal of Clinical Microbiology*, *42*(3), 1285-1287. https://doi.org/10.1128/jcm.42.3.1285-1287.2004.

Chakraborty, T., Huhle, B., Bergbauer, H., & Goebel, W. (1986). Cloning, expression, and mapping of the *Aeromonas hydrophila* aerolysin gene determinant in *Escherichia coli* K-12. *Journal of Bacteriology*, *167*(1), 368-374. https://doi.org/10.1128/jb.167.1.368-374.1986.

Chandrarathna, H. P. S. U., Nikapitiya, C., Dananjaya, S. H. S., Wijerathne, C. U. B., Wimalasena, S. H. M. P., Kwun, H. J., & De Zoysa, M. (2018). Outcome of co-infection with opportunistic and multidrug-resistant *Aeromonas hydrophila* and *A. veronii* in zebrafish: Identification, characterization, pathogenicity, and immune responses. *Fish and Shellfish Immunology*, *80*, 573-581. https://doi.org/10.1016/j.fsi.2018.06.049.

Citterio, B., & Biavasco, F. (2015). *Aeromonas hydrophila* virulence. *Virulence*, *6*(5), 417-418. https://doi.org/10.1080/21505594.2015.1058479.

Clinical and Laboratory Standard Institute (CLSI). (2016). *Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria* (3rd ed.). Clinical and Laboratory Standard Institute, Pennsylvania, USA.

Daskalov, H. (2006). The importance of *Aeromonas hydrophila* in food safety. *Food Control*, *17*(6), 474–483. https://doi.org/10.1016/j.foodcont.2005.02.009.

dela Cruz, T. E. E., & Torres, J. M. O. (2012). *Gelatin hydrolysis test protocol*. American Society for Microbiology, Washington DC.

Devi, R., Khan, M. I. R., Choudhury, T. G., Pradhan, S. K., & Kamilya, D. (2024). Characterisation of pathogenic *Aeromonas veronii* and *Aeromonas* media isolates from aquaculture system: Virulence, antibiotic susceptibility, and host mortality assessment. *Indian Journal of Fisheries*, *71*(2), 78-85. https://doi.org/10.21077/ijf.2024.71.2.144222-10.

Dooley, J. S., & Trust, T. J. (1988). Surface protein composition of *Aeromonas hydrophila* strains virulent for fish: identification of a surface array protein. *Journal of Bacteriology*, *170*(2), 499-506. https://doi.org/10.1128/jb.170.2.499-506.1988.

Finney, D. J. (1952). *Probit Analysis*. Cambridge University Press, Cambridge.

Harley, J. P., & Prescott, L. M. (2002). *Laboratory Exercises in Microbiology* (5th ed.). The McGraw-Hill Companies, New York.

Hatha, M., Vivekanandhan, A. A., Joice, G. J., & Christol. (2005). Antibiotic resistance pattern of motile aeromonads from farm-raised freshwater fish. *International Journal of Food Microbiology*, *98*(2), 131–134. https://doi.org/10.1016/j.ijfoodmicro.2004.05.017.

Hamdan, R. H., Daud, H. M., Ong, B. L., Abdelhadi, Y. M., Hamid, N. H., Manaf, S. R., Faten, A. M. N., Kuttichantran, S., & Alsaid, M. (2015). Virulence genes detection of *Aeromonas hydrophila* originated from diseased freshwater fishes. *Advances in Environmental Biology*, *9*(22), 22-26.

Heuzenroeder, M. W., Wong, C. Y. F., & Flower, R. L. P. (1999). Distribution of two hemolytic toxin genes in clinical and environmental isolates of *Aeromonas* spp.: correlation with virulence in a suckling mouse model. *FEMS Microbiology Letters*, *174*(1), 131-136. https://doi.org/10.1111/j.1574-6968.1999.tb13559.x.

Hossain, S., De Silva, B. C. J., Dahanayake, P. S., & Heo, G. J. (2018). Characterization of virulence properties and multi drug resistance profiles in motile *Aeromonas* spp. isolated from zebrafish (*Danio rerio*). *Letters in Applied Microbiology*, *67*(6), 598-605. https://doi.org/10.1111/lam.13075.

Huys, G., Kesters, I., Coopman, R., Janssen, P., & Kersters, K. (1996). Genotypic diversity among *Aeromonas* isolates recovered from drinking water production plants as revealed by AFLP™ analysis. *Systematic and Applied Microbiology*, *19*, 428-435. https://doi.org/10.1016/S0723-2020(96)80073-7.

Janda, J. M., & Abbott, S. L. (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, *23*(1), 35-73. https://doi.org/10.1128/cmr.00039-09.

Johnson, M. (1993). The veterinary approach to channel catfish. In L. Brown (Ed.), *Aquaculture for Veterinarians: Fish Husbandry and Medicine* (pp. 249-270). Pergamon Press, Oxford.

Kerigano, N. K., Chibsa, T. R., Molla, Y. G., Mohammed, A. A., Tamiru, M., Bulto, A. O., Wodaj, T. K., Gebreweld, D. S., & Abdi, A. K. (2023). Phenotypic, molecular detection and antibiogram analysis of *Aeromonas hydrophila* from *Oreochromis niloticus* (Nile Tilapia) and Ready-To-eat fish products in selected Rift Valley lakes of Ethiopia. *BMC Veterinary Research, 19*(1), Article 120. https://doi.org/10.1186/s12917-023-03684-3.

Kingombe, C. I. B., Huys, G., Tonolla, M., Albert, M. J., Swings, J., Peduzzi, R., & Jemmi, T. (1999). PCR detection, characterization, and distribution of virulence genes in *Aeromonas* spp. *Applied and Environmental Microbiology*, *65*(12), 5293-5302. https://doi.org/10.1128/AEM.65.12.5293-5302.1999.

Kumar, C. B., Kumar, A., Paria, A., Kumar, S., Prasad, K. P., & Rathore, G. (2022). Effect of spatio temporal variables, host fish species and on farm biosecurity measures on the prevalence of potentially pathogenic *Aeromonas* species in freshwater fish farms. *Journal of Applied Microbiology*, *132*(3), 1700-1712. https://doi.org/10.1016/j.aquaculture.2024.740808.

Kumar, C. B., & Rathore, G. (2024). Assessment of freshwater fish farms for the identification of the geographical areas harbouring antimicrobial resistance. *Aquaculture*, *586*, Article 740808. https://doi.org/10.1111/jam.15330.

Leung, K. Y., & Stevenson, R. M. W. (1988). Characteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. *Microbiology*, *134*(1), 151-160. https://doi.org/10.1099/00221287-134-1-151.

Li, J., Ma, S., Li, Z., Yu, W., Zhou, P., Ye, X., Islam, M. S., Zhang, Y. A., Zhou, Y., & Li, J. (2021). Construction and characterization of an *Aeromonas hydrophila* multigene deletion strain and evaluation of its potential as a live-attenuated vaccine in grass carp. *Vaccines*, *9*(5), Article 451. https://doi.org/10.3390/vaccines9050451.

Lim, J., & Hong, S. (2020). Characterization of *Aeromonas salmonicida* and *A. sobria* isolated from cultured salmonid fish in Korea and development of a vaccine against furunculosis. *Journal of Fish Diseases*, *43*(5), 609-620. https://doi.org/10.1111/jfd.13158.

MacFaddin, J. F. (1980). *Biochemical Tests for Identification of Medical Bacteria* (2nd ed.). Williams & Wilkins Co., Baltimore, Maryland.

Martin-Carnahan, A., & Joseph, S. W. (2005). Aeromonadales ord. nov. In D. J. Brenner, N. R. Krieg, J. T. Staley, G. M. Garrity, D. R. Booe, P. Vos, M. Goodfellow, F. A. Rainey, & K. H. Schleifer (Eds.), *Bergey's Manual of Systematic Bacteriology* (pp. 556-587). Springer, New York.

Monir, M. S., Bagum, N., Kabir, S. M. L., Borty, S. C., & Ud Doulah, M. A. (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*(1), 17-24. https://doi.org/10.3329/aajfss.v1i1.55757.

Muziasari, W. I., Pitkänen, L. K., Sørum, H., Stedtfeld, R. D., Tiedje, J. M., & Virta, M. (2016). The resistome of farmed fish feces contributes to the enrichment of antibiotic resistance genes in sediments below Baltic Sea fish farms. *Frontiers in Microbiology*, *7*, Article 2137. https://doi.org/10.3389/fmicb.2016.02137.

Ørmen, Ø., & Østensvik, Ø. (2001). The occurrence of aerolysin positive *Aeromonas* spp. and their cytotoxicity in Norwegian water sources. *Journal of Applied Microbiology*, *90*(5), 797-802. https://doi.org/10.1046/j.1365-2672.2001.01309.x.

Park, S. M., Kim, H. W., Choi, C., & Rhee, M. S. (2021). Pathogenicity and seasonal variation of *Aeromonas hydrophila* isolated from seafood and ready-to-eat sushi in South Korea. *Food Research International*, *147*, Article 110484. https://doi.org/10.1016/j.foodres.2021.110484.

Pemberton, J. M., Kidd, S. P., & Schmidt, R. (1997). Secreted enzymes of *Aeromonas*. *FEMS Microbiology Letters*, *152*(1), 1-10. https://doi.org/10.1111/j.1574-6968.1997.tb10401.x.

Pradhan, S. K., Devi, R., Khan, M. I. R., Kamilya, D., Choudhury, T. G., & Parhi, J. (2023). Isolation of *Aeromonas salmonicida* subspecies *salmonicida* from aquaculture environment in India: Polyphasic identification, virulence characterization, and antibiotic susceptibility. *Microbial Pathogenesis*, *179*, Article 106100. https://doi.org/10.1016/j.micpath.2023.106100.

Roges, E. M., Gonçalves, V. D., Cardoso, M. D., Festivo, M. L., Siciliano, S., Berto, L. H., Pereira, V. L. A., Rodrigues, D. P., & de Aquino, M. H. C. (2020). Virulence-associated genes and antimicrobial resistance of *Aeromonas hydrophila* isolates from animal, food, and human sources in Brazil. *BioMed Research International*, *2020*, Article 1052607. https://doi.org/10.1155/2020/1052607.

Saleh, A., Elkenany, R., & Younis, G. (2021). Virulent and multiple antimicrobial resistance *Aeromonas hydrophila* isolated from diseased Nile Tilapia Fish (*Oreochromis niloticus*) in Egypt with sequencing of some virulence-associated genes. *Biocontrol Science*, *26*(3), 167-176. https://doi.org/10.4265/bio.26.167.

Samayanpaulraj, V., Sivaramapillai, M., Palani, S. N., Govindaraj, K., Velu, V., & Ramesh, U. (2020). Identification and characterization of virulent *Aeromonas hydrophila* Ah17 from infected *Channa striata* in river Cauvery and *in vitro* evaluation of shrimp chitosan. *Food Science & Nutrition*, *8*(2), 1272-1283. https://doi.org/10.1002/fsn3.1416.

Semwal, A., Kumar, A., & Kumar, N. (2023). A review on pathogenicity of *Aeromonas hydrophila* and their mitigation through medicinal herbs in aquaculture. *Heliyon*, *9*(3), Article e14088. https://doi.org/10.1016/j.heliyon.2023.e14088.

Sen, K., & Rodgers, M. (2004). Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water utilities: a PCR identification. *Journal of Applied Microbiology*, *97*(5), 1077-1086. https://doi.org/10.1111/j.1365-2672.2004.02398.x.

Sha, J., Pillai, L., Fadl, A. A., Galindo, C. L., Erova, T. E., & Chopra, A. K. (2005). The type III secretion system and cytotoxic enterotoxin alter the virulence of *Aeromonas hydrophila*. *Infection and Immunity*, *73*, 6446-6457. https://doi.org/10.1128/iai.73.10.6446-6457.2005.

Shameena, S. S., Kumar, K., Kumar, S., Kumar, S., & Rathore, G. (2020). Virulence characteristics of *Aeromonas veronii* biovars isolated from infected freshwater goldfish (*Carassius auratus*). *Aquaculture*, *518*, Article 734819. https://doi.org/10.1016/j.aquaculture.2019.734819.

Smith, P. (2006). Breakpoints for disc diffusion susceptibility testing of bacteria associated with fish diseases: a review of current practice. *Aquaculture*, *261*(4), 1113-1121. https://doi.org/10.1016/j.aquaculture.2006.05.027.

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

Stratev, D., & Odeyemi, O. A. (2016). Antimicrobial resistance of *Aeromonas hydrophila* isolated from different food sources: A mini-review. *Journal of Infection and Public Health*, *9*(5), 535-544. https://doi.org/10.1016/j.jiph.2015.10.006.

Swain, P., Nayak, S. K., Sahu, A., Mohapatra, B. C., & Meher, P. K. (2002). Bath immunisation of spawn, fry and fingerlings of Indian major carps using a particulate bacterial antigen. *Fish and Shellfish Immunology*, *13*(2), 133-140. https://doi.org/10.1006/fsim.2001.0388.

Vilches, S., Jimenez, N., Tomás, J. M., & Merino, S. (2009). *Aeromonas hydrophila* AH-3 type III secretion system expression and regulatory network. *Applied and Environmental Microbiology*, *75*(19), 6382-6392. https://doi.org/10.1128/AEM.00222-09.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*(2), 697-703. https://doi.org/10.1128/jb.173.2.697-703.1991.

Wilson, K. (2001). Preparation of genomic DNA from bacteria. *Current Protocols in Molecular Biology*, *56*(1), 2-4. https://doi.org/10.1002/0471142727.mb0204s56.

Yesmin, S., Rahman, M. H., Hussain, M. A., Khan, A. R., Pervin, F., & Hossain, M. A. (2004). *Aeromonas hydrophila* infection in fish from swamps in Bangladesh. *Pakistan Journal of Biological Sciences*, *7*(3), 409-411. https://doi.org/10.3923/pjbs.2004.409.411.

Yogananth, N., Bhakyaraj, R., Chanthuru, A., Anbalagan, T., & Nila, K. M. (2009). Detection of virulence gene in *Aeromonas hydrophila* isolated from fish samples using PCR technique. *Global Journal of Biotechnology & Biochemistry*, *4*, 51-53.

Zhang, Y. L., Arakawa, E., & Leung, K. Y. (2002). Novel *Aeromonas hydrophila* PPD134/91 genes involved in O-antigen and capsule biosynthesis. *Infection and Immunity*, *70*(5), 2326-2335. https://doi.org/10.1128/iai.70.5.2326-2335.2002.