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Evaluation of *in vivo* and *in vitro* Pathogenicity of *Aeromonas veronii* Isolated from Freshwater Fishes of Andhra Pradesh

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Abstract

Aeromonas spp. are responsible for causing hemorrhagic septicemia resulting in significant mortalities in carp aquaculture. Among the different species, *Aeromonas veronii* is considered as a significant pathogen and is second only to *A. hydrophila* in causing severe economic losses to freshwater fish farmers. The present study was carried out to isolate and identify *A. veronii* from freshwater fishes in Andhra Pradesh, India. In addition, both *in vivo* and *in vitro* pathogenicity assays were performed to assess its virulence. A total of 95 *A. veronii* strains were meticulously isolated from 80 [*Labeo rohita* (n=40), *Catla catla* (n=40)] fish samples and subjected to biochemical tests (API 20E system), PCR confirmation (amplification of 16S rRNA gene), and virulence gene profiling. The strains exhibited a genetic diversity ranging from four to eight virulence genes, with a prominent genotype (ahy^+, ahp^+, $enol^+$, lip^+, alt^+, hly^+) observed in 28% of the isolates. *In vivo* pathogenicity analysis indicated that 72% of the strains exhibited hemolytic activity, which was strongly associated with the presence of aer^+, hly^+, and $ahyB^+$ genes. Furthermore, *in vivo* challenge experiments demonstrated that even *Aeromonas* strains with as few as four virulence genes (ahy^+, $enol^+$, aer^+, alt^+) were capable of inducing over 90% mortality. These findings strongly imply that the pathogenicity of *A. veronii* is more significantly influenced by the synergistic effect of virulence genes rather than their total number. The high virulence of *A. veronii* poses a serious threat to the freshwater aquaculture, underscoring the need for enhanced monitoring and management strategies.

Keywords: *Aeromonas veronii*, rohu, catla, virulence genes, pathogenicity

Introduction

Aquaculture plays a significant role in global food production, particularly in Asia. Global aquaculture production reached an unprecedented 130.9 million tonnes (MT) in 2022, comprising 94.4 MT of aquatic animals and 36.5 MT of algae (FAO, 2024). India ranks second in global fish production, primarily through the cultivation of major carps (FAO, 2022). Andhra Pradesh leads in fisheries production with 4.8 MT, of which 2.314 MT come from carps (DoF, 2022). The major cultivable carps are *Labeo rohita, Catla catla, Cirrhinus mrigala*, and *Cyprinus carpio* which are favoured for their fast growth rate, consumer preference, high market value (Manam & Quraishi, 2024) and also constitute a major source of protein. Consequently, the shift of culture systems from traditional to intensified methods has led to a rise in disease outbreaks, particularly bacterial infections, which account for approximately 25% of the total diseases reported (Tyagi et al., 2022). Among the most common diseases affecting freshwater fish is hemorrhagic septicemia (Mukherjee et al., 2017), caused by various mesophilic *Aeromonas* species. Aeromonads are primarily Gram-negative, rod-shaped, motile bacteria that inhabit freshwater environments such as groundwater, lakes, rivers, and both chlorinated and untreated drinking water (Pianetti et al., 2012; Govender et al., 2021). Under stressful conditions, these opportunistic pathogens

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can infect fish, causing primary diseases or secondary infections. The key pathogenic species within the genus *Aeromonas* includes *A. hydrophila, A. caviae, A. schubertii*, and *A. jandaei*. Notably, *A. veronii* is known for causing pandemics and epidemics in freshwater fish, leading to significant economic losses in the aquaculture sector (Liu et al., 2024).

Additionally, Aeromonads are also considered major human pathogens, causing gastroenteritis, pneumonia, septicemia, and wound infections, particularly in immunocompromised patients (Wimalasena, Shin, Hossain, & Heo, 2017). Multiple studies have identified virulence factors such as flagella, hemolytic or aerolysin enzymes, exotoxins, and extracellular enzymes (lipase, serine protease) as primary determinants of pathogenicity (Sreedharan, Philip, & Singh, 2013; Li, Ni, Liu, & Lu, 2011; Tyagi et al., 2022). These factors aid bacterial infiltration, replication, defense against the host, and can cause structural alterations, disruption of organ functions, or disease development within the host (Zepeda-Velazquez et al., 2017). *A. veronii,* possessing one or more of these virulence factors, is particularly pathogenic (Sreedharan et al., 2013). Enterotoxins produced by *Aeromonas* species can lead to fluid accumulation (Albert et al., 2000), while skin lesions are linked to enolase or serine protease (Pessoa et al., 2020), plasma membrane digestion to lipase (Chuang, Chiou, Su, Wu, & Chang, 1997), dysentery infections to flagella (Kirov et al., 2002), epithelial cell damage to aerolysin (Wang et al., 2003), and erythrocyte lysis to hemolysin (Nawaz et al., 2010). Hemolytic and proteolytic activities of *Aeromonas* spp. are closely associated with these virulence genes (Singh, Mani, Chaudhary, & Somvanshi, 2011). Therefore, to effectively control diseases in fish culture systems, pathogen identification and characterization are crucial.

Hence, this study aimed to isolate and characterize *A. veronii* from diseased freshwater fish such as rohu and catla samples from Andhra Pradesh, India. The distribution of virulence genes and their relationship with hemolytic, proteolytic, and lipolytic activities were assessed. Additionally, an *in vivo* pathogenicity study was conducted to evaluate the potential virulence of *A. veronii* strains with different virulence gene profiles in freshwater fish.

Material and Methods

A total of 80 diseased and moribund *Labeo rohita* (n=40, 630±50 g) and *Catla catla* (n=40, 600±20 g) were collected from 30 farms in SPSR Nellore, Andhra Pradesh, India (2022-2023). Pathological signs and necropsy conditions were recorded per Noga (2010), and samples were transported aseptically alive for further analysis.

Based on the severity of infection, the kidney, spleen, liver, and skin lesions were collected as inoculums using sterile cotton swabs. Initially, the samples were enriched in alkaline phosphate medium with ampicillin (30 μg/mL) and streaked onto Rimler-Shotts (RS) agar plates, which were incubated at 32°C for 18-24 hours (El Latif, Elabd, Amin, Eldeen, & Shaheen, 2019). Pure cultures were obtained by repeated streaking and preserved at -20°C in 30% glycerol stock (Ramesh & Souissi, 2018). The pure cultures were maintained on slants and regularly subcultured onto fresh slants every 15 days to ensure viability and purity. Gram staining and motility tests were initially performed for identification, followed by biochemical tests, including oxidase, citrate, indole, H_2S, methyl red, starch hydrolysis, carbohydrate fermentation, esculin hydrolysis, Voges-Proskauer, malonate utilization, and salicin fermentation. Reconfirmation was carried out using the API 20E kit (HiMedia, India).

Aeromonas isolates underwent species confirmation via biochemical tests and amplification of 16S rRNA species specific gene using PCR (Polymerase Chain Reaction), employing a positive control (*Aeromonas veronii* ATCC 9071). Among the isolated strains, one displaying similar phenotypic characteristics was provisionally identified through sequencing analysis using NCBI-BLAST and was assigned the gene accession number MZ540305. Nine virulence genes namely aerolysin (*aer*), enterotoxin (*alt*), elastase (*ahy*), enolase (*enol*), DNase (*exu*), lipase (*lip*), flagellin (*fla*), hemolysin (*hly*), and serine protease (*ahp*) were targeted. Primer sizes, thermal cycling conditions, and gene sequences are provided in Table 1. DNA was extracted using a Genei kit (Bangalore) as per protocols of the manufacturer. Briefly, each strain was mixed in 110 μL of extraction solution, incubated at 35°C for 20 min, and centrifuged at 10,000 rpm for 10 min. The supernatant (100 μL) was mixed with 100% ethanol and centrifuged under the same conditions. The DNA pellet was washed with ethanol (95% and 70%). After air drying, the pellet was dissolved in 50 μL of nuclease-free water and stored at -20°C. PCR was performed with 25 μL reaction mixture consisting of master mix (12.5 μL), forward and reverse primers (1 μL each), DNA template (2 μL), and nuclease-free sterile distilled water (8.5 μL), with 35 cycles renging from 94°C (4 min) to 72°C (10 min). Electrophoresis was conducted on a 1.2% agarose gel stained with ethidium bromide. *A. veronii* (ATCC 9071) served as a positive control, and nuclease-free water as a negative control.

The pathogenicity of *A. veronii* strains was examined by assessing β-hemolytic, proteolytic, and lipolytic activity using 5% sheep blood agar (SBA), skim milk agar (SMA), and olive oil-nutrient agar with rhodamine B (ONR) (HiMedia, India), respectively. The assays were performed following the methods of Hoel, Vadstein, and Jakobsen (2017), Wassif, Cheek, and Belas (1995), & Pessoa et al. (2020), with slight modifications. *A. veronii* strains cultured on TSA slants were incubated onto SBA, SMA, and ONR media and incubated at 37°C for 24-48 h. Hemolysis was indicated by clear zones on SBA, proteolysis by clear zones on SMA, and lipolysis by orange fluorescence under UV light.

To address the relationship between the virulence gene profiles and the pathogenicity of *A. veronii*, a challenge study was conducted over 7 days duration in rohu and catla using five strains with different gene profiles as described by Li et al. (2011). A total of 600 healthy fishes i.e 300 rohu (mean size 200.5 ±4 g), 300 catla (mean weight 180.6±5 g); were acclimatized for 15 days. Prior to acclimatization, the fish were disinfected by dipping in 2 mg/L $KMnO_4$ for 10 minutes (Barkoh, Smith, & Southard, 2010). During the acclimatization period, the fish were fed on a commercial fish diet containing 35% protein at a feeding rate of 3% of body weight per day.

The bacterial cell suspension was prepared as per Doan et al. (2018) with slight modifications. Freshly overnight-grown cultures (2 mL) in Trypticase soya broth (TSB) (HiMedia, India) were centrifuged, and resuspended in phosphate buffered solution (PBS, pH -7.4). The optical density (0.5) was measured at 600 nm (LABMAN, India), and bacterial counts were determined by serial dilution using the spread plate method (Mallik et al., 2020).

Before conducting the main experiment, a preliminary challenge test was performed to assess the pathogenicity of *A. veronii* in fish. Fish were injected intraperitoneally with bacterial suspensions at different concentrations, ranging from 10^4 to 10^8 cfu/mL, and the LD_{50} was calculated using the method of Reed and Muench (1938). The results indicated an LD_{50} value of 10^5 cfu/mL, meaning that at this concentration, 50% of the infected fish die. Based on this, the concentration of 10^5 cfu/mL was selected for the main pathogenicity study. Prior to injection, fish were anesthetized with MS-222 (150 mg/L) (Dawood, Gewaily, & Sewilam, 2022). Seven groups were tested: (1) sham control (saline injection), (2) uninfected control, and (3-7) injected with *A. veronii* strains carrying different virulence profiles (Table). Groups 3, 4, 5, 6 and 7 were injected with 0.2 mL of bacterial suspension at a concentration of 2.6 x 10^5 cfu/mL, 3.2 x 10^5 cfu/mL, 2.8 x 10^5 cfu/mL, 4.2 x 10^5 cfu/mL and 3.5 x 10^5 cfu/mL, respectively, per fish. Freshly dead and moribund fish were collected for re-isolation and PCR confirmation of virulence genes. The experiment was maintained in four replicates, each in a 90 L tank containing five fish.

Mortality rates and virulence gene profiles were analyzed using ANOVA (SPSS 2022) at a significance level of 5% ($P < 0.05$). Correlation analysis (R-studio) assessed the relationship between *in vitro* and *in vivo* pathogenicity at the 0.05 significance level (2-tailed) by Spearman's rho. A cluster dendrogram was also constructed to evaluate *Aeromonas* associations in fish.

Results and Discussion

The diseased fishes in the current study exhibited clinical signs such as gill discoloration, dropsy, scale loss, fin/tail rot, pop eye, and hemorrhages (Fig. 1 & Fig. 2), which fully corroborate observations reported in previous studies (Ünver & Bakýcý, 2021). Furthermore, the liver and kidney were identified as the main target organs affected by *A. veronii*, consistent with findings by Mohanty, Mishra, Das, Jena, and Sahoo, 2008; Sayuti et al. 2021. Dendrogram (Fig. 3) analysis showed a high prevalence of pathogenic strains in the kidney of both rohu and catla. Among the *Aeromonas* genus, species such as *A. hydrophila, A. veronii, A. caviae*, and *A. jandaei* are major pathogens in aquaculture, causing hemorrhagic septicemia and skin ulcers in fish (Wang, Hou, Rasooly, Gu, & He, 2021). Similarly, in humans, few Aeromonads including *A. hydrophila, A. caviae*, and *A. veronii* are the common etiological agents of gastroenteritis (Yuwono, Wehrhahn, Liu, Riordan, & Zhang, 2021).

Historically, *A. hydrophila* was considered the primary cause of hemorrhagic septicemia, however, *A. veronii* has now emerged as the dominant etiological agent in cyprinids (Ran et al., 2018; Li et al., 2024). This study identified *A. veronii* in 90% (n=95) of strains isolated from rohu and catla farms in Andhra Pradesh, confirming its significant role in hemorrhagic septicemia. Supporting evidence from Cai, Wu, Jian, Lu, and Tang (2012) & Eissa, El Lamei, Ismail, Youssef, and Mansour (2016) also attributes hemorrhagic septicemia in fish to *A. veronii*. Virulent aeromonads have been identified in various fish hosts, including zebrafish (Li et al., 2011), common carp (Hu, Wang, Pan, Lu, & Liu, 2012), milkfish (Simon, Lalitha, & Joseph, 2016), rohu (Sahoo et al., 2011), red tilapia (Sayuti et al., 2021), and pangasius (Kumar et al., 2022), as well as in human infections (Parker & Shaw, 2011; Ahmed, Ismaiel, Zeid, Ibrahim, & Enan, 2021).

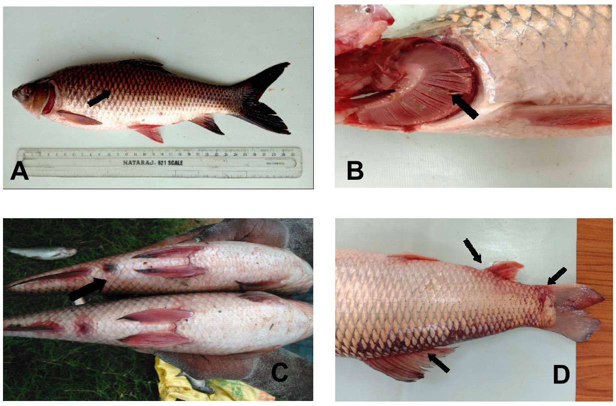

Fig. 1. Gross pathological symptoms of diseased *Labeo rohita*. A. Hemorrhages on body and in the eye (Black arrows); B. Pale gills (Black arrow); C. Hemorrhagic vent (Black arrows); and D. Hemorrhages on the fins, base of the fins and Tail rot (Black arrows).

Among the Indian Major Carps (IMC), rohu is the most popular species due to its fast growth rate and high flesh quality (Mohanty et al., 2008). However, its susceptibility to diseases is increased by factors such as poor-quality seed, inadequate feed, and high stocking densities (Vivekanand, Kamal, Tarkeshwar, Jaspreet, & Kumar, 2022). In this study, of the 90% (n=95) of *A. veronii* strains isolated, 66.3% (n=63) were from 40 diseased rohu samples, and 33.68% (n=32) from 40 diseased catla samples, indicating a greater susceptibility of rohu to the *Aeromonas* infections.

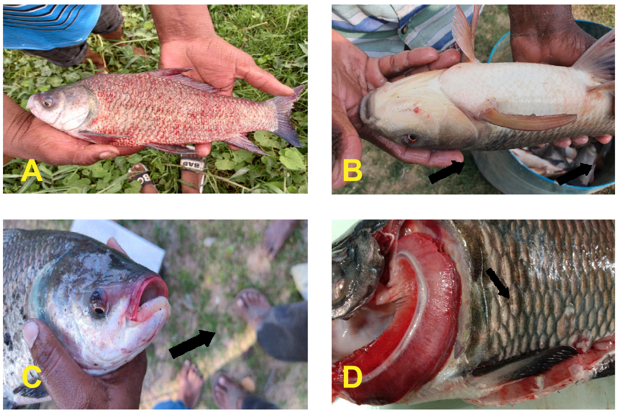

Fig. 2. Gross pathological symptoms of diseased *Catla catla*. A. Hemorrhages on the body surface (Black arrows); B. Skin leisons (Black arrow); C. Hemorrhagic mouth (Black arrows); and D. pale gills (Black arrows)

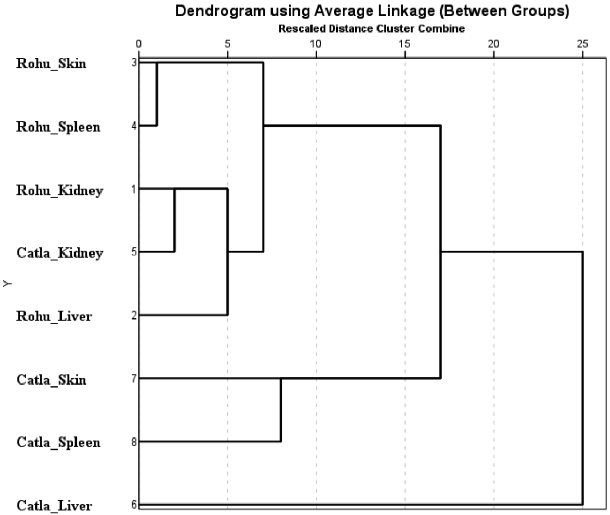

Fig. 3. Dendrogram showing similarity of *Aeromonas* prevalence between rohu and catla: rohu kidney (Cluster 1) and catla kidney (Cluster 5) are positioned in separate branches but two clusters exhibit a common linkage at a higher hierarchical level.

The biochemical characteristics of *A. veronii* strains are given in Table 2. These findings are consistent with previous reports (Hickman-Brenner et al. 1987; Abbott, Cheung, & Janda, 2003; Behera et al. 2023), indicating that the isolated *A. veronii* strains are lethal pathogens capable of utilizing various substrates for growth, although this can vary depending on geographic location (Behera et al., 2023). The API 20E results also confirmed *A. veronii* with 90–98% similarity. Further PCR amplification of 16S rRNA

Table 1. Details of the primers used in this study

Primer	Sequence (5′-3′) (Forward & Reverse)	Size (bp)	Cycling conditions			Reference
			Denaturation	Annealing	Extension	
16S rRNA	F: AGAGTTTGATCATGGCTCAG R: GGTTACCTTGTTACGACTT	1509	94°C/60 sec	59°C/60 sec	72°C/5 min	Borrell, Acinas, Figueras, & Martinez Murcia (1997)
aer	F: CCTATGGCCTGAGCGAGAAG R: CCAGTTCCAGTCCCACCACT	431	94°C/30 sec	55.5°C/30 sec	72°C/30 sec	Mansour, Mahfouz, Husien, & El-Magd (2019)
alt	F: TGACCCAGTCCTGGCACGGC R: GGTGATCGATCACCACCAGC	442	94°C/30 sec	63°C/50 sec	72°C/30 sec	Nawaz et al. (2010)
hly	F: GGCCGGTGGCCCGAAGATACG GG R: GGCGGCGCCGGACGAGACGGGG	592	94°C/30 sec	62°C/30 sec	72°C/ 2 min	Zhu et al. (2007)
lip	F: CACCTGGTTCCGCTCAAG R: GTACCGAACCAGTCGGAGAA	247	95°C/45 sec	58°C/30 sec	72°C/30 sec	Nawaz et al. (2010)
ahy	F: ACACGGTCAAGGAGATCAAC R: CGCTGGTGTTGGCCAGCAGG	540	94°C/30 sec	60.6°C/30 sec	72°C/30 sec	Mansour et al. (2019)
ahp	F: ATTGGATCCCTGCCTATCGCT R:TCAGTTCAGCTAAGCTTGCAT CCGTGCCGTATTCC	911	94°C/30 sec	55°C/30 sec	72°C/30 sec	Zhu et al. (2007)
enol	F: ATGTCCAAGATCGTTAAAGTGAT R: TTAAGCCTGGTTCTTCACTTCTT	1302	94°C/30 sec	55°C/50 sec	72°C/1 min	Sha et al. (2005)
fla	F: GATTGGTATTGCCTTGCAAG R: GMYTGGTTGCGRATGGT	608	94°C/30 sec	55°C/30 sec	72°C/1 min	Nawaz et al. (2010)
exu	F: CCGTGCCAGGACTGGGTCTT R: GATTGGTATTGCCTTGCAAG	323	94°C/30 sec	61°C/30 sec	72°C/1 min	Nawaz et al. (2010)

gene (Fig. 4) also confirmed 95 (90%) isolates as *A. veronii*, 7 isolates (6.7%) as *A. caviae*, and 3 isolates (2.8%) as *A. hydrophila.*

The pathogenicity of aeromonads is primarily attributed to their hemolytic, enterotoxic, proteolytic, and lipolytic activities, which facilitate bacterial attachment, colonization, and subsequent tissue damage in both fish and humans (Daskalov, 2006). The major virulence factors in *Aeromonas* include enterotoxins, endotoxins, hemolytic and other proteolytic enzymes (Hu et al., 2012; Nawaz et al., 2010; Li et al., 2011; Li et al., 2024). Therefore, detecting these virulence genes and their pathogenic patterns is crucial for effective disease control (Li et al., 2011). *A. veronii* strains in the present study showed heterogenous distribution of virulence genes, with each strain carrying between four to eight genes. The distribution of these virulence genes is shown in Table 3, and PCR amplification of all the nine virulence genes are depicted in Fig. 4. Among the nine virulence genes, *ahy* (89%), *ahp* (86%), and e*nol* (84%) were the most prevalent. Other genes such as *lip* (77%), *aer* (76%), *alt* (67%), *fla* (63%), and *hly* (52%) were less common. The most common genotype detected was *ahy*$^+$ *ahp*$^+$ *enol*$^+$ *lip*$^+$ *alt*$^+$ *hly*$^+$ (28%) followed by *ahy*$^+$ *ahp*$^+$ *enol*$^+$ *lip*$^+$ *aer*$^+$ *alt*$^+$ *fla*$^+$ *hly*$^+$ (16%), *ahy*$^+$ *ahp*$^+$ *enol*$^+$ *lip*$^+$ *aer*$^+$ *alt*$^+$ *fla*$^+$ (13%), *ahp*$^+$ *enol*$^+$ *aer*$^+$ *alt*$^+$ (10%), and *ahy*$^+$ *enol*$^+$ *aer*$^+$ *alt*$^+$ (8%). Serine proteases and elastase genes degrade the mucosal layer of epithelial tissues, causing skin lesions and activating other virulence factors like aerolysin (Nawaz et al., 2010; Pessoa et al., 2020). Enolase, a glycolytic enzyme, aids bacterial survival by degrading blood plasma (Fernández-Bravo & Figueras, 2020). The lipase genes, hydrolyzes triglycerides, damaging the intestinal epithelium (Beaz-Hidalgo & Figueras, 2013). Aerolysin genes causes cellular damage (Nawaz et al., 2010). Cytotoxic enterotoxin genes are linked to diarrhea and fluid secretion (Ottaviani et al., 2011). Flagellin genes are associated with dysenteric infections (Sha et al., 2002). Hemolysin genes are cytotoxic and contribute to human diseases (Chen et al., 2014).

The *in vitro* pathogenicity results are given in Table 3. All 95 isolates exhibited at least one enzymatic activity i.e., proteolytic (84%), lipolytic (75%), or â-hemolytic (72%). The production of hemolytic and proteolytic toxins by *Aeromonas* is considered a hallmark of pathogenicity (Santos, Gonzaìlez, Otero, & Garciìa-Loìpez, 1999; McMahon, 2000). In this study, 72% of *A. veronii* strains exhibited hemolytic

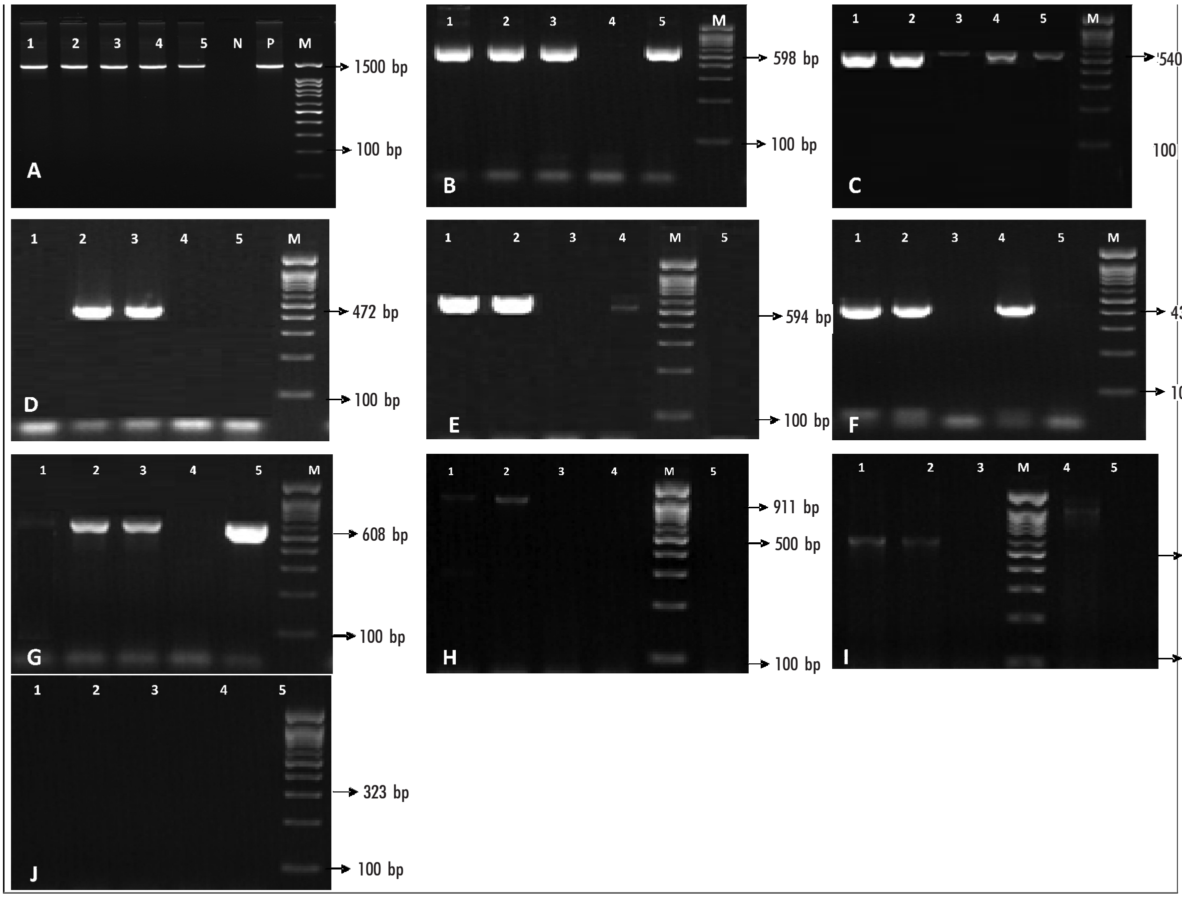

Fig. 4. PCR amplification of genes of *A. veronii* isolated from freshwater fishes

A) 16S rRNA (1500 bp) Lane M: 100 bp ladder; Lane N: Negative control; Lane P: Positive control; Lane 1-5 *A. veronii* isolates.

B) Enolase (598 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates.

C) Elastase (540 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates.

D) Enterotoxin (472 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates.

E) Lipase (594 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates.

F) Aerolysin (431 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates.

G) Flagellin (608 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates.

H) Serine protease (911 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates.

I) Haemolysin (597 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates.

J) DNase (323 bp) Lane M: 100 bp ladder; Lane 1-5 *A. veronii* isolates activity and carried the *aer*$^+$, *alt*$^+$, *hly*$^+$ and *ahp*$^+$ virulence genes. The high hemolytic activity indicates their potential to cause lysis of red blood cells through the production of hemolysins and aerolysin (Janda & Abbott, 2010). Both aerolysin and hemolysin genes induce pore formation in infected cells (Heuzenroeder, Wong, & Flower, 1999), leading to fluid accumulation. Sreedharan et al. (2013) also reported high hemolytic activities from *A. veronii* strains that possessed both aerolysin and hemolysin genes. Furthermore, the protease enzyme exhibits caseinolytic activity and can cause discoloration and degradation of mucus (Beaz-Hidalgo & Figueras, 2013). In the present study, proteolytic activity was found in 84% of *A. veronii* strains, indicating high production of caseinase.

The present study findings align with the report of Erdem, Kariptas, and Kaya (2010), who reported protease activity in 94% of *Aeromonas* strains. Moreover, a statistically significant association was observed between β-hemolytic activity and the presence of aerolysin/hemolysin and serine protease genes, strongly indicating the role of serine protease in the activation of aerolysin. This finding is consistent with Abrami et al. (1998), who stated that serine protease is necessary for aerolysin activation. Additionally, lipolytic activity correlated with the presence of lipase enzyme (Chacón, Figueras, Castro-Escarpulli, Soler, & Guarro, 2003). However, Da Silva Custódio (2009) suggested that, lipolytic activity may occur even in the absence of lipase genes. Interestingly, bacterial isolates in the present study showed 75% lipolytic activity, and all strains harboured the lipase gene. Furthermore, hemolytic, proteolytic, lipolytic activities were observed in 46% of the *A. veronii* isolates; hemolytic, proteolytic activities in 57%, and hemolytic and lipolytic activities in 7% of the isolates.

Several pathogenicity studies have suggested that pathogenicity of *Aeromonas* spp. is complex and may involve a wide range of virulence genes acting either singly or in combination (Sha et al., 2009). The *in vivo* pathogenicity findings are provided in Table 4. High mortalities were found in challenged rohu (100%) compared to catla (90–95%). No mortality was observed in Group 1 (sham control) and Group 2 (control). Additionally, strains of *A. veronii* harboring the *aer*$^+$ *alt*$^+$ *enol*$^+$ *fla*$^+$ *lip*$^+$ *ahy*$^+$ *ahp*$^+$ *hly*$^+$ and *aer*$^+$ *alt*$^+$ *enol*$^+$ *ahp*$^+$ gene profile exhibited equal mortality rates. The present study findings suggest that the pathogenicity of *A. veronii* is not solely dependent on the number of virulence genes present but rather on the specific types of genes involved. For example, in the present study, the strain AVR5, which harboured *aer*$^+$, *alt*$^+$, *enol*$^+$, *fla*$^+$, *lip*+, *ahy*$^+$, *ahp*$^+$, and *hly*$^+$ genes caused high mortality rates of 95-100% in infected fish. Similarly, strains AVR23 and AVR60, carrying only four virulence genes, still induced 90-100% mortality, emphasizing that combination of virulence genes plays crucial role in

Table 2. Biochemical reactions of *A. veronii* isolated from freshwater fishes

Biochemical test	Results	Biochemical test	Results
Gram Staining	Gram –ve	Lysine decorboxylase	+
Motility	Motile	Ornithine	+
Oxidase	+	Arginine	-
Citrate	+	Maltose fermentation	+
Indole	+	Sorbitol	-
H_2S production on triple sugar iron agar	-	Mannose	+
Esculin	+	Glucose	+
Methyl Red	+	Sucrose	+
Voges-Proskauer	+	Lactose	-
Malonate	-	Fructose	+
Starch	-	Raffinose	-
Salicin	+	Gas production	+

Table 3. Virulence gene distribution and phenotypic characterization of virulence genes in *A. veronii*

A. veronii	Virulence gene distribution pattern in *A. veronii*								
	ahy	*ahp*	*enol*	*lip*	*aer*	*alt*	*fla*	*hly*	*exu*
Total strains =95	85 (89%)	$82^{**/***}$ (86%)	80 (84%)	74^{+} (77%)	$73^{**/***}$ (76%)	64^{***} (67%)	60 (63%)	50^{**} (52%)	0%
	Phenotypic determination of virulence genes								
Total strains =95			Hemolytic **				69 (72%)		
Total strains =95			Proteolytic ***				80 (84%)		
Total strains =95			Lipolytic^{+}				72 (75%)		

Note: *ahy*: elastase; *ahp*: serine protease; *enol*: enolase; *lip*: lipase; *aer*: aerolysin; *alt*: enterotoxin; *fla*: flagellin; *hly*: hemolysin; *exu*: DNase.
Correlation is significant at the 0.05 level (2-tailed) (Samples with hemolytic activity harbored *aer, hly, ahp genes*), * Correlation is significant at the 0.05 level (2-tailed) (samples with proteolytic activity harbored *aer, alt, ahp*), $^{+}$Correlation is significant at the 0.05 level (2-tailed) (samples with lipolytic harbored lipase gene).

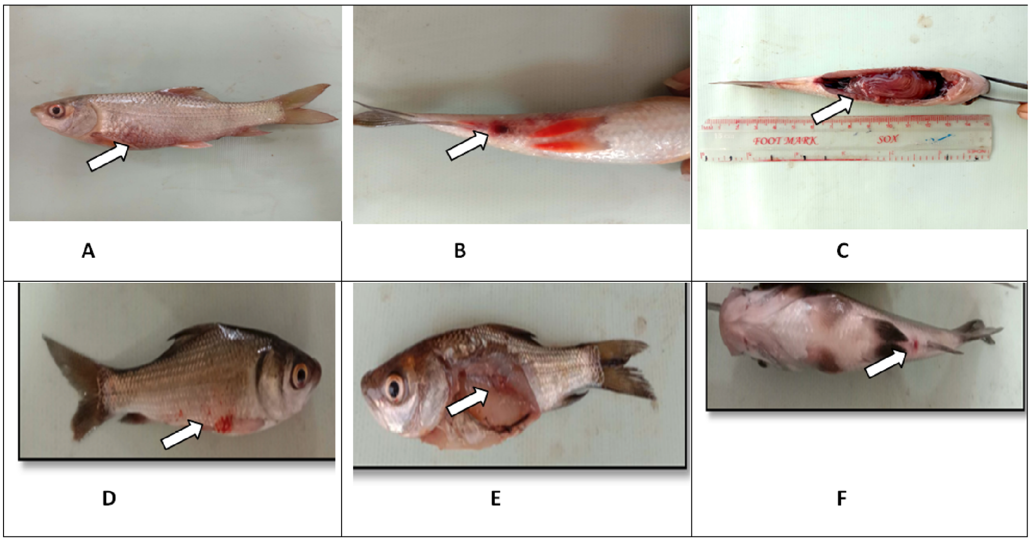

Fig. 5. Gross pathological symptoms of challenged fishes. A. Rohu: Hemorrhages on ventral body surface (White arrows); B. Rohu: Hemorrhagic vent (White arrows); C. Rohu: Fluid accumulation in the abodomen parts (White arrow); D. Catla: Hemorrhages on ventral body surface (White arrows); E. Catla: Pale kidney (White arrows); F. Hemorrhagic vent (White arrow).

pathogenicity. Previous studies have highlighted the importance of certain virulence genes such as aerolysin, enterotoxin, hemolysin, lipase, and serine protease in *Aeromonas* pathogenicity (Nawaz et al., 2010; Li et al., 2011; Das, Kumar, Das, & Nayak, 2023). The present findings align with these reports, indicating that the presence of these genes, particularly aer^{+}, alt^{+}, $enol^{+}$, and ahp^{+} or ahy^{+}, significantly contributes to the pathogenic potential of *A. veronii*. Moreover, challenged fishes exhibited clinical symptoms similar to naturally infected fish, including hemorrhagic septicemia manifestations such as hemorrhages on the body surface, vent, eye, mouth, tail rot, dropsy and paleness of gills and internal organs. The pathological symptoms in challenged rohu and catla are depicted in Fig 5.

The present study concludes that *A. veronii* poses significant pathogenic risks in freshwater fish culture, primarily driven by key virulence factors such as aerolysin, hemolysin, protease, and lipase. Notably, the pathogenicity depends more on the specific virulence genes present rather than their number. Strains with a combination of aer^{+}, alt^{+},

Table 4. *In vivo* pathogenicity test of *A. veronii* strains challenged in rohu and catla

Group	Strain No.	Genotype	No. of animals stocked	LD_{50}	Mortality (Rohu) (%)								Mortality (Catla)								Re-isolated strain virulence genotype
					12 h	24 h	36 h	48 h	60 h	72 h	3-7 days	Mort- ality rate (%)	12 h	24 h	36 h	48 h	60 h	72 h	3-7 days	Mort- ality rate (%)	
	Mortality rate (%)																				
Group 1	Sham control	-	20	-	0	0	0	0	0	0	0	0^a	0	0	0	0	0	0	0	0^a	-
Group 2	Control	-	20	-	0	0	0	0	0	0	0	0^a	0	0	0	0	0	0	0	0^a	-
Group 3	AVR5	aer^+ alt^+ $enol^+$ fla^+ lip^+ ahy^+ ahp^+ hly^+	20	$2.6x10^5$	0	0	3 (15%)	4 (20%)	4 (20%)	6 (30%)	3 (15%)	100^b	0	0	3 (15%)	3 (15%)	4 (20%)	4 (20%)	5 (25%)	95^b	Similar to original strains
Group 4	AVC2	aer^+ alt^+ $enol^+$ fla^+ lip^+ ahy^+ ahp^+ hly^-	20	$3.2x10^5$	0	0	2 (10%)	3 (15%)	4 (20%)	4 (20%)	7 (35%)	100^b	0	0	1 (5%)	3 (15%)	3 (15%)	6 (30%)	6 (30%)	95^b	Similar to original strains
Group 5	AVC32	alt^+ $enol^+$ lip^+ ahy^+ ahp^+ hly^+ fla^- aer^-	20	$2.8x10^5$	0	0	3 (15%)	4 (20%)	4 (20%)	5 (25%)	4 (20%)	100^b	0	0	2 (10%)	3 (15%)	3 (15%)	5 (25%)	5 (25%)	90^b	Similar to original strains
Group 6	AVR20	aer^+ alt^+ $enol^+$ ahy^+ ahp^- $hly^-$$fla^-$ lip^-	20	$4.2x10^5$	0	0	2 (10%)	3 (15%)	5 (25%)	7 (35%)	3 (15%)	100^b	0	0	2 (10%)	4 (20%)	5 (25%)	6 (30%)	1(5%)	90^b	Similar to original strains
Group 7	AVR23	aer^+ alt^+ $enol^+$ ahp^+ fla^- lip^- ahy^- hly^-	20	$3.5x10^5$	0	0	1 (5%)	2 (10%)	2 (10%)	6 (30%)	9 (45%)	100^b	0	0	1 (5%)	3 (15%)	3 (15%)	7 (35%)	4 (20%)	90^b	Similar to original strains

Note: The mean with different superscript letter in each column represents significant difference at $P < 0.05$ level.

enol$^+$, and *ahp*$^+$ genes exhibited high mortality rates in infected fish. These findings underscore the complex interplay of virulence factors in *A. veronii*, emphasizing the importance of vigilant monitoring and targeted management strategies in freshwater aquaculture to mitigate the risks from disease outbreak, safeguard fish health, and address potential zoonotic threats. Further research is essential to elucidate the mechanisms underlying virulence and develop effective control measures.

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