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Detection of Virulence Genes, Antimicrobial Susceptibility and Pathogenicity of *Aeromonas veronii* Isolates from *Labeo rohita* and *Catla catla*

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ABSTRACT

Background: *Aeromonas veronii* is the most common bacterium responsible for diseases in freshwater fish rearing systems. Multiple factors can be involved in the virulence processes of *Aeromonas* bacteria. Hence, the purpose of the present investigation was to evaluate the virulence genes, antimicrobial susceptibility and pathogenicity of *A. veronii* isolated from freshwater fishes.

Methods: In this investigation, we isolated *A. veronii* from cultured freshwater fishes, *Labeo rohita* and *Catla catla*. *A. veronii* was identified by bacterial staining and culture characteristics. In addition, Polymerase chain reaction (PCR) was used to evaluate the distribution of nine virulence genes including aerolysin, cytotonic enterotoxin (442, 272 bp), elastase, enolase, flagellin, lipase, serine protease and DNase.

Result: A total of 88 *A. veronii* strains were isolated, which includes 56 strains from rohu and 33 strains from catla. The strains were Gram-negative, rod-shaped, motile bacteria and the colonies are yellow on blood agar. All the *A. veronii* strains were positive for at least one or more virulence genes tested. The isolates carried more virulence genes, especially in the combination of aer, alt, ela, lip, AhyB genes were found to be more virulent. Antimicrobial susceptibility to 17 antibiotics was determined and the strains of *A. veronii* showed 100% resistance to tetracycline, pencillin and β-lactum group of antibiotics. In addition, multiple antibiotic resistance (MAR) indexes ranged from 0.24 to 0.76, suggesting that they originated from high risk contaminated zones were (W. Godavari and Nellore districts) antimicrobials are often used.

Key words: *Aeromonas veronii,* Antimicrobial susceptibility test, *Catla catla*, *Labeo rohita,* Pathogenicity studies, Virulence genes.

INTRODUCTION

Aeromonas veronii is one of the significant pathogenic bacteria among the Aeromonads, causes a number of infections in fish (Silver *et al*., 2011). *A. veronii* has been associated with ulcerative syndrome, fin rot and hemorrhagic septicemia in freshwater fish (Mallik *et al*., 2020). In recent years, there have been an increasing number of cases of large-scale *A. veronii* outbreaks, resulting in serious losses to the aquaculture industry and threatening food safety (Zhang *et al*., 2019). This bacterium has a strong ability to adapt to the external environment and produce a wide range of virulence factors leading to various diseases (Song *et al.,* 2018). *A. veronii* is cytotoxic and haemolytic, with virulence genes coding for enterotoxins (act and alt), haemolytic toxins (aerA and hlyA), type III secretion systems, cholesterol aceyltransferase and a type IV pilus (Sreedharan *et al*., 2012).

In similar study, hemolytic and cytotoxic capabilities, as well as pathogenicity were found to be correlated with the presence of virulence genes in *Aeromonas* strains (Zheng *et al*., 2019). The Aquaculture development has been accompanied by an increase in the occurrence of bacterial infections and antibiotic overuse has resulted in antibiotic resistance (Li *et al.,* 2020). The antibiotic residues in aquatic animals and their products pose a risk to human health (Castro-Escarpulli *et al*., 2003). Therefore, the goal of the current study was to verify the existence of most common

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Aeromonas species of *A. veronii* strains in freshwater fish culture farms, the presence of virulence genes, antimicrobial susceptibility investigations and evaluate the potential pathogenicity of these isolates in fishes.

MATERIALS AND METHODS

Sample collection and Identification of *Aeromonas* species

A total of 50 diseased fish samples (25 *Labeo rohita*; 25 *Catla catla*) were collected from cultured farms located in different areas of Andhra Pradesh, India from 2021 to 2022. The bacteriological work was carried at Fisheries College, Muthukur, Nellore district. A total of 88 *Aeromonas veronii viz*., 56 strains were isolated from rohu, 33 from catla fish. All the strains were isolated from the kidney, gills and liver of diseased fishes based on the severity of infection. The samples of fish were pre-enriched in alkaline peptone water broth for 8 h at 37°C and loop resulting sample were sub-cultured on a blood agar plate (Hi-media, India) with 20% ampicillin and then incubated at 16-24 h at 37°C (Zhou *et al*., 2019). The selected colonies were confirmed by biochemical characterization with gram staining, oxidase, catalase, Indole, methyl red test, urease test, haemolysin production, VP test, O/129 sensitivity reduction of nitrate to nitrite, H_2S production, lysine decarboxilase and arginine dihydrolase reactions. The species identification of *Aeromonas* was followed by (Hickman-Brenner *et al*., 1987; Martin-Carnahan and Joseph, 2005). The confirmation of isolated *A. veronii* was performed using an AP1 20NE Kit (Biomerieux).

Molecular identification of virulence genes

The presence of 9 virulence genes in isolated *A. veronii* was determined by the use of PCR. The chromosomal DNA from *A. veronii* was extracted using the genomic DNA purification kit (Bangalore Genei, Bangalore) following the manufacturer's protocol. Isolates of *A. veronii* strains were subjected to identify the nine virulence genes *viz*., aerolysin (*Aer*), cytotoxic enterotoxin (*alt*), DNase (*exu*), elastase (*ahyâ*), enolase (*enol*), lipase (*lip*), flagellin (*fla*) and serine protease (*ahp*) using reported primers (Table 1). The partial virulence genes were amplified using the universal primers in a 25 µL reaction volume of 100 ng of template DNA, ten picomoles of forward and reverse primer, 250 µM dNTPs, 1U of Taq polymerase, 1X Taq buffer with 3 mM $MgCl_2$. The amplification procedure consisted of an initial denaturation step at 94°C for 3 min, followed by 30 cycles with denaturation at 94°C for 30 s, at annealing at 55.5°C for 30 s for the aerolysin gene, serine protease, flagellin or 60°C for 30 s for the cytotonic eneterotoxin gene, elastase, enolase, Lipase, DNase genes or 62°C for 30 s for haemolysin and extension at 72°C for 30 s for aer, alt, lip, AhyB, Ahp or 1 min for Fla, DNase, Enol, HlyA. A final extension step was carried out at 72°C for 10 min. Aliquots from amplification reactions were analyzed by 1-5% agarose gel electrophoresis and viewed under UV light.

Antibiotic susceptibility test

The antibiotic susceptibility tests were performed against 17 potential antibiotics by use of the Mullen Hinton Agar (MHA) by disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2018), as shown in Table 2. All the discs were purchased from Himedia, Indian scientific, India. The procedure for conducting susceptibility test was described in the previous studies by Lavanya *et al.* (2021). The diameter of the zone of inhibition (Table 1) was measured according to the antibiotic resistance interpretation chart CLSI (2018). Multiple antibiotic resistance (MAR) of *A. veronii* and MAR index were calculated as per Orozova *et al*. (2010).

$$\text{MAR index} = \frac{\text{Number of antibiotics to which the bacterium is resistant}}{\text{The total number of antibiotics tested}}$$

Ethics statement

Animals are used in accordance with Indian legislation. The treatment and care of animals used in this study were in accordance with the guidelines for the care and use of animals in scientific research issued by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA, Ministry of Environment and Forests (Animal Welfare Division), Government of India). The ethics committee of Sri Venkateswara Veterinary University (SVVU, 2019), Tirupati Andhra Pradesh, India, authorized the study.

Pathogenicity experiments

The pathogenicity test was performed in both healthy rohu (300 numbers) and catla (300 numbers) (mean weight of 25±4 g) fishes with *Aeromonas veronii* isolate having 8-1 nos of virulence genes. All the fishes were transported to wet lab and were treated with 2 ppm potassium permanganate for 10 min (Barkoh *et al*., 2009), were further acclimatized for 15 days. To fulfill the Koch's postulates, randomly selected healthy fishes were tested for *Aeromonas* bacteria. The challenge test was carried out in HDPE tanks of 60 cm × 40 cm × 30 cm (L × W × H) size. The fish were stocked at 10 numbers for each tank. The bacterial cell suspension preparation was performed as described by Mallik *et al*. (2020) with some modifications. Briefly, the *A. veronii* isolates were streaked on TSA plates and incubated for 18-24 h at 28°C. A single representative colony was picked and inoculated into two 150 ml Erlenmeyer flasks containing 20 ml Tryptone Soya Broth TSB (HI-Media, India) each. The flasks were incubated in a rotary shaker with 90 rpm for 16-20 h at 28°C. the bacterial log phase was measured at 600 nm followed by centrifugation at 10,000 rpm for 10 min and the pellet was washed with phosphate buffer saline. At last, the bacteria pellet was re-suspended in 0.85% PBS solution. The pathogenicity study was divided into three categories *viz*., first category for *A. veronii* injection, second category for sterile normal saline injection as sham control and third category as control with no injection.

The each rohu and catla fishes were injected with 0.2 ml of 10^8 CFU/ml *A. veronii* bacterial cell suspension. The second group of fishes were injected with 0.2 ml of PSB solution. Prior to the bacterial challenge, all the fishes were anesthetized using tricaine methanesulfonate (MS-222, Sigma, 150 mg/L) (Das *et al*., 2019). After the challenge, all the experimental category fishes were observed for 96 h at 12 h intervals to study the clinical signs and mortality rates along with sham control and control groups. All the challenged group fishes were observed for mortality for 96 h (Sung *et al*., 2000).

Statistical analysis

IBM SPSS (Statistical Package for Social Sciences) software version 19.0 by one-way ANOVA was used for statistical analysis.

RESULTS AND DISCUSSION

The clinical signs of diseased rohu and catla fish showed haemorrhages on body surface, fin bases, anus, around mouth, hemorrhagic eye, dropsy, tail rot and fin rot and fluid accumulation in intestinal parts. The biochemical test results showed that *A. veronii* was Gram-negative, slightly yellowish colony (0.5-3.0 mm diameter), rod shaped, β haemolytic of 5% sheep blood agar, motile. Further it was positive for catlase, citrate, indole, VP, MR, esculin, O/129 sensitive and other decorboxilase test. A total of 88 strains of *A. veronii* were isolated from diseased rohu and catla. Among 88 strains 55 isolates were isolated from diseased rohu and 33 isolates were from diseased catla. Significantly ($p<0.05$) higher prevalence of *A. veronii* was isolated from rohu when compared to catla.

A. veronii is a Gram-negative, rod-shaped bacterium that is facultatively anaerobic (Li *et al*., 2020). The biochemical evaluvation of *A. veronii* revealed β-haemolysin activity (Skwor *et al*., 2014), resistant to O/129, gas production positive from D- glucose and it has grown without addition of NaCl (Hickman-Brenner *et al*., 1987). Other biochemical parameters also showed positive results for lysine, ornithin decorboxylase and negative for arginine dihydrolase (Hickman-Brenner *et al*., 1987; Mallik *et al*., 2020). In the current investigation, *A. veronii* isolates also exhibited positive findings for the aforementioned reactions. Simultaneously, according to the prevalence of *A. veronii* isolates more strains were isolated from rohu (62.5%) fish when compared with catla (37.5%), it can be seen that rohu is the most edible fish than catla.

In this study, virulence genes were identified in *A. veronii* strains obtained from rohu and catla fishes of Andhra Pradesh, India. Because of their rapid findings, PCR testing have been employed to determine the distribution of virulence genes (Yu *et al*., 2015). Because of the complexities of *Aeromonas* pathogenesis, no one suspected virulence associated factor can be identified as being responsible for specific symptom or disorder (Albert *et al.* 2000). All 88 *A. veronii* strains were tested for PCR amplification of nine virulence genes. The frequency of virulence genes distribution in *A.veronii* isolate was shown in Table 3. All the strains were positive for at least one or more virulence genes tested. The eight virulence genes were present in 9/88 (10.2%) of isolates. Seven virulence genes are present in 6/88 (6.8%); six genes in 10/88 (11.3%); five genes in 34/88 (38%.6%); four genes in 14/88 (15.9%); three genes in 7/88 (7.9%); two gene in 5/88 (5.6%); one gene in

Table 1: Primers used in PCR for virulence genes expression of *Aeromonas veronii.*

Virulence gene	Gene code	Primer sequence (5′-3′)	Product size (bp)	Reference
Aerolysin	Aer431F	F: CCTATGGCCTGAGCGAGAAG	431	Mansour *et al.* (2019)
	Aer431R	R: CCAGTTCCAGTCCCACCACT		
Cytotonic	Alt442F	F: TGACCCAGTCCTGGCACGGC	442	Nawaz *et al.* (2010)
enterotoxin	Alt442R	R: GGTGATCGATCACCACCAGC		
	Alt272F	F: AGGATGCCCTCAACACCATC	272	U-taynapun *et al.* (2020)
	Alt272R	R: GCTCTGTTTCAGGTTGTCGC		
Haemolysin	HlyA597F	F: GGCCGGTGGCCCGAAGATACGGG	597	Sreedharan *et al.* (2012)
	HlyA597R	R: GGCGGCGCCGGACGAGACGGGG		
Lipase	Lip594F	F: GACTCCCTCAAGGACAGCAG	594	U-taynapun *et al.* (2020)
	Lip594R	R: AGAGGCTTTCAGGGCATTG		
Elastase	AhyB540F	F: ACACGGTCAAGGAGATCAAC	540	Mansour *et al.* (2019)
	AhyB540R	R: CGCTGGTGTTGGCCAGCAGG		
Serine protease	Ahp1011F	F: ATTGGATCCCTGCCTATCGCTTCAGTTCA	911	Zheng *et al.* (2012)
	Ahp1011R	R: GCTAAGCTTGCATCCGTGCCGTATTCC		
Enolase	Enol598F	F: CGCCGACAACAACGTCGACATC	598	Kumar *et al.* (2020)
	Enol598R	R: CTTGATGGCAGCCAGAGTTTCG		
Flagellin	Fla608F	F: TCCAACCGTYTGACCTC	608	Nawaz *et al.* (2010)
	Fla608R	R: GMYTGGTTGCGRATGGT		
DNase	Exu323F	F: AGGACATGCACAACCTCTTCC	323	Nawaz *et al.* (2010)
	Exu323R	R: GATTGGTATTGCCCTTGCAACG		

3/88 (3.4%). Surprisingly DNase gene was absent in all the tested *A. veronii* stains. Significantly more prevalence of each virulence gene present in *Aeromonas veronii* strains isolated from rohu when compared with catla. In addition, among the isolated strains, 85.22% (75/88) carried Lip gene; 81% (72/88) carried AhyB gene; 75% (66/88) carried alt gene; 65.9% (58/88) carried each of aer and Ahp genes; 59% (52/88) carried Fla gene; 47% (42/88) carried Enol gene; and 10% (9/88) carried HlyA genes.

According to some reports, the key virulence factors in *Aeromonas* spp. include aer, alt, act, ahp, lip, ela (Hu *et al*., 2012; Oliveira *et al*., 2012). In accordance with previous findings (Albert *et al*., 2000; Sechi *et al*., 2002; Nawaz *et al*., 2010; Hu *et al*., 2012; Mallik *et al*., 2020), we discovered significant variation in the distribution of virulence genes among the *A. veronii* strains. All the *A. veronii* isolates from rohu and catla fish had one or more virulence genes in various combinations. This present study found that the carrying rate of lip gene (85.22%) in *A. veronii* was higher than other virulence genes. According to some studies, lipase is found in all *Aeromonas* spp., which correlates with lipase activity in fish (Anguita *et al*., 1993; Merino *et al*., 1999; Chacon *et al*., 2003). Castro-Escarpulli *et al*. (2003) recovered 75% of the lipase gene in *A. veronii bv. sobria* from frozen fish. Tyagi *et al*. (2022) and Youssef *et al*. (2022) discovered a lipase gene in *A. veronii* isolated from *L. rohita* and tilapia. Lipases are essential for bacterial feeding (Pemberton *et al*., 1997), although some studies have found that *A. hydrophila* with mutant lipase gene lowers lethality, which shows that the role of lipase as virulence factor (Merino *et al*., 1999). Furtermore, Aeromonads express four different types of extracellular lipases (lip, lipH3, pla and plc) that actively contribute in the modification of the host plasma membrane and thus increasing the severity of infection (Pemberton *et al*., 1997). The second most common virulence gene (87%) was elastage, however its involvement in disease has not been well investigated. Further, elastase is a zinc metalloprotease, is an important virulence component in the organism's pathogenesis (Cascon *et al.,* 2000). Elasage has been identified in many *Aeromonas* spp.

Table 2: Antibiotics: Antibiotic resistance interpretation chart CLSI (2018).

Antibiotics with code	Antibiotic class	Disc content	Diameter of zone of inhibition (mm)		
		(µg/disc)	R (<)	IM	S (>)
Amikacin (AK)	Aminoglycosides	30	14	15-16	17
Amoxyclav (AMX)	B-lactams	30	13	14-17	18
Ampicillin (AMP)	Pencillin	10	13	14-16	17
Chloramphenicol (C)	Phenolic	30	12	13-17	18
Ciprofloxacin (CIP)	Quinolones	5	15	16-20	21
Co-trimoxazole (CoT)	Sulfones	25	10	11-15	16
Doxycycline hydrochloride (DO)	Diaminopyramidines	30	10	11-13	14
Enrofloxacin (EX)	Fluroquinolones	10	15	16-20	21
Erythromycin (E)	Macrolids	15	13	14-22	23
Gentamicin (GEN)-	Aminoglycosides	10	12	13-14	15
Nalidixic acid (NA)	Quinolones	30	13	14-18	19
Neomycin (N)	Aminoglycosides	30	12	13-16	17
Nitrofurantoin (NIT)	Nitrofuran	300	14	15-16	17
Novobiocin (NV)	Fluoroquinolones	30	12	13-16	17
Oxacillin (O)	Pencillin	5	17	-	18
Oxytetracycline (OX)	Tetracycline	30	11	12-14	15
Trimethoprim (TR)	Diaminopyramidines	5	10	11-15	16

Table 3: Distribution of virulence genes in *Aeromonas veronii* isolated from Rohu and catla of Andhra Pradesh.

Positive virulence genes in *Aeromonas veronii*	Total stains= 88		Percentage (%)
	Rohu (n= 55)	Catla (n= 33)	
Aer,Alt, AhyB, Enol, HlyA, Ahp, Lip, Fla	6	3	10.2
Aer,Alt, AhyB, Enol, Ahp, Lip, Fla	4	2	6.8
Aer,Alt, AhyB, Ahp, Lip, Fla	5	5	11.3
Aer,Alt, AhyB, Ahp, Lip	22	11	38.6
AhyB, Enol, Fla, Lip	8	6	15.9
Alt, Enol, Fla	5	3	7.9
Enol, Fla	3	2	5.6
Lip	2	1	3.4

(U-taynapun *et al*., 2020; El-Gohary *et al*., 2020; Shuang *et al.* (2020). The cytotoxic enterotoxins and aerolysins play critical roles in infection establishment, which trigger fluid accumulation in animals (Nawaz *et al*., 2010). In the current study, *A. veronii* had 75% of alt genes and 65% aer genes and ahp genes, respectively. Aerolysin and other extracellular enzymes are activated by the serine protease, thus impacting the overall pathogenicity aeromonads (Cahill, 1990). Furthermore, fla was found in 59% of *A. veronii* isolate and this gene important for motility, colonization and aeromonads with flagella are related with dysenteric infections (Kirov *et al*., 2002). In the current investigation, 47% *A. veronii* strains have enolase genes, with surface expression varying depending on cellular conditions and the capasity of enolase to operate as a plasminogen receptor (Fontan *et al*., 2000). The Hly A gene was determined to be the least prevalent in the current research isolated (10%). The genes encoding Aer, Alt, AhyB, Ahp and Lip gene were found in high abundance in the *A. veronii* strains. The DNase gene is responsible for DNA hydrolysis which was not found in this study. The absence of DNase gene in *A. veronii* was reported by (Nawaz *et al*., 2010, Wimalasena and Heo, 2021). In contrast, greater frequency of DNase was found in *Aeromonas* from Malaysia (Khor *et al*., 2015) and South Korea (Yi *et al*., 2013). This might be due to the uniqueness of the host and sample source.

The 88 isolates of *Aeromonas veronii* were tested for antibiotic susceptibility against 17 antibiotics. All the stains showed 100% resistance to pencillin group; ampicillin, β-lactum group; amoxyclav and tetracycline group; oxytetracyclin. Followed by, 77.27% resistance to erythromycin; 65.9% resistance to chloramphenicol; 54.54% resistance to novobiocin, doxycyclin hydrochloride; 51.13% resistance to nitrofuratoin, oxacillin, amikacin; 46.59% resistance to ciprofloxacin; 45.45% resistance to neomycin; 43.18% resistance to trimethoprim; 39.77% resistance to co-trimazole; 31.81% resistance to nalidixic acid; 28.4% resistance to enrofloxacin and 22.72% resistance to gentamycin. Statistical analysis by Pearson chi-square displayed that antibiotic resistance to ampicillin, amoxyclav and oxytetracycline was significantly high (p<0.05) in comparison to the other 14 antibiotics tested. Further, the strains showed significantly more susceptibility to gentamycin, enrofloxacin and nalidixic acid. The multiple antibiotic resistance patterns of 88 strains of *A. veronii* showed MAR index ranged between 0.29 to 0.76. All the strains were found to be multiple antibiotic resistant.

The antibiotics oxytetracycline, tetracycline and ampicillin are widely used by Indian fish farmers to manage bacterial infections in fish farms due to their broad-spectrum action (Shahi *et al.,* 2013). However, because of the *A. veronii* strains in this study are resistance to the majority of antibiotics in the pencillin group; ampicillin (10 µg), β-lactum group; Amoxiclave (30 µg), tetracycline group; oxytetracyclin (30 µg), there is a possibility that using these antibiotics may result in the development of antimicrobial resistance. The present study agreed with (Yucel and Beyatii, 2004; Guz and Kozinska, 2004 and Hassan *et al*., 2017). Furthermore, the current investigation discovered that *A. veronii* is susceptible to antibiotics of aminoglycosides group; gentamycin (10 µg), fluroquinolones group; endrofloxacin (10 µg) and quinolones group; nalidixic acid (30 µg), there is a possibility that these antibiotics could control the *A. veronii* infections in fish farms. Hassan *et al.* (2017) also found *A. veronii* to be sensitive to nalidixic acid, however Miao *et al*. (2023) discovered resistance to gentamycin and enrofloxacin. Antimicrobial resistance has become a serious issue in aquaculture due to abuse of antibiotics not only as disease control agents but also as growth boosters. This practice may lead to development of Multiple antibiotic resistance (MAR) in *Aeromonas*. MAR value greater than 0.2 suggests the presence of high-risk sources of antibiotics contamination and indiscriminate use (Krumperman, 1985). The current research strains had MAR index value ranging from 0.29 to 0.76, which is completely in accordance with that statement. In addition Shameena *et al.* (2019) also reported more than 0.4 MAR value and Dhanapala *et al.* (2021) recorded 0.54 MAR for *A. veronii*.

The experimentally challenged fishes showed symptoms like erratic swimming behavior, lethargy and hemorrhagic vent post 24-48 h of challenge. Further, hemorrhages on body, eye, swollen and reddish anal region, pale kidney, tail rot and accumulation of bloody fluid in visceral cavity were also observed (Plate 1 and 2). The mortality rates of challenged rohu with *A.veronii* stains were shown in Table 4. The *A. veronii* having 5-8 virulence genes recorded 93.33-100% mortality within 96 h. However, there was no significant difference in the mortality rate of rohu challenged with *A. veronii* having 8 to 5 virulence genes. Significantly lower (p>0.05) mortality rates 43.33-56.67% were recorded in *A. veronii* strains having 1-4 genes. Furthermore, we found that the *A. veronii* strain with combination of aer, Alt, AhyB, Ahp and Lip virulence gene are in highly virulent nature when compared with other gene combinations and found more than 93.33% mortality rate within 96 h of post challenge.

The mortality rates of challenged catla with *A. veronii* stains were shown in Table 5. The mortality rates of challenged catla were lower when compared with challenged rohu fish. The *A. veronii* having 4-8 virulence genes recorded with 73.33-93.33% mortality. However, there was no significant difference in the mortality rate of catla challenged with *A. veronii* having 8 to 5 virulence genes. Significantly lower (p>0.05) mortality rates recorded at 23.33-43.33% in Catla fish challenged with *A. veronii* strains having 1-4 genes.

The presence of virulence factors, particularly those associated with extracellular products, is critical for bacterial pathogenicity (Jutfelt *et al*., 2008). Li *et al.* (2011) screened different *A. hydrophila* isolates for aerolysin (aerA), cytotoxic enterotoxin (alt) and serine protease (ahp) genes and discovered a significant correlation between the presence of these genes and bacterial pathogenicity. Furthermore,

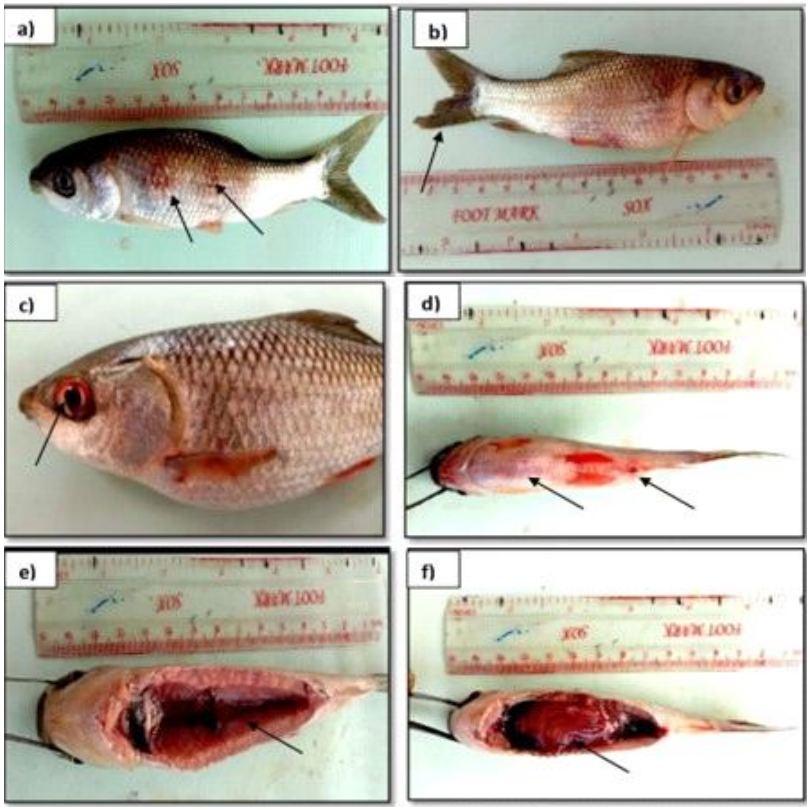
several research have found a link between the number of virulence genes and their pathogenicity potential (Albert *et al*., 2000; Sha *et al*., 2002; Chang *et al*., 2008). However, oliveira *et al*. (2012) found no statistically significant difference between the presence of virulence genes and mortality rate in tilapia challenged with *A. hydrophila*. Our

Table 4: Moratlity rate (mean±SD) of *Labeo rohita* being challenged with *A. veronii.*

Aeromonas species	Mortality rate of challenged fish at different time intervals					Mortality (%)
	Number of dead/Number of challenged fish (%)					
	0-12 h	12-24 h	24-48 h	48-72 h	72-96 h	
Lip	-	1.00^{a}±1.00	1.67^{a}±0.57	0.67^{a}±0.57	1.00^{a}±1.00	43.33^{a}±4.21
Enol, Fla	-	1.00^{a}±1.00	2.00^{a}±0.57	1.67^{b}±1.00	0.67^{a}±0.57	53.33^{a}±3.13
Alt, Enol, Fla	-	1.00^{a}±1.00	2.00^{a}±0.57	1.67^{b}±1.00	0.67^{a}±0.57	53.33^{a}±3.13
AhyB, Enol, Fla, Lip	-	1.00^{a}±1.00	2.00^{a}±0.57	2.00^{b}±1.00	0.67^{a}±0.57	56.67^{a}±4.13
Aer, Alt, AhyB, Ahp, Lip	-	2.33^{b}±0.57	2.67^{b}±0.57	2.67^{b}±1.00	1.67^{b}±0.57	93.33^{b}±5.77
Aer, Alt, AhyB, Ahp, Lip, Fla	-	3.67^{c}±0.57	3.33^{c}±0.57	2.00^{b}±0.00	0.67^{a}±1.15	96.67^{b}±5.09
Aer, Alt, AhyB, Enol, Ahp, Lip, Fla	-	5.00^{d}±1.00	3.33^{c}±0.57	1.67^{a}±1.15	-	100.0^{b}±0.00
Aer, Alt, AhyB, Enol, HlyA, Ahp, Lip, Fla	-	5.00^{d}±1.00	3.33^{c}±0.57	1.67^{a}±1.15	-	100.0^{b}±0.00
Sham control group	-	-	-	-	-	00.00%
Control group	-	-	-	-	-	00.00%

Note: Triplicates were maintained for each group with 10 fish/replication.

The mean with different superscript letter in each column represents significant difference. The multivariate ANOVA was done at P<0.05 for separation of means. The significant difference between treatment groups means were tested using Duncan's multiple range test (Duncan, 1995).



a) Ulcers and hemorrhages on body surface; b) Trail rot; c) Drpsy and hemorrhagic eye; d) Reddish vent and hemorrhages on ventral side; e) Pale kidney; f) Bloody fluid accumulation in intestine.

Plate 1: Clinical and pathological signs observed in experimentally challenged *L. rohita* with *A. veronii.*

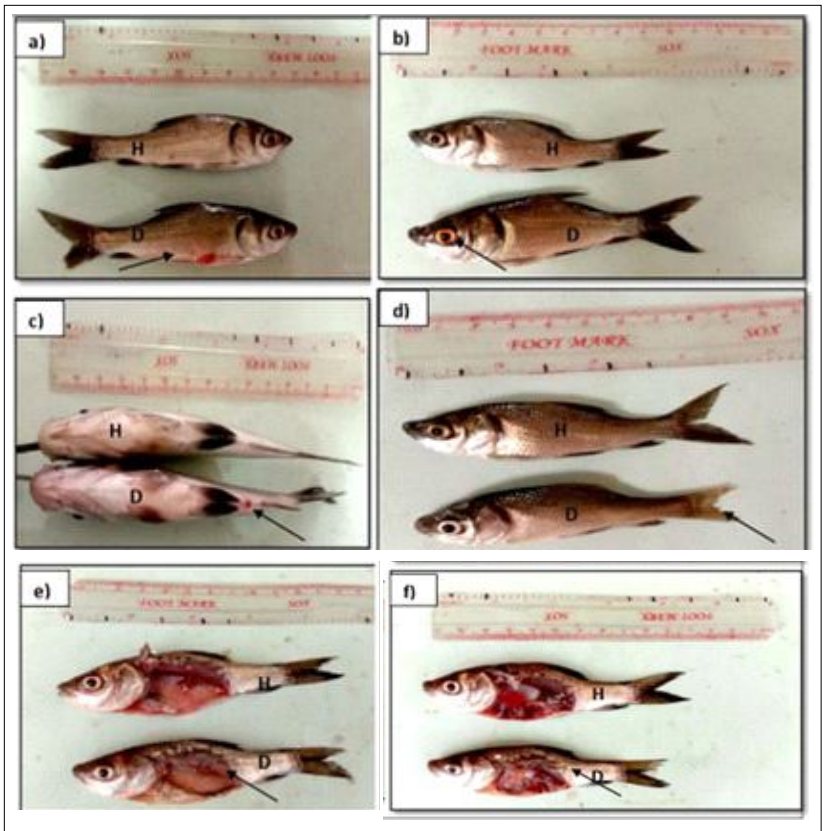
study, on the other hand used pathogenicity detection to determine the existence of virulence genes in *A. veronii* isolates indicated a strong correlation between the presence of number of virulence gene and mortality (%) in rohu and catla fish. The strains that had eight to seven virulence genes revealed the highest mortality (100%) in rohu and 93.33% mortality in catla fish, followed by strains with six to five virulence genes revealed 96-83% mortality in rohu and 83-73%

Table 5: Moratlity rate (mean±SD) of *Catla catla* being challenged with *A. veronii.*

Aeromonas species	Mortality rate of challenged fish at different time intervals					Mortality (%)
	Number of dead/Number of challenged fish (%)					
	0-12 h	12-24 h	24-48 h	48-72 h	72-96 h	
Lip	-	0.00^{a}±0.00	0.67^{a} ±0.57	0.67^{a}±0.57	1.00^{a}±1.00	23.33^{a}±2.21
Enol, Fla	-	0.00^{a}±0.00	1.67^{a} ±0.57	0.67^{a}±0.57	1.00^{a}±1.00	33.33^{a}±2.21
Alt, Enol, Fla		0.00^{a}±0.00	1.67^{a} ±0.57	0.67^{a}±0.57	1.00^{a}±1.00	33.33^{a}±2.21
AhyB, Enol, Fla, Lip	-	1.00^{a}±1.00	1.67^{a} ±0.57	0.67^{a}±0.57	1.00^{a}±1.00	43.33^{b}±4.21
Aer, Alt, AhyB, Ahp, Lip	-	1.33^{b}±0.57	2.67^{b} ±0.57	1.67^{b}±1.00	1.67^{b}±0.57	73.33^{c}±5.77
Aer, Alt, AhyB, Ahp, Lip, Fla	-	2.33^{b}±0.57	2.67^{b} ±0.57	1.67^{b}±1.00	1.67^{b}±0.57	83.33^{c}±5.77
Aer, Alt, AhyB, Enol, Ahp, Lip, Fla	-	2.33^{b}±0.57	2.67^{b} ±0.57	2.67^{b}±1.00	1.67^{b}±0.57	93.33^{c}±5.77
Aer, Alt, AhyB, Enol, HlyA, Ahp, Lip, Fla	-	2.33^{b}±0.57	2.67^{b}±0.57	2.67^{b}±1.00	1.67^{b}±0.57	93.33^{c}±5.77
Sham control group	-	-	-	-	-	00.00%
Control group	-	-	-	-	-	00.00%

Note: Triplicates were maintained for each group with 10 fish/replication.

The mean with different superscript letter in each column represents significant difference. The multivariate ANOVA was done at P<0.05 for separation of means. The significant difference between treatment groups means were tested using Duncan's multiple range test (Duncan, 1995).



a) Hemorrhages on body surface; b) Hemorrhagic eye; c) Hemorrhagic vent; d) Trail rot; e) Pale kidney; f) Pale liver.

Plate 2: Clinical and pathological signs observed in experimentally challenged *Catla catla* with *A. veronii.*

mortality in catla. The stains with four to one virulence genes showed 56.67-43.33% mortality in rohu and 43-23% mortality in catla. It was also shown that strains having AhyB, enol, fla and lip genes had lower mortality when compared to other strains with different virulent genes. Furthermore, an integrated involvement of wide variety of virulence genes also plays an important role in the establishment of infection as noticed in the case of *A. veronii* strains with 8 to 7 genes causing 100% mortality in rohu and 93% mortality in catla. This study is agreed with Li *et al.* (2020), the study confirms that the number of virulence genes carried by *A. veronii* (aer+ ser+ act+ Aha+ exu+ lip+) positively correlated with the pathogenicity of *A. veronii*. similarly, Pattanayak *et al.* (2020) also revealed that *A. hydrophila* with aer, hly, Alt, outer membrane protein TS (Omp TS), ahp, fla, lip and type 3 secretion system showed 100% mortality in challenged rohu.

CONCLUSION

A. veronii isolates of this present study were found to be virulent based on biochemical and *in-vitro* challenge experiment studies. All virulence factors except DNase were expressed phenotypically in the *A. veronii* strains, which also possessed heterogenic virulence gene distribution. Our findings reveal that more significance of *A. veronii* infections in rohu fish when compared to catla. The *A. veronii* strains studied here had more virulence genes especially Aer, Alt, AhyB, Ahp, Lip combination genes that are more pathogenic nature to fish. The current isolates tested positive for antibiotic resistance to tetracycline, β-lactum group and pencillin groups of antibiotics. Aminoglycosides and fluoroquinolones, on other hand, continue to be effective antibiotic groups for treating diseases in fish. Furthermore, multiple antibiotic resistance is a key issue that highlights the importance of monitoring antibiotic usage in fish culture.

Conflict of interest: None.

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