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Characterisation of pathogenic *Aeromonas veronii* and *Aeromonas media* isolates from aquaculture system: Virulence, antibiotic susceptibility and host mortality assessment

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Abstract

This study presents the identification, virulence characterisation and antibiotic susceptibility of *Aeromonas veronii* COFCAU_AV1 and *Aeromonas media* COFCAU_AM, isolated from diseased *Clarius batrachus* and aquaculture pond water. Strain identification employed physiological, biochemical and 16S rRNA gene sequence analyses. Both strains demonstrated hemolytic and slime-forming activities and produced DNase, amylase, lipase, gelatinase and caseinase. The LD_{50} doses in *Labeo rohita* fingerlings were $10^{6.8}$ and 10^{7} cells $fish^{-1}$ for *A. veronii* and *A. media*, respectively. *A. veronii* harboured virulence genes *aerA, act, alt, ast, exsA, ascC* and *lip*, while *A. media* had *aerA* and *lip*. *A. veronii* exhibited high resistance to penicillin, erythromycin, vancomycin and nalidixic acid, but high sensitivity to streptomycin, kanamycin and chloramphenicol. *A. media* showed strong resistance to penicillin and ampicillin but was highly sensitive to chloramphenicol, nalidixic acid and ciprofloxacin. Both strains demonstrated potential to cause mortality and morbidity in *L. rohita*, with *A. veronii* exhibiting comparatively higher virulence. This study also reports the pathogenicity of *A. media* strain in Indian major carps for the first time.

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Introduction

Aquaculture is a rising food production sector that is afflicted with disease-related issues, arising from its commercialisation and intensification. Diseases have become a primary constraint that is causing hindrance to the advancement and long-term viability of aquaculture operations globally (Smith, 2006). Significant production losses are increasingly being documented owing to a number of pathogenic bacteria, which are accountable for significant mortalities in a number of fishes at various stages of their growth and development (Yesmin *et al.,* 2004). The majority of bacterial infections in fish are mainly induced by the members of the genera including *Citrobacter, Aeromonas, Vibrio, Edwardsiella, Pseudomonas* and *Flavobacterium*. In freshwater tropical environments, motile aeromonads (*Aeromonas* spp.) are the most often reported bacteria associated with fish diseases (Karunasagar *et al.,* 2003). A number of species belonging to the *Aeromonas* genus *viz.*, *A. hydrophila*, *A. veronii*, *A. caviae*, *A. sobria*, *A. salmonicida* and *A. bestiarum* have been reported to cause disease in a variety of fish species (Bandeira *et al.*, 2021).

A. veronii is a potentially important bacterial pathogen responsible for causing mortality in several fish species (Silver *et al.,* 2011). Several clinical signs including fin/tail rot, ulcer, abdominal swelling, haemorrhages and exophthalmia are exhibited by fish infected with the bacterium. *A. veronii* demonstrated notable haemolytic and cytotoxic properties and harbours

various potential virulence genes that code for several lethal factors including haemolytic toxins, enterotoxins, type IV pilus, cholesterol acyltransferase and type III secretion systems (Sreedharan *et al.,* 2013). *A. media* is a non-motile, Gram-negative and rod-shaped bacterium, isolated frequently from the aquatic environment (Gibson *et al.,* 1998). Till now, there are only a few studies on *A. media*-mediated infection in aquatic animals such as zebra mussel (*Dreissena polymorpha*) (Gu *et al.,* 2001), sea cucumber (*Apostichopus japonicus*) (Gaoxue *et al.,* 2007) and Chinese sucker (*Myxocyprinus asiaticus*) (Huang *et al.,* 2013). *A. media* is biologically and biochemically unique and not closely related to other *Aeromonas* spp. (Allen *et al.,* 1983). They can be segregated using different biological and biochemical tests. There is not much relevant research and information available on the virulence potential of this species in finfish.

A broad spectrum of virulence determinants in various *Aeromonas* spp. play a significant role in the infection process (Kingombe *et al.,* 1999; Albert *et al.,* 2000). Bacterial virulence factors are primarily responsible for the pathogenicity mechanisms and are associated with the bacterial growth, penetration and evasion of the immune system of the host (Vilches *et al.,* 2004). To understand the mechanisms of pathogenesis and epidemiological distribution of *A. veronii* and *A. media*, it is very essential to evaluate the presence of virulence determinants in these isolates. *A. veronii* meagerly induce disease in freshwater fish species leading to substantial economic losses. However, not much information is available regarding the pathogenicity of *A. media* in fish. In the present study, two bacterial strains, *A. veronii* and *A. media,* isolated from a diseased fish and aquaculture pond water, respectively, were identified and characterised for their virulence properties and antibiogram profile.

Materials and methods

Bacterial isolation

Diseased *Clarias batrachus*, showing haemorrhagic lesions and dropsy, from a local aquaculture farm and water samples (500 ml) from a nearby pond with diseased fish were collected and promptly transported to the laboratory for bacterial isolation. The fish was disinfected, dissected and its kidney was inoculated in *Aeromonas* isolation medium (HiMedia, India). The water samples were diluted and inoculated in *Aeromonas* isolation medium and incubated for 24-48 h at 30°C. The presumptive *Aeromonas* colonies were randomly picked and streaked onto TSA plates to obtain pure culture.

Physiological, biochemical and molecular characterisation

Pure cultures of two isolates designated as COFCAU_AV1 and COFCAU_AM were subjected to physiological (Gram staining and motility by hanging drop method) and a series of conventional biochemical tests (Table 1) (Austin and Austin, 2007). For molecular characterisation, bacterial isolates were subjected to PCR analysis using 16S rRNA gene specific universal primers as described by Pradhan *et al.* (2023). Purification of the PCR products was done using PCR purification kit (Thermo Fisher Scientific, USA) and sequenced (Bioserve Biotechnologies Pvt. Ltd., India). The sequence data were analysed using BLAST (Basic Local Alignment Search Tool) algorithm with the help of Chromas software (Technelysium Pvt. Ltd., Australia). The genetic divergence was measured and a phylogenetic tree describing the evolutionary relationship was generated by the neighbor-joining method using the MEGAX software.

Phenotypic determination of virulence

Bacterial isolates were tested for their ability to produce different exoenzymes *viz.,* gelatinse, caseinase, lipase, Amylase and DNase (Pradhan *et al.,* 2023). The haemolytic activity of the isolates was assessed by inoculating them on blood agar medium supplemented with 5% fish blood (Pradhan *et al.,* 2023). Other virulence properties examined were slime formation and swarming activity as described by Torlak *et al.* (2017) and Kirov *et al.* (2002), respectively.

Virulence gene detection

The PCR amplification of ten putative virulence genes *viz*., aerolysin (*aerA*), cytotoxic (*act*) and cytotonic enterotoxin (*ast* and *alt*), lipase (*lip*), haemolysin (*hlyA*), elastase (*ela*), transcriptional regulator (*exsA*) of type-three secretion system (T3SS), and outer and inner membrane ring of T3SS (*ascV* and *ascC*) was carried out employing the methods as described in the preceding sub-section. The primer sequence, product size and annealing temperature of the target genes are listed in Table 2.

Evaluation of *in vivo* pathogenicity of *A. veronii* and *A. media* in fish

Healthy *L. rohita* fingerlings (8.06±1.94 g, length 8.02±1.6 cm) were acclimatised in 500 l fibre-reinforced plastic tanks, fed twice daily. Daily water exchange (20-25%) facilitated waste removal. Fish were divided into five experimental groups, each injected with 100 µl of varying bacterial concentrations (10^4-10^8 cells ml^{-1}), while a control group received PBS (pH 7.4). Fish health was monitored for two weeks and LD_{50} values were calculated using the Reed and Muench method (Reed and Muench, 1938). Bacteria were re-isolated from deceased/moribund fish to confirm infection. A separate experiment involved injecting the LD_{50} dose into *Cirrhinus mrigala* and *Labeo catla*, recording clinical symptoms and mortality under similar conditions.

Antibiotic susceptibility

Antibiotic susceptibility of *A. veronii* and *A. media* was evaluated using the disc diffusion method on Mueller-Hinton agar plates (Bauer *et al.,* 1966). Overnight cultures were swabbed on plates and antibiotic-impregnated discs (HiMedia) were placed aseptically, followed by incubation at 30°C for 24 h. Results were interpreted according to the 'Clinical and Laboratory Standard Procedure' guidelines for 15 antibiotics *viz*, polymyxin-B, gentamicin, kanamycin, azithromycin, chloramphenicol, tetracycline, ciprofloxacin, amoxiclav, ampicillin, penicillin, erythromycin, vancomycin, rifampicin, nalidixic acid and streptomycin.

Table 1. Summary of phenotypic characterisation of *A. veronii* COFCAU_AV1 and *A. media* COFCAU_AM

Biochemical characteristics	*A. veronii* (COFCAU_AV1)	*A. media* (COFCAU_AM)
Gram stain	-	-
Motility	+	-
Oxidase	+	+
Catalase	+	+
Indole	+	+
Methyl red test	+	+
Voges-Proskauer reaction	+	-
H_2S production	-	-
Citrate utilisation	+	+
Triple sugar iron	Yellow slant and yellow butt	Yellow slant and yellow butt
Nitrate reduction test	+	+
Urease test	+	-
Esculin hydrolysis test	-	+
Gas production from glucose	+	-
Acid production from:		
Maltose	+	+
Lactose	+	+
Mannitol	+	+
Sucrose	+	+
Sorbitol	-	-
Inositol	-	-
Arabinose	+	+
Dextrin	-	+
Xylose	-	-
Rhamnose	+	-
Raffinose	-	-
Decarboxylation of:		
Ornithine	-	-
Lysine	+	-

Table 2. Primer details of target genes for PCR analysis

Gene	Primer sequence (5'-3')	Annealing temperature (°C)	Size (bp)	References
aerA	F- CCCGCCGATCTGCAACCGGG R- CTGGTCTGGATAGACGGGCTCTGCC	58ºC for 30 s	489	Ormen and Ostensvik (2001)
act	F- AGAAGGTGACCACCAAGAACA R- AACTGACATCGGCCTTGAACTC	52ºC for 30 s	232	Kingombe *et al.* (1999)
ast	F- TCTCCATGCTTCCCTTCCACT R- GTGTAGGGATTGAAGAAGCCG	60ºC for 30 s	331	Kingombe *et al.* (1999)
alt	F- TGACCCAGTCCTGGCACGGC R- GGTGATCGATCACCACCAGC	58ºC for 30 s	442	Kingombe *et al.* (1999)
hlyA	F- GGCCGGTGGCCCGAAGATACGGG R- GGCGGCGCCGGACGAGACGGG	55ºC for 30 s	597	Heuzenroeder *et al.* (1999)
lip	F- ATCTTCTCCGACTGGTTCGG R- CCGTGCCAGGACTGGGTCTT	55ºC for 30 s	382	Sen and Rodgers (2004)
ela	F- ACACGGTCAAGGAGATCAAC R- CGCTGGTGTTGGCCAGCAGG	56ºC for 30 s	513	Sen and Rodgers (2004)
exsA	F- TACCACAGAGAAGGGCGATA R- GCGAGCAGAAACAGCAACT	55ºC for 30 s	435	Lim and Hong (2020)
ascV	F- ATGGACGGCGCCATGAAGTT) R- TATTCGCCTTCACCCATCCC	55ºC for 30 s	710	Chacon *et al.* (2004)
ascC	F- GCATTGGAGCAACAGTCCCA R- CCTTCAATCCCCTTGCGAT	52ºC for 30 s	476	Lim and Hong (2020)

Results and discussion

Phenotypic and molecular identification of the bacterial strains

In this study, two bacterial strains *A. veronii* COFCAU_AV1 and *A. media* COFCAU_AM were isolated from diseased fish and aquaculture pond water, respectively. The identity of the isolates was confirmed based upon their distinctive physiological and molecular properties. The strain COFCAU_AV1 was a typical rod-shaped, Gram negative and motile bacterium. The biochemical tests revealed that the strain was positive and negative for 16 and 8 biochemical reactions, respectively. The other strain COFCAU_AM was also Gram negative and rod-shaped but non-motile. In a total of 24 biochemical reactions, the strain was positive for 13 and negative for 11 reactions, respectively. The biochemical and physiological features of the test strains were consistent with those of *A. veronii* and *A. media* as delineated in Bergey's Manual of Systematic Bacteriology (Martin-Carnahan and Joseph, 2005). *A. veronii* exhibited positive glucose fermentation, Voges-Proskauer test, and negative H_2S production, which are some of the representative reactions for the identification of *A. veronii*. Similarly, non-motility, glucose fermentation with no gas production and negative decarboxylation reaction of amino acids *viz.,* lysine and ornithine, exhibited by *A. media* strain confirmed their distinct identity (Martin-Carnahan and Joseph, 2005). The results of the biochemical tests (Table 2) and molecular analyses by 16S rRNA gene amplification confirmed the strains COFCAU_AV1 and COFCAU_AM as *A. veronii* and *A. media*, respectively.

Besides biochemical tests, molecular analysis by sequencing of the 16S rRNA gene also confirmed their identity as *A. veronii* and *A. media*. BlLAST search of 16S rRNA gene sequence of *A. veronii* strain by NCBI nBLAST revealed 99.89% similarity with that of the *A. veronii* strain CRC6 (MT231935) whereas, *A. media* was 100% homologous with *A. media* strain L34 (KU179358). The GenBank accession numbers (MK907586 and MK907592) of the strains were obtained after submitting the 16S rRNA gene sequence to the 'National Center for Biotechnology Information' database. Fig. 1a and b depicts phylogenetic trees constructed with the neighbor-joining method using the MEGAX software.

Phenotypic determination of virulence and virulence genes detection

The results of the phenotypic determinants of virulence and virulence genes detection are presented in Tables 3 and 4, respectively. Determining the production of exoenzymes and toxins by a bacterium is a direct method of demonstrating the pathogenic potential of a bacterium (Sreedharan *et al.,* 2013). In the current work, the strain COFCAU_AV produced hydrolytic enzymes *viz.,* lipase, caseinase, amylase, gelatinase and DNase while COFCAU_AM produced amylase, lipase, and DNase, which indicated their potential to produce putative virulence factors. The pathogenicity of *Aeromonas* spp. is attributed to the involvement and phenotypic expressions of different enzymes secreted (Castro-Escarpulli *et al.,* 2003). Both *A. veronii* and *A. media* exhibited haemolytic activity which is one of the crucial virulence factors that signify the pathogenicity of aeromonads (Santos *et al*., 1999). The activity is attributed to the ability of the strains to produce both haemolysin and aerolysin toxins which contribute to virulence by creating pores in the infected cells (Heuzenroeder *et al.,* 1999). In this study, the haemolysis of fish blood by the test strains indicated their potential to secrete haemolytic factors. The test bacterial isolates were also capable to produce slime. Slime production assists in the adherence of microorganisms to certain host tissues which aids in the subsequent production of microbial colonies (Tenover *et al.,* 1998). The slime-forming ability of the strains was also indicative of the pathogenic attributes.

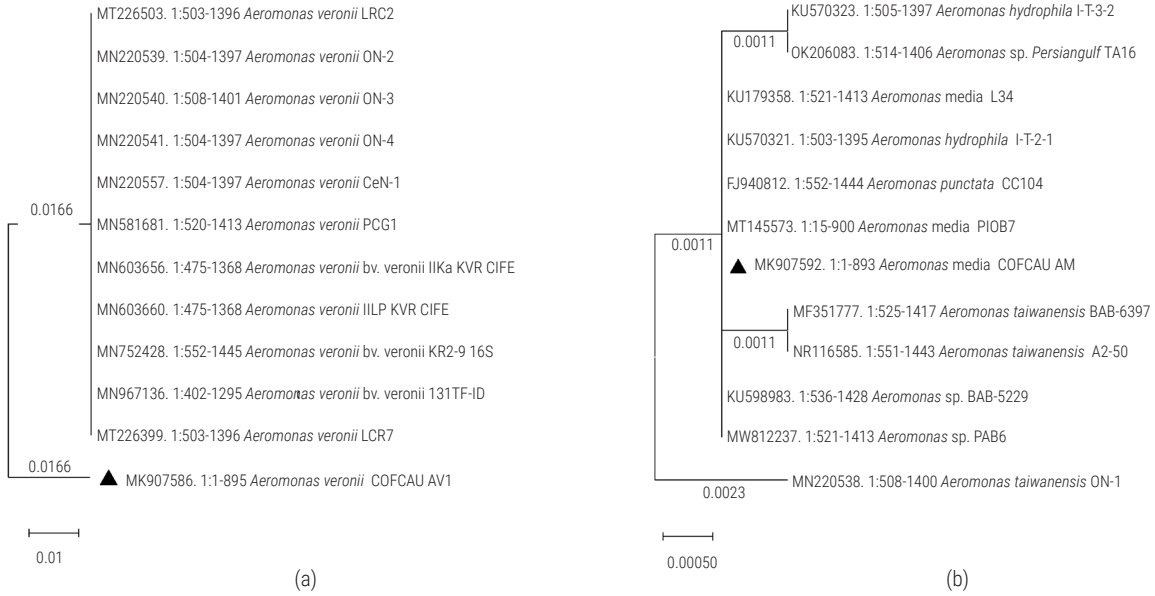

Fig. 1. Phylogenetic tree based on partial 16s rRNA gene sequence of (a) *A. veronii* strain COFCAU_AV1 (▲) and (B) *A. media* strain COFCAU_AM (▲). The tree was constructed using neighbour-joining algorithms with genetic distance calculated according to Kimura's 2-parameter method of MEGAX software

Table 3. Phenotypic determination of *A. veronii* COFCAU_AV1 and *A. media* COFCAU_AM

Phenotypes	Results	
	A. veronii	*A. media*
Gelatinase	+	-
Amylase	+	+
Lipase	+	+
DNase	+	+
Caseinase	+	-
Hemolytic	+	+
Swarming	+	-
Slime production	+	+

Table 4. Distribution of major virulence genes in *A. veronii* COFCAU_AV1 and *A. media* COFCAU_AM

Gene	Amplicon size (bp)	Isolates	
		A. veronii	*A. media*
aerA	489	+	+
act	232	+	-
ast	331	+	-
alt	442	+	-
hlyA	597	-	-
Lip	382	+	+
Ela	513	-	-
exsA	435	+	-
ascV	710	-	-
ascC	476	+	-

The pathogenicity potential of certain *Aeromonas* species is associated with multiple virulence genes encoding several secreted extracellular enzymes and toxins (Janda, 2002). The test strains, *A. veronii,* and *A. media* were examined for various virulence genes including *ast, act, aerA, alt, hlyA, ela, exsA, ascC, ascV* and *lip*. Among these, seven genes *i.e., aerA*, *act, alt, ast, exsA, ascC* and *lip* genes were present in *A. veronii* whereas only two genes *viz., aerA* and *lip* were present in *A. media*. One of the major virulence toxins contributing to the pathogenicity of numerous *Aeromonas* strains is aerolysin (*aerA*), which exhibit both haemolytic and cytolytic activities (Epple *et al.,* 2004). The *act* gene encodes a Type II-secreted pore-forming cytotoxic enterotoxin that has cytotoxic, enterotoxic and haemolytic properties and is responsible for several other lethal biological activities (Sha *et al.,* 2005). The heat-labile (*alt*) and heat-stable (*ast*) enterotoxins are mainly responsible for evoking severe diarrhea in many hosts (Albert *et al.,* 2000; Sha *et al.,* 2005). In this study, *A. veronii* showed presence of the *ast, alt* and *act* genes. However, they were absent in the other test strain *A. media*. The absence of *ast* gene in *A. veronii* isolates was also demonstrated in another study (Shreedharan *et al.,* 2013). A single gene *i.e., alt* was only detected among three genes (*ast, alt, and act*) tested in *A. media*, isolated from fresh retail ready-to-eat sushi (Hoel *et al.,* 2017). The geographic distribution of the isolates may be the reason for these variations in the expression of discrete toxin gene incidence (Albert *et al.,* 2000). In the present study, the expression of haemolytic activity may be attributed to the presence of *aerA* and *act* gene in *A. veronii* and *aerA* gene in *A. media,* respectively. The lipase activity, as confirmed phenotypically can be corroborated by the confirmation of the presence of *lip* gene in both bacterial strains. *A. veronii* bv. *sobria* harbouring different extracellular lipase genes (*lip, lipH3, pla, plc* and *GCAT*) had lipolytic activity (Castro-Escarpulli *et al.,* 2003). Among the three T3SS genes studied, *A. veronii* possess *exsA* and *ascC* genes. The *exsA* gene encodes a transcriptional regulator that plays an essential role in the activation of the T3SS (Shrestha *et al*., 2015) and the inhibition of host macrophage functions (Tian *et al*., 2019).

In vivo pathogenicity of *A. veronii* and *A. media*

The LD_{50} of *A. veronii* and *A. media* were $10^{6.8}$ and 10^{7} cells $fish^{-1}$, respectively, inducing mortality in 50% of the challenged *L. rohita*. Similarly, the LD_{50} of *A. veronii* bv. *veronii* in goldfish was calculated as 1.6×10^{6} CFU per fish (Han *et al.,* 2008). If the LD_{50} values of a bacterial strain fall within the range of 10^{6} to 10^{7} CFU g^{-1} fish, it can be regarded as moderately virulent (Mittal *et al.,* 1980). Therefore, the LD_{50} of *A. veronii* and *A. media* in *L. rohita* fingerlings indicated that both strains were moderately virulent.

The first mortality of fish occurred on 1st day after injection. The clinical signs also began to appear one day after the injection in most of the experimentally infected fishes. *A. veronii* injected *L. rohita* exhibited haemorrhages on the ventral body and fin bases, protruded and haemorrhagic anus, and abdominal dropsy. *L. rohita* infected with *A. media* showed slight haemorrhages in the fin base and scale pockets, excess mucus secretion, reddish vent, and abdominal dropsy. Internally, the liver and kidney presented a pale appearance with enlarged size in some cases. Injection of the two bacteria in *L. catla* and *C. mrigala* resulted in pathological signs, almost like that of *L. rohita*. The clinical signs observed in the IMCs challenged by *A. veronii* were similar to those recorded in other fishes such as goldfish (Lu *et al.,* 2016a), channel catfish (Hoai *et al.*, 2019) and crucian carp (Chen *et al.,* 2019).

The mortality percentages recorded in *L. catla* exposed to *A. veronii* and *A. media*, were 63 and 53% respectively. In the case of *C. mrigala* the values were 55 and 38%, respectively. *C. mrigala* was less susceptible to both the bacteria, compared to *L. rohita* and *L. catla*. Although both the test strains produced almost identical clinical signs during this study, they were more prominent in *A. veronii*-infected fish. The *A. media* strain was less virulent in all three IMCs compared to the *A. veronii* strain. Moreover, *C. mrigala* was least susceptible to both strains, compared to the other IMCs. This study demonstrated that both pathogens could induce mortality and morbidity in all three commercially important IMCs.

Antibiotic susceptibility

The test isolates in this investigation displayed varying antibiotic resistance profiles. The finding of the antibiotic susceptibility test is summarised in Table 5. *A. veronii* presented high susceptibility to streptomycin, kanamycin and chloramphenicol while high resistance to penicillin, erythromycin, vancomycin and nalidixic acid. The bacterium had intermediate break-point values to ampicillin and ciprofloxacin. In a previous investigation, erythromycin, and penicillin resistance of *A. veronii* was also observed (Sreedharan *et al.,* 2013). On the other hand, *A. media* strain was resistant to tetracycline, penicillin, erythromycin, vancomycin and rifampicin whereas, susceptible to chloramphenicol, gentamicin, tetracycline, streptomycin, nalidixic

Table 5. Antibiotic sensitivity pattern of *A. veronii* COFCAU_AV1 and *A. media* COFCAU_AM

Antibiotics (Concentration in µg)	*A. veronii*		*A. media*	
	Zone of inhibition (mm)	Sensitivity	Zone of inhibition (mm)	Sensitivity
Amoxyclav (30)	16	S	20	S
Ampicillin (10)	16	I	0	R
Penicillin G (10)	0	R	0	R
Vancomycin (30)	0	R	13	R
Polymyxin-B (300)	13	R	13	R
Gentamicin (10)	16	S	19	S
Kanamycin (30)	19	S	19	S
Streptomycin (10)	18	S	15	S
Tetracycline (30)	<10	R	22	S
Chloramphenicol (30)	25	S	35	S
Rifampicin (5)	<10	R	12	R
Ciprofloxacin (5)	26	I	30	S
Nalidixic Acid (30)	0	R	29	S
Azithromycin (15)	<10	R	16	I
Erythromycin (15)	0	0	12	R

S: Susceptible; R: Resistant, I: Intermediate

acid, ciprofloxacin, kanamycin and amoxiclav. The *A. media* strain isolated from river water had susceptibility to oxytetracycline, chloramphenicol, chlortetracycline, nitrofurantoin, gentamicin, tetracycline, kanamycin and neomycin but not to ampicillin, cloxacillin, novobiocin, penicillin G, or sulfafurazole (Allen *et al.,* 1983). Sensitivity to various antibiotics such as chloramphenicol, cefalotin, cefotaxime, cefixime, netilmicin, gentamicin and azithromycin was observed in *A. media* KC-2 strain isolated from a diseased koi carp (Lu *et al.,* 2016b).

The antibiotic resistance pattern displayed by these test strains may be attributed to the presence of antibiotic-resistance genes present in plasmids. This antibiotic susceptibility study on the test isolates can serve as a guideline regarding the timing and requirement of any potential antibiotic treatment during infection by these bacteria. On the other hand, the observed antibiotic resistance emphasises the importance of judicious use and selection of the antibiotic in the event of disease outbreaks by isolated strains.

From this study, it can be stated that both *A. veronii* and *A. media* were moderately virulent to IMCs. Possibly, this is the first study that reports the pathogenicity of an *A. media* strain in Indian major carps. Multiple virulence genes and phenotypic expressions of various virulence factors have been displayed by both isolates, which indicated their potential in establishing infection in fish hosts. The antimicrobial susceptibility pattern of the bacterial isolates signifies the emergence and spread of antibiotic-resistant bacteria and resistance genes in the aquatic environment and the health risk associated with it. The present findings also revealed that *A. veronii* was more pathogenic than *A. media* uncler experimental conditions as it exhibited more virulence-related factors.

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