Indian J. Fish., 69(3): 91-99, 2022
DOI: 10.21077/ijf.2022.69.3.122636-11

Molecular characterisation of *Flavobacterium columnare* associated with columnaris disease in freshwater fishes with emphasis on virulence and antibiotic resistance pattern

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ABSTRACT

Flavobacterium columnare is a major aquaculture pathogen that causes columnaris disease. However, only very limited studies have been conducted on its virulence and drug resistance pattern. Three pathogenic bacterial isolates of *F. columnare* (CIFRI-RCM8, CFCCSL66 and CFCCO41) were obtained from diseased fish (*Labeo rohita*, *L. catla* and *Clarias magur*) of culture farms in Odisha and West Bengal, India. Clinical signs such as tail rot, skin ulcer and gill discolouration, as well as soft ulceration across the entire body, irrespective of the fish size were noticed. The virulence of the isolates was confirmed by a recursive *in vitro* experiment. The pathological changes in infected *L. rohita* were studied to confirm the pathogenicity. Considerable difference in virulence was noticed when fishes were challenged with different *F. columnare* strains. Among the isolates, CIFRI-RCM8 was the most virulent, whereas CFCCO41 was the least virulent one. All the isolates were found to be multidrug-resistant showing resistance to cefuroxime, ampicillin, cephalothin, amoxyclav, cefalexin, ceftazidime, carbenicillin, trimethoprim, clindamycin, ticarcillin and penicillin-G. The study suggested that *F. columnare* is one of the most important etiological agents of disease in freshwater fishes for which appropriate management protocols needs to be developed.

Keywords: Antibiotics, Columnaris disease, *Flavobacterium columnare*, Molecular characterisation, Virulence

Introduction

Columnaris disease is one of the top three diseases causing significant economic loss in the aquaculture industry. The disease is caused by *Flavobacterium columnare*, a Gram negative rod shaped pathogenic bacteria that are commonly distributed in fresh and marine aquatic environments including fish, food, water and soil (Bernardet and Bowman, 2006; Singh *et al*., 2021). The bacteria causes diseases in numerous fish species associated with cold and warm water aquaculture (Starliper and Schill, 2011; Faisal *et al.,* 2017). The majority of fish species like *Labeo rohita*, *L. catla*, *Oncorhynchus mykiss*, *Carassius auratus*, *Clarias magur*, *Anabas testudineus*, *Oreochromis* sp. and *Ictalurus punctatus* get severely affected by columnaris disease which leads to mass mortality (Verma and Rathore, 2013; Loch and Faisal, 2015; LaFrentz *et al.,* 2018). Columnaris disease is a major contributing factor for the mortality of fish in India, China, Japan, Korea and other Asian countries (Nayak *et al.,* 2014; Loch and Faisal, 2015; LaFrentz *et al.,* 2018). Recently, the disease has been reported in Himalayan and sub-Himalayan fish hatcheries and culture systems, causing enormous death in fry and fingerlings with ulceration and saddleback lesions on the dorsal side of the body, including the head region (Singh *et al.,* 2021). The clinical sign of columnaris disease is manifested as gill, skin and fin lesions (Bernadet and Bowman 2006; Declercq *et al.,* 2021). Although the bacterium was characterised in several fish species from different geographical regions, the virulence of columnaris disease is not fully understood because of difficulties in reproducing experimental infection (Declercq *et al.,*2015). Hence, most of the studies have been based on natural outbreaks of columnaris disease. Generally, the disease outbreaks are associated with high stocking densities, temperature variance and high organic load (Wakabayashi, 1991; Decostere *et al.,* 1999; Declercq *et al.,* 2015). Most likely this disease is associated with poor environmental conditions causing stress, which is common in commercial aquaculture systems with maximum production level (Moore *et al.,* 1990; Wakabayashi, 1991). However, this bacteria has been found in tropical and sub-tropical water all over the world and has shown pathogenicity when the water temperature exceeds 20°C (Rucker *et al.,* 1954; Declercq *et al.,*2013a). Difference in virulence pattern among *F. columnare* isolates has been observed in different fish species (Decostere *et al.,* 1999; Declercq *et al.,*2013a).

Some of the virulent strains of *F. columnare* can induce mortality within the host and produce typical clinical signs on the body surface; whereas other virulent isolates are capable to kill the fish species at low temperature without any external manifestation (Nayak *et al.,* 2014; Declercq *et al.,* 2021).

In the current investigation, *F. columnare* was isolated and identified from *L. rohita*, *L. catla* and *Clarias magur* using morphological and molecular methods. To evaluate pathogenicity and virulence pattern, *in vitro* testing and *in vivo* challenge experiments were also conducted. Further, in this study, we also attempted to describe the virulent isolates in terms of their drug-resistance profile.

Materials and methods

Collection of diseased fish

Clinically disease manifested fishes from ponds and different wetlands of Odisha and West Bengal, India, were collected for disease surveillance and diagnosis purposes. During the investigation, mostly diseased fish species namely rohu (*L. rohita*), catla (*L. catla*) and walking catfish (*C. magur*) were collected and transported to the laboratory for pathogen identification. All the moribund fishes were showing various signs like gill discolouration, tail rot and skin ulcer resembling the typical signs of columnaris like disease. The live fish were handled as per the guidelines approved by ICAR-Central Inland Fisheries Research Institute, Institutional Animal Ethics Committee (IAEC). The external body surface, gill and internal organs of each fish were examined critically for the presence of any parasites.

Isolation of F. columnare from diseased fishes

The body surface of diseased fish specimens were disinfected using cotton swab of 70% ethanol to avoid microbial contamination. After aseptic dissection of sick fishes, organs like gill, ulcerated skin, liver and kidney were used for isolation of bacteria. Sterile loop was used to inoculate the tissue samples on nutrient agar (Hi-media, India) plates. All the streaked plates were incubated at 30^0C for 36-48 h. After incubation, the bacterial growth pattern was observed and dominant bacterial colonies were sub-cultured and purified on nutrient agar media. Three strains *viz*, CIFRI-RCM8, CFCCSL66 and CFCCO41 were isolated from freshwater fish species *viz, L. rohita*, *L. catla* and *C. magur,* respectively. The pure cultures were streaked onto Anacker and Ordal's (AO) agar media (Anacker and Ordal, 1955) and were incubated for 24 h at 30°C.

Molecular characterisation

Pure cultures of these three bacterial isolates (CIFRI-RCM8, CFCCSL66 and CFCCO41) were grown in Anacker and Ordal's (AO) broth and the genomic DNA extraction was performed as per manufacturer's instructions using column based bacterial genomic DNA extraction kit (Hi-media, HTBM008, India). PCR amplification was done using universal primers-8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Eden *et al.,* 1991) and species-specific primer Col-72F (5′-GAA GGA GCT TGT TCC TTT-3′) and Col-1260R (5′-GCC TAC TTG CGT AGT G-3′). The PCR reaction mixtures (50 µl) comprised 25 µl Taq PCR Master Mix, 2X (Qiagen, India), 18 µl of RNase-free water, 1.5 µl of both the primers and 50-100 ng of DNA template. The reaction was carried out in a Thermal cycler (G8800 Sure Cycler, Agilent, USA) under PCR cycling conditions as described previously (Malick *et al.,* 2020). Amplicons were checked by electrophoresis in 1.2% agarose gels.

Phylogenetic analysis

PCR products were purified and sequenced at Agrigenome Labs Pvt. Ltd, India. Assembly of the consensus sequence of the 16S rRNA gene of the bacterial isolates was done using DNA baser software and the sequences were compared with NCBI's publicly available GenBank database using the BLAST program. The nucleotide sequences of the three bacterial isolates CIFRI-RCM8, CFCCSL66 and CFCCO41 were deposited in NCBI database. The sequences of our isolates and closely related 16S rRNA sequences of *F. columnare* (ATCC 49512), *F. psychrophilum* (ATCC 49418), *F. branchiophilum* (QI14), *Chryseobacterium balustinum* (LMG 8329), *C. bernardetii* (G229), *C. gleum* (LMG 8334) and *C. indologenes* (DSM 16777T) were retrieved from the GenBank (NCBI) for the construction of phylogenetic tree. By using Molecular Evolutionary Genetics Analysis (MEGA-X) software the tree was prepared using the ClustalW utility through the Neighbor-joining (NJ) method with 1000 replicates (Saitou and Nei, 1987; Kumar *et al.,* 2018).

Antimicrobial susceptibility test

The bacteria were subjected to antimicrobial sensitivity test using Kirby-Bauer disc diffusion method on Mueller-Hinton agar plates (Farmer, 2004). Pure cultures of the three isolates were inoculated in diluted (1:5) Muller Hinton Broth (DMHB) and kept overnight at 30^0C under shaking. The bacterial culture (100 µl) was spread evenly on the Muller-Hinton agar (Himedia, India) plates and allowed to dry. The different class of antibiotics (aminoglycosides, penicillins, β-lactams, macrolides, cephalosporins, chloramphenicol, quinolones/fluoroquinolones, sulphonamides, lincosamides, tetracyclines, nitrofurans carbapenems and glycopeptides) were placed on the inoculated plates. The plates were

carefully incubated at 30^{0}C and the diameter of zones of inhibition was measured after 24 h of incubation and interpreted as resistant, intermediate and susceptible as per the non-Enterobacteriaceae protocol of Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2011).

In vitro virulence study

Congo red binding assay

Congo red binding assay was performed by adding 0.03% Congo red in autoclaved nutrient agar media. Bacterial isolates were spotted on agar plates and incubated at 37^{0}C for 36 to 48 h. Pathogenic bacteria absorbed the dye and appeared as deep red raised colonies after 36-48 h.

Gelatinase assay

Gelatinase assay was performed by spot inoculation of the three isolates onto Nutrient Agar (Himedia, India) plates containing 0.4% gelatin. The agar plates were incubated for 24 h at 37^{0}C. Following the incubation period, the plates were saturated with 15% mercuric chloride solution and left to stand for 5-10 min. The surplus solution from the plates was removed and the clean zone surrounding the colonies was measured.

Experimental challenge and LD_{50} determination

Healthy *L. rohita* fingerlings weighing 25-30 g were collected from a registered fish farm and transported to the ICAR-CIFRI hatchery unit for experimental use. The fishes were acclimatised for two weeks before the experiment. They were maintained in fiberglass reinforced plastic (FRP) tanks having aeration facility and fed on commercial feed at the rate of 4% body weight daily. To determine the virulence and LD_{50} of the bacteria, *L. rohita* fingerlings were challenged with the bacterial isolates by intramuscular route. The fishes were distributed in 32 tanks (10 fishes per tank) of 50 l capacity each. The experiment was carried out in duplicates. All three bacterial cultures at varying concentrations of 10^4, 10^5, 10^6, 10^7 and 10^8 cells per fish were used for challenging *L. rohita*. The fishes were injected with 100 µl each of the respective bacterial culture and control fishes were injected with phosphate-buffered saline (PBS, pH 7.2) at room temperature (28±2^{0}C). Fish mortality was recorded at 12 h intervals for 7 days to calculate the lethal dose (LD_{50}) as per Reed and Muench (1938). Moribund fishes were collected for microbiological analysis to confirm the presence of the challenged bacterium.

Results

Gross pathology and isolation

Collected fishes had ulcers on their bodies, as well as haemorrhagic patches. Tail rot and red spots were also observed on the caudal peduncles. No gross lesions could be noticed in the internal organs. The fish exhibited uneasiness and sluggish movements. The colonies of bacteria were noticed as semi-translucent and yellow in Anacker and Ordal's (AO) agar plates after 36-48 h of growth and all three isolates (CIFRI-RCM8, CFCCSL66 and CFCCO41) also showed gliding motility.

16S rRNA gene sequencing and phylogeny for identification of the isolates

Molecular characterisation of the three isolates revealed that these bacteria (CIFRI-RCM8, CFCCSL66 and CFCCO41) have maximum sequence similarity with *F. columnare* followed by isolates *F. psychrophilum*, *F. branchiophilum*, *C. balustinum*, *C. bernardetii, C. gleum* and *C. indologenes* (Fig. 1). Overall phylogeny of the isolates appears to be closely related within the *F. columnare* species and the partial 16S rRNA gene sequences of the three isolates *viz*, CIFRI-RCM8, CFCCSL66 and CFCCO41 were deposited in the GenBank and assigned the accession codes MK770609, KF051089 and KF051085, respectively.

Antimicrobial resistance profile

The antibiotic resistance pattern of the individual pure cultures are shown in Table 1 and were analysed as per the non-Enterobacteriaceae protocol of CLSI guidelines (CLSI, 2011). The growth of bacterial culture in 1:5 diluted Muller Hinton Broth (DMHB) was observed and the isolates were tested with 40 antibiotics for detection of drug resistance pattern after 24 h incubation in 1:5 diluted Muller Hinton Agar (DMHA) plates. The results revealed that all the three isolates were completely resistant to cefuroxime, ampicillin, cephalothin, clindamycin, ticarcillin, amoxyclav, cefalexin, ceftazidime, carbenicillin, trimethoprim and penicillin-G. Nevertheless, all of the isolates were positive for antibiotic susceptibility as such to amikacin, azithromycin, ciprofloxacin, co-trimoxazole, ceftriaxone, cefotaxime, cefoperazone, cefadroxil, chloramphenicol, erythromycin, furazolidone, gentamycin, gatifloxacin, doxcycline hydrocloride, imipenem, kanamycin, levofloxacin, meropenem, norfloxacin, nitrofurantoin, netillin, nalidixic acid, ofloxacin, oxytetracycline, piperacillin, streptomycin, tetracycline, tobramycin and vancomycin

Virulence study

All the three bacterial isolates were positive for Congo red binding assay (Fig. 2). Colonies developed red colouration after 24 h of incubation. In the gelatinase test, all three isolates produced enzymes that hydrolyzes gelatin. A clear zone was observed around the colonies (Fig. 3).

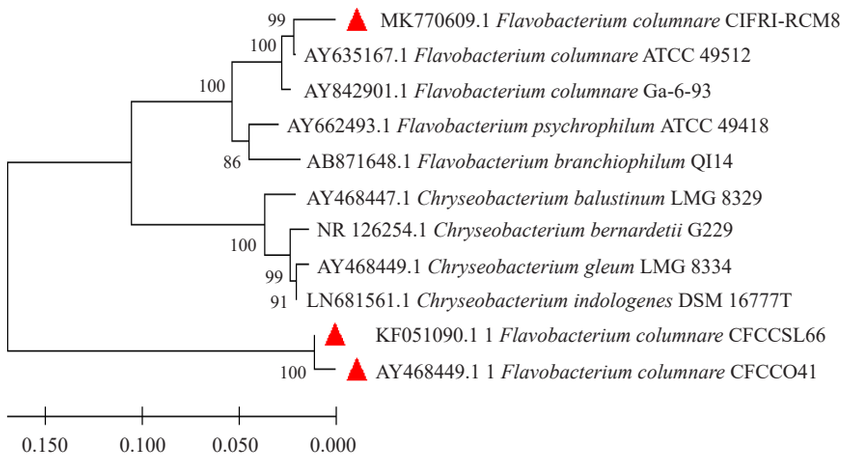

Fig. 1. Phylogenetic tree based on the partial 16S rRNA gene sequence of the *F. columnare* isolates along with other *Flavobacterium* spp. and *Chryseobacterium* spp.

Table 1. Antibiotic resistance profiles of *F. columnare* isolates recovered from freshwater fishes

Antimicrobial agent	Concen-tration (µg per disc)	Zone of inhibition (mm)			Susceptibility			Standard diameter of the inhibition zone (mm)		
		CIFR-RCM8	CFCC SL66	CFCC O41	CIFR-RCM8	CFCC SL66	CFCC O41	R	I	S
Amikacin	30	27	25	24	S	S	S	≤14	15-17	≥18
Ampicillin	10	0	0	0	R	R	R	≤13	14-16	≥17
Amoxyclav	30	0	0	0	R	R	R	≤13	14-17	≥18
Azithromycin	15	24	15	20	S	I	S	≤14	15-19	≥20
Cefalexin	30	0	0	0	R	R	R	≤11	20-12	≥21
Ciprofloxacin	5	27	29	26	S	S	S	≤10	11-15	≥16
Cephalothin	30	0	0	0	R	R	R	≤14	15-17	≥18
Co-trimoxazole	25	16	19	12	S	S	I	≤10	11-15	≥16
Ceftriaxone	30	18	19	25	I	I	S	≤13	14-20	≥21
Ceftazidime	30	0	0	0	R	R	R	≤14	15-17	≥18
Cefotaxime	30	18	10	13	I	S	S	≤14	15-22	≥23
Cefuroxime	30	0	0	0	R	R	R	≤14	15-17	≥18
Cefoperazone	75	18	25	22	R	S	S	≤15	16-20	≥21
Cefadroxil	30	25	27	20	S	S	S	≤14	15-19	≥20
Chloramphenicol	30	30	27	24	S	S	S	≤12	13-17	≥18
Carbenicillin	100	18	20	15	R	R	R	≤18	19-23	≥24
Clindamycin	2	12	15	16	R	R	R	≤15	16-18	≥20
Doxcycline HCl	30	26	22	28	S	S	S	≤12	13-15	≥16
Erythromycin	15	24	21	18	S	I	I	≤13	14-23	≥24
Furazolidone	50	19	22	20	S	S	S	≤12	13-16	≥17
Gentamycin	10	29	26	27	S	S	S	≤12	13-14	≥15
Gatifloxacin	5	23	26	20	S	S	S	≤14	15-17	≥18
Imipenem	10	26	27	24	S	S	S	≤16	17-21	≥22
Kanamycin	30	23	19	22	S	S	S	≤13	14-17	≥18
Levofloxacin	5	22	25	26	S	S	S	≤13	14-16	≥17
Meropenem	10	33	35	28	S	S	S	≤14	15-17	≥18
Norfloxacin	10	18	15	12	S	I	R	≤12	13-16	≥17
Nitrofurantoin	300	21	20	18	S	S	S	≤14	15-16	≥17
Netillin	30	30	25	24	S	S	S	≤12	13-14	≥15
Nalidixic acid	30	23	19	24	S	S	S	≤13	14-18	≥19

Contd.....

Antimicrobial agent	Concen-tration (µg per disc)	Zone of inhibition (mm)			Susceptibility			Standard diameter of the inhibition zone (mm)		
		CIFR-RCM8	CFCC SL66	CFCC O41	CIFR-RCM8	CFCC SL66	CFCC O41	R	I	S
Ofloxacin	5	23	25	22	S	S	S	≤12	13-15	≥16
Ox tetracycline	30	17	21	25	I	S	S	≤14	15-18	≥19
Penicilin-G	10	0	0	0	R	R	R	≤15	16-20	≥21
Piperacillin	100	25	23	24	S	S	S	≤17	18-20	≥21
Sreptomycin	10	19	13	16	S	I	S	≤11	12-14	≥15
Ticarcillin	75	0	0	0	R	R	R	≤19	20-22	≥23
Trimethoprim	5	0	0	0	R	R	R	≤10	11-15	≥16
Tetracycline	25	20	23	19	S	S	S	≤14	15-18	≥19
Tobramycin	10	26	22	18	S	S	S	≤12	13-14	≥15
Vancomycin	30	19	16	22	S	I	S	≤14	15-16	≥17

R=Resistant; I=Intermediate; S=Sensitive

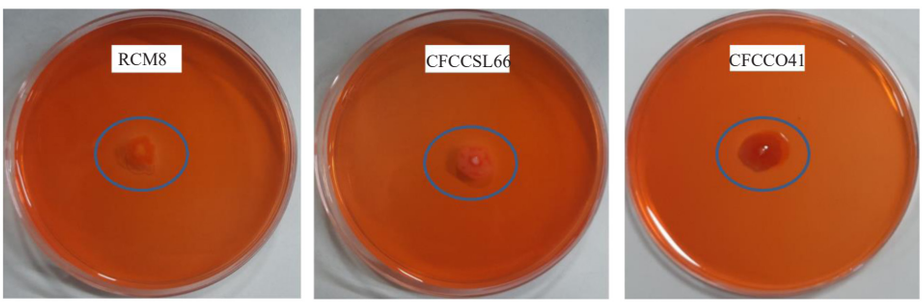

Fig. 2. Deep red raised colonies of *F. columnare* observed in Congo red binding assay

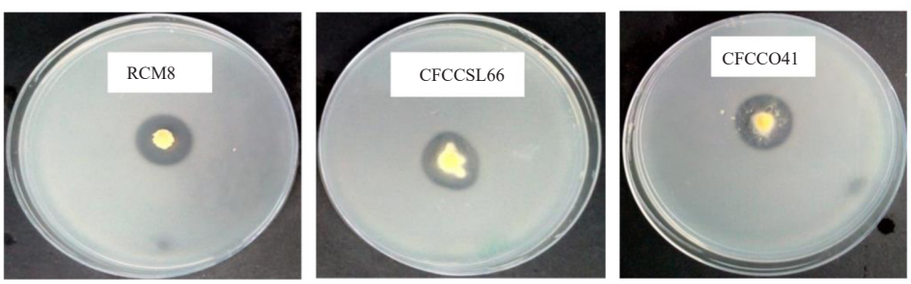

Fig. 3. Clear zone around the colonies showing gelatinase producing activity of *F. columnare*

The challenged fishes were reluctant to swim with reduced feed consumption, redness on the body surface, gill lesions and rotting of the tip of the caudal fin started to develop on the 3rd day. In our study, isolate CIFRI-RCM8 was found highly virulent with the appearance of symptoms within 72 h of injection and caused 100% mortality and LD_{50} value of 1.8×10^5 CFU per fish was calculated in *L. rohita.* In the case of the remaining two isolates (CFCCSL66 and CFCCO417) fishes showed initial reduced feed intake, followed by normal swimming and feeding activity and death after 3 days of challenge with mortality ranging from 80-95% within 7 days. The mortality data were recorded in CFCCSL66 and CFCCO417 isolates and the LD_{50} value of isolates to *L. rohita* were 7.9×10^6 CFU per fish and 5.7×10^6 CFU per fish, respectively (Fig. 4). None of the control fishes died in any of the experiments. *F. columnare* were recovered from gill tissue of moribund fishes and matched with the original isolate used for challenge study which was confirmed using molecular method of identification.

Discussion

Due to changing environmental conditions and continuing pollution of the aquatic ecosystem, there has been substantial impact on fish health, leading to an increase in bacterial and fungal infections (Johnson and

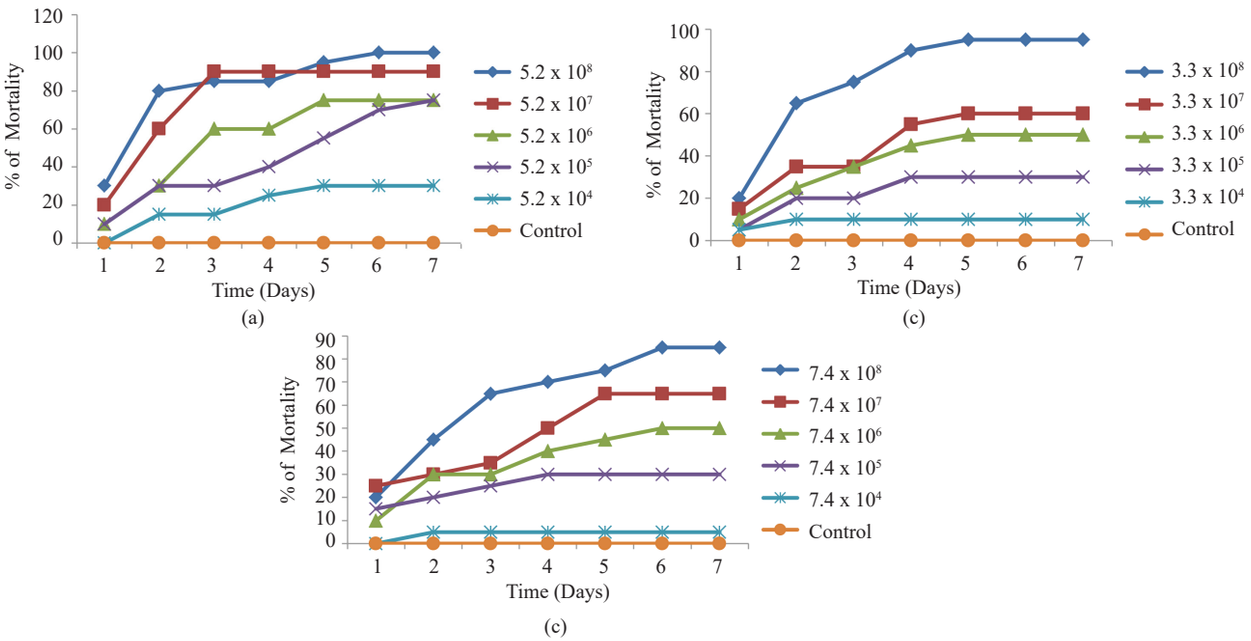

Fig. 4. Cumulative mortality curves for the determination of LD_{50} values in *L. rohita* challenged with *F. columnare*

Paull, 2011; Ruzauskas *et al.,* 2016). Bacterial diseases are the most serious threats to freshwater fish production in India (Das and Mishra, 2014). Several bacterial species that cause diseases in fish include *Aeromonas hydrophila*, *F. columnare, Acinetobacter* sp., *Chryseobacterium* sp. and *Pseudomonas* sp. (Nieto *et al.,* 1984; Walczak *et al.,* 2017; Malick *et al.,* 2020). Primary infection by *F. columnare* has resulted in a huge financial loss for freshwater fish culture (Das and Mishra, 2014; Sarker *et al.,* 2017). *Flavobacterium* sp. frequently proliferates in the skin, gill and digestive system of healthy or diseased aquatic hosts, as a component of mixed bacterial flora (Bernardet and Bowman, 2006; Loch and Faisal, 2015). *F. columnare* is one of the most important pathogenic species that causes columnaris disease (Figueiredo *et al.,* 2005; Declercq *et al.,* 2013a; Lafrentz *et al.,* 2018). The multifaceted pathogenicity caused by *Flavobacterium* sp. is attributed to a variety of virulence factors. They may produce a wide range of biologically active chemicals, including lipopolysaccharides, extracellular proteins and outer membrane proteins, as well as external factors like enzymes and toxins.

The accurate identification of the pathogen and understanding of the underlying mechanism of pathogenesis is essential for effective disease control. This experiment was conducted to characterise the virulence and antibiotic resistance of the pathogen. Three isolates were recovered from diseased rohu (*L. rohita*), catla (*L. catla*) and walking catfish (*C. magur*). *In vitro* virulence investigations showed that our isolates were able to uptake Congo red dye and showed gelatinase activity, which supports the previous study of Santos *et al.* (1999). These tests proved *F. columnare* as a carrier of the virulence factors. However, these properties have distinguished *F. columnare* from other non-pathogenic bacteria (Berkhoff and Vinal, 1986; Bernardet, 1989*).*

Based on morphological and 16S rRNA gene sequence analysis, three isolates were identified as *F. columnare.* The results indicated that identifying the 16S rRNA gene can be effective in detecting *F. columnare* infection in fish (Bader *et al.,* 2003; LaFrentz *et al.,* 2017). There was maximum similarity (99.66%) between our isolate and reference isolate (ATCC-49512) of *F. columnare*. The 16S rRNA partial sequence data and phylogenic analysis revealed that our isolates CIFRI-RCM8, CFCCSL66 and CFCCO417 were distinct from other *Flavobacterium* sp. and *Chryseobacterium* species. The phylogenetic grouping of the isolates with the reference strain showed that the isolates were more closely related to *F. columnare* at the genetic level (Triyanto and Wakabayashi, 1999; Suomalainen *et al.,* 2006).

The challenge experiment validated the *in vivo* pathogenicity of *F. columnare* to *L. rohita*. Considering the high virulence in *L. rohita* it was further proved that CIFRI-RCM8 is the most virulent isolate whose LD_{50} value was 1.8×10^5 CFU per fish, while the LD_{50} value of isolates CFCCSL66 and CFCCO417 were 7.9×10^6 and 5.7×10^6 respectively. These findings suggested that all of the *F. columnare* isolates were capable of infecting *L. rohita*

fish and have pathogenicity similar to columnaris disease. Variety of clinical signs due to *F. columnare* infection have been recorded including gill lesions in *L. catla*, *Cyprinus carpio*, *C. auratus*, rainbow trout, *C. magur* and *L. rohita* (Decostere *et al.,* 1998; Verma and Rathore, 2013; Patra *et al*., 2016). The experimental infectivity investigation demonstrated abnormal swimming behaviour, sudden jumping, subsequent sinking and death. Additionally, organs such as the gill and tail were found to be infected. *F. columnare* caused mortality in Zebrafish with LD_{50} of 3.2×10^6 cells ml^{-1} as previously reported by Moyer and Hunnicutt (2007). According to our previous study (Dash *et al.,* 2009), a dose of 6×10^4 cells ml^{-1} in rohu fry could result in 50% mortality. Sarker and Abraham (2019) reported that in abrasion-immersion challenge trials, *F. columnare* SGM4 induced considerable mortalities (45%) in *C. magur* at 7.2×10^6 cells ml^{-1}. Using an immersion assay, the LD_{50} values of *F. columnare* in *Cirrhinus mrigala* fry were found to range from 3.0×10^5 to 9.0×10^6 cfu ml^{-1} (Nayak *et al.* 2014). From the study, it is evident that *F. columnare* isolates have varying degrees of pathogenicity as reported by Singh *et al.* (2021).

The antimicrobial sensitivity study exhibited no zone of inhibition, particularly for cefuroxime, ampicillin, cephalothin, amoxyclav, cefalexin, penicilin-G, ceftazidime, trimethoprim and ticarcillin for all three isolates. However, all the isolates were susceptible to most of the other tested antibiotics. According to Suomalainen *et al.* (2006) and Nayak *et al.* (2014), most of the *F. columnare* isolates were susceptible to oxytetracycline, tetracycline, streptomycin, nitrofurantoin, kanamycin, gentamycin, erythromycin and chloramphenicol. But, another study by Declercq *et al.* (2013b) suggested that *F. columnare* was resistant to oxytetracycline followed by chloramphenicol and nitrofurantoin. The variation in the resistance pattern of our isolates with that reported for others may be due to the use pattern of antibiotics in particular geographic locations. Further, the resistance pattern varies among the isolates due to acquired genetic resistance, however, this needs further investigation.

In this study, the presence of virulence factors in *F. columnare* was suggestive of its potential for pathogenicity and to cause health problems in *L. rohita, L. catla* and *C. magur.* Further, antibiotic resistance and virulence characteristics of this bacterium indicated the importance of this pathogen in fish disease and freshwater aquaculture. Antibiotic resistance and early detection of the specific pathogen might be an aid in controlling the disease with suitable management practice.

Acknowledgments

The authors thank the Director, ICAR-Central Inland Fisheries Research Institute, West Bengal, for providing the necessary facilities to carry out the work. The research work was supported by the Rajiv Gandhi National Fellowship from the University Grants Commission, Government of India awarded to the first author.

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Date of Receipt : 26.03.2022
Date of Acceptance : 23.08.2022