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Isolation and characterization of *Flavobacterium columnare* from freshwater ornamental goldfish *Carassius auratus*

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Abstract

Filamentous bacteria overlaying ulcerated area on the body surface were observed in the wet-mout preparation from a moribund goldfish with saddle back appearance. The causative agent was identified as *Flavobacterium columnrae*, on the basis of biochemical test, species - specific polymerase chain reaction (PCR) and sequencing of 16S rDNA gene with the universal bacterial primers. Furthermore, the strain (ING-1) attributed to genomovar II in 16S rDNA PCR-restriction fragment length polymorphism (PCR-RFLP) and sequence analysis. In phylogenetic analysis, the strain ING-1, produced typical columnaris disease symptoms in rohu (*Labeo rohita*) fingerlings within 10 days. This is a new record about molecular detection and identification of *Flavobacterium columnare*, occurring naturally on a new host *Carassius auratus* in India.

Key words

Bioassay, *Carassius auratus*, *Flavobacterium columnare*, Genomovar, PCR-RFLP

Introduction

Aquarium keeping is amongst the most popular hobbies with millions of enthusiasts worldwide (Livengood and Chapman, 2011). Among ornamental fish, the goldfish, *Carassius auratus*, is the most commonly kept in garden ponds and of international significance. Despite the wide international culture and distribution of goldfish, few bacterial diseases have also been reported and characterized (Citarasu *et al*., 2011). Among bacterial diseases, columnaris disease is regarded as a predominant disease of ornamental fish (Decostere *et al*., 1999 a,b; Schneck and Caslake, 2006). Columnaris disease debilitates a wide ecological and phylogenetic spectrum of temperate and tropical freshwater fishes (Bernardet *et al*., 1996; Olivares-Fuster *et al*., 2007; Bullard *et al*., 2011). Moreover, various commercially important fish species, such as salmonids, eels, carps, goldfish, tilapia and channel catfish are susceptible to this disease, (Řehulka and Minařík, 2007; Soto *et al*., 2008; Suomalainen *et al*., 2009). The aetiological agent of this disease is a Gram-negative, filamentous, yellow pigmented bacterium named *Flavobacterium columnare*, considered to be ubiquitous in the aquatic environment worldwide.

Host range of *F. columnare* is continuously expanding and includes a wide spectrum of temperate and tropical freshwater fish species of Asia, North America and Europe (Wakabayashi, 1993; Shotts and Starliper, 1999; Olivares-Fuster *et al*., 2007). The latest fish species to be affected by this pathogen is striped catfish, *Pangasianodon hypothalamus* from Vietnam (Tien *et al*., 2012) and bluegill, *Lepomis macrochirus* from the United States of America (Bullard *et al*., 2013). This pathogen also possesses a high degree of intra-species heterogeneity (Triyanto and Wakabayashi, 1999). Three genomovars (I, II and III) are exhibited on the basis of restriction fragment length polymorphism (RFLP) of 16S rDNA (La Frentz *et al*., 2013). Studies showed incongruity in the virulence properties of these genomovars of *F. columnare* (Arias *et al*., 2004; Thomas-Jinu and Goodwin, 2004; Darwish and Ismaiel, 2005). Moreover, genomovar II appears to be more pathogenic for channel catfish, *Ictalurus punctatus* (Rafinesque) as compared to genomovar I (Shoemaker *et al*., 2008). This shows that molecular identification and genetic characterization of *F. columnare* from a new

geographical location is very important. *F. columnare* was isolated recently from *Catla catla* in India (Verma and Rathore, 2013). However, the pathogen is not yet reported from the ornamental fish species in India. The present study report isolation of *F. columnare* from ornamental goldfish (*Carassius auratus*) in India.

Materials and Methods

Disease investigation : Freshwater goldfish (average size 10.56±1.3 cm), showing 'saddle back appearance' and yellowish deposition on gills were collected from an aquarium in Lucknow, Uttar Pradesh, India. Tentative diagnosis of columnaris disease was established by microscopic observation. Cytological examination of smears from skin and fins was also done by Romanowsky staining for demonstration of bacilli. Representative skin and gill samples from the affected fish were fixed in buffered 10% formalin. Tissues were processed in a routine manner. For pathological examination tissue section were cut at 5µm thickness, and stained with Hematoxylin-Eosin and Mac Callum-Goodpasteur.

Bacterial isolation and biochemical identification : White deposits from skin surface were streaked on Shieh agar and incubated at 28°C for 72 hr. Yellow rhizoids, adherent to agar surface colonies with spreading margins were subcultured and Gram stained to check purity. The isolate was identified as *F. columnare* on the basis of growth in the presence of neomycin and polymyxin B, presence of flexirubin-type pigment, chondroitinase production, congo red binding and production of a diffusible gelatin-degrading enzyme (Griffin, 1992). Phenotypic characterization of the isolate was also done following the protocol of Bernardet (1989). Susceptibility of the strain to antibiotics was tested as described by Michel *et al*., (2002), using Shieh agar. Identified bacterial isolate was termed as ING-1 and stored at -80°C for further characterization.

Molecular confirmation of the bacterial isolate

Genomic DNA isolation: Genomic DNA from freshly grown broth culture of strain ING-1 was isolated according to the protocol of Murmur (1961), with minor modifications. In brief, the cells were pelleted and resuspended in an equal volume of TES buffer (50 mM Tris buffer, 1 mM EDTA, 8.56% saccharose) pH 8.0 and sodium dodecyl sulphate (SDS) was added to the mixture. The solution was treated once with chloroform-isoamyl alcohol (24/1; v/v) and once with a mixture of phenol, chloroform and isoamyl alcohol (25/24/1; v/v/v). DNA was precipitated by an equal volume of isopropanol, and dissolved in 1x Tris-EDTA buffer and stored at -20^{0}C for further use.

16S rDNA based identification : For 16S rDNA based identification, genomic DNA was amplified in PCR using bacterial universal primers, 20F 5'- AGA GTT TGA TC(A C)TG GCT CAG-3' (position at 8-27 *E. coli* numbering) and 1500R 5'- CGA TCC TAC TTG CGT AG-3' (position at 1510-1492 *E. coli* numbering) (Weisburg *et al*., 1991). PCR amplification was performed with final volumes of 50 µl, using 0.4 µl of Taq DNA polymerase (5 U µl^{-1} Fermentas), 10 mM dNTPs (Fermentas), 1.5 mM $MgCl_2$ Fermentas), 10 pM of each primer and 100 ng of purified genomic DNA. Subsequently, the sample was subjected to a pre-heating cycle at 95°C for 5 min. Each amplification cycle consisted of DNA denaturation at 95°C for 1 min, primer annealing to the template at 52°C for 1 min, followed by primer extension at 72°C for 1.5 min. The 35th cycle involved a primer extension at 72°C for 10 min followed by an infinite period at 4°C. PCR product was separated on a 0.8% agarose gel with ethidium bromide (0.5 μg m l^{-1}) at a constant voltage of 7V cm^{-1}. The amplification product was purified and sequenced. Sequence data were compiled and sequence similarities were calculated with Lasergene software (DNASTAR, Madison, USA). The nucleotide substitution rate was calculated and a distance matrix tree was constructed by the neighbor-joining method (Saitou and Nei, 1987), using the program CLUSTAL W (Thompson *et al.,* 1994). Alignment positions with gaps and unidentified bases were not considered in the calculations. Phylogenetic tree was constructed by using MEGA ver 5 software (Tamura *et al*., 2011). The topology of tree was evaluated by bootstrap analysis with 1,000 replicates

Species confirmation by PCR : Presence of *F. columnare* specific fragment was studied with *F. columnare* positive strains. PCR amplification of partial 16S rDNA was carried out by using previously described two sets of primer pair Col F/Col R (5'-CAG TGG TGA AAT CTG GT-3' /5'-GCT CCT ACT TGC GTA GT-3') and FvpF1/FvpR1: (5'-GCC CAG AGA AAT TTG GAT-3' /5'-TGC GAT TAC TAG CGA ATC C-3'). The PCR conditions were as follows: initial denaturation step at 94°C for 5 min, 30 cycles at 94°C for 30 sec, 45°C/59°C for 30 sec, 72°C for 2 min with a final extension of 72°C for 8 min, respectively (Darwish *et al*., 2004; Bader *et al*., 2003). The PCR products were electrophoresed on 1% agarose gel.

Genomovar ascription : Genomovar of strain ING-1 was confirmed by 16S rDNA PCR-RFLP. For this, previously amplified 16S rDNA was digested with restriction endonuclease *Hae*III and electrophoresed on 3% agarose gel to obtain genomovar specific profile (Triyanto and Wakabayashi, 1999; LaFrentz *et al*., 2014).

Bioassay : Bath immersion method was conducted to determine the virulence potential of ING-1 as per the protocol of Thomas-Jinu and Goodwin (2004). Briefly, rohu juveniles (n=20, 65.8±3.9 gm) were collected and acclimatized at 28ºC in the National Bureau of Fish Genetic Resources, Lucknow fish farm, maintained and handled as per guidelines of use of fish in research (UFR, 2004). The fish were divided in two groups (1 test and 1 control) and each group comprising 10 fish was maintained separately in fiber-reinforced plastic tanks (1.2×1×0.8m^3) containing 500 l of water. For pathogenicity trial, strain ING-1 was inoculated in 750 ml of Shieh broth and incubated at 28°C in a

shaker incubator to obtain a turbidity of 0.30-0.35 at 550nm. Bath immersion was done in three 50 l glass aquarium tanks as triplicates; each containing ten fingerlings. For preliminary experiment, each tank was filled with 6 L of water at 28°C and 200 ml of bacterial culture was added. After a hr of exposure, the volume of water in each tank was increased nearly to 20 l (Verma and Rathore, 2013). The pathogen was re-isolated from the gills of the moribound fish to confirm Koch's postulate. The control fish were kept seprately under similar conditions as the experimental fish except that they were exposed to sterile Shieh broth culture.

Results and Discussion

Presumptive diagnosis of columnaris disease was established by "saddleback appearance" on the body surface of the diseased fish (Fig. 1). In microscopic observation, typical bacterial haystacks were observed from scrapings of skin surfaces and fins. In Romanowsky staining, Gram negative long rod bacterium was demonstrated in the smears of skin of the naturally infected goldfish. Additionally, similar bacilli were also observed in MacCallum- Goodpasteur staining of histological section of diseased gills and skin (Fig. 2). The causative bacterium was Gram negative long rods and possessed all the five characteristics of *F. columnare* suggested by Griffin (1992), to differentiate it from other yellow pigmented Gram negative long rods bacteria. The other phenotypic characteristics of the strain ING-1 were similar to the biochemical tests of *F. columnare* as reported by Bernardet *et al.* (1996). On the basis of these peculiar phenotypic traits and histopathological examination, isolate ING-1 was identified as *F. columnare*. Reference strain of *F. columnare* (LMG-10406), *F. aquatile* (LMG 4008) obtained from BCCM™/LMG Bacteria Collection, University of Ghent, Belgium and *F. columnare* (Verma and Rathore, 2013) were included in the study for comparison. The colony of strain ING-1 was rhizoid in shape and adhered to the agar surface, absorbed congo red dye, produced flexirubin pigment, gelatinase and chondroitinase enzymes. Other biochemical traits of ING-1 strain are included in Table 1.

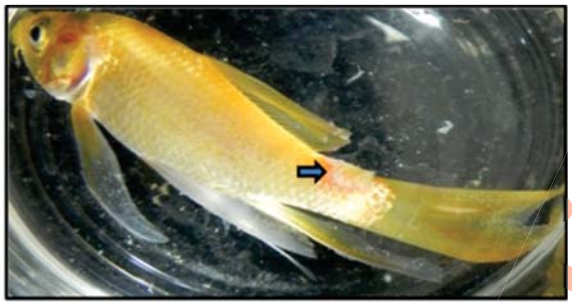

Fig. 1 : Diseased ornamental gold fish *Carassius auratus* showing saddleback appearance: A presumptive clinical sign of columnaris disease caused by *Flavobacterium columnare*. Arrow indicates dense mass of the bacteria

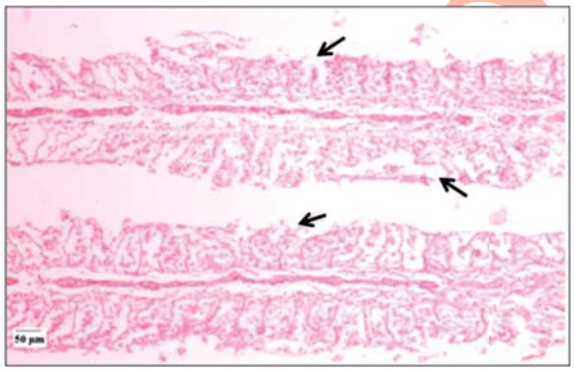

Fig. 2 : Histopathological examination of the gills of the ornamental gold fish infected with *Flavobacterium columnare*: The arrows indicate necrosis of gill lamellae

In antibiotic susceptibility testing, the strain ING-1 was found to be susceptible for ampicillin, erythromycin and oxytetracycline, but resistant to tobramycin, neomycin and polymixin B (Table 2). Definitive identification of the strain was confirmed by observing *F. columnare* specific amplicons. Both the primer sets (ColF/ColR and FvpF1/FvpR1) produced specific amplicons of 675 bp and 1193 bp, respectively (Fig. 3 and Fig. 4). The 16S rDNA sequence of ING-1 showed highest (≥ 99%) sequence similarity with *F. columnare* strains RDC-1 (Verma and Rathore, 2013). It also showed higher similarity with other published *F. columnare* strains isolated from different geographic locations. In PCR-RFLP of 16S rDNA, restriction profile of strain

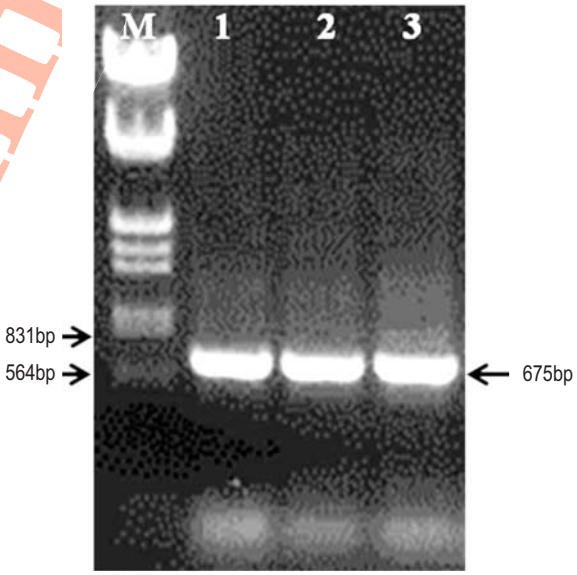

Fig. 3 : PCR amplification using *F. columnare* specific primers (ColF and Col R) for the confirmation of the *F. columnare* strain ING-1. Lane M-lambda DNA *EcoR*I *Hind*III double digested marker (Fermentas); Lane 1-*F. columnare* reference strain LMG10406; Lane 2-*F. columnare* strain RDC-1; Lane 3- *F. columnare* strain ING-1

Table 1 : Biochemical tests of *Flavobacterium columnare* ING-1 strain isolated from ornamental goldfish and other reference strains

Biochemical Tests	*F. columnare* goldfish isolate	*F. columnare* strain RDC-1	*F. columnare* LMG 10406	*F. aquatile* LMG 4008
Flexirubin pigment	+	+	+	-
Congo red absorption	+	+	+	-
Cytochrome oxidase	+	+	+	-
Catalase	+	+	+	+
Nitrate reduction	+	-	+	-
Chondroitinase production	+	+	+	-
Production of H_2S	+	+	+	+
Citrate utilization	-	-	-	
Starch hydrolysis	-	-	-	+
Esculin hydrolysis	-	-	-	+
Gelatin hydrolysis	+	+	+	+
Tyrosine hydrolysis	-	-	-	+
Tributyrine hydrolysis	-	-	-	+
Acid from glucose	-	-	-	+
Voges-Proskauer reaction	-	-	-	-
Growth in 0% NaCl	+	+	+	+
0.5% NaCl	+	+	+	+
1% NaCl	-	-	-	-
Growth at 15°C	-	-	-	-
25°C	+	+	+	+
30°C	+	+	+	+
37°C	+	+	+	+

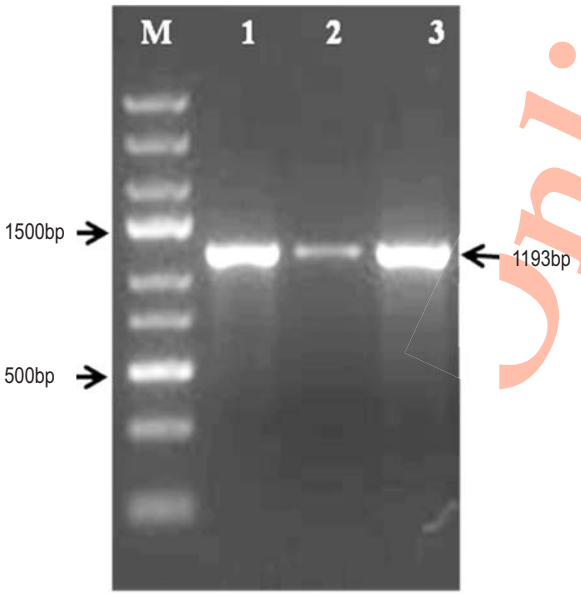

Fig. 4 : *Flavobacterium columnare* specific PCR amplification using FvpF1 and FvpR1 primers: Lane M- Expressed DNA ladder (Fermentas); Lane 1-*F. columnare* strain RDC-1; Lane 2- *F. columnare* reference strain LMG 10406; Lane 3 - *F . columnare* strain ING-1

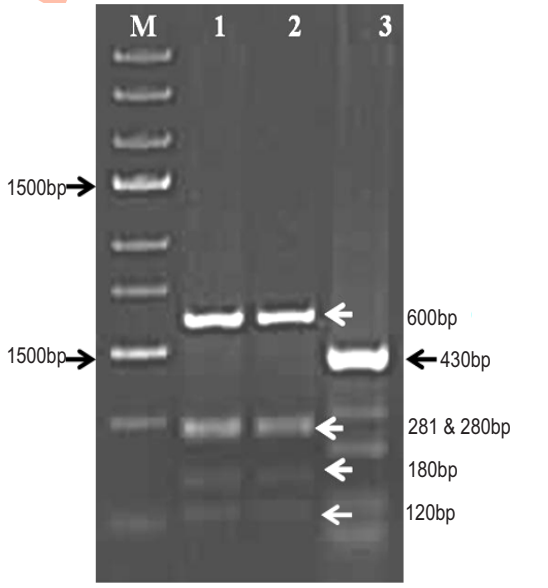

Fig. 5 : Genomovar confirmation of the ornamental gold fish isolate of *F. columnare* strain ING-1 by restriction digestion of amplified 16S rDNA with *Hae*III enzyme. Lane M: Express DNA ladder (Fermentas); Lane 1: Strain ING-1 (genomovar II); Lane 2: Strain RDC-1 (genomovar II); Lane 3: reference strain LMG-10406 (Genomovar I)

Table 2 : Antimicrobial susceptibility of *Flavobacterium columnare* ING-1 strain isolated from ornamental gold fish

Drug	Disc content (µg)	Inhibition diameter (mm)[2]	Break point for resistance (mm[2])	Status
Ampicillin	10	43–45	19	S
Gentamycin	15	15–16	19	R
Erythromycin	15 i.u.	27–36	22	S
Polymyxin B	50	8-10	15	R
Streptomycin	10 i.u.	26–28	15	S
Oxytetracycline	30 i.u	40–42	19	S
Neomycin	30 i.u.	12–13	17	R
Kanamycin	10	10-13	18	R
Chloramphenicol	30	32-34	8	S
Tobramycin	15	10-12	19	R

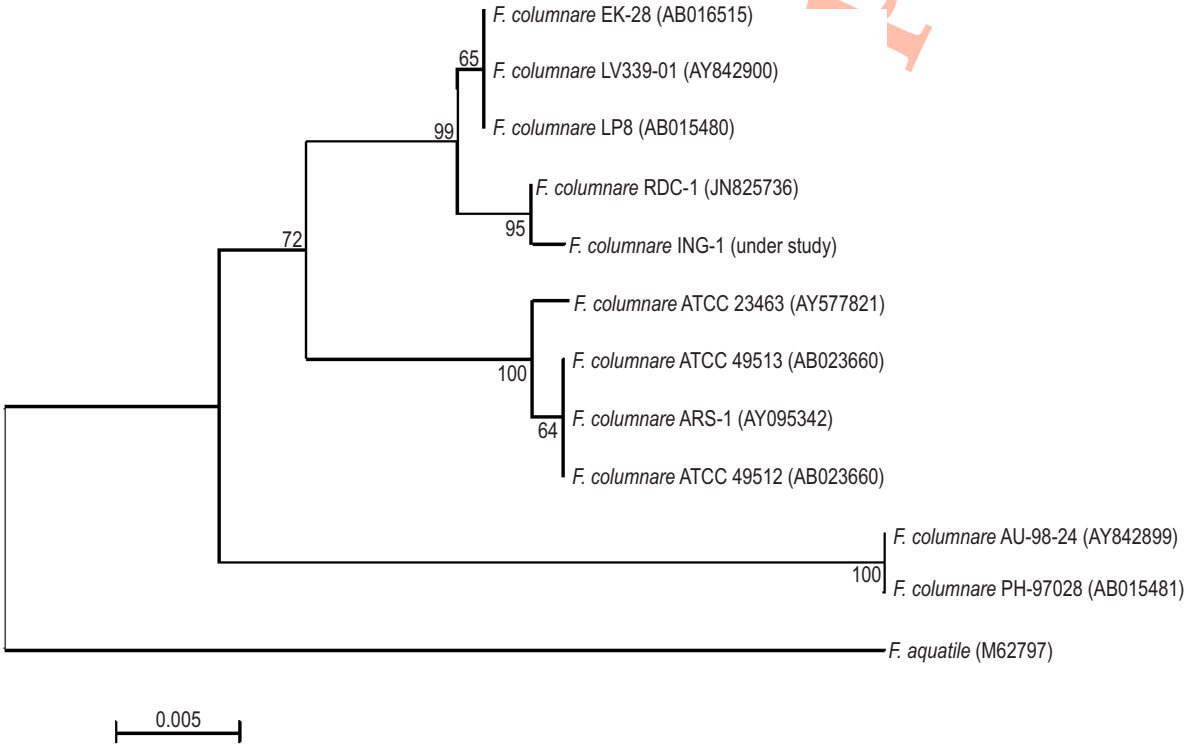

Fig. 6 : 16S rDNA gene based phylogenetic analysis of *Flavobacterium columnare* strain ING-1 showing phylogenetic relationship with other strains reported from India and abroad. The dendrogram was generated by the MEGA v 5.2 using the Neighbour-joining method with 1000 replicate bootstraps. *F. aquatile* (M62797) was used as references for out group source for rooting of the dendrogram. The studied *F. columnare* (ING-1) strain is highlighted in bold.

ING-1 corresponded to Genomovar II (Fig. 5) of Triyanto and Wakabayashi, (1999). Phylogenetic tree based on 16S rDNA sequences showed clustering of strain ING-1 the other *F. columnare* strains belonging to Genomovar II (Fig. 6). In bioassay, goldfish strain ING-1 was found to be pathogenic for rohu fingerlings in bath immersion challenge. All the experimental rohu fingerlings showed the sign of columnaris disease 72 hr post exposure. 70% mortality was observed within 10 days in 2.6 x 10^6 cfu ml^{-1} dose of strain ING-1. However, no mortality was observed in control group. *F. columnare* was re-isolated from the gills of moribund experimental fingerlings, which indicated that this pathogen was responsible for mortality.

Natural infection of *F. columnare* in goldfish indicated that goldfish are susceptible to morbidity and mortality associated with an Asian genomovar of *F. columnare* (Michel *et al*., 2002) Hence,

it is recommended that fish aquaculturists should avoid polyculture including goldfish because this fish may function as a carrier population for this pathogen (Bullard *et al*., 2013). Information on fish–host distribution and geographical distribution of *F. columnare* suggested that genomovar II is the most virulent genomovar among all the genomovars infecting warm-water fish species (Triyanto and Wakabayashi, 1999; Arias *et al*., 2004; Olivares-Fuster *et al*., 2007, 2011; Shoemaker *et al*., 2008). Therefore, this information is critically important to those attempting to control columnaris disease in warm-water aquaculture settings.

F. columnare strain (RDC-1) isolated from food fish (*Catla catla*) was described under Genomovar II, which is also referred to as Asian genomovar (Verma and Rathore, 2013) Findings of the present study are consistent with the previously reported strain RDC-1. Hence, it is suggested that genomovar II strains of *F. columnare* are prevalent in India. Moreover, it has also been established that Genomovar II of *F. columnare* is diversifying its host range; as environmental conditions of our country seem to be favorable for this strain. Apart from Genomovar II (Asian genomovar) which other Genomovar of *F. columnare* are present in India, is still unknown.

The present study findings indicated that *F. columnare* strain (ING-1) isolated from ornamental fish belonged to the most virulent Genomovar (Asian genomovar) of *F. columnare*. Since the ornamental fish trade will persist, the risk of major disease incursions and new emerging diseases will keep threatening the sector. Hence, appropriate health management measures should be maintained and effectively implemented and efforts to be directed to prevent entry of the emerging diseases into the system.

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