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Molecular characterization of *Flavobacterium columnare* isolated from a natural outbreak of columnaris disease in farmed fish, *Catla catla* from India

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Flavobacterium columnare **is currently one of the important bacterial pathogens causing columnaris disease in several farmed fish species across diverse geographies. A presumptive columnaris disease outbreak in farmed catla, *Catla catla* (Hamilton), was investigated with the aim of isolating and identifying the causative pathogen. *F. columnare* (strain RDC-1) was isolated from gills of infected fish and identified by conventional biochemical methods, and through species specific polymerase chain reaction and sequencing of the 16S rDNA for molecular identification. Strain RDC-1 belonged to genomovar II with ≥99% similarity to available 16S rDNA sequences of *F. columnare*, and also shared ≥70% DNA-DNA relatedness with known strains of *F. columnare*. Bath immersion studies of RDC-1 showed development of columnaris disease in catla fingerlings within 7 days, with a cumulative mortality of 83.3%. This is the first molecular confirmation of *Flavobacterium columnare* as a fish pathogen of farmed *Catla catla* in India.**

Key Words——*Catla catla*; DNA-DNA hybridization; *Flavobacterium columnare*; genomovar

Introduction

Flavobacterium columnare, an aetiological agent of "columnaris disease," is one of the important bacterial pathogens of freshwater fish (Bernardet et al., 1996; Shoemaker et al., 2008). Columnaris disease is manifested by the appearance of greyish white areas of erosion surrounded by a reddish hyperemic zone on the body surfaces, usually described as saddle-back appearance or extensive gill necrosis (Decostere et al., 1998; Tripathi et al., 2003). Diagnosis of columnaris disease is generally based on isolation of the pathogen and biochemical characterization. However, isolation and identification of *F. columnare* is difficult and uncertain using standard methods (Groff and LaPatra, 2000). This is due to its slow growth rate and its inhibition by other competitive and opportunistic bacteria during the cultivation process (Tiirola et al., 2002). Differentiation of *F. columnare* from other yellow pigmented bacteria by biochemical methods is not entirely certain as evident by renaming of *F. columnare* ATCC 43622 to *F. johnsoniae* at a later date (Darwish et al., 2004). Hence, molecular identification methods such as species-specific polymerase chain reaction (PCR) based on 16S rDNA are needed for confirmatory identification of *F. columnare* (Bader et al., 2003; Darwish et al., 2004).

The host range of *F. columnare* is continuously expanding and includes a wide spectrum of temperate

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and tropical freshwater fish. The latest fish species to be affected by this pathogen are 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. Based on restriction fragment length polymorphisms (RFLP) of 16S rDNA, three genomovars (I, II & III) of *F. columnare* are known (Triyanto and Wakabayashi, 1999). Studies show that 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.

India is the second largest aquaculture producer in the world with a production of 4.46 million ton (FAO, 2010). Carp-based freshwater aquaculture comprising mainly three Indian major carps, viz., catla, rohu *Labeo rohita* (Hamilton), and mrigal *Cirrhinus mrigala* (Hamilton), contributes about 97.5% of the total aquaculture production. Bacterial disease outbreaks coupled with environmental stressors pose a major threat to the growth of freshwater aquaculture farming and at times these disease investigations remain limited to gross clinical signs or a few presumptive laboratory tests. Columnaris disease was first reported from India by Kumar et al. (1986) and again by Dash et al., in 2009. Both the studies were based on isolation of yellow pigmented bacteria from diseased fish and presumptive identification of a pathogen on the basis of a few phenotypic traits. The present work describes the isolation and molecular confirmation of *F. columnare* from a natural outbreak of columnaris disease in farmed catla from India.

Materials and Methods

Disease investigation. Moribund catla (500−600 g) showing prominent yellowish-white lesions in gills and fin erosions were obtained from a commercial farm in Lucknow, Uttar Pradesh, India. The farm reported nearly 30% mortality in the pond within a week of the disease outbreak. Tentative diagnosis of columnaris disease was established by microscopic observation of typical bacterial haystacks in wet mounts from scrapings of skin, gills, and fins. Cytological examination of smears from skin, gills, and fins was also done by Romanowsky staining for demonstration of bacilli. Representative gill samples from affected fish were fixed in buffered 10% formalin. Tissues were processed in a routine manner for pathological examination, cut at 5-mm thickness, and stained with Maculum-Goodpasteur stain for demonstration of bacilli in gill sections.

Bacterial isolation and identification. A yellowish deposit from gills was streaked on Shieh agar and incubated at 28°C for 72 h. Yellow rhizoid colonies with spreading margins were subcultured and Gram stained to check purity. The isolate was provisionally 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). Chondroitinase production was demonstrated by the plate method as per the procedure described earlier (Xiao et al., 2009). Other phenotypic characteristics of the isolate were carried according to Bernardet (1989). An antimicrobial susceptibility test of the isolate was carried out by the disc diffusion method on Mueller-Hinton Agar. The identified bacterial isolate was termed RDC-1 and stored frozen at −80°C for further molecular characterization.

Molecular confirmation of RDC-1.

Species specific PCR: Genomic DNA was isolated from freshly grown broth culture of RDC-1 by the phenol-chloroform method and used as a template in PCR using two different species-specific primer sets, i.e. ColF/ColR (Darwish et al., 2004) and FvpF1/FvpR1 (Bader et al., 2003) for molecular confirmation of RDC-1.

16S rDNA based identification and genomovar confirmation: For bacterial identification, 16S rDNA of RDC-1 was amplified by PCR using universal primers 20F and 1500R (Weisburg et al., 1991). The obtained PCR product (∼1,500 bp) was gel purified, ligated into pTZ57R/T cloning vector (Fermentas, Burlington, ON) and sequenced on an automated ABI Sequencer using M13 sequencing primers. Bacterial identity of the 16S rDNA sequence of RDC-1 was deduced by BLAST search to ascertain its closest related sequences. A total of nine closest 16S rDNA sequences of *F. columnare*, comprising three genomovars, were aligned using the multiple sequence alignment program CLUSTAL W (Thompson et al., 1994). After removing non-base characters and ambiguous bases, 1,458 nucleotides were used for phylogenetic sequence analysis

using MEGA version 5 (Tamura et al., 2011). A tree was constructed by the neighbor-joining method and tree topology was evaluated by bootstrap analysis of 1,000 datasets. The nucleotide sequence of 16S rDNA of RDC-1 has been assigned GenBank accession number JN825736.

For confirmation of the genomovar, amplified 16S rDNA of RDC-1 was digested with restriction endonuclease *HaeIII* and electrophoresed on 2% agarose gel to obtain a genomovar-specific profile (Triyanto and Wakabayashi, 1999).

DNA-DNA hybridization. For DNA-DNA hybridization, the following type or reference strains were obtained from the BCCM^{TM}/LMG Bacteria Collection, University of Ghent, Belgium: *F. columnare* LMG 10406 (=ATCC 49512 (T)), *F. aquatile* LMG 4008 (T), *F. hibernum* LMG 21424 (T), *F. hydatis* LMG 8385 (T), *F. johnsoniae* LMG 1341 (T), *F. micromati* LMG 21919 (T), *F. psychrophilum* LMG 13179 (T) and *F. succinicans* LMG 10402 (T). In addition, a strain of *F. columnare* (PB06-113) was kindly provided by Dr. Andrew E. Goodwin (UAPB, Pine Bluff, AR). PCR-RFLP confirmed that this strain belonged to genomovar II and was also used for this study.

Genomic DNA relatedness of RDC-1 with *F. columnare* strains as well as other reference *Flavobacterium* species was examined using microplate quantitative DNA-DNA hybridization as described by Ezaki et al. (1989) except for colorimetric detection (Kusunoki et al., 1991) using streptavidin-alkaline phosphatase and pNPP as a substrate. Briefly, genomic DNA from each of the reference *Flavobacterium* species or strains (Marmur, 1961) was heat-denatured and immobilized to microwells by simple dry-adsorption. Dry-adsorption was conducted by adding DNA solution (each 300 ng/50 μl) to micro-wells, evaporating at 37°C overnight and heating to dryness at 60°C for 2 h on polystyrene plates (Hirayama et al., 1996). Genomic DNA of RDC- 1 was sonicated and labeled with photobiotin (Vector Laboratories) following the manufacturer's protocol and used as a probe for hybridization with reference species or strains. The hybridization time and temperature were 4 h and 42°C in 50% formamide, respectively. The percentage DNA similarity was calculated as $100\{(I_{test} - I_{blank})/(I_{ref} - I_{blank})\}$; where I_{test} is the intensity of hybridization between the stain to be tested and reference strain, I_{ref} is the intensity of hybridization of the reference strain with itself and I_{blank} is the background hybridization (Christensen et al., 2000). Each cross-hybridization was performed in duplicate with four replicates.

Virulence testing. Bath immersion was conducted to determine the virulence of RDC-1 as per the procedure described by Thomas-Jinu and Goodwin (2004). A single colony of RDC-1 was inoculated to 250 ml of Shieh broth and incubated at 28°C in a shaker incubator to obtain a turbidity of 0.30−0.35 at 550 nm. A week prior to the experimental infection, catla fingerlings of an average length of 8−10 cm were acclimatized at 28°C and fed daily with commercial feed. Bath immersion was done in three 50 L glass aquarium tanks as triplicates; each containing ten fingerlings. For the preliminary experiment, each tank was filled with 6 L of water and 200 ml of bacterial culture was added. After a 1 h bath exposure, fish were transferred to a fresh tank filled with 20 L water, aerated and held under observation. This resulted in 100% mortality within 48 h in all three tanks. Thereafter the experiment was repeated under similar conditions, but with a reduced dose of 30 ml culture. Fish were observed for 7 days and % cumulative mortality was calculated. Fin clippings from moribund fish of the test group were screened for appearance of typical haystacks in wet mounts. Ten fish were kept separately as a control under similar conditions as the experimental fish but were exposed to sterile Shieh broth.

Results

Disease investigation and bacterial identification

Presumptive diagnosis of columnaris disease was established on the basis of prominent yellowish-white deposits in the gills of diseased fish (Fig. 1), and haystack appearance of bacilli in the wet mounts of gills, skin and fins. However, another distinctive feature of columnaris disease, i.e. saddleback appearance, was not noted in any of the diseased fish. Romanowsky staining of the smears from skin, gills, and fins revealed the presence of typical long thin bacilli indicating extensive bacterial growth on the body surface. Similar bacilli were observed in Macullum-Goodpasteur staining of histological sections of diseased gills, along with erosion of secondary gill lamellae and leukocyte infiltration (Fig. 2). Bacterial isolation from gills yielded a pure culture of flat, yellow, rhizoid colonies having root-like structures that strongly adhered to the agar surface. This strain was termed RDC-1. The adherence property of RDC-1 was more pronounced in

broth culture grown in a glass flask under shaking conditions, where an accumulation of bacteria formed a ring on the upper level of the broth culture. Strain RDC-1 was positive for all the five characteristics of *F. columnare* suggested by Griffin (1992), to differentiate it from other yellow pigmented Gram negative long rods. Of these tests, chondroitinase production was visualized as a clear zone of nearly 22 mm around the bacterial growth on a Shieh agar plate containing chondroitin sulfate. The other phenotypic characteristics of RDC-1 were similar to the biochemical tests of *F. columnare* as reported by Bernardet and Grimont (1989). Thus, strain RDC-1 was biochemically identified as *F. columnare*. The reference strain of *F. columnare* (LMG-10406) was included in the study for comparison. An antimicrobial susceptibility test of RDC-1 showed resistance to polymixin B, neomycin, kanamycin and tobramycin. The strain was susceptible to ampicillin, erythromycin, streptomycin, oxytetracycline and chloramphenicol.

Fig. 1. Diseased catla, *Catla catla* (Hamilton), fish showing prominent yellowish deposits in the gills.

A presumptive clinical sign of columnaris disease caused by *Flavobacterium columnare*.

Molecular confirmation of RDC-1

Molecular identification of RDC-1 was done by species-specific PCR and 16S rDNA sequencing. The primer sets (ColF/ColR & FvpF1/FvpR1) produced specific amplicons of 675 bp and 1,193 bp, respectively. The 16S rDNA sequence of RDC-1 showed 99.7% similarity with *F. columnare* strains LP-8 and LV-339 and 99.3% with EK-28. It also showed $\geq$ 97% similarity with other published *F. columnare* strains isolated from different geographic locations. In the 16S-PCR-RFLP, the restriction profile of RDC-1 corresponded to genomovar II (Fig. 3) of Triyanto and Wakabayashi (1999). The phylogenetic tree based on the 16S rDNA sequences showed the clustering of RDC-1 with *F. columnare* strains belonging to genomovar II (Fig. 4).

DNA-DNA hybridization

DNA-DNA hybridization was done to confirm the DNA relatedness of RDC-1 to two strains of *F. columnare* and eight other *Flavobacterium* species. Strain RDC-1 was found to show highest DNA homology

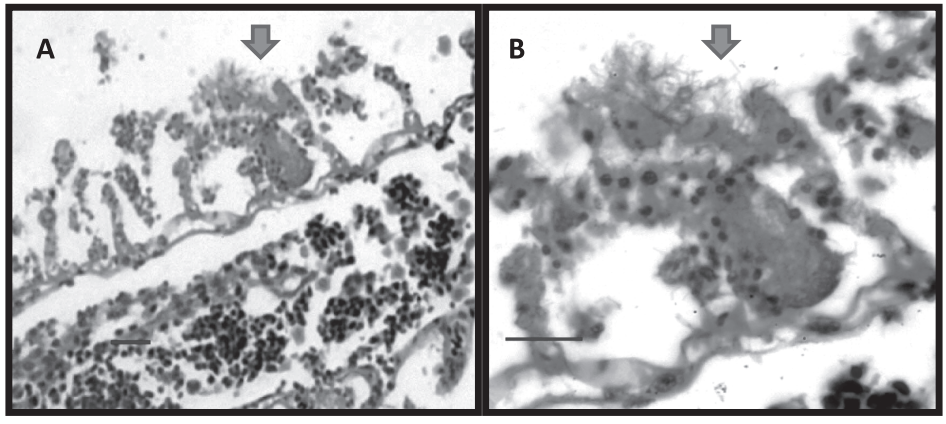

Fig. 2. Histological section of diseased gills from catla, *Catla catla* (Hamilton), infected with columnaris disease.

Erosion of secondary gill lamellae, leucocyte infiltration and bacterial mass can be easily seen (Arrows) in Macullum-Goodpasture staining of infected gill tissue at lower magnification (A). Long, thin bacterial cells seen in gill lamellae at higher magnification (B). Bar represents 20 μm.

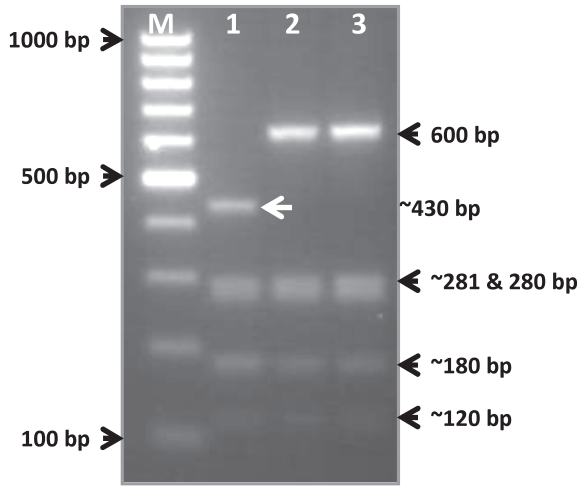

Fig. 3. Genomovar confirmation of *F. columnare* strain RDC-1 by restriction digestion of 16S rDNA with *Hae* III.

Lane M: 100 bp ladder; Lane 1: Type strain LMG-10406=genomovar I (white arrow); Lane 2: strain RDC-1=genomovar II; Lane 3: strain PB06-113=genomovar II.

(78%) with strain PB06-113 and 53% homology with LMG 10406 strain of *F. columnare*. It showed less (48–32%) homology with other species of genus *Flavobacterium* (Fig. 5).

Virulence testing

Strain RDC-1 was pathogenic to catla fingerlings by the bath immersion method. The initial experiment indicated that 1-h exposure with 200 ml broth culture of RDC-1 (equivalent to 1.8×10^7 cfu ml $^{-1}$ in 6 L water) caused 100% mortality within 48 h of bath treatment. However, a reduced dosage of 30 ml culture (equivalent to 2.6×10^6 cfu ml $^{-1}$ in 6 L water) resulted in a cumulative mortality of 83.3% within 7 days of infection (Fig. 6). No mortality was observed in the control group. Fin clippings from the moribund fish showed the appearance of typical haystacks in wet mounts, indicating experimental columnaris infection in the test group.

Discussion

With the intensification of stocking rates and suboptimal water quality parameters, Indian freshwater aquaculture is witnessing the emergence of several bacterial diseases. Columnaris disease is a serious, contagious and stress-induced infection caused by *F. columnare*, considered to be an opportunistic pathogen of freshwater fish, having significant economic impact. Early detection and exact identification of this pathogen would prevent its spread and reduce economic losses to fish farmers (Panangala et al., 2007). In this study, a presumptive columnaris disease outbreak in catla from a grow-out fish pond having nearly 30% mortality was investigated, with the aim of isolating and identifying the causative pathogen. *F. columnare* was isolated from gills of infected fish and identified by conventional biochemical and molecular methods. Phe-

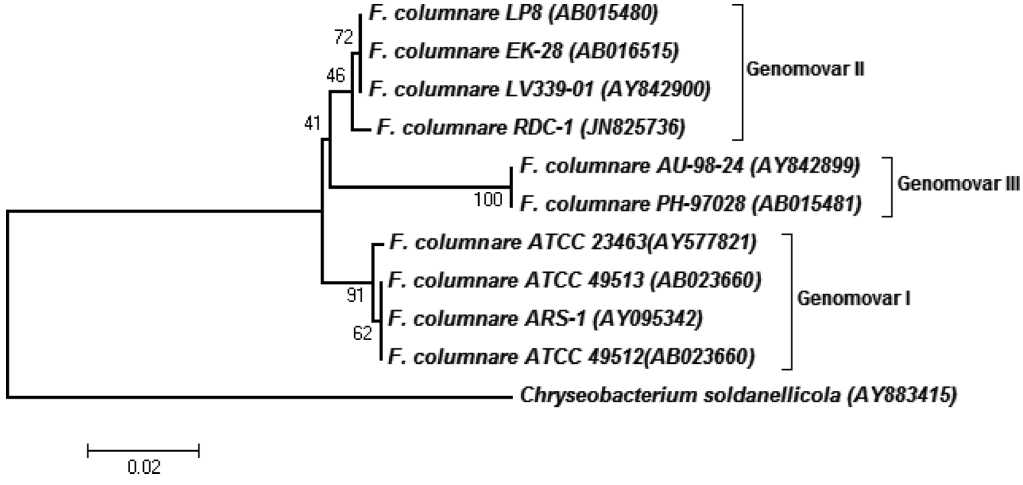

Fig. 4. Neighbor-Joining phylogenetic tree based on 16S rDNA sequences of strain RDC-1 and other *F. columnare* strains representing different genomovars.

Chryseobacterium soldanellicola (AY883415) was used as out group for rooting the tree. Numbers at nodes are bootstrap values from 1,000 replicates. Bar indicates substitution per nucleotide position.

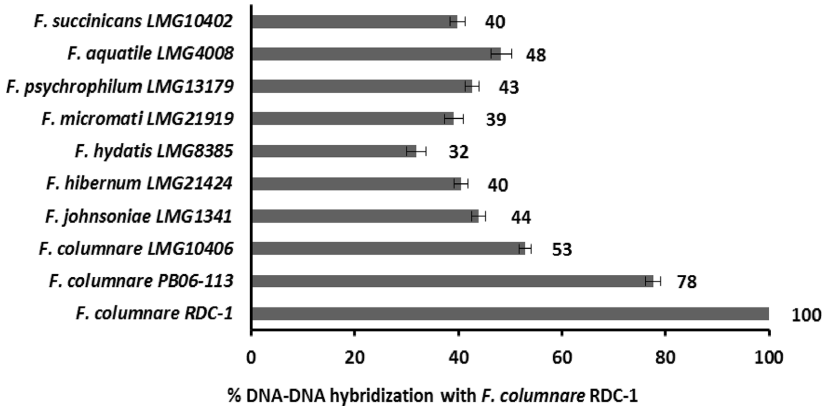

Fig. 5. Homology values of *F. columnare* RDC-1 with other reference *F. columnare* strains and some *Flavobacterium* species based on DNA-DNA similarity.
The bars at the tops of the columns indicate standard deviations.

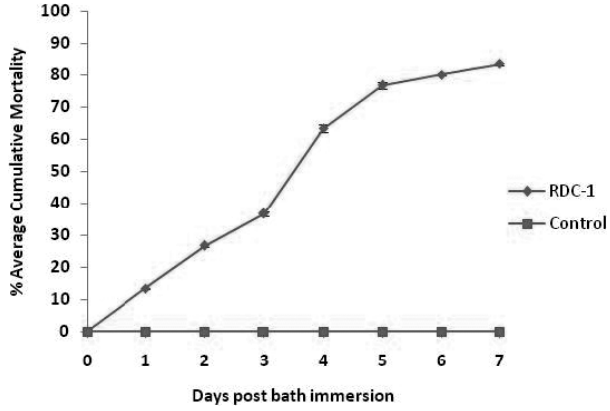

Fig. 6. Mean cumulative mortality of catla, *Catla catla* (Hamilton), fingerlings following bath immersion with *F. columnare* RDC-1 strain (genomovar II) after 7 days of treatment.
Infectious dose was 2.6×10^6 cfu ml $^{-1}$.

notypic traits of this isolate resembled type strain of *F. columnare* reported by Bernardet et al. (1996). This strain, RDC-1, showed ≥99% similarity of 16S rDNA to Japanese strains of *F. columnare*, LP-8 and EK-28. Species identification has been already defined as ≥ 99% similarity of the 16S rDNA gene sequence to the sequence of its closest bacterial relative in the GenBank database (Drancourt et al., 2000). In addition to 16S rDNA gene sequencing, DNA-DNA hybridization is another recommended method for defining bacterial species (Martinez-Murcia, 1999), wherein a relative binding ratio of ≥70% under optimal conditions is considered to be the border line for species differentiation (Wayne et al., 1987). Strain RDC-1 showed 78% DNA relatedness to *F. columnare* (PB06-113); thereby fulfilling the other recognized criterion for species identification. As per the given results, RDC-1 conforms to the above empirical cut-off points and provides vital evidence for its molecular identification as *F. columnare*. To the best of our knowledge, this is the first study on molecular confirmation of *F. columnare* from India.

Genetic variability in the 16S rDNA gene sequences and/or DNA-DNA hybridization similarities form the basis for classifying the strains of *F. columnare* into three genomic groups termed as genomovars (Triyanto and Wakabayashi, 1999). On the basis of variability in the 16S rDNA, RDC-1 was ascribed to genomovar II, which was previously referred as the Asian genomovar (Michel et al., 2002).

In pathogenicity trials, strain RDC-1 resulted in a cumulative mortality of 83.3% to catla fingerlings within 7 days of a bath immersion. Previous studies have used abrasion (Bader et al., 2006), temperature stress (Thomas-Jinu and Goodwin, 2004) or feed deprivation (Shoemaker et al., 2003) to induce mortality in channel catfish fingerlings by immersion bath challenge of *F. columnare*. In an another study, the disease was reproduced in channel catfish fry by bath immersion without any stress factors, wherein the cumulative mortality ranged from 92 to 100% on challenge with genomovar II isolates of *F. columnare* in 14 days (Shoemaker et al., 2008). In our study also, genomovar II strain RDC-1 was found to be highly virulent to catla fingerlings in the absence of any stress factors. This also proves that the pathogenic strain (RDC-1) of *F. columnare* was the etiological agent responsible for columnaris disease outbreak in farmed catla. Our future studies will focus on development of a vaccine against columnaris disease for protection of Indian Ma-

jor Carps, using the RDC-1 strain of *F. columnare* in India.

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