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Rapid Detection of *Flavobacterium columnare* Infection in Fish by Species-specific Polymerase Chain Reaction

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Abstract

This study describes rapid detection of *Flavobacterium columnare* induced columnaris disease in cultured freshwater fish, viz., *Labeo rohita, Ctenopharyngodon idella, Puntius* sp. and *Anabas testudineus* by species-specific polymerase chain reaction. Gill discolouration, yellow necrotic areas, white patches on gill, saddle back and erosion of scales were the prominent clinical signs in all diseased fish, except *Puntius* sp., which had typical signs of ulcer at the base of dorsal fin. Of the nine disease cases, eight were found columnaris positive through culture independent species-specific PCR. The two sets of *F. columnare* specific primers such as ColF, ColR and Col72F, Col1260R yielded amplicons of around 675 bp and 1000 bp, respectively in all positive samples. Phylogenetically, the nucleotide sequences of the positive samples namely, C1 and RG1 formed monophyletic group with *F. columnare*, thus confirmed the infection as columnaris.

Keywords: *Flavobacterium columnare*; Columnaris; Species-specific PCR; Phylogeny

Introduction

Flavobacterium columnare, the causative agent of columnaris, belongs to the family Flavobacteriaceae [1]. Most freshwater fish (cultured and wild) are considered susceptible to *F. columnare* [1-3]. It has a worldwide distribution, causes remarkable economic losses, and severely affects cold and warm freshwater fish such as *Oreochromis niloticus* [4], *Clarias batrachus and Labeo rohita* [5], *Ictalurus punctatus* [6], *Catla catla* [7], *Anabas testudineus* [8], *Oncorhynchus mykiss* [9] and *Carassius auratus* [10]. The presumptive diagnosis of columnaris based on the clinical signs of host and the characteristic morphologic features of *F. columnare* and the definite diagnosis are more extensive and laborious that requires sophisticated laboratory testing. Molecular analysis is one of the major parts of definitive diagnosis of columnaris. Polymerase chain reaction (PCR) based diagnostic tests have been used for the detection of *F. columnare* nucleic acid in infected skin samples and in bacterial cultures [11]. Several *F. columnare* specific PCR protocols have been developed [11-13]. These techniques use species-specific primers to amplify the 16S rRNA gene fragment and to differentiate closely related bacteria (*F. psychrophilum*, *F. aquatile*, *F. branchiophilum*) and other fish pathogens like *Edwardsiella* sp., *Aeromonas* sp. and *Streptococcus iniae*. The sensitivity of the protocol, determined by spiking fish tissues with *F. columnare*, was reported to range from 30 to 59 colony forming units/mg of tissue [11].

Metagenomics is defined as the culture-independent genomic analysis of an assemblage of microorganisms [14]. A TaqMan-based real-time PCR targeting a 113 bp nucleotide region of the chondroitin AC lyase gene of *F. columnare* has also been developed [15] for the detection and quantification of *F. columnare* in tissues (blood, gills and kidney) of infected fish. West Bengal is one of the most productive states in India from aquaculture point of view. With the intensification in culture, there has been an increase in the incidence of diseases in cultured fish. Dash et al. [16] reported the prevalence of bacterial gill disease (16.14 ± 2.24%) and columnaris disease (13.80 ± 1.56%) in cultured carps of West Bengal along with other diseases. In a recent study, *F. columnare* was isolated and characterized genotypically from *Clarias batrachus* cultured in West Bengal [17]. The aim of this study was to explore the usefulness of culture independent analysis by species-specific polymerase chain reaction to identify *F. columnare* infection rapidly.

Materials and Methods

Sample collection

During the routine fish disease surveillance in the winter periods of 2014 and 2015, nine disease cases – three from Mymensingh, Bangladesh (Cases 1-3), five from West Bengal, India (Cases 4-8) and one from Lembuchera, Tripura, India (Case 9) - with white patches on gill, tail rot, skin discoloration, scale loss, skin sloughing, and emaciation (Table 1) were examined as per Heil [18] and OIE [19]. The samples include five lots of *Anabas testudineus* of Mymensingh, Bangladesh (Cases 1-3) and Baruipur, South 24 Parganas district, West Bengal, India (Cases 4-5), two lots of *Labeo rohita* - one each from Bagnan, Howrah district (Case 6) and Bamanghata, South 24 Parganas district (Case 7), one lot of *Ctenopharyngodon idella* from Chakgaria, Kolkata (Case 8), West Bengal, India and one *Puntius* sp. (Case 9) from Lembuchera, West Tripura district, Tripura, India (Table 1). At site, the behavioral abnormalities, gross and clinical signs of diseased fish were recorded. On each sampling day, a lot comprising infected and weak fish (n=5) with typical disease symptoms and also apparently healthy fish (n=5) from unaffected ponds were brought to the laboratory in oxygen filled polythene bags separately for further analysis. In case of freshly dead fish (n=5) desirable tissue portions were dissected, pooled and fixed in 70% alcohol at site for culture independent analysis by species-specific PCR. At laboratory, the infected fish were dissected aseptically and the affected tissues (0.1-0.2 g from each fish) were cut and pooled. The pooled samples were fixed separately in 70% alcohol. A total of 23 pooled samples of diseased fish comprising different body

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Case	Date of sampling	Place	Location (Latitude and Longitude)	Fish species and size	Mortality (%)	Examined tissues	PCR results
1	10.10.14	Kalibari, Mymensingh, Bangladesh[a]	Lat.24˚43'00"N Long. 90˚10'11"E	*Anabas testudineus* (95-122 g)	35	Gill Muscle	+ +
2	10.10.14	Tarakanta, Mymensingh, Bangladesh[a]	Lat.24˚52'26"N Long. 90˚25'10"E	*Anabas testudineus* (96-128 g)	40	Gill Muscle Kidney Spleen	+ + - -
3	10.10.14	Ishwarganj, Mymensingh, Bangladesh[a]	Lat.24˚41'36"N Long. 90˚35'48"E	*Anabas testudineus* (90-112 g)	50	Gill Muscle	+ +
4	07.10.15	Baruipur, South 24 Parganas, West Bengal, India	Lat.22˚21'54"N Long. 88˚25'28"E	*Anabas testudineus* (40-56 g)	22	Gill Muscle Kidney	+ + +
5	17.10.15	Baruipur, South 24 Parganas, West Bengal, India	Lat.22˚21'48"N Long. 88˚25'32"E	*Anabas testudineus* (45-60 g)	30	Gill Muscle Kidney Spleen	+ + + +
6	04.12.15	Bagnan, Howrah, West Bengal, India	Lat.22˚28'56"N Long. 87˚56'31"E	*Labeo rohita* (105-120 g)	95#	Gill Muscle Kidney	+ - -
7	04.12.15	Bamanghata, South 24 Parganas, West Bengal, India	Lat. 22˚29'89" N Long. 88˚29'33"E	*Labeo rohita* (140-200 g)	1.5	Gill** Muscle	+ -
8	11.12.15	Chakgaria, Kolkata, West Bengal, India	Lat. 22˚28'47"N Long. 88˚24'04"E	*Ctenopharyngodon idella* (15-25 g)	Negligible	Gill Muscle*	+ +
9	23.12.15	Lembuchera, West Tripura, Tripura, India	Lat. 23˚54'20"N Long. 91˚18'31"E	*Puntius* sp. (≈ 200 g)	ND	Muscle	-

[a]Samples were collected, processed and supplied by M/S Quality Feeds Limited, Dhaka - 1230, Bangladesh. Freshly preserved samples (within 2 days of collection) in 70% alcohol were used for laboratory analysis; **: Nucleotide sequence code: RG1; *: Nucleotide sequence code: C1; Muscle=Skin and muscle tissue; ND: No data; #: Kidney myxoboliasis.

Table 1: Sampling details of diseased fish and identification of *Flavobacterium columnare* by species-specific polymerase chain reaction.

parts such as skin and muscle tissue (9), kidney (4), gill (8) and spleen (2) were collected and processed analysis. From the healthy fish (cases 1-8), only gill samples (0.1-0.2 g from each fish) were collected, pooled for each case and fixed as above. The case 9 had only one 70% alcohol fixed muscle tissue sample (*Puntius* sp.) received from the College of Fisheries, Lembuchera, Tripura, India.

DNA isolation and PCR detection of *Flavobacterium columnare*

The DNA isolation was done by using NucleoSpin tissue kit (Clontech, Takara) and stored at -20˚C for PCR work. The PCR of species-specific 16S rRNA gene fragment was performed with two sets of primers namely ColF 5'-CAGTGGTGAAATCTG-GT-3', ColR 5'-GCTCCTACTTGCGTAGT-3' [12] and Col-72F 5'-GAAGGAGCTTGTTCCTTT-3', Col-1260R 5'-GCCTACTTGCG-TAGTG-3' [13] for the detection of *F. columnare* infection. A 25 µL PCR mixture contained 2.5 mM $MgCl_2$, 10X reaction buffer, 10 nmole each 200 µM dNTPs, 10 pmole of each primer, 2 units of Taq DNA polymerase (Takara) and 50 ng template DNA. Amplification was done by initial denaturation at 94˚C for 30 sec followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 54˚C for 1 min and extension at 72˚C for 1 min. The final extension was at 72˚C for 5 min. The amplicons were examined and visualized by electrophoresis in 2.0% agarose gel in TAE buffer. The gel was stained with ethidium bromide and viewed in Gel Doc System (G:Box Syngene UK). Documentation of columnaris positive DNA samples was by demonstrating 675 bp and 1000 bp bands on agarose gel, respectively for ColF and ColR as well as Col-72F and Col-1260R primers.

Sequencing and phylogenetic analyses

After PCR amplification, two amplicons of *F. columnare* specific primers ColF and ColR, viz., C1 of case 8 - DNA isolated from the muscle tissue of caudal peduncle region of *C. idella* and RG1 of case 7 - DNA isolated from the gill of *L. rohita* were sequenced at the Genomics Division, Xcelris Labs Ltd, Ahmedabad, India. The edited sequences were compared against the GenBank database of the National Centre for Biotechnology Information (NCBI) by using the BLAST (Basic Local Alignment Search Tool) program (http://blast.ncbi.nlm.nih.gov). For the construction of phylogenitic tree, besides the edited sequences of C1 and RG1, 20 more gene sequences comprising 10 *F. columnare*, 4 *Flavobacterium* sp., 5 strains of Gram negative long rods, viz., *Flectobacillus roseus, Chryseobacterium indologenes, Tenacibaculum maritimum* and *Sphingobacterium thalpophilum* and a Gram positive strain *Bacillus cereus* were selected from the NCBI GenBank database. Data analysis and multiple alignments were performed by using ClustalW 1.6 (MEGA6). The evolutionary history was inferred using the Neighbor-Joining method [20]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [21]. The evolutionary distances were computed using the Kimura 2-parameter method [22]. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 [23]. The nucleotide sequences of the amplicon C1 (Accession number KX452119) and amplicon RG1 (Accession number KX452118) have been deposited in NCBI GenBank.

Results

All the infected fish, except the case 9 (*Puntius* sp.), exhibited white patches on gill, tail rot, skin discoloration, scale loss, skin sloughing, and emaciation, resembling columnaris infection. Mortalities were severe in *Anabas testudineus,* ranging from 22 to 50% of the total population. On the other hand, it was negligible to 1.5% in carps, except the case 6, where the *L. rohita* experienced 95% mortality due to kidney myxoboliasis (Table 1). All the gill samples and 6 out of 9 muscle tissue samples were PCR positive (Figures 1A and 1B). The gill and muscle tissue samples of all *A. testudineus* were PCR positive. The

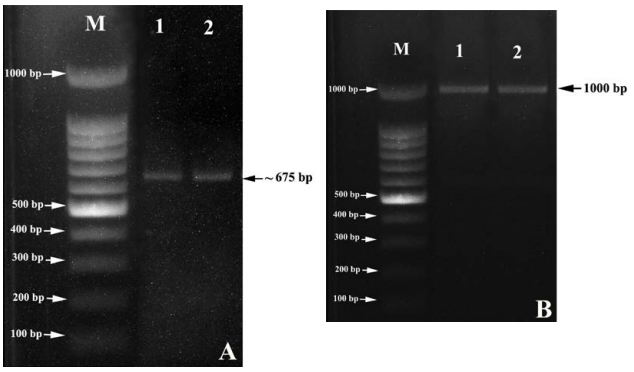

Figure 1: Agarose gel (2%) showing *Flavobacterium columnare* specific 16S rDNA gene fragment amplicons of primers (A): ColF, ColR and (B): Col72F, Col1260R. Lane M: 100 bp molecular weight DNA marker; Lane 1: RG1 - DNA isolated from the gill of *Labeo rohita*; Lane 2: C1 - DNA isolated from the muscle tissue of caudal peduncle region of *Ctenopharyngodon idella*.

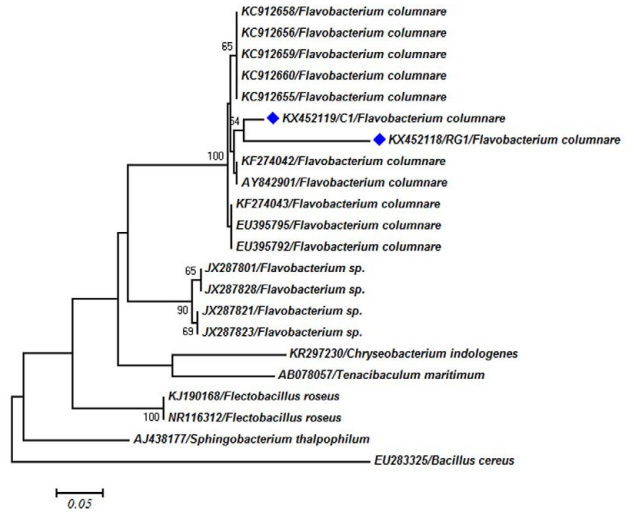

Figure 2: Molecular phylogenetic tree produced by Neighbor-Joining method. Numbers at nodes indicate bootstrap confidence values (1000 replicates). The GenBank accession number is provided for each species. Units of evolutionary distance are in the number of base substitutions per site.

internal organs of *A. testudineus* from Baruipur were found to be PCR positive for *F. columnare.* Of the nine fish disease cases, eight were confirmed as *F. columnare* infection by species-specific PCR (Table 1). The gill samples of all apparently healthy fish were PCR negative. The edited gene sequences of C1 and RG1 were of size 675 bp. The *Puntius* sp., which exhibited the clinical signs of ulcer disease, was negative for *F. columnare*. In phylogenetic tree, the nucleotide sequences of C1 and RG1 formed a monophyletic branch and clustered with *F. columnare* with 98% sequence similarity. The gene sequences of C1 and RG1 were closely related to *F. columnare* KC912655 (97%); and *F. columnare* KF274042 (96%), respectively (Figure 2).

Discussion

The culture independent PCR method with species-specific primers was found to be more sensitive for the detection of *F. columnare* infection in fish than the standard culture techniques because *F. columnare* is often overgrown by the fast-growing bacteria such as *Pseudomonas* spp. and others [24]. In our earlier studies, we could isolate only one *F. columnare* (accession number KU851952) and many related *Flavobacterium* spp. [17,25] out of 55 samples. Molecular characterization by culture independent species-specific PCR revealed *F. columnare* infection in all diseased *A. testudineus and carp samples collected in West Bengal, India and Bangladesh.* Likewise, *F. columnare* infection was reported in *A. testudineus [8], Labeo rohita* [5] *by culture based techniques* and from *Ctenopharyngodon idella* [26] by species-specific PCR. Although reports on *Flavobacterium* infection in *Puntius* sp. are available [8], our observation with *Puntius* sp. resembling the clinical signs of ulcer from Lembuchera, Tripura was PCR negative, when tested using *F. columnare*-specific primers. It contradicted the earlier report on the observations and identification of *F. columnare* from ulcers [3].

Of the four kidney samples processed, two cases of *A. testudineus were* PCR positive, indicating the systemic *F. columnare* infection, which is most often found with acute infections [27]. Likewise, the analysis of two spleen samples of *A. testudineus* yielded one PCR positive. On the other hand, all the gill samples and six out of nine skin and muscle tissues were PCR positive. These observations corroborate the findings of Welker et al. [11], who opined that detection of *F. columnare* in the gill was more sensitive than in the muscle and other internal organs. Adherence to the gill is an important aspect of the pathogenesis of columnaris [28]. The gills may, therefore, be a good choice for detection of *F. columnare* in fish as water is actively pumped across gill lamellae. The gill samples of apparently healthy fish were, however, PCR negative. Though few earlier studies reported systemic infection with *F. columnare* [29-31], according to many studies columnaris disease is more renowned as cutaneous disease [1,3,32] and gill disease [7,10]. Bader et al. [33] demonstrated mucus to be the best location for molecular detection of *F. columnare* and blood the worst. Phylogenetic analysis based on the nucleotide gene sequences indicated that the novel sequences of C1 and RG1 belonged to the family Flavobacteriaceae, phylum Bacteroidetes, and fell within the evolutionary radiation of the genus *Flavobacterium*. The 16S rDNA gene sequences of all *F. columnare* strains clustered together with C1 and RG1 and formed a monophyletic group, which support their correct diagnosis. The gene sequences of both C1 and RG1 were clustered together with 98% DNA similarity, which further enlighten the accuracy of the claim.

Conclusion

The results of the present study demonstrated the usefulness of culture independent analysis by species-specific polymerase chain reaction to detect columnaris disease using *F. columnare*-specific primers. It has more potency to save time and reduce labour over the culture dependent methods.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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