



Isolation and immunohistochemical identification of *Flavobacterium psychrophilum* from the tissue of catfish, *Clarias batrachus*

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Abstract

This study deals with the identification of bacteria in paraffin embedded tissue of Indian catfish, *Clarias batrachus* experimentally infected with *F. psychrophilum* to ascertain its precise role in the occurrence and state of carrier stage of coldwater disease in asymptomatic fishes. Significant ($P < 0.05$) mortality was recorded in *C. batrachus* of this region due to persistent occurrence of creamish/yellowish with non smooth edged colonies of rod shaped flexible gram negative bacterium, *F. psychrophillum*. Serodiagnosis made through polyclonal antiserum dilutions (raised against H antigen of Aqb Fb – 6 isolate in rabbit) revealed that staining intensity of this bacterium was dilution dependent as tissue and smears treated with 1:300 and 1:500 antiserum dilutions conferred close relationship at immunofluorescence level with *F. psychrophilum* and stained deeply in the tissues and smear prepared from liver, kidney and intestine of experimentally infected *C. batrachus*. Result of this study would be helpful in identifying the fish pathogenic bacteria in carrier fish where mortality occurs without explicit clinical signs of disease.

Key words

Clarias batrachus, *Flavobacterium psychrophilum*, Immunofluorescence, Polyclonal antiserum

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Introduction

Indian catfish, *Clarias batrachus* has significant role in average human diet of local people of this region. But its production has been impeded due to persistent occurrence of *F. psychrophillum* infection with cultured as well as feral freshwater fish of Nanak sagar and Sarda reservoirs (Prasad, 2009; Prasad *et al.*, 2011 and Verma *et al.*, 2011). The ubiquitous distribution of this bacterium in various aquatic ecosystems (Nematollahi *et al.*, 2003 and Nilsen *et al.*, 2011) and its severe consequences on fish production has been noted (Toranzo *et al.*, 2004; Suomalainen *et al.*, 2005). In fact *Myxobacterium sp.* referring to *Myxobacter* belongs to *Cytophaga / Flexibacter* (Bruno, 1992) or *Flavobacterium* has been rechristened as *F. psychrophilum* (Bernardet *et al.*, 2002). Isolation and characterization of *Myxobacterium sp.* using selective medium (Durmaz *et al.*, 2012) and accomplishing biochemical tests (Austin and Austin, 2007) have been made. Its flexible characters in terms of their physico-chemical requirements generally lead to improper diagnosis and use of therapeutic agents resulting into a great loss to fish industry (Barnes and Brown, 2011). Moreover, its extreme high (10^{10} cfu ml⁻¹)

dosage leads to the development of clinical signs of coldwater disease as it has been noted in case of rainbow trout, *Oncorhynchus mikis* (Michel and Gracia, 2003). Therefore, it is imperative to diagnose this disease at the initial stage of development so that an appropriate strategy can be developed to mitigate the occurrence of cold water diseases in cultured fish.

The conventional methods employed to identify this bacterium seem to be cumbersome. On the other hand, immunofluorescence technique has been found to be useful (Panangala *et al.*, 2006 and Misaka and Suzuki, 2007) in finding out the possibility of presence of this bacterium in carrier fish where clinical signs are sometimes less distinctive. This technique provides additional information on the cellular localization of this bacterium in which polyclonal mono-specific antisera raised against the targeted bacterium is used to stain the histological sections (Hibi *et al.*, 2007 and Misaka *et al.*, 2008) and smears prepared from 18–24 old bacterial colonies.

To ascertain its precise role in the occurrence of disease it is necessary to identify whether the asymptomatic fishes are a

carrier of the bacterial pathogen or not. Since the persistent occurrence of this bacterium was recorded in dead/ moribund fishes, therefore this study was planned to identify the bacterium in paraffin embedded experimentally infected tissue of catfish *C. batrachus* with *F. psychrophillum* and compared with its smear preparation.

Materials and Methods

Bacterial isolation and identification: Bacteriological samples were obtained by inserting sterilize inoculating loop into the lesion, liver, kidney and intestine of symptomatic fish (exhibiting overt sign of disease) and streaked onto selective medium supplemented with casein (0.3% w/v), tryptone (0.2% w/v), yeast extract (0.05% w/v), calcium chloride (0.03% w/v) and agar (1.00% w/v) and sterilized with antibiotics solution (Erythromycin,

10 $\mu\text{g ml}^{-1}$ and Neomycin sulphate 10 $\mu\text{g ml}^{-1}$ or polymyxin sulphate, 256 IU ml^{-1}). In case of moribund fish organs like liver, kidney, spleen and necrotized tissues were dissected out and preserved in 0.3% NaCl solution, homogenized in the same solution, centrifuged at 5000 rpm and supernatants were used for the isolation of bacteria on Sheih medium (Decostere *et al.*, 1997) supplemented with polymyxin sulphate 256 IU ml^{-1} . All the inoculated plates were incubated at $20 \pm 2^\circ\text{C}$ temperature for about 18–24 hr and isolated strains were identified (Verma *et al.*, 2007) on the basis of selected biochemical tests.

Experimental infection of fish : Fifty *C. batrachus* with average length of 23 ± 4 cm and weight 90 ± 5 g were collected from Nanak Sagar reservoir, Udham Singh Nagar, Uttarakhand, India and checked for injury and disease conditions and those found healthy were used for further study. Fish were first treated with

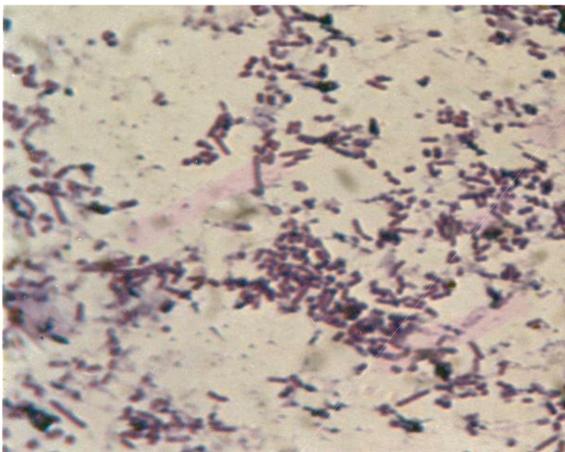


Fig. 1 : Showing Gram stained *F. psychrophillum* in smear of pure culture (X 1000)

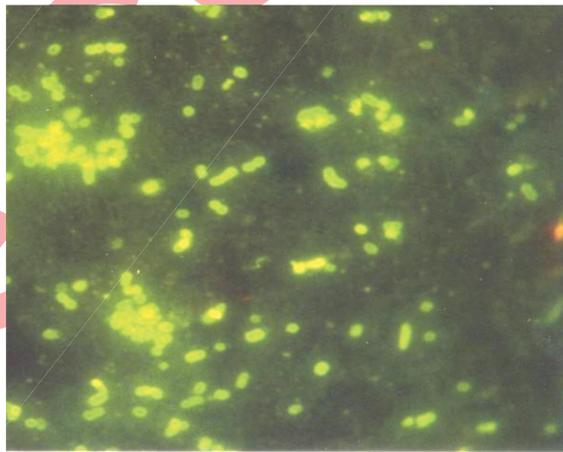


Fig. 2 : Showing brilliantly stained *F. psychrophillum* in smear of pure culture treated with anti serum dilution 1:300 (X 1000)

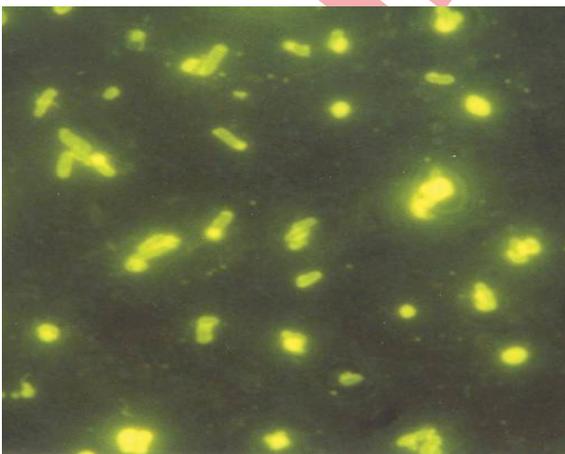


Fig. 3 : Brilliantly stained selected *F. psychrophillum* cells in smear of pure culture treated with anti serum dilution 1: 500 (X 1000)

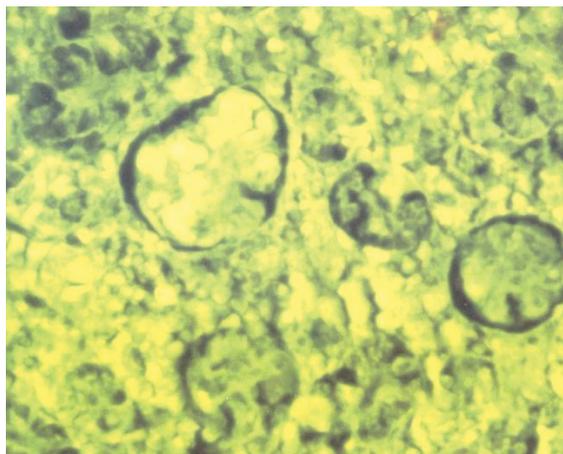


Fig. 4 : Showing marked tubules and lymphoid cells of kidney of control fish *C. batrachus* (X 1000)

0.01% KMnO_4 solution for 5 min to remove parasitic infection, if any and then kept @ 25 fish/tank in plastic pools (1m X 1m) filled with 300 l of non-chlorinated freshwater and acclimatized for 10 days. To check the lethal dose (LD_{50}) of bacterium, a set of ten healthy *C. batrachus* were subjected to intramuscular inoculation @ $2 \times 10^{5-7}$ cfu ml^{-1} live cells of *Flavobacterium* (Aqb Fp 6 isolate) and the values were determined (Verma *et al.*, 2011). Further, forty fishes were divided into two sets, one with triplicate comprising 10 ($n = 10$) *C. batrachus* in each replicate and inoculated with 0.2 ml of bacterial suspension (2×10^5 cfu ml^{-1}) and the fish of the other set ($n=10$) received 0.5 ml of sterilized PBS only and served as control. They were transferred to glass aquaria (12 X 12 X 36") filled with non chlorinated tap water (70 l) in which temperature was maintained at 20 to 25°C by using thermostat (Sobo, China). The experiment was conducted for 30 days and tissue samples of experimentally infected fishes along with control were collected aseptically at 0, 7, 14, 21 and 30 days

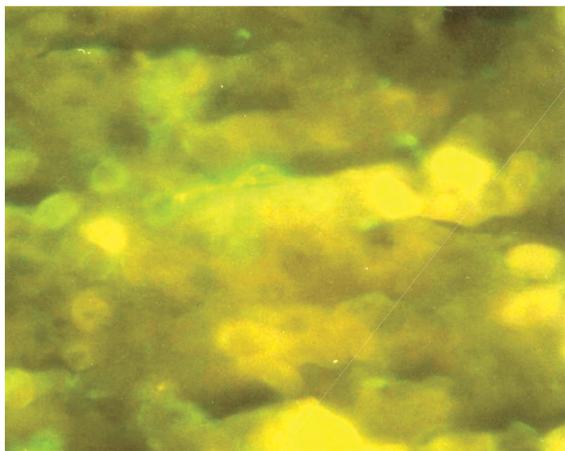


Fig. 5 : Showing brilliantly stained *F. psychrophillum* (arrow) in the kidney of *C. batrachus* treated with anti serum dilution 1:300 (X1000)

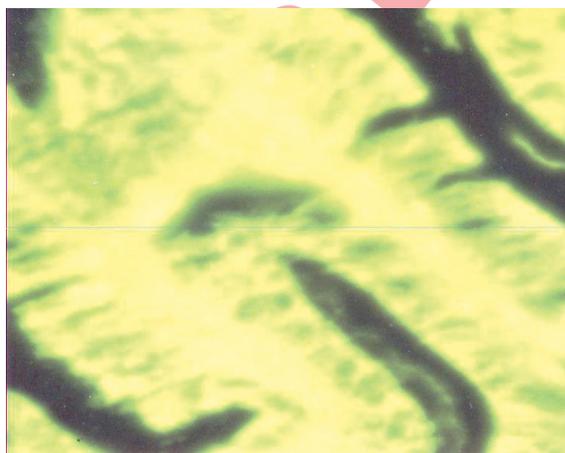


Fig. 6 : Showing marked epithelial cells of intestine of control fish (X1000)

interval. During the experiment fishes were fed @ 5% of their body weight with laboratory prepared pelleted feed containing 35% protein (comprising rice bran 25, wheat bran 25, mustard oil cake 22, fish meal 26 and mineral mixture 2%) as per Pearson's square method, once a day and the residuals were removed after 48hr by siphoning. During the experiment 25% water in plastic pools was changed daily to maintain the physico-chemical parameters, pH and dissolved oxygen.

Preparation of polyclonal antiserum : Polyclonal antiserum or hyper immune serum was developed in pathogen free rabbit by immunizing intravenously with H antigen of *F. psychrophillum* Aqb Fp 6 @ 0.5, 1.0, 2.0, 4.0 and 4.0 ml at 3 day interval. H antigens were prepared (Edward and Ewing, 1996) with slight modifications and serum obtained (Verma *et al.*, 2007) were kept in vials and preserved at -80°C for further application. During the experiment rabbits were fed with laboratory prepared basal diet (wheat bran 40, maize crushed 30, soya been de-oiled cake 28, mineral mixture 2 and salt 1% and green grasses) in a day.

Fixation of tissue samples and immunofluorescence staining : Tissues from heart, liver, kidney and intestine of challenged *C. batrachus* along with the control one were removed aseptically on 0, 7, 14, 21 and 30 day. The small tissues fixed in 10% formalin rinsed in tap water for about 18–24 hr were embedded in paraffin wax according to the standard procedure and cut at 4 to 6 μm thickness. The microtome sectioned tissues were firstly dipped in water bath ($40-45^\circ\text{C}$) and then placed immediately on clean slides for immunohistochemistry according to Panangala *et al.* (2006) with slight modification (Verma and Prasad, 2008).

Slides fixed sections of liver, kidney and intestine were deparaffinised by putting them in xylene for about 30 min (two changes) and hydrated by putting successively through

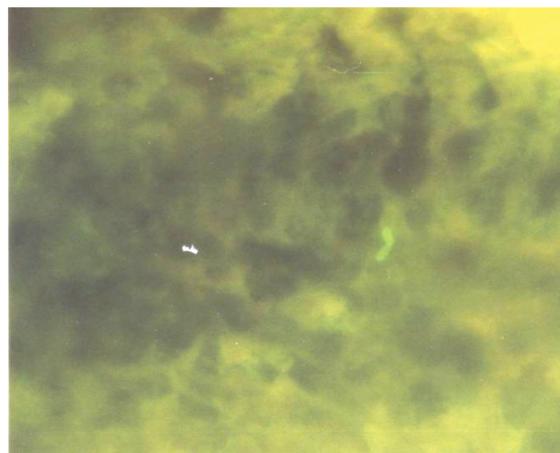


Fig. 7 : Showing ablaste stained *F. psychrophillum* (arrow) in the intestine of recipient fish treated with anti serum dilution 1: 300 (X 1000)

descending grades of alcohol and distilled water and finally washed in PBS for 10 min. Thereafter, 200–400 µl of diluted (1:300 in PBS) polyclonal antiserum of *Flavobacterium* (Aqb Fp 6) were added on fixed tissue and incubated at 32°C for about 2hr in humid chamber. Further, slides were rinsed thrice in PBS for about 10 min each by putting in coupling jars. Tissues were finally flooded with diluted (1:150) goat anti rabbit IgG FIT conjugate and incubated at 32°C for about 1hr in humid chamber followed by subsequent 3 washings in PBS for about 10 min each. Likewise, smears of bacterial inocula of broth cultured *F. psychrophilum* prepared on sterilized slides were air dried and flooded with chilled acetone for about 3 to 5 min followed by further air drying. The polyclonal antiserum prepared against H antigen of *Flavobacterium* (Aqb Fp–6) was diluted and then 200–400 µl of diluted (1:100, 1:200, 1:300, 1:500 and 1:1000 in PBS -pH 7.5) monospecific polyclonal antiserum was added and then slides were mounted (Verma *et al.*, 2008) with 50% buffered glycerine (50% PBS + 50% glycerin) and examined under fluorescent microscope (Olympus) by using X 100 oil immersion objective.

Results and Discussion

Persistent occurrence of *F. psychrophilum* with diseased *C. batrachus* of Nanak Sagar and Sarda reservoir was recorded in comparison with other wild fish. This implies that catfishes are more prone to *F. psychrophilum* infection in the reservoirs investigated. Moreover, the overt symptoms such as appearance of white lesions around the caudal fin and erosion of underlying muscles exhibited by naturally and experimentally infected catfishes of the present investigation have analogy to progression made in salmonid fish (Nematollahi *et al.*, 2003), Atlantic salmon, *Salmo salar* (Cipriano, 2005); chum salmon, *Oncorhynchus keta* (Misaka and Suzuki, 2007) and Indian catfish, *C. batrachus* (Verma *et al.*, 2007 and 2011).

Development of colourless / creamish – yellowish raised, convex, irregular without rhizoid edged bacterial colonies was noted. The bacteriological examination revealed the presence of long slender, Gram-negative rod-shaped bacteria (Fig. 1). Most of the isolates formed colonies at 20 ± 2°C temperatures within 18–24 hr but grew slowly at 15°C. The young colonies (16–18 hr old) were colourless while the older (72 hr old) ones were yellow orange or bright yellow on the selective medium. Misaka *et al.* (2008) and Ronnholm and Wiklund (2010) also reported similar attributes in *F. psychrophilum*. Morphologically, most of the isolates were identical in terms of physical and biochemical characteristics except Fp5–Fp8 as they formed colonies at 22°C but less at 20°C. Cepeda *et al.* (2004) and Barnes and Brown (2011) could also record variation in terms of temperature requirement for the growth of *F. psychrophilum* isolates. However, Hesami *et al.* (2008) reported that the optimum growth temperature of this bacterium was 15°C. The positive biochemical characteristics (motility, catalase reaction, H₂S production, degradation of casein, gelatin, Tributyrin utilization of lactose) and

negative responses (gram staining, nitrate reduction, indole production, citrate utilization etc.) exhibited by the isolates Fp1–Fp8 are in accordance with Durmaz *et al.* (2012).

The LD₅₀ concentration of Fp 6 isolate of *F. psychrophilum* was enumerated to 2×10⁶ cfu ml⁻¹ at 20 ± 2°C. The fishes experimentally infected with Fp 6 isolate of *F. psychrophilum* were marked with the symptoms of white lesions on the ventro-lateral side and ulcerations and dark pigmentation near the caudal peduncle along with loss of fins on the onset of 18–25 day infection. The inoculated bacterium was reisolated from the symptomatic fish when grown on selective medium. Appearance of explicit symptoms in experimentally infected fish and recovery of inoculated bacterium revealed its virulent nature and would be responsible for the initiation of coldwater disease in the susceptible fish. This is in accordance with those of Misaka *et al.* (2008) and Ronnholm and Wiklund (2010). Moreover, Michel and Gracia, (2003) noted clinical signs of coldwater disease in rainbow trout, *Oncorhynchus mikis* inoculated with extreme by high (10¹⁰ cfu ml⁻¹) dosage of bacterium.

There was positive response for both bacteriological culture and IFA test in the organs of 30 day challenged *C. batrachus* and pure culture of bacterial colonies originated from infected fishes. Conversely, the control fish were negative in terms of bacteriological culture and IFA test. The dilutions 1:200 and 1:500 of polyclonal antiserum (prepared against H antigen of Aqb Fb – 6 isolate by immunization of rabbit) conferred close relationship at immunofluorescence level with *F. psychrophilum* and stained deeply (Fig. 2 and 3). The formaline fixed and paraffin wax embedded tissues (kidney, liver and intestine) of experimentally infected fishes were also confirmatory for IFA test. The dilutions 1:300 and 1:500 of polyclonal antiserum exhibited cross reactivity with the bacterial cells in them. The rod shaped bacterial cells were found to be well preserved and brilliantly stained. (Fig. 4, 5, 6, 7). The *in situ* localization of *F. psychrophilum* in kidney and intestine of 30 day recipient fish was similar and prominent in almost all groups of challenged fish. Hibi *et al.* (2007), Misaka and Suzuki (2007) and Misaka *et al.* (2008) reported that positive IFA test implied that these bacterial cells were taken up by phagocytic cells which were comparable with the smear prepared from the necrotized organs and from pure culture. Isolation of *F. psychrophilum* from infected tissue and internal organs (kidney, liver and intestine) of experimentally infected fishes and positive reactivity for IFA test revealed that this bacterium was invasive in nature. However, Austin and Austin (2007) reported that this bacterium only infected gills and skin.

There was negative immunostaining in the tissue of experimentally infected fish kept for 7 days but positive in those kept for 14, 21 and 30 days. This finding elucidates that colonization of *F. psychrophilum* in different organs of fish takes at least 4–7 days which is comparable with the bacterial smear and concurs contemplation of Madetoja and Wiklund (2002),

Panangala *et al.* (2006), Hibi *et al.* (2007) and Misaka *et al.* (2008) and Verma and Prasad (2008). They reported that cultivation of organisms from infected fish might be negative while there are still a chances of positive immunohistochemistry and have acquiescence with the present findings. Similarly, if fish are not used for the isolation of organisms and put into any fixative, it would not be possible to cultivate the bacterium from such tissue in such situation the bacterial cells can still be detected by this method which has also affirmation with the present finding.

Based on the above data it can be concluded that detection and tissue localization of bacterial cells using IFA test would be highly sensitive and reliable for diagnosis of bacterial fish disease where mortality occurs without apparent symptoms.

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