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**Original Research Article**

# Characterization, pathogenicity, antibiotic sensitivity and immune response of *Flavobacterium columnare* isolated from *Cirrhinus mrigala* and *Carassius auratus*

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**A B S T R A C T**

An attempt has been made to study the pathogenicity, immune response and antibiotic sensitivity of four strains of *Flavobacterium columnare* obtained from the diseased fishes *Cirrhinus mrigala* and *Carassius auratus*. Study on virulence was attempted with pure cultures of myxobacteria. Response of the strains to twenty-three drugs was observed. Hyperimmune serum was raised in healthy *C. mrigala* and serological relationship of isolated bacteria was determined by agglutination test. Agglutination titre values revealed that *F. columnare* obtained from goldfish were more virulent and pathogenic in comparison to that obtained from mrigal. $LD_{50}$ values of the four strains ranged from $3.0X10^5$ to $9X10^6$cfu/ml. All the four strains were found to be sensitive to amikacin, bacitracin, ampicillin and nitrofurazone. Three strains exhibited sensitive nature to kanamycin, norfloxacin and streptomycin. *F. columnare* study was found to be most sensitive to aquaneem concentration (an herbal product) used in this, indicating its potential as an alternative therapeutic agent in commercial aquaculture.

**Keywords**

*Flavobacterium columnare*, Pathogenicity, Immune response, Antibiotics, *Cirrhinus mrigala*, *Carassius auratus*

## Introduction

The spectrum of fish disease and understanding their epizootiology can be singly or cumulatively correlated with the aquatic environment in which they live, host specificity of the fish and causative factors like interaction between the pathogen and the ecosystem itself. Industry-oriented intensive aquaculture has expanded rapidly and it has led to increase in infection caused by *Flavobacterium* bacteria in freshwater fishes (Hawke and Thune, 1992; Soltani *et al.*, 1995; Dash, 2002; Bader, *et al.*, 2003; Jinu and Goodwin, 2004; Figueiredo *et al.*, 2005). Yellow pigmented filamentous, Gram negative bacteria belonging to genera *Flavobacter* (Formerly known as *Flexibacter)* have been found to be present in connection with external symptoms on fish like gill erosion, fin or tail rot or saddle-like skin lesion (Rintamaki-Kinnunen *et al.*, 1997; Austin and Austin, 1999). Mortality of fish has occurred due to presence of gliding bacteria in freshwaters (Pacha and Ordal,

1970; Farkas and Olah, 1986; Holt *et al.,* 1993; Austin and Austin, 1999; Frerichs and Roberts, 1989; Noga, 2000). Columnaris is a ubiquitous disease of freshwater fishes (Kumar *et al.,* 1986; Triyanto and Wakabayashi, 1999; Austin and Austin, 1999; Bader *et al.,* 2003; Jinu and Goodwin, 2004) and *Flavobacterium columnare* has been reported worldwide (Dash, 2002; Stringer-Roth *et al.,* 2002; Arias *et al.,* 2004; Figueiredo *et al.,* 2005; Panangala *et al.,* 2006; Panangala *et al.,* 2007). *Myxobacteria* have been reported to be pathogenic to goldfish causing gill disease (Ostland *et al.,* 1997; Soltani *et al.,* 1995), to black molies and platys (Decostere *et al.,* 1998). Typically, but not always, pathogens of this group cause external infection in fish involving the skin and gills (Carson *et al.,* 1993; Thune, 1993; Decostere *et al.,* 1999; Noga, 2000). Environmental conditions are frequently crucial in the expression of pathogenicity by these species; water temperature in particular has a major influence in the occurrence of disease caused by *F. columnare, Cytophaga psychrophila* and *F. maritimus* (Wakabayashi, 1993; Wood and Yasutake, 1957; Wakabayashi, 1991; Kinnunen *et al.,* 1997; Decostere *et al.,* 1998). The disease was first described in cultivated carp in Northern Europe in 1970s and revealed that the disease particularly affected the gills of carps and goldfish held in earthen ponds (Hoole *et al.,* 2001).

The biochemical and physiological characteristics of this genus were studied extensively (Bullock, 1972; Griffin, 1992; Shotts and Starliper, 1999; Holt *et al.,* 1994, Decostere *et al.,* 1997; Plumb, 1999; Austin and Austin, 1999; Jinu and Goodwin, 2004; Griffin (1992) described *Flavobacterium columnare* as Gram-negative motile rods, typically 0.5 μm wide and 1.0–3.0 μm long. Its growth on solid media is typically yellow to orange pigmented and its colonies are translucent, circular, convex, smooth and shiny with entire edges. The virulence among *F. columnare* strains has been reported (Decostere *et al.,* 1998; Dash, 2002). Serological studies have been carried out by (Dash 2002; Panangala *et al.,* 2006). Using agglutination procedures, they have demonstrated the presence of common antigens among different strains of *F. columnare*. Slide agglutination test with rabbit serum and *F. columnare* was suggested to be a definitive diagnosis method for detection of Columnaris disease (Snieszko and Bullock, 1976; Dash, 2002). According to (Lehman *et al.,* 1991) slide agglutination is a rapid and confirmatory test for the identification of related bacteria *Cytophaga psychrophila* affecting eels and cyprinids. Vasquez Barnas (1991) performed sero-agglutination test to confirm diagnosis of two Myxobacterial species.

In the present study, attempts were made to isolate *Flavobacterium columnare* from the carp *Cirrhinus mrigala* and goldfish *Carassius auratus*. Pathogenicity was attempted with pure cultures of *Myxobacteria* isolated from carp and goldfish. To detect the immune response in carps, serum was examined for presence of agglutinin against *Flavobacterium columnare* from carp and goldfish isolates. Drug sensitivity tests were also performed taking twenty-three drugs supplied by HiMedia, India. Aquaneem, an herbal product of Kamala Chemicals, Calcutta was also tried in this study.

## Materials and Methods

### Collection of diseased samples

During nursery phase rearing of mrigal *Cirrhinus mrigala,* symptoms like tail rot and fin rot, erosion of gill lamellae and dark

patchy lesions were observed. During the same period, similar lesions were also observed in goldfish *Carassius auratus* both in aquarium stock of three shops as well as in some domestic aquaria at Bhubaneswar, Orissa, India. Samples were collected from these sources and gross patho-morphological lesions were recorded at the site itself. Infected fishes were brought to the laboratory and kept in 40 L fibreglass reinforced plastic jars for further clinical and bacteriological observations. About 30% mortality was observed within seven days from each of the nursery pond and aquarium. Water samples were collected for water quality analysis (parameters including temperature, pH, alkalinity, hardness and dissolved oxygen) as per the standard protocol (APHA, AWWA, WPCF, 1989). The behavioural changes and gross pathological observations in the spontaneous cases were recorded from time to time.

**Isolation and cultivation**

All culture media were obtained from Hi-Media, Mumbai, India and tests were conducted between temperatures 25°C–37°C unless specified otherwise. For isolating gliding bacteria from fish samples, scrapped material collected from external lesions was suspended in tryptone broth. The broth cultures were incubated at 25°C, 30°C and 37°C. To minimize the risk of being overpopulated by opportunistic pathogens and contaminating water bacteria, decimal dilutions of the inoculum were streaked separately on Tryptone Agar (TA) medium. Pure cultures were maintained in cytophaga agar slants.

**Biochemical studies**

Biochemical tests were conducted at $30^0$C using protocols described by Pacha and Porter (1968) and Bernardet (1989). The presence of catalase was tested with hydrogen peroxide and cytochrome oxidase activity was determined by using oxidase discs (Hi- Media, India). The presence of urease was detected by inoculating cultures into urease broth followed by incubation. Indole formation, citrate utilization, methyl red (MR) test and Voges-Proskauer (VP) tests were also performed. Nitrate reduction was determined by nitrate broth and carbohydrate usage was determined by triple sugar iron agar test and by using sugar discs in phenol red broth base.

Production of extracellular galactosamine glycan was demonstrated as described by McCurdy (1969). Tryptone broth was supplemented with 0.4% dextrose. Before inoculation, the pH was recorded to be 7.2; after inoculation and incubation on a rotary shaker for five days, the pH was again determined. Tryptone broth was supplemented with 0.05% ferric ammonium citrate and 0.1% aesculin (Hi-Media). The aesculin solution was separately neutralized and decontaminated through filtration. A brown-black discoloration of the culture plates within five days after inoculation was considered as a positive reaction. Tryptone broth was overlaid with 5–6 ml of the same medium supplemented with 0.5% L-tyrosine. The culture plates were examined after five days of inoculation for disappearance of tyrosine crystals.

**Morphology and motility**

Because of reports on fixation and staining artifacts (Stainer, 1947), shape of the living *Myxobacteria* cells was determined in wet mounts. The strains were grown in tryptone broth for 24h and then examined through a phase contrast microscope. For reproducing the flexing movements, the strains were grown on fish tissue (Bootsma, 1973). This medium is a modification of Garnjohst

(1945) for inducing the formation of Myxobacterial resting structures. It was prepared by boiling pieces of carp sample as well as goldfish muscles (approximately 100 mg each) and then several changes made in tap water (pH 7.7–8.0). Each piece of extracted muscle was then transferred to a tube containing 5 ml of tap water, ground with a glass rod and autoclaved for 15 min at 120°C individually. Wet mounts were prepared from inoculated medium after 24h of incubation and subsequently examined as described above.

## Growth kinetics

The culture broths specified were incubated at 25°C, 30°C and 37°C. Growth was measured turbidometrically after 48h of incubation using a Nephelometer Brand without colour filter. In addition, all strains were tested for growth in TSA broth at 37°C after five days of incubation. The sodium chloride tolerance of carp and goldfish isolates was determined with 0.1–1% NaCl (analytical grade, BDH). Growth was determined after five days of incubation by comparing the optical density of inoculated tubes with that of the uninoculated controls. Pellicle formation within 5 days was indicative of growth. All tests were performed in triplicate.

## Starch hydrolysis

Starch hydrolysis was demonstrated as described by Bullock (1972) using analytical grade soluble starch (Hi-Media). This test was performed by spot inoculating the bacterial culture into Petri plates containing Nutrient Agar with addition of 1% soluble starch. It was followed by incubation at 37°C for 24h. After incubation, the plates were flooded with iodine solution and allowed to stand for 5 minutes, thereafter the solution was decanted. If the starch were hydrolysed, a clear zone would be formed around the bacterial colonies. Reddish brown area around the colonies indicates partial hydrolysis of starch.

## Casein hydrolysis and hydrolysis of chicken egg protein

Casein hydrolysis was performed by spot inoculating the bacterial culture into culture plates containing nutrient agar supplemented with 1% casein. It was incubated at 37°C for 24–48h. After incubation, the plates were flooded with acidic mercuric chloride and allowed to stand for 5–10 minutes. Thereafter the solution was decanted. Casein hydrolysis was observed as formation of a clear zone around the bacterial colonies. In case of a negative reaction, the unhydrolyzed casein will form a white precipitate leading to opacity. The zone diameter was measured and zone ratio was calculated by dividing diameter zone of activity with diameter of bacterial smear.

## Gelatin hydrolysis

The Smith's modification of Frazier's method (Conn *et al.,* 1957) was used to determine gelatin hydrolysis. This test was performed by spot inoculating the bacterial culture into culture plates containing Nutrient agar supplemented with 0.4% gelatin. It was followed by incubation at 37°C for 24–48h. After incubation, the plates were flooded with acidic Mercuric chloride solution and were allowed to stand for 5–10 minutes. Thereafter the solution was decanted. Appearance of a clear zone around the colonies indicated hydrolysis of gelatin caused by enzyme gelatinase secreted by the bacteria. In case of a negative reaction, unhydrolysed gelatin forms a white precipitate leading to opacity.

## Drug sensitivity

Drug sensitivity tests were performed using Kirby-Bauer disc sensitivity method (Bauer *et al.*, 1966). Overnight bacterial culture in tryptone broth was spread over the diagnostic sensitivity test media and sensitivity disc purchased from Hi-Media, India and some prepared in laboratory were used for the test. The antibiotics used were Amikacin(Ak), Amoxycillin(Am), Ampicillin(A), Bacitracin(Ba), Cefuroxime(Ce), Chlorotetracycline(Ct), Cloxacillin(Cx), Cephalexin(Cp), Cephalothin(Ch), Ciprofloxacin(Cf), Chloramphenicol(C), Co-Trimoxazole(Co), Flumequine(Fm), Gentamycin(G), Erythromycin(E), Penicillin-G(P), Novobiocin(Nb), Trimethoprim(Tr), Nalidixic acid(Na), Oxytetracycline(O), Tetracycline(T), Ofloxacin(Of), Neomycin(N), Nitrofurazone(Nf), Norfloxacin(Nx), Polymyxin B(Pb) and the vibriostatic compound O/129 (Himedia, India). Inhibition zone diameters were recorded after 48h of incubation at $30^0$C. Zones of inhibition produced after definite period of incubation were measured and based on the observations, the bacteria were classified into resistance, intermediate and sensitive groups. Altogether twenty-two different drug discs were used in this test. The four bacterial samples/isolates have been treated with each of the antibiotics individually.

## Pathogenicity

Immersion challenge method was used to determine the virulence of the representative isolate. Groups of mrigal fry (average weight 1–1.5g, 20 fish per Fibreglass reinforced plastic (FRP) tank each containing static underground water of 30 l capacity) were challenged with 10-fold dilution of the isolate ($10^4$, $10^5$,$10^6$,$10^7$ cfu/ml) in sterile phosphate buffered saline (Sodium chloride, 8g/l; potassium chloride, 0.2g/l; potassium dihydrogen orthophosphate, 0.2g/l; disodium hydrogen phosphate, 1.15g/l) at room temperature ($28\pm2^0$C). Aeration was provided in the tanks. Each experiment included a control group where fry-staged fishes were kept without adding the inoculum in the tanks. During the experiment, fishes were monitored twice daily and dead or moribund fishes were sampled for the presence of challenged bacterium. The experiment was carried out for a period of 96h. $LD_{50}$ values were calculated as per the method of Reed and Muench (1938). Carp and goldfish isolates were tested for their pathogenicity to mrigal to verify Koch's postulate.

## Antigens

Antigens were prepared using a modification of the method adopted by Anacker and Ordal (1959). A loopful of bacterial culture from slant was inoculated aseptically into 100ml of tryptone soya broth and incubated at 30°C for 24 h. After the specified period of incubation, the cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed thrice with phosphate buffered saline (PBS, pH 7.2). Finally the pellet was suspended in PBS and the volume adjusted to 3ml. Formaldehyde 0.3% (v/v) was added to the suspension and the set up was left overnight for inactivation of bacteria.

The formalin-inactivated cells were harvested on the next day by centrifugation at 10,000 rpm for 10 minutes at 4°C. It was washed thrice with PBS (pH 7.2) and then reconstituted in PBS. The suspension was dispensed into required length of dialysis membrane (Hi-Media), which was presoaked for 10–15 min in PBS and both

the open ends were securely tightened by thread. It was dialysed against PBS for 12–18 hrs. After dialysis, the antigen was recovered and volume was adjusted to 3 ml using PBS. It was properly labeled and stored at -20°C for future use. Viability of the cells if any, was checked upon tryptone soya agar plates. Portions of the formalin-killed bacterial cells were sonicated at 50 Hz for 10 minutes with a five minute interval between two cycles in the sonicator (Artek Sonic Dismembrator, Model 150). After sonication, the suspension was centrifuged at 10,000 rpm for 15 min. The supernatant as antigen was collected carefully and stored at -20°C for further use.

**Antiserum**

Twenty advanced and healthy *Cirrhinus mrigala* fingerlings with an average body weight of 50±10 g were collected from farm ponds of Central Institute of Freshwater Aquaculture (CIFA) and were stocked in four Fibre reinforced plastic tanks each of 1000 l capacity. Continuous aeration was provided. The fish were fed twice a day at 2–3% of body weight with formulated carp feed obtained from the Institute. Fifty percent water replacement from the tanks was done daily with bore well water. The animals were acclimatized for seven days prior to immunization.

Formalin-inactivated bacterial antigen (200:1) was added to 1.8 ml of PBS (pH 7.2) to make a final volume of 2 ml. It was mixed thoroughly with Freund's complete adjuvant (FCA) in ratio 2:1 to form a homogenous emulsion. Emulsion of antigen with Freund's incomplete adjuvant (FIA) was also prepared in the similar manner.

**Immunization dose**

The fish were injected intraperitoneally with emulsion of antigen and FCA at a dose rate of 0.1 ml per fish. The animals were boostered on the $7^{th}$ and $14^{th}$ day of immunization with the same dose of emulsion of adjuvant and antigen but FIA was used instead of FCA. The final dose of immunization was administered on $36^{th}$ day. Blood from individual surviving animal was collected from fish by caudal vein puncture method on $42^{nd}$ day of immunization. The blood samples were collected in a sterile glass tube and allowed to clot at room temperature for 30 min to 1h. The clot was ringed to promote contraction. The tubes were left at 4°C overnight in a slanting position. On the next day, centrifugation was done at 1500 rpm for 15 minutes and serum was collected and kept individually in Eppendorf tubes. It was stored at -20°C until further use.

**Agglutination test**

Slide agglutination test (Bullock, 1972) was performed to detect serological relationship of the isolated bacteria. On a clean slide, 1:10 dilution of formalin-killed bacterial suspension was taken along with PBS. To it, a hyper immune serum of fish was added and the slide was gently rocked to mix the antibody with the bacterial suspension. Appearance of floccules or clumping of bacterial antigen along with the clearing of the solution within five minutes was considered as positive result. A control set was simultaneously prepared without addition of serum.

Agglutination titres were determined as per the method of Miguel *et al.* (1994). Serum sample of fish was serially diluted two-fold steps with sterile PBS on a clean glass slide. Equal volumes of formalin-killed bacterial suspensions and diluted sera were then mixed. After 3–4 minutes of gentle rocking, the glass slides were allowed to stand at room temperature for 5 min and cell

agglutination was observed. Agglutination titre was determined as the inverse of the highest dilution of serum causing agglutination of the bacterial cells.

## Results and Discussion

### Symptomatology and gross pathology

The fishes showed ulceration on the body surface with haemorrhagic spots. The ulcers were also found at caudal peduncles. In some cases erosion of tail region and fins were observed. Fishes showed restlessness and lethargic movements.

### Biochemical Indices

There were no significant differences between the four strains (Table 1). Production of $H_2S$, aesculin hydrolysis and starch hydrolysis appeared to be suitable tests for distinguishing isolates. All the strains have been found to be proteolytic in nature. All the strains were uniform in their biochemical properties. The four strains showed positive reaction for catalase, oxidase, nitrate reductase, motility and utilization of glucose, dextrose, sucrose, glycerol, mannitol and maltose and also hydrolysis to casein, gelatin and starch. None of the isolates produced gas, $H_2S$, indole, urease, lysine, ornithine and arginine decarboxylase, cellulase and lipase or acid from mellibiose, inulin, dulcitol or cellobiose.

Enzymatic activity of the isolates was measured taking the ratio between diameter of zone of activity and diameter of the smear. All the isolates showed positive enzymatic activities. The colony morphology, gram staining and other biochemical parameters are enumerated in the Table 1 and from which the isolates were grouped. Forty isolates (24 carp isolates and 16 gold fish isolates) of *Flavobacterium columnare* were characterized biochemically and were characterized into four strains based on their biochemical properties.14 isolates of carp were grouped to MS 2 and 10 isolates were grouped to MS 9. Similarly 9 gold fish isolates were grouped as MS 8 and 7 isolates as MS 10.

### Cell morphology and motility

Bacterial cells of all strains have been observed to be long, filamentous, flexible, weakly refractable, Gram-negative rods. Most isolates exhibited flexing movements, sometimes associated with the formation of mounds when grown on fish tissue (Table 1). The colonies were semi-translucent and pale yellow in colour. Cells when viewed under oil immersion objective after Gram staining were long slender rods with rounded edges. Cells in most of the culture turned coccoid after 4–5 days of incubation. No fruiting bodies or microcysts were obtained after three weeks of incubation in any of the isolates tested. All four isolates exhibited gliding motility over cytophaga agar but were non-motile in hanging drop preparation.

### Growth under various environmental conditions

No significant differences in growth were observed between the four strains at different environmental conditions. Positive growth was recorded for 40 numbers of isolates on cytophaga agar, Nutrient agar and tryptone soya agar. However growth was poor on TSA and Nutrient agar as compared to cytophaga agar. Maximum bacterial growth was recorded at 30°C. All the four strains grew in broth containing 0.5% NaCl, none of them grew at 0.5% NaCl.

## Pathogenicity and $LD_{50}$

Mrigal fingerlings challenged with varying concentration of bacterial cultures showed mortality after 24 h. Bacteria recovered from dead fish showed positive agglutination with antisera raised in *C. mrigala*. $LD_{50}$ values for the four isolates varied between $3.0X10^5$ and $9X10^6$ cfu/ml. The physiochemical properties of the source water was temperature $27.5\pm2.0^0$C, dissolved oxygen 5.2±0.8mg/l, pH 7.4±0.4, alkalinity 112±6.05mg/l and total hardness 79.4±6.4mg/l, respectively. Gold fish isolates produced higher percentage of mortality (80–90%) as compared to the carp isolates (30–50%) at $10^7$ cfu/ml concentration.

## Slide agglutination

Slide agglutination with polyclonal hyper immune sera showed positive reaction for both carp isolates and goldfish isolates. All the four isolates showed positive to both the reference strains of carp and gold fish. The isolates were found to be serologically homologous. The protein concentration of the 0.5ml emulsified with FCA and FIA was found to be 0.5g/ml. No mortalities were found in fish during immunization trial. Agglutination titre was found to be 256 in case of carp strains and 512 in case of gold fish strains (Fig 1)**.**

## Drug sensitivity

Detailed results of experiment on drug sensitivity are presented in Table 2. Bacterial growth observed in individual culture plates treated with antibiotics at the end of 24 h of incubation was sufficient for visual observation of drug sensitivity. All the four isolates were sensitive to Amikacin, Bacitracin, Ampicillin, Nitrofurazone. 75% of the isolates were sensitive to Cephalothrin and Isolistin. 50% of the isolates were susceptible to Novobiocin, Carbenilin, Cloxacillin and Erythromycin.

*F. columnare* is an established pathogen of fish and the causative agent of Columnaris disease (Bullock *et al.*, 1971; Decostere *et al.*, 1999). It has been reported from a many parts of the world. The occurrence of columnaris disease in carp (*Cyprinus carpio*) was reported by Davis, 1922; Isom 1960; Bootsma and Clerk 1976 and in rohu (*Labeo rohita)* by Kumar *et al.* (1986), pathogenicity tests with carp and goldfish, producing ulcers surrounded by a red zone under the test conditions (Dyar and Ordal 1949).

In this context, it is observed in this study or findings of previous studies that clinical signs of Columnaris disease in carp largely depend on the virulence of *F. columnare* isolates, the individual resistance of the carp and certain environmental factors (Bootsma and Clerk 1976). It must be emphasized that ulcers surrounded by a red zone are only produced by virulent *F. columnare* isolates in healthy mrigal that are kept in higher water temperatures. In our study, the isolation of *F. columnare* was done mostly from necrotic gill, skin lesions of *Cirrhinus mrigala* and *Carassius auratus*.

The biochemical, physiological, cellular, colonial characteristics of all the four isolates were consistent with those of *F. columnare* previously reported (Bullock, 1972; Dash *et al.*, 2008). The colonies were flat, spreading, pale yellow and rhizoid edges denoting typical Flavobacterial character as reported by Bullock (1972); Shamsudin and Plumb (1996) and subsequently by Dash *et al.* (2008). All isolates were gram negative, slender rods with rounded edges, thus being consistent to earlier reports on cell morphology of

*Myxobacteria* (Shamsuddin and Plumb, 1996; Dash *et al.*, 2008). The physiological properties of the four isolates were similar in the environmental condition from which they were isolated. Dash *et al.* (2008) reported similar type of observation while working with seven isolates of *F. columnare* isolated from carps, catfish and goldfish. *Myxobacteria* can tolerate up to 3% concentration of sodium chloride (Shamsuddin and Plumb, 1996). *F. columnare* isolated from *Catla catla, Labeo rohita, C. mrigala, C. auratus, Anabas testudineus* and *Clarias batrachus* could tolerate up to 2% sodium chloride (Dash *et al.*, 2008).

Four isolates were identified to be *F. columnare*. These isolates are rhizoidal, yellow and formed flat colonies in solid media, reduction of nitrate and hydrolysis of gelatin and casein. Apart from these characteristics, the isolates showed positive reaction to catalase, oxidase and motility and no utilization of amino acids. Similar type of properties of *F. columnare* was previously reported by Pyle and Shotts (1980) and Dash *et al.* (2008). Acids from sugars were reported positive for *F. columnare*. Glucose was used oxidatively in all isolates. These findings are in accordance with the findings of Bullock (1972) and Dash *et al.* (2008). The production of enzymes that degrade proteins like casein and gelatin and also that hydrolyze starch was common to all four isolates. Similar types of results were also reported by Newton *et al.* (1997). The pattern of enzyme activity could be due to the variation in the physiological properties of the isolates and the media used along with the incubation time.

Moyer and Hunnicutt (2007) reported that $LD_{50}$ dose of *F. columnare* was found to be 3–20X$10^6$ cells/ml when challenged to Zebra fish by injection and bath infection. Dash *et al.* (2008) also reported a dose response of 6X$10^4$ cells/ml could cause 50% mortality in rohu fry (1–1.5 gm/12–15 days). In the present study the $LD_{50}$ value was found to range from 3.0X$10^5$ to 9.0X$10^6$ cfu/ml. Such a dose response for those concentrations to produce 50% mortality is supposed to be highly virulent.

The agglutination tests revealed an antigenic relationship between carp and goldfish isolates. The titre however, may contain slight inaccuracies because the antigen suspensions were only roughly standardized. Huang *et al.* (1983) studied the serological behaviour of eleven stains of *Myxobacteria* pathogenic to various fishes in China. Observations of study conducted by Huang *et al.* (1983) can be highlighted and made relevant to present observations, if possible. Bullock (1972) indicated presence of common antigens between various Myxobacterial isolates isolated from salmonids. Anacker and Ordal (1959) reported that all strains of carp isolates were agglutinated by unadsorbed sera of rabbits immunized subcutaneously with a mixture of antigen and Freund's complete adjuvant.

However, it has been revealed in this study that goldfish isolates were more virulent and pathogenic as noticed from the pathogenicity traits. Similarly, the agglutinating titre was higher in goldfish isolates as compared to carp isolates. Myxobacterial samples isolated from goldfish and subsequently the antigen prepared produced high antibody titre than Mrigal isolates, because the former could interact with comparatively much lesser amount of antibody to form Ag-Ab complex.

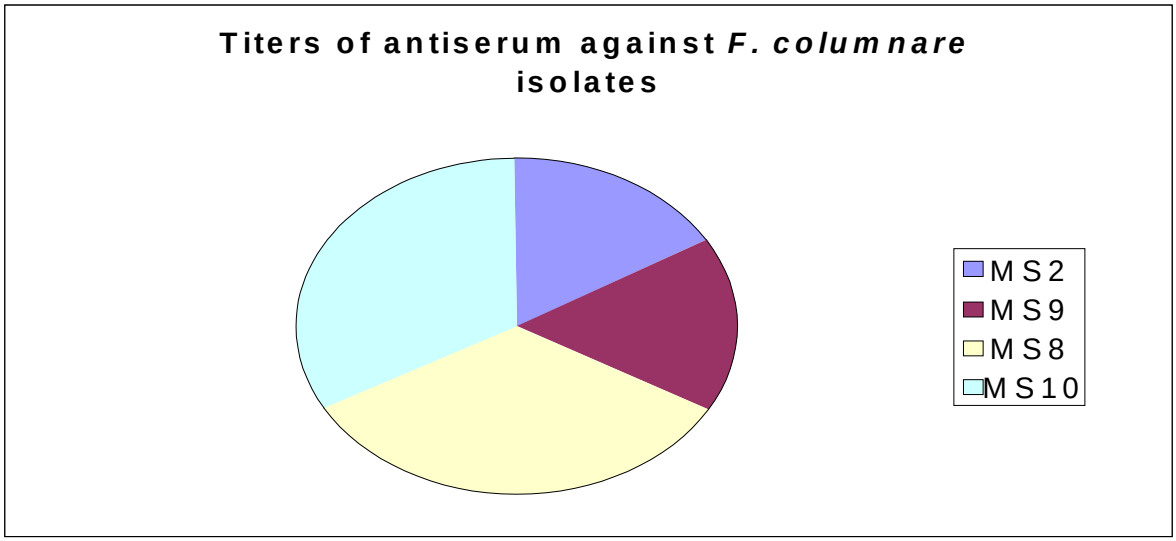
**Table.1** Biochemical properties of *Flavobacterium columnare* isolated from Carp and Gold fish

| **Strain No** | **MS9** | **MS2** | **MS10** | **MS8** |
|---|---|---|---|---|
| **Tests** | | | | |
| Catalase | + | + | + | + |
| Oxidase | + | + | + | + |
| $H_2S$ | + | – | + | – |
| Gas | – | – | – | – |
| Motility | + | + | + | + |
| Indole | – | – | – | – |
| Nitrate | + | + | + | + |
| Citrate | + | + | – | – |
| Urease | – | – | + | – |
| MR | – | – | – | – |
| O/F | +/- | +/- | +/- | +/- |
| VP | – | – | – | – |
| Casein | + | + | + | + |
| Gelatin | + | + | + | + |
| Starch | – | – | – | – |
| Lysine | – | – | – | – |
| Arginine | – | – | – | – |
| Ornithine | – | – | – | – |
| Glucose | + | + | + | + |
| Sucrose | + | + | + | + |
| Lactose | + | + | + | + |
| Dextrose | + | + | + | + |
| Fructose | + | + | + | + |
| Melibiose | – | – | – | – |
| Inulin | – | – | – | – |
| Glycerol | + | + | + | + |
| Maltose | + | – | + | + |
| Mannitol | + | + | + | + |
| Dulcitol | – | – | – | – |
| Cellobiose | – | – | – | – |
| Tyrosine | + | + | + | – |
| Congo red | + | + | + | + |
| Chicken egg hydrolysis | + | + | + | + |
| Growth with 0.5% NaCl | + | + | + | + |
| Growth at $30^0$C | + | + | + | + |
| Flexing movement on fish tissues | ++ | ++ | ++ | ++ |

**Table.2** Drug sensitivity pattern of *Flavobacterium columnare* strains against each antibiotic

| Antibiotic | Sensitivity | Resistant | Intermediate | Total strains |
|---|---|---|---|---|
| Penicillin (P) | 0 | 0 | 4 | 4 |
| Oxytetracycline (O) | 0 | 0 | 4 | 4 |
| Trimethoprim(Tr) | 0 | 0 | 4 | 4 |
| Amikacin(Am) | 4 | 0 | 0 | 4 |
| Bacitracin(B) | 4 | 0 | 0 | 4 |
| Ampicillin(Amp) | 4 | 0 | 0 | 4 |
| Novobiocin(Nb) | 2 | 2 | 0 | 4 |
| Carbenicillin(Cb) | 2 | 2 | 0 | 4 |
| Chlorotetracycline(Ct) | 0 | 0 | 4 | 4 |
| Gentamycin(G) | 0 | 0 | 4 | 4 |
| Neomycin(N) | 0 | 2 | 2 | 4 |
| Co-trimazole(Co) | 0 | 0 | 4 | 4 |
| Chloramphenicol(C ) | 0 | 0 | 4 | 4 |
| Streptomycin(S) | 0 | 1 | 3 | 4 |
| Cephalothin (Ch) | 3 | 1 | 0 | 4 |
| Colistin (CL) | 3 | 1 | 0 | 4 |
| Kanamycin(K) | 0 | 1 | 3 | 4 |
| Norfloxacin(Nx) | 0 | 1 | 3 | 4 |
| Ciprofloxacin(CF) | 0 | 0 | 4 | 4 |
| Furazolidone (Fr) | 0 | 2 | 2 | 4 |
| Nitrofurazone (Nr) | 4 | 0 | 0 | 4 |
| Cloxacillin (Cx) | 2 | 2 | 0 | 4 |
| Erythromycin | 2 | 2 | 0 | 4 |
| Aquaneem | 4 | 0 | 0 | 4 |

**Fig.1** Titers of antiserum against *F. columnare* isolates



Sulphonamides, Oxytetracycline and Oxolinic acid are also used against columnaris disease caused by *F. columnare* (Wakabayashi, 1991; Hawke and Thune, 1992; Austin and Austin, 1993). According to Prost (1977), use of Oxytetracycline is the most effective control measure for *F. columnare* bacteria. Kuo and Chung (1994) reported *F. columnare* as being most sensitive to Norfloxacin, Flumequine and Oxolinic acid than to Neomycin sulphate, Chloramphenicol and Sulphamethazone. According to Kumar *et al.* (1986), prophylactic antibiotic treatment with Streptomycin sulphate and Penicillin may prove successful in preventing the occurrence of columnaris disease. They also suggested that oral administration or bathing of fishes in antibiotic solution rather than parenteral injection would be more practical and feasible means of prophylactic treatment of fish. Amin *et al.* (1988) reported that *F. columnare* was sensitive to erythromycin. In the present experiment, out of the twenty-four drugs used, eight exhibited a clear zone for each of the four isolates. Three isolates exhibited sensitive nature to Kanamycin, Norfloxacin and Streptomycin and these might be considered promising for therapy. Aquaneem concentration used was found to be most sensitive to *Myxobacteria* as indicated by the zone of inhibition formed.

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