Pathology of *Flavobacterium* sp. KG3 in Experimentally Challenged Ornamental Goldfish *Carassius auratus* (L.)

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**ABSTRACT**

**Objective:** This study investigated the pathogenicity and pathology of the gill rot-associated bacterium *Flavobacterium* sp. KG3 in experimentally challenged *Carassius auratus*.

**Material and Methods:** The pathogenicity of *Flavobacterium* sp. KG3 was assessed by intraperitoneal injection (i/p) and abrasion-bath treatment followed by histopathology.

**Results:** *Flavobacterium* sp. KG3 challenge caused extensive damages to the gills and internal organs of *C. auratus*, resulting in cellular and tissue-level alterations. The i/p challenge resulted in significant mortalities, with an LD$_{50}$ value of $2.5 \times 10^5$ CFU/fish. The abrasion-bath challenge resulted in 60% mortality at $6.0 \times 10^6$ cells/mL in 5 days. The kidney of the challenged *C. auratus* exhibited extensive haemorrhages, polymorphic and constricted nephritic tubules, fibrosis, glomerulopathy, degeneration of nephritic tubular epithelium, disruption of blood vessels, cellular and nuclear hypertrophy, granuloma formation, necrosis of haematopoietic area, vacuolation in haematopoietic tissue, widening of lumen, and thickening of the luminal lining.

**Conclusion:** Like other recognized bacterial pathogens, *Flavobacterium* sp. KG3 was moderately virulent to *C. auratus* and can produce systemic pathology in the gills, muscle, spleen, and kidney.

**Keywords:** *Carassius auratus*, *Flavobacterium* sp., *Flavobacterial* infection, virulence, histopathology

**INTRODUCTION**

Aquarium keeping is amongst the most popular hobbies with millions of enthusiast’s worldwide (1). Among ornamental fish, goldfish *Carassius auratus* is the most common and of international significance. Ornamental fish are cultured on a large scale in various localities of West Bengal, India in earthen ponds and cemented tanks. Diseases of infectious and non-infectious origin are affecting the ornamental fish production and the livelihood of aquarists. A variety of diseases including bacterial and viral diseases have been documented in goldfish (2-4). Among the bacterial diseases, flavobacteriosis caused by *Flavobacterium* spp. is regarded as a predominant disease of ornamental fish (5). They are Gram-negative rods with 0.3-0.5 µm in diameter and 1.0-40.0 µm in length and known for their opportunistic pathogenic role in fish (6). Diseases caused by *Flavobacterium* columnare, *F. psychrophilum*, *F. branchiophilum*, and other *Flavobacterium* spp. have been documented frequently in fish as primary or opportunistic pathogens (6-11). The number of formally described species of the genus *Flavobacterium* has rapidly expanded from 26 (7) to over 100 (11).

Over the years, there are increasing incidences of flavobacterial infection in cultured Indian fish. Bacterial gill disease by *F. branchiophilum* in Indian major carps (12), *F. columnare* infection in *Catla catla* (13), *Carassius auratus* (14) and *Labeo rohita*, *Ctenopharyngodon idella* and *Anabas testudineus* (15), and infection by *Flavobacterium* spp.
in Carassius auratus (4) were documented in India. They were also isolated in healthy carps (16). The reports on the pathogenic potential of these bacteria on cultured fish and their systematic pathology are scanty. In an earlier study, we reported the phenotypic and molecular characterization and virulence of gill rot associated Flavobacterium sp. KG3 (17). The present study describes the pathogenicity of Flavobacterium sp. KG3 in goldfish Carassius auratus as well as the histopathological alterations in different organs.

MATERIAL AND METHODS

Bacterial Strain

The bacterial strain Flavobacterium sp. KG3 (NCBI GenBank accession number KP997186) used in this study was isolated from the diseased Catla catla gill (17). It was maintained as a glycerol stock in the Department of Aquatic Animal Health, Faculty of Fishery Sciences, Kolkata, India, whose phenotypic and molecular characteristics are described in our earlier report (17).

Pathogenicity of Flavobacterium sp. KG3 in Carassius auratus

The pure culture of Flavobacterium sp. KG3 was revived from the glycerol stock at -20°C and maintained on cytophaga agar (CA) slant. The cell suspension of this strain was prepared and suitably diluted up to 10⁻⁹ in sterile physiological saline. The number of cells/mL of suspension was determined by spread plating on CA (17).

The experimental goldfish (3.85±0.66 g; 7.99±1.12 cm) were procured from Piyarapur (Lat. 22⁰47'49"N; Long 88⁰18'18"E), Hooghly district, West Bengal, India. They were packed in oxygen filled polythene bags and brought to the laboratory within 2 hours of collection. At the laboratory, the fish were immersed in 5 ppm KMnO₄ solution for 15 min and transferred to the fiberglass reinforced plastic (FRP) tanks of 500L capacity at the rate of 75 numbers/tank. The weak fish were removed immediately. All fish were maintained in the FRP tanks for 20 days and fed daily with pellet feed twice daily at 2% of the body weight. The challenge experiments were carried out in glass aquaria (60×30×30 cm), after thorough washing and drying. The glass aquaria were filled with 30L of bore-well water and conditioned for three days. Each aquarium was stocked with 10 healthy goldfish and acclimatized for 3 days with continuous aeration. The fish were fed twice daily with pellet feed at 2% of the body weight and maintained under optimal condition. The wastes and faecal matter were siphoned off and 50% of the water exchanged on alternate days.

The pathogenicity of Flavobacterium sp. KG3 was tested by intraperitoneal injection (i/p) and abrasion-bath treatment in duplicate. Aliquots (0.1 mL each) of Flavobacterium sp. KG3 cell suspensions from 10⁻⁶ to 10⁻¹ dilutions were intraperitoneally (i/p) injected, i.e., between the pelvic fins and anal vent to get 10⁸-10⁵ cells/fish, respectively. The control fish were given 0.1 mL each of sterile saline (i/p). The abrasion-bath treatment was done as described previously (17). In brief, the scales of all the fish from each aquarium were scrapped off gently with a scalpel from caudal peduncle to the pectoral fin (abraded). The abraded fish from each aquarium were immersed for 60 min in a suspension (1000 mL containing 6.0×10⁶ cells of Flavobacterium sp. KG3/mL). All the fish were then transferred to the respective aquaria containing 30L water. The control group was neither abraded nor challenged. The fish groups were maintained in the respective aquaria for 28 days. The signs of infection, behavioural abnormalities and mortality were recorded daily. Reisolation of Flavobacterium sp. KG3 from the gills and kidney of freshly dead fish was on CA followed by phenotypic confirmation.

Histopathology

Bouin’s solution was used to fix the gill, muscle, kidney and spleen samples of Flavobacterium sp. KG3 challenged Carassius auratus. The 24 h fixed samples were processed by standard techniques and embedded in paraffin wax. Thin sections of 5 μm thickness were prepared and stained with Haematoxylin and Eosin (18).

RESULTS

Pathogenicity of Flavobacterium sp. KG3 in Carassius auratus

The gross and clinical signs observed in the experimentally challenged C. auratus were lethargy, sluggishly, erratic movement,
Sp. KG3 was determined to be $2.5 \times 10^5$ CFU/fish. The abraded fish cultured fish as well as the tissue level changes in the infected made to assess the pathogenic potential of these bacteria on a major cause for concern. Therefore, further attempts were pathogens of winter disease in freshwater aquaculture and is established that Flavobacterium spp. are emerging as major fish localities of West Bengal, India (4,15,17). The surveillance results the cultured food fish and ornamental fish species from diverse trial infection with mild to moderate mortalities were recorded in 2015 (November-February), increasing incidences of flavobacte-

**DISCUSSION**

During the surveillance work on the winter diseases in 2014 and 2015 (November-February), increasing incidences of flavobacterial infection with mild to moderate mortalities were recorded in the cultured food fish and ornamental fish species from diverse localities of West Bengal, India (4,15,17). The surveillance results established that Flavobacterium spp. are emerging as major fish pathogens of winter disease in freshwater aquaculture and is a major cause for concern. Therefore, further attempts were made to assess the pathogenic potential of these bacteria on cultured fish as well as the tissue level changes in the infected fish. In the present study, cent percent mortality was observed in goldfish challenged with Flavobacterium sp. KG3 at a level of $10^6$ CFU/fish in 24 h. About 80-90% mortalities were recorded at $10^6-10^7$ CFU/fish within 60 h of the challenge. The LD$_{50}$ value of Flavobacterium sp. KG3 was determined to be $2.5 \times 10^5$ CFU/fish, which imply that the tested bacterium was moderately virulent to C. auratus as per the degree of virulence (19). Flavobacterium sp. KG3 also resulted in 60% mortality within 5 days of challenge in abraded and bath treated C. auratus. These results suggested that physical or mechanical injuries may facilitate the entry of Flavobacterium sp. KG3 to effect significant mortalities in goldfish. In our earlier study, the same bacterium, when challenged by abrasion-bath treatment in C. catla fingerlings at 4.7×10^6 cells/mL, resulted in 2.8 times higher mortality (56.7%) within 5 days of challenge than the unchallenged and abraded catla (20%) at 24-28 °C (17). These results corroborate the earlier observations made in zebrafish Danio rerio with abrasion-bath experiments using F. columnare (5), which recorded LD$_{50}$ values of 1.1×$10^6$-1.1×$10^7$ CFU/mL. However, their challenge experiments with the same strain by intramuscular and intraperitoneal injection yielded much higher LD$_{50}$ values (3.2×$10^9$ CFU/fish and 4.2×$10^{10}$ CFU/fish, respectively) than those observed in the present study by i/p route (2.5×$10^5$ CFU/fish). The results implied that Flavobacterium spp. have varying degrees of pathogenic potential on fish with challenge routes. Irrespective of the challenge route, our strain Flavobacterium sp. KG3 demonstrated its virulence and pathogenic potential. Contrarily, in suspension challenge study with F. branchiophilum in intact L. rohita fingerlings mortalities ranging from nil at $10^6-10^8$ CFU/mL to 80% at $10^{11}$ CFU/mL were recorded (12). The results of the present study, thus, demonstrated that with skin injuries or breach of the immune barrier the Flavobacterium sp. KG3 can cause mortalities in fish as with other potential bacterial pathogens. The above results, thus, indicated that Flavobacterium sp. KG3 may be involved in the pathogenesis of goldfish in union with adverse environmental conditions and/or injuries. The haemorrhagic lesions on the internal organs of challenged goldfish were indicative of septicemia condition.
The histopathological observations in *Flavobacterium* sp. KG3 challenged goldfish also demonstrated extensive damages in the gills and internal organs. The gills of experimentally challenged *C. auratus* had extensive necrosis and hyperplasia, inflammation of cartilaginous tissue similar to those reported earlier (18,20,21). In our earlier study, cartilaginous tissue inflammation, mucus secretion, loss of gill lamellar structure, necrosis of gill filament with the associated reduction in the number of lamellae per filament, obliteration of interlamellar water channels, and fusion of lamellae were noted in naturally infected *C. catla* exhibiting gill rot, from where the *Flavobacterium* sp. KG3 was isolated (17). Likewise, columnaris diseased fish recorded congestion of blood vessels, dissociation of surface epithelium of the lamellae from the capillary bed probably due to the accumulation of oedematous fluid and scattered areas of haemorrhage with globose masses of blood cells (20). On the other hand, proliferative branchitis consisting of epithelial hyperplasia of the gill lamellae and interlamellar space resulting in lamellar fusion was observed during the flavobacterial infection (21). In the present study, the gills of *C. auratus* exhibited extensive necrosis with other cellular and tissue level alterations, which are histologically similar to those of several earlier studies (13,14,20,22).

In the kidney, severe nephritic cellular and tissue level alterations including granuloma formation were observed, thus indicating the systemic pathogenic potential of *Flavobacterium* sp. KG3. Similarly, granulomatous lesions in the kidney of *Mollensia sphenops* affected with *Flavobacterium* sp. was reported (23). Contrarily, in an experimental study with *F. columnare*, kidney lesions were localized in the glomerulus (20). In support of the present study, renal tubular degeneration and proteinaceous casts in the tubular lumen, focal renal tubular degeneration, and necrosis, melanomacrophage, hyperplasia, tubular degenerative changes, necrosis and edema within the renal interstitium of the kidney of an injected (i/p) fish with *Flavobacterium* were noted (21). According to Ferguson (22), inspection of the Hema(toxylin and Eosin or Giemsa stained sections from the affected tissue can reveal typical long and slender bacterial cells, where they appear bluish-purple or blue, respectively. But, the present experiment and also in earlier studies (20,24), the stained tissue sections failed to reveal bacteria in the internal organs. Also, the challenged *C. auratus* exhibited diffused muscle bundle, sarcosylis along with haemocyte infiltration; whereas several earlier studies noted severe haemorrhagic muscle with degeneration of the myofibers (21), and degeneration of muscle fibers and necrosis (20).

Histopathological alterations such as necrosis, loosely packed white pulp with vacuolated cells, melanomacrophage aggregate and necrosis were noted in the spleen of challenged *C. auratus*. The presence of melanomacrophage aggregate in the spleen is indicative of immune reactions to ward-off the bacterial challenge. An early study observed no bacilli or microscopic lesions in the liver, spleen and anterior kidney of *Flavobacterium* infected koi carp (25). However, in the present study, microscopic lesions were noted in all internal organs, which confirm that *Flavobacterium* sp. KG3 has the ability to cause systemic infection. In agreement with this study, a friable and swollen spleen or splenomegaly in columnaris-like diseased fish was reported (21).

**CONCLUSION**

The histopathological observations of the present study, in general, presented the fact that *Flavobacterium* sp. KG3 can induce pathogenesis both externally and internally in experimentally challenged fish. Since the flavobacterial infection is severe during the winter months appropriate preventive or stress mitigation measures such as avoidance of crowding, use of probiotics, immunomodulators, vaccines or development of disease-resistant stocks, etc are recommended to manage the flavobacterial infection or winter diseases.

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**Conflict of Interest:** The authors have no conflict of interest to declare.

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**REFERENCES**


