ISTANBUL UNIVERSITY PRESS

RESEARCH ARTICLE

Eur J Biol 2018; 77(2): 76-80

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

Sudeshna Sarker, Thangapalam Jawahar Abraham*, Sayani Banerjee, Harresh Adikesavalu

Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, West Bengal, India

ORCID IDs of the authors: T.J.A. 0000-0003-0581-1307.

Please cite this article as: Sarker S, Abraham TJ, Banerjee S, Adikesavalu H. Pathology of *Flavobacterium* sp. KG3 in Experimentally Challenged Ornamental Goldfish *Carassius auratus* (L.). Eur J Biol 2018; 77(2): 76-80.

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 2.5×10^5 CFU/fish. The abrasion-bath challenge resulted in 60% mortality at 6.0×10^6 cells/mL in 5 days. The kidney of the challenged *C. auratus* exhibited extensive haaemorrhages, polymorphic and constricted nephritic tubules, fibrosis, glomerulopathy, degeneration of nephritic tubular epithelium, disruption of blood vessels, cellular and nuclear hypertrophy, granuloma formation, necrosis of haaematopoietic area, vacuolation in haaematopoietic 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.

Address for Correspondence: Thangapalam Jawahar Abraham E-mail: abrahamtj1@gmail.com
Received: 11.10.2018 **Accepted:** 03.12.2018

in *C. 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 *C. autarus* 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 characterizations 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. 22°47′49″N; Long 88°18′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 $KMnO_4$ 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 (n=14) were filled with 30L each 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 10^0 to 10^{-3} dilutions were intraperitoneally (i/p) injected, i.e., between the pelvic fins and anal vent to get 10^8-10^5 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 6.0×10^6 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 *C. 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, sluggishness, erratic movement,

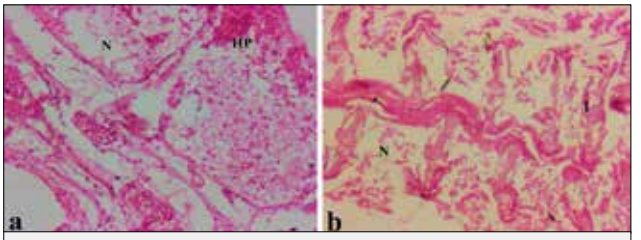

Figure 1. a, b. Photomicrograph of the gill of *Flavobacterium* sp. KG3 challenged *Carassius auratus* showing a) extensive necrosis (N) and hyperplasia (HP) [X200]; b) extensive necrosis (N) and inflammation of cartilaginous tissue (I) [X100]. H&E staining

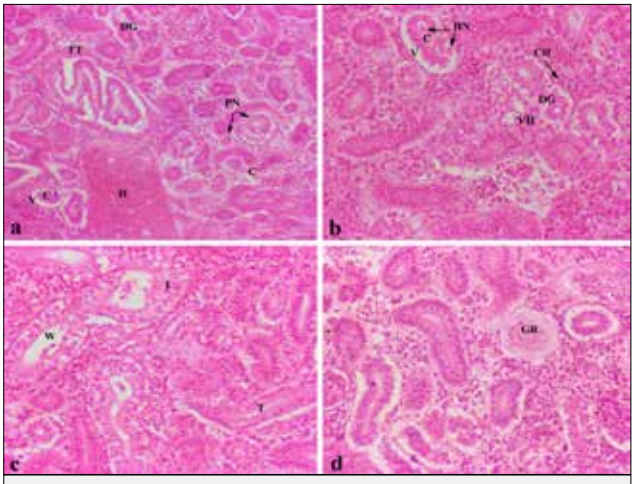

Figure 2. a-d. Photomicrograph of the kidney of *Flavobacterium* sp. KG3 challenged *Carassius auratus* showing a) extensive haemorrhages (H), polymorphic nephritic tubules (PN), fibrosis around the inflamed nephritic tubules (FT), constricted nephritic tubules (C) with vacuolated surrounding (V), degeneration of nephritic tubular epithelium (DG) [X100]; b) vacuolation in haematopoietic tissue (VH), constricted nephritic tubules (C) with vacuolated surrounding (V), cellular (CH) and nuclear hypertrophy (HN), degeneration of tubular epithelium (DG) [X200]; c) nephritic tubule inflammation (I), widen lumen (W) and thickening of luminal lining (T) [X200]; d) granuloma-like formation (GR) [X200]. H&E staining

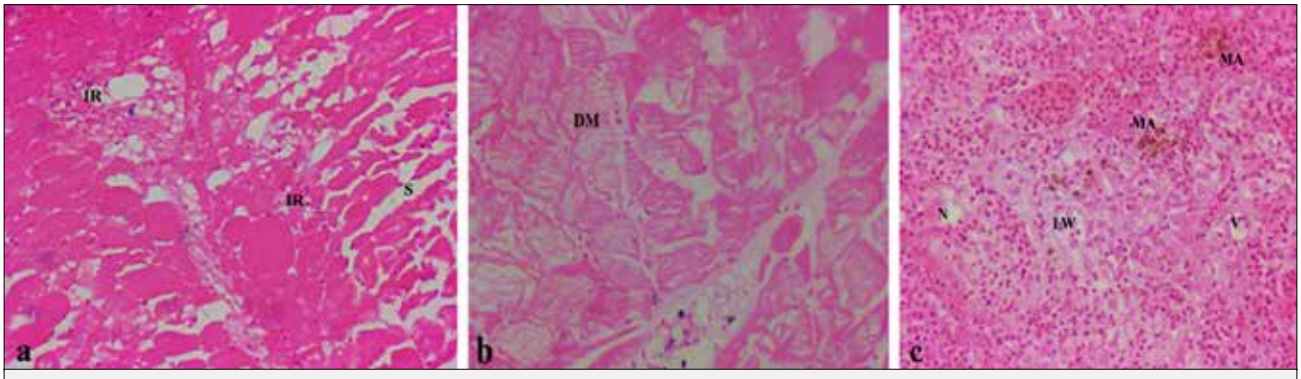

Figure 3. a-c. Photomicrograph of the muscle of *Flavobacterium* sp. KG3 challenged *Carassius auratus* showing a) sarcolysis (S) along with haemocyte infiltration (IR); b) diffused muscle bundle (DM). c) Photomicrograph of the spleen of *Flavobacterium* sp. KG3 challenged *Carassius auratus* showing melanomacrophage aggregate (MA), necrosis (N), loosely packed white pulp (LW) with vacuolated cells (V) [X200]. H&E staining

hanging, anorexia, white patches on the gills, excessive mucus secretion, caudal peduncle lesions, tail rot, cutaneous haemorrhages, scale loss, ulceration in the abraded area, skin discoloration, skin peeling, fluid-filled peritoneal cavity and intestine, pale and discoloured kidney and liver, and haemorrhagic internal organs. About 80-100% mortalities were noted in challenged *C. auratus* at 10^6-10^8 CFU/fish levels. The LD_{50} dose of *Flavobacterium* sp. KG3 was determined to be 2.5×10^5 CFU/fish. The abraded fish recorded 60% mortality at a challenge dose of 6.0×10^6 cells/mL.

Histopathology

The gills of experimentally challenged *C. auratus* exhibited extensive necrosis, hyperplasia (Figure 1a) and inflammation of cartilaginous tissue (Figure 1b). In kidney, extensive haemorrhages, polymorphic nephritic tubules, fibrosis around the inflamed nephritic tubules, constricted nephritic tubules with vacuolated surrounding, degeneration of nephritic tubular epithelium (Figure 2a), vacuolation in haematopoietic tissue, cellular and nuclear hypertrophy, degeneration of tubular epithelium (Figure 2b), nephritic tubule inflammation, widen lumen and thickening of luminal lining (Figure 2c) and granuloma formation (Figure 2d) were observed. The challenged goldfish exhibited sarcolysis along with haemocyte infiltration (Figure 3a) and diffused muscle bundle (Figure 3b). Histopathological alterations such as melanomacrophage aggregate, necrosis and loosely packed white pulp with vacuolated cells were noted in the spleen of challenged *C. auratus* (Figure 3c).

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 10^8 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 2.5×10^5 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^{10} 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^{12} 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 *Molliensia 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 Hematoxylin 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, sarcolysis 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 was 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.

Ethics Committee Approval: All the experimental protocols with goldfish as an experimental animal were approved by the Ethical Committee, WBUAFS, Kolkata, India.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - T.J.A; Design - T.J.A; Supervision – T.J.A., S.B.; Resource - T.J.A., S.S., H.A.; Materials - S.S., H.A.; Data Collection and/or Processing - S.S., S.B., H.A.; Analysis and/or Interpretation - S.S., S.B., H.A., T.J.A.; Literature Search - S.S., S.B.; Writing - S.S., T.J.A.; Critical Reviews - T.J.A.

Acknowledgement: The authors thank the Vice-Chancellor, West Bengal University of Animal and Fishery Sciences, Kolkata for providing necessary facilities to carry out the work.

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

Financial Disclosure: The research work was supported by the Indian Council of Agricultural Research (Grant F. 10(12)/2012-EPD dated 23.03.2012), Government of India, New Delhi under the Niche Area of Excellence program.

REFERENCES

1. Livengood EJ and Chapman FA. The Ornamental Fish Trade: An Introduction with Perspectives for Responsible Aquarium Fish Ownership. University of Florida, IFAS Extension, 2011, pp. 1-8.
2. Citarasu T, Alfred DK, Velmurugan S, Thanga VV, Kumaran T, Michael BM, et al. Isolation of *Aeromonas hydrophila* from infected ornamental fish hatchery during massive disease outbreak. Int J Curr Res 2011; 2(1): 37-41.
3. Sahoo PK, Swaminathan TR, Abraham TJ, Kumar R, Pattanayaka S, Mohapatra A, et al. Detection of goldfish haematopoietic necrosis herpes virus (Cyprinid herpesvirus-2) with multi-drug resistant *Aeromonas hydrophila* infection in goldfish: First evidence of any viral disease outbreak in ornamental freshwater aquaculture farms in India. Acta Trop 2016; 161: 8-17.
4. Sarker S, Patra A, Adikesavalu H and Abraham TJ. Flavobacteriosis in cultured freshwater ornamental telescopic eye goldfish *Carassius auratus*. Int J Curr Microbiol Appl Sci 2016; 5(4): 39-46.
5. Moyer TR, Hunnicutt DW. Susceptibility of zebrafish *Danio rerio* to infection by *Flavobacterium columnare* and *F. johnsoniae*. Dis Aquat Organ 2007; 76(1): 39-44.

6. Bernardet JF and Bowman JP. Genus I. *Flavobacterium* Bergey et al. 1923, emend. Bernardet et al. 1996. Whitman W, editor, Bergey's Manual of Systematic Bacteriology; Vol. 4. 2nd edition, 2011 The Williams and Wilkins Co., Baltimore, MD.
7. Bernardet JF and Bowman JP. The genus *Flavobacterium*. Dworkin M, Falkow S, editors. The Prokaryotes: A Handbook on the Biology of Bacteria, Vol. 7. 2006 Springer-Verlag, New York, NY, pp. 481-531.
8. Austin B and Austin DA. Bacterial Fish Pathogens. Diseases of Farmed and Wild Fish. 5th edition. 2012 Springer-Praxis in Aquaculture in Fisheries, Praxis Publication Ltd., Chichester, UK, p. 457.
9. Starliper CE and Schill WB. Flavobacterial diseases: columnaris disease, coldwater disease, and bacterial gill disease. Woo PTK, Bruno DW, editors. Fish Diseases and Disorders. 2nd edition. Vol. 3. 2012 CAB International, Wallingford, UK, pp. 606-31.
10. Good C, Davidson J, Wiens GD, Welch TJ, Summerfelt S. *Flavobacterium branchiophilum* and *F. succinicans* associated with bacterial gill disease in rainbow trout *Oncorhynchus mykiss* (Walbaum) in water recirculation aquaculture systems. J Fish Dis 2015; 38(4): 409-13.
11. Loch TP, Faisal M. Emerging flavobacterial infections in fish: A review. J Adv Res 2015; 6(3): 283-300.
12. Swain P, Mishra S, Dash S, Nayak SK, Mishra BK, Pani KC, et al. Association of *Flavobacterium branchiophilum* in bacterial gill disease of Indian major carps. Indian J Anim Sci 2007; 77(7): 646-9.
13. Verma DK, Rathore G. Molecular characterization of *Flavobacterium columnare* isolated from a natural outbreak of columnaris disease in farmed fish, *Catla catla* from India. India. J Gen Appl Microbiol 2013; 59: 417-24.
14. Verma DK, Rathore G, Pradhan PK, Sood N, Punia P. Isolation and characterization of *Flavobacterium columnare* from freshwater ornamental goldfish *Carassius auratus*. J Environ Biol 2015; 36(2): 433-9.
15. Patra A, Sarker S, Banerjee S, Adikesavalu H, Biswas D, Abraham TJ. Rapid detection of *Flavobacterium columnare* infection in fish by species-specific polymerase chain reaction. J Aquac Res Dev 2016; 7: 445.
16. Verma DK, Rathore G. New host record of five *Flavobacterium* species associated with tropical freshwater farmed fishes from North India. Brazilian J Microbiol 2015; 46(4): 969-76.
17. Sarker S, Abraham TJ, Banerjee S, Adikesavalu H and Patra A. Characterization, virulence and pathology of *Flavobacterium* sp. KG3 associated with gill rot in carp, *Catla catla* (Ham.). Aquaculture 2017; 468(1): 579-84.
18. Roberts RJ. Fish Pathology. 4th edition, 2012 Wiley-Blackwell, UK, p.581
19. Pu JY, Huang XX, Lu CP. Virulence detection of *Streptococcus suis* type 2 in zebrafish. Sci Agricult Sin 2007; 40: 2655-8.
20. Pacha RE, Ordal EJ. Histopathology of experimental columnaris disease in young salmon. J Comp Pathol 1967; 77(4): 419-23.
21. Loch TP. Identification of novel flavobacteria from Michigan and assessment of their impacts on fish health. Ph.D. Thesis, Michigan State University, East Lansing 2012, p.270.
22. Ferguson HW. Systemic Pathology of Fish: A Text and Atlas of Normal Tissues in Teleosts and their Responses in Disease. 2nd edition, 2006 Scotian Press, London, p.367.
23. Kluge JP. A granulomatous disease of fish produced by flavobacteria. Path Vet 1965; 2(6): 545-52.
24. Morrison C, Cornick J, Shum G, Zwicker B. Microbiology and histopathology of saddle-back disease of underyearling Atlantic salmon, *Salmo salar* L. J Fish Dis 1981; 4: 243-58.
25. Tripathi NK, Latimer KS, Gregory CR, Ritchie BW, Wooley RE, Walker RL. Development and evaluation of an experimental model of cutaneous columnaris disease in koi *Cyprinus carpio*. J Vet Diagn Invest 2005; 17(1): 45-54.