Pathogenicity and pathology of *Chryseobacterium* sp. PLI₂ in experimentally challenged ornamental goldfish, *Carassius auratus* (L.)

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ABSTRACT

The pathogenicity and pathology of *Chryseobacterium* sp. PLI₂ in experimentally challenged *Carassius auratus* goldfish were studied. *Chryseobacterium* sp. PLI₂ produced typical chryseobacteriosis symptoms in intraperitoneally challenged and abrasion-bath treated *C. auratus*. The LD₅₀ was determined to be $3.13 \times 10^7$ cells/fish. It caused 40% mortality within 3 weeks in abraded fish when challenged at a level of $\approx 2.50 \times 10^7$ cells/mL. The challenged fish were lethargic, anorectic and exhibited erratic movement. With disease progression, they all had white patches on the gills, excessive mucus secretion, caudal peduncle lesions, focal cutaneous haemorrhages, haemorrhagic eye and opercula, scale loss, skin discoloration, skin peeling, emaciation, pale kidneys and black spleen. Histologically, inflammation of the cartilaginous tissue, fusion of the lamellae and extensive necrosis were noticed in the gills. The spleen showed sinusoidal dilation, melamomacrophage aggregate, marginated leucocytes with a hypertrophied nucleus and necrotized areas. Granuloma-like formations, inflamed renal tubules and glomerulus, extensive necrosis, mild melamomacrophage aggregate, acentric nucleus, constricted renal tubules with a vacuolated surrounding, hypoplastic haematopoietic tissue, degeneration of tubular epithelium with proteinaceous casts in the tubular lumen, and fibrosis around the renal tubules were observed in the kidneys. The challenge experiment indicated that *Chryseobacterium* sp. can cause systemic disease in goldfish.

Key words: *Carassius auratus*; *Chryseobacterium* sp.; pathogenicity; histopathology; granuloma
Introduction

Worldwide, ornamental fish keeping is considered the 2nd most popular hobby, second only to photography. Over 2,500 species are involved in the global ornamental fish industry, of which about 30 freshwater fish species dominate the global market (DEY, 2016). At present, ornamental fish keeping has developed into an extensive and global component of international trade worth millions of dollars. In India, a variety of freshwater and marine ornamental fish are available (GHOSH et al., 2003; MINISEKHARAN and RAMACHANDRAN, 2012). Since 1985 the value of international trade in exports of ornamental fish has increased at an average annual growth rate of approximately 14%. About 90% of Indian ornamental fish were traded from Kolkata port, followed by 8% from Mumbai and 2% from Chennai (GHOSH et al., 2003; MINISEKHARAN and RAMACHANDRAN, 2012). India’s overall ornamental fish trade was worth about 1.06 million US$, and its share in the global ornamental fish trade was <1% (RANI et al., 2014).

There has been a considerable economic loss in Indian ornamental fish aquaculture due to the frequent occurrence of diseases. A variety of diseases, including bacterial and viral diseases, have been reported and characterized in goldfish (CITARASU et al., 2011; SAHOO et al., 2016; SARKER et al., 2016). Susceptibility to Chryseobacterium infection in freshwater angelfish, Pterophyllum scalare (KAYIŞ and ER, 2014), puffer fish Arothron hispidus (CAMPBELL et al., 2008) and yellow perch Perca flavescens (PRIDGEON et al., 2013) have been documented. In recent years, an increasing number of reports is emerging on the infections caused by Chryseobacterium sp. (ABRAHAM et al., 2017) and members of the closely related genus Flavobacterium in cultured freshwater finfish species from India (VERMA et al., 2015; PATRA et al., 2016; SARKER et al., 2017). However, no reports are available on the infection caused by Chryseobacterium in ornamental fish cultured in India, and its pathogenic potential. In this communication, the pathology of Chryseobacterium sp. PLI₂ in Carassius auratus goldfish cultured in West Bengal, India is reported.

Materials and methods

Bacterial strain and preparation of bacterial cell suspensions. Chryseobacterium sp. PLI₂ (NCBI GenBank accession number KP898212), isolated from pacu Piaractus brachypomus fry, was obtained from the collection of the Department of Aquatic Animal Health, Faculty of Fishery Sciences, Kolkata, India. The phenotypic and molecular characterizations of Chryseobacterium sp. PLI₂ are described by ABRAHAM et al. (2017). Chryseobacterium sp. PLI₂, maintained on cytophaga agar (CA) slants, were streaked onto CA plates and incubated at 30 °C for 24 h to obtain young discrete colonies. One young colony was picked aseptically, transferred to 10 mL of cytophaga broth [CB]
(SONG et al. 1988) and incubated at 30 °C for 24 h. This 24 h old culture was then transferred to 500 mL CB and re-incubated at 30 °C for 48 h. The cells were harvested by centrifugation at 7500 rpm for 20 min at 25 °C. The cell pellets were washed twice by centrifugation with sterile physiological saline, re-suspended in 10 mL sterile saline, and used immediately. A portion of the cell suspension was suitably diluted up to 10^9 in sterile saline, and the number of cells/mL of suspension was determined by spread plating on CA, followed by incubation at 30 °C for 48h.

Experimental fish. Healthy experimental goldfish Carassius auratus (3.85 ± 0.66 g; 7.99 ± 1.12 cm) were procured from a grow-out pond in Piyarapur (Lat. 22⁰47’49”N; Long 88⁰18’18”E), Hooghly district, West Bengal, India, and brought to the laboratory in oxygen filled polythene bags. On reaching the laboratory, the fish were disinfected in 5 ppm potassium permanganate solution for 15 min and maintained in fibreglass reinforced plastic (FRP) tanks of 500 L capacity at 75 fish/tank. All the fish were maintained in FRP tanks under optimal conditions for 20 days and fed twice daily with pellet feed at 2% of the body weight. The waste and faecal matter were siphoned out, and 50% water exchange was undertaken on alternate days.

Pathogenicity of Chryseobacterium sp. PLI_2. Pathogenicity of Chryseobacterium sp. PLI_2, was tested by two methods, viz., abrasion-bath treatment, i.e., immersion of abraded C. auratus in Chryseobacterium sp. PLI_2 cell suspension (ADIKESAValu et al., 2015), and by intraperitoneal injection (ABRAHAM et al., 2016) in duplicate. Twelve glass aquaria (60 × 30 × 30 cm), after thorough washing and drying, were filled with 30L each of clean bore-well water and conditioned for 3 days. The healthy goldfish were stocked at 10 fish/ aquarium and acclimatized for 3 days with continuous aeration. Prior to the challenge, one day starved fish were anaesthetized using clove oil at 50 µL/L water. Briefly, aliquots (0.1 mL each) of Chryseobacterium sp. PLI_2 cell suspensions from 10^6 to 10^3 dilutions were injected intraperitoneally (i/p) so as to obtain 10^10 - 10^7 cells/fish, respectively. For the challenge by immersion of abraded fish in Chryseobacterium sp. PLI_2 suspension, the scales of all the fish from each aquarium were scrapped off gently with a scalpel from the caudal peduncle to the pectoral fin. All the abraded fish were immersed in a bacterial suspension containing ≈2.50×10^7 cells of Chryseobacterium sp. PLI_2/mL for 30 min. After the bath treatment, all the fish, along with the contents of the bath were transferred to the respective aquaria. The control group was neither abraded and bath treated nor injected with Chryseobacterium sp. The challenged and control fish groups were maintained in the respective aquaria for 28 days under optimal conditions. The challenged fish were given ad libitum access to pellet feed. The external signs of infection, behavioural abnormalities and mortality were recorded daily. The bacterium Chryseobacterium sp. was re-isolated from the gills and kidney of freshly dead or infected
fish on CA, and confirmed phenotypically. The LD50 value was calculated following REED and MUENCH (1938), to determine pathogenicity.

**Histopathology.** The affected body parts of challenged *C. auratus* were fixed in Bouin’s solution for 24 h. The fixed gills, kidney, and spleen samples were processed by standard techniques and embedded in paraffin wax. Thin (5 μm) sections were prepared and stained with haematoxylin and eosin (PRESNELL and SCHREIBMAN, 1997).

**Results**

*Pathogenicity of Chryseobacterium sp. PLI₂ on Carassius auratus.* The gross and clinical signs started to appear on and from the 2nd day after the challenge. These included lethargy, sluggish behaviour, erratic movement, hanging, being anorectic, white patches on the gills, excessive mucus secretion, caudal peduncle lesions, focal cutaneous haemorrhages, opercula haemorrhage, scale loss, skin discoloration, skin peeling, emaciation, haemorrhagic spots on the eyes, pale kidney and black spleen in the *C. auratus* i/p experimentally challenged with *Chryseobacterium* sp. PLI₂. The mortality was about 60 - 80% in i/p challenged *C. auratus* at 10⁷ - 10¹⁰ cells/fish levels. The mortalities were high between 7 and 14 days after the injection and subsided thereafter. The LD50 value of *Chryseobacterium* sp. PLI₂ was determined to be 3.13×10⁷ cells/fish. The abraded fish had scale loss, skin discoloration, skin peeling, reddening and lesions in the abraded area. The strain PLI₂ caused 40% mortality within 21 days in abraded fish when challenged at a level of ≈2.50×10⁷ cells/mL for 30 min.

*Histopathology of experimentally challenged Carassius auratus.* The gills of the challenged *C. auratus* had cartilaginous tissue inflammation, lamellar fusion and necrosis (Fig. 1).

The kidneys of the challenged *C. auratus* exhibited cellular and nuclear hypertrophy, inflammation of renal tubules and glomerulus, epithelial cells devoid of a nucleus, mild melanomacrophage aggregate, epithelial cells devoid of a nucleus (Fig. 2), granuloma-like formations, degeneration of the renal tubular epithelium, and necrosis (Fig. 3).
Fig. 1. Photomicrograph of the gill tissues of *Chryseobacterium* sp. PLI$_2$ challenged *Carassius auratus* showing inflammation of cartilaginous tissue (I), fusion of gill lamellae (FL) and necrosis (N). H&E; ×100.

Fig. 2. Photomicrograph of the kidney of a *Chryseobacterium* sp. PLI$_2$ challenged *Carassius auratus* showing cellular hypertrophy (CH), nuclear hypertrophy (HN), inflammation of renal tubules (I), mild melanomacrophage aggregate (MA), inflamed glomerulus (IG), and epithelial cells devoid of a nucleus (EN). H&E; ×200.
Fig. 3. Photomicrograph of the kidney of *Chryseobacterium* sp. PLI, challenged *Carassius auratus* showing granuloma-like formations (GR), degeneration of renal tubular epithelium (DG), nuclear hypertrophy (HN) and necrosis (N). H&E; ×200.

Fig. 4. Photomicrograph of the spleen of *Chryseobacterium* sp. PLI, challenged *Carassius auratus* showing sinusoidal dilation (SD), melanomacrophage aggregate (MA), marginated leucocytes (ML) with the hypertrophied nucleus (HN) and necrotized area (N). H&E; ×200.
In addition, there were haematopoietic tissue necrosis, constriction of the renal tubule with a vacuolated surrounding, hypoplastic haematopoietic tissue, proteinaceous casts in the tubular lumen and fibrosis around the renal tubules (Figure not shown). The spleen showed sinusoidal dilation, melanomacrophage aggregate, marginated leucocytes with a hypertrophied nucleus and necrotized areas (Fig. 4).

**Discussion**

During the fish disease surveillance program, chryseobacterial infection in pacu *P. brachypomus* fries and a few commercial ornamental fish species (ABRAHAM et al., 2017) were observed, which necessitated this challenge experiment to assess the pathogenic potential of *Chryseobacterium* sp. The initial challenge trials (i/p) identified that the *Chryseobacterium* sp. from goldfish was avirulent (>5.00×10⁹ cells/fish) and, therefore, *Chryseobacterium* sp. PLI₂ was used in this study. In *C. auratus* experimentally challenged with *Chryseobacterium* sp. PLI, the observed gross and clinical signs were similar to several earlier reports (LOCH, 2012; PRIDGEON et al., 2013; LOCH and FAISAL, 2015a,b; WEERAPORNPRASIT et al., 2015; ABRAHAM et al., 2017). Eighty per cent mortality was observed in fish challenged i/p with *Chryseobacterium* sp. PLI₂ at 2.50×10¹⁰ cells/fish. The LD50 value of *Chryseobacterium* sp. PLI₂ was determined to be 3.13×10⁷ cells/fish, which is lower than the report by LOCH and FAISAL (2015b), who demonstrated an LD50 value of 4.50×10⁸ cells/fish. The challenge trials indicated that *Chryseobacterium* sp. was moderately virulent to goldfish. In our earlier challenge experiments, *Chryseobacterium* sp. PLI₂ caused substantial mortalities (25%) in abraded-bath (2.2×10⁷ cells/mL) treated pacu fries, with pale and inflamed kidneys (ABRAHAM et al., 2017). In earlier studies, members of the genus *Chryseobacterium*, such as *C. indologenes* were also confirmed to be pathogenic to *P. flavescens* (PRIDGEON et al., 2013), and *C. piscicola* was suggested to be moderately virulent to *Salmo salar* (ILARDI et al., 2010). In this study, the immersion of abraded goldfish at 2.5×10⁷ cells/mL resulted in 40% mortality, thus suggesting that *Chryseobacterium* sp. PLI₂ requires a breach of a primary barrier to initiate disease processes and mortality.

Histopathologically, cartilaginous tissue inflammation, fusion of lamellae and necrosis were observed on the gills, which corroborates several earlier studies on chryseobactériosis (MUDARRIS and AUSTIN, 1992; LOCH, 2012; LOCH and FAISAL, 2015a,b), where necrosis, epithelial hyperplasia of the gill lamellae and interlamellar space, lamellar fusion, monocytic infiltrate, and mucus cell hyperplasia within the primary lamellae were noted. The histopathological alterations observed in the kidneys suggested that *Chryseobacterium* sp. can elicit pathological changes similar to those of known fish pathogens. WEERAPORNPRASIT et al. (2015) reported granuloma formations in the kidneys, as also in this study. Observations such as degeneration and necrosis of renal tubular epithelial cells, necrosis of interstitial tissue within the posterior
kidney, dilatation and necrosis of glomeruli, congestion, and oedema within the kidneys were also reported (MUDARRIS and AUSTIN, 1992; LOCH, 2012; LOCH and FAISAL, 2014; 2015a,b; WEERAPORNPRASIT et al., 2015). In an earlier study necrosis of glomeruli, congestion, and oedema within the kidneys were also reported (MUDARRIS and AUSTIN (1992), but no pathological changes in either the heart or spleen of fish with chryseobacteriosis were reported. In contrast, the present study recorded sinusoidal dilation, melanomacrophage aggregate, marginated leucocytes with a hypertrophied nucleus, necrotized areas and foamy appearance in the spleen of the challenged C. auratus. In support of our study, some recent reports have also revealed pathological changes in Chryseobacterium infected spleen (LOCH, 2012; WEERAPORNPRASIT et al., 2015). Our results, thus, demonstrated that Chryseobacterium sp. has the pathogenic potential to cause systemic changes in challenged goldfish. The histopathological evidence further indicated that the inflammation type following induced chryseobacteriosis in C. auratus was granulomatous in nature.

**Conclusion**

The results of the present study, in general, revealed that Chryseobacterium sp. can elicit pathogenesis both on the external body parts as well as in internal organs, thus proving its capability to be a causative agent of systemic disease in ornamental fish. With the intensification of global aquaculture operations and the movement of live ornamental goldfish throughout the world, the emergence of Chryseobacterium as a systemic fish pathogen is a serious cause for concern. Further investigations are, however, required to fully elucidate the pathogenic mechanisms of Chryseobacterium in goldfish, for proper disease management measures.

**Conflicts of interest**

The authors declare that there is no conflict of interest.

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704

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SAŽETAK
U ovome je radu analizirana patogenost i patološke promjene u zlatnih ribica Carassius auratus pokusno inficiranih bakterijom Chryseobacterium sp. PLI2. Lezije tipične za krizeobakteriozu razvile su se u intraperitonealno inficiranih i abrazijskom kupkom tretiranih riba. Određena je LD50 od 3,13×10⁷ stanic/ribi, koja je uzrokovala 40 % pomora unutar tri tjedna u riba inficiranih dozom od 2,50×10⁷ stanic/mL. Zaražene su ribe bile letargične, anoreksične i ataksične. S razvojem bolesti sve su razvile bijele mrlje po škrgama, intenzivan sluzavi sekret, lezije na stražnjim pedunkulima, žarišna krvarenja po koži, krvarenja u oku i škrznim poklopcima, ljuštenje kože i mršavljenje, a utvrđeni su i blijedi bubrezi i tamno obojena slezena. Histološki je na škrgama utvrđena upala hrskavičnog tkiva, spajanje lamela i proširene nekroze. U slezeni je zamijećeno proširenje sinusoida, nakupljanje melanomakrofaga, marginacija leukocita s hipertofičnom jezgrom i nekrotičnim područjima. U bubrezima je uočeno formiranje granuluma te upalne promjene u tubulima i glomerulima, proširene nekroze, blago nakupljanje melanomakrofaga, acentrične jezgre, suženje tubula s vakuoliziranjem okolinom, hipoplazija hematopoetskog tkiva, distrofija tubularnog epitela s talozima proteina u lumenu i fibroza okolnog područja. Pokus je dokazao da Chryseobacterium sp. može uzrokovati opću bolest u zlatnih ribica.

Ključne riječi: Carassius auratus; Chryseobacterium sp.; patogenost; patohistologija; granulom