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Short communication

Identification and pathogenicity of *Plesiomonas shigelloides* in Silver Carp

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ABSTRACT

Plesiomonas shigelloides was isolated from diseased silver carp in Kolkata Wetland, West Bengal, India. It was associated with mortality of Silver Carp, *Hypophthalmichthys molitrix,* which is the most widely cultivable fish species in fresh water aquaculture. The *P. shigelloides* was identified based on the biochemical and molecular characterization and the pathogenicity was studied on *H. molitrix*. The 16S rRNA gene sequence of the present isolate (NCBI GenBank Accession Number- KX986915) revealed that, the isolate was 100% identical with *P. shigelloides* (NCBI GenBank Accession Number- MF100995). *P. shigelloides* killed 10–100% fish in experimental infection and the LD_{50} value was calculated as 1.3×10^5CFU per fish with intraperitoneal injection. The challenged fish had subcutaneous haemorrhagic ulcers and reddening at injection sites, however there was no clinical sign observed in the gill. The histology of experimentally challenged *H. molitrix* showed the kidney alterations and exhibited shrinkage of epithelial cells in renal tubule and enlargement of glomerular cells.Liver showed focal accumulation of edematous fluid, necrosis and infiltration of heterophils indicative of bacterial infection. The pathology and pathogenesis studies of this pathogen in inland open waters like wetlands and lakes would help in management of the outbreak of disease in these aquatic ecosystems.

1. Introduction

The planktivorous filter feeding Silver Carp (*Hypophthalmichthys molitrix)* is one of the Carp species, has great potential in aquaculture in the developing countries for its faster growth rate and lower feeding cost in comparison with other fish species (Shagar and El-Refaee, 2012). However, the intensification and expansion of aquaculture using Silver Carp which is having higher susceptibility to various bacterial pathogens, causing severe threat to this industry in India and Bangladesh (Mishra et al., 2017). Several bacterial infections in conjunction with environmental factors result as prime cause for mass mortalities and poor growth thus, affecting the yield and marketability of this fish (Bagum et al., 2013).

Plesiomonas shigelloides is a motile, facultative anaerobic, gram-negative aquatic bacterium recently recognized as potential human and animal pathogen classified in the family. Enterobacteriaceae and is the only oxidase-positive member of this family (Chen et al., 2013). Earlier reports on concurrent infection with this bacterium and other microorganisms like *Aeromonas*spp.,*Vibrio* spp., and *Edwardsiella tarda* indicated that water is the environmental niche for *P. shigelloides* (Butt et al., 1997). Fresh and estuarine waters are primary reservoir for this bacterium which is mostly reported from tropical and subtropical countries. Thus various poikilotherms and homeotherms, aquatic organisms, water dwelling reptiles and birds can harbor this bacterium. High mortality in trout due to *P. shigelloides* alone (Cruz et al., 1986) and along with other bacteria like *Flavobacterium* spp. and *Aeromonas hydrophilla* were also reported (Vladik and Vitovec, 1974). This pathogen was identified as one of the main pathogen to cultured sturgeons in Beijing area (Wang et al., 2013). Recently, *P. shigelloides* was also isolated from clinical cases of fishes during mass mortality of *Ctenopharyngodo nidellus* (Hu et al., 2014) and *Orechromis nilotica* (Liu et al., 2015) and proved to be highly pathogenic for these cultured fishes. Similar type of findings was also reported by Nisha et al. (2014), where 100% mortality recorded in cichlid ornamental fish due to infection caused by *P. shigelloides*. Finally, there is paucityon clear information regarding zoonotic importance of *P. shigelloides*, with the exception of few piscine species and frequency of the species caused infection in nonhuman vertebrates or other animals (Janda et al., 2016). This investigation was conducted to find out the role of *P. shigelloides* as causative agent of mass mortality in Silver Carp (*H.molitrix)* in a sewage fed Wetland at Kolkata, West Bengal, India during summer season through microbiological, molecular and clinico-pathological evaluation.

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Fig. 1. Protruded eye and diffused haemorrhagic spots on the body surface of the Silver Carp, *Hydpophthalmichthys molitrix*.

2. Materials and methods

2.1. Anamnestic data collection, physico-clinical examination of diseased fish and aquatic environment

Eco Park Lake, Kolkata, West Bengal, India (N 22°35′56″; E 88°28′01″) is a sewage fed recreational Aquatic Park which is used for aquaculture purpose. The fish species like, *Labeo rohita, Catla catla*, *Cirrhinus mrigala, Labeo bata* and *Hypophthalmichthys molitrix* are generally cultured in this Wetland. In the month of May 2016 disease outbreak occurred in *H. molitrix* (Silver carp) in the wetland. During the period of disease investigation and through passive data collection, 60% Silver carp mortality waso bserved. Live fishes were collected by netting for clinical examination. Most of the survived fishes were showing prominent clinical signs of haemorrhagic spots on the body surface (Fig. 1). Diseased fishes were brought to the laboratory in live condition for isolation of pathogenic bacteria and histopathological examination of internal organs.

2.2. Bacteria isolation

The moribund fishes with reddish lesions in the body were used for bacterial isolation. Fishes were anesthetized by using an overdose of clove oil (Merck, Germany) (50 μl/l). The fishes were cleaned with alcohol and then dissected. Samples of liver and kidney were taken aseptically and plated directly on Tryptic Soya Agar (TSA, Hi-media). The plates were incubated at 37°C for 24 h. Single colony isolates were selected and re-streaked on fresh TSA plates to obtain a pure culture. The pure culture was grown in Tryptic Soya Broth (TSB) and maintained as glycerol stock at −20 °C for further use in biochemical and molecular characterization, antimicrobial sensitivity and challenge study.

2.3. Biochemical characterization of bacterial isolate

The isolated strain was primarily characterized by Gram-staining. ONPG (β-galactosidase), lysine utilization, urease, nitrate reduction, Voges Proskauer's (VP), malonate utilization, esculin hydrolysis, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose and lactose oxidase, ornithine utilization, phenylalanine deamination, H_2S production, citrate utilization, methyl red and indole test were performed (Krovacek et al., 2000). The biochemical charecterstics of the isolate was compared with Bergey's manual of systemic bacteriology (Holt et al., 1994).

2.4. Detection of hemolysis by solid-phase assays

Solid hemolysis assay was performed by streaking *Plesiomonas shigelloides* strains on blood agar plates (blood agar base, Hi-media) supplemented with 5% sheep red blood cells (RBCs) to screen their hemolytic activity. A lucid hemolytic zone within the bacterial growth zone was scored as positive.

2.5. DNA isolation and 16S rRNA gene amplification

Bacterial genomic DNA was extracted by Sarkosyl method (Sambrook and Russel, 2001). The primers chosen for amplification of 16S rRNA gene were UFF2 5′-GTTGATCATGGCTCAG-3′ as forward primer and URF2 5′-GGTTCACTTGTTACGACTT-3′as reverse primer (Behera et al., 2017). The PCR amplification of the 16S rRNA gene was performed using 16S rRNA specific primers with the thermal cycler Gene Amp PCR system 9700 (ABI, USA). The final volume of the PCR reaction mixture (50 μl) was composed of 100 ng of isolated genomic DNA, 1U Taq DNA polymerase (Sigma, USA), 5 μl of 10× PCR buffer, 1 μl of 50 mM $MgCl_2$, 1 μl of 10 mMdNTP (Sigma, USA), and 1 μl of 10 pmol of each primer (Sigma, USA). The thermal condition was set with initial denaturation for 2 min. at 95 °C, 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 60 s and extension at 72 °C for 90 s with final extension for 7 min. at 72 °C. PCR product was visualized on 1.8% agarose gel (Behera et al., 2018; Das et al., 2018).

2.6. Molecular identification of the bacterium

The PCR product was sequenced in both directions using an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, CA) to check the validity of the sequence data. The forward and reverse sequences of the Eco3 were aligned using the software Bio Edit version 7.0.0. The sequence of forward stand was proofread using the sequence of complementary stand. 1407 bp of 16S rRNA sequence of Eco3 was then compared to sequences available in GenBank using the NCBI–BLAST program facility (http://www.ncbi.nlm.nih.gov/BLAST). 16S rRNA sequences of most known *Plesiomonas* sp. were retrieved from NCBI GenBank and phylogenetic analysis was carried out by applying the neighbour-joining and maximum-likelihood algorithms to ensure coherency of the clusters formed. Bootstrapping was performed (10,000 replications) using the MEGA 6 program (Tamura et al., 2013).

2.7. Experimental challenge

Live, apparently healthy *H. molitrix* weighing of about 15–20 g were procured from a local hatchery and were acclimatized under laboratory conditions for 15 days by providing commercially available fish diet twice daily at the rate of 2% of their body weight. The fish were randomly assigned to eight experimental groups in triplicates for challenge study. A total number of 270fishes were equally divided in 27groups with 10fishes in each 200 l capacity tanks (1× 3 tank as control and 8 × 3 for experimental challenges).

2.8. LD_{50} determination

The bacteria were sub-cultured in 10 ml of Brain Heart Infusion (BHI) broth and the suspension was incubated at 37 °C for 24 h. After incubation, the culture was centrifuged for 5 min at 5000 rpm. The supernatant was discarded and pellets were washed twice with normal saline (NS) solution and finally resuspended in 10 ml of NS. Taking 1 ml of cell suspension dilution was made up to 10^{-7} in NS and the number of cells/ml was determined by spread plate method after incubation at 37 °C for 24 h. The fish were injected with 0.2 ml of bacterial suspension intraperitoneally with final concentration of 2.5×10^9, 2.5×10^8, 2.5×10^7, 2.5×10^6, 2.5×10^5, 2.5×10^4 2.5×10^3 and 2.5×10^2 CFU ml^{-1} in respective group. The control group was injected with 0.2 ml of saline water. The experiment was carried out in triplicate. Fish mortality was recorded every 24 h for 7 days. The injected bacterial isolate as a pathogen was re-isolated from the blood and liver of moribund fish to satisfy Koch's postulates. The cumulative mortality and lowest bacterial dose which will cause 50% mortality (LD_{50}) in Silver Carp was calculated using the method described by Reed and Muench (1938).In order to satisfy Koch's postulate the bacteria was reisolated and identified from the kidney, blood and liver of