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Note

Association of *Enterobacter cloacae* in the mortality of *Pangasianodon hypophthalmus* (Sauvage, 1878) reared in culture pond in Bhimavaram, Andhra Pradesh, India

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ABSTRACT

Enterobacter cloacae, associated with mortality of freshwater reared *Pangasianodon hypophthalmus* in Bhimavaram, Andhra Pradesh, is reported for the first time in India. The prominent clinical signs of infected fish were reddish lesions near the pectoral fin and belly regions. The bacterium isolated from the infected fish was identified as *E. cloacae* by biochemical tests and 16SrDNA sequencing. The clinical signs could be reproduced in healthy fish following injection of the bacterial isolate. The results of this study provided strong evidence to link *E. cloacae* with the mortality of cultured *P. hypophthalmus.*

Keywords: Bacterial infection, *Enterobacter cloacae*, Mortality, *Pangasianodon hypophthalmus*

The freshwater catfish *Pangasianodon hypophthalmus,* indigenous to Mekong Delta in Vietnam, was introduced in India *via* Bangladesh. This exotic species has very good potential for inland aquaculture, with fast growing ability and good market value. Though *P. hypophthalmus* is highly resistant to diseases and can also tolerate poor water quality, compared to other freshwater fish species, emerging reports suggest that several diseases affect this fish, resulting in poor growth and mortality. Ferguson *et al*. (2001) described the pathology observed in clinically sick animals as severe multifocal necrotising bacterial infection and named the disease as bacillary necrosis of pangasius (BNP). The causative agent of BNP has been identified as *Edwardsiella ictaluri* (Crumlish *et al.,* 2002). Infections by *Aeromonas hydrophila* resulting in heavy mortality of cultured fish in Vietnamese *P. hypophthalmus* production system have also been reported (Subagja *et al.,* 1999). *A. hydrophila*, the aetiological agent of motile aeromonad septicemia (MAS), causes diseases in wide range of freshwater fish species (Newman, 1993), and is often associated with the infections of stressed or immunocompromised hosts (Roberts, 1993).

With the increase in aquaculture of *P. hypophthalmus* in India, the outbreak and spread of bacterial infections is a major concern. Recently, routine examination of farmed *P. hypophthalmus* in Bihmavaram region of Andhra Pradesh, India, indicated *E. cloacae* as the probable cause for mortality. *E. cloacae*, member of the family Enterobacteriaceae, is widely distributed in nature and also found in feces of humans and animals. *E. cloacae*, is an opportunistic pathogen of humans (Flyan *et al.,* 1987; Gaston 1988; Kanemitsu *et al.,* 2007) and other organisms, such as fish (Troast, 1975; Hansen *et al.,* 1990) and strains resistant to multiple antibiotics have been reported. In the study reported here, we present strong evidence to link *E. cloacae* with the mortality of cultured *P. hypophthalmus*. In this preliminary report, we describe results of microbiological investigations of a *P. hypophthalmus* infection that led to the isolation of *E. cloacae* as the causative agent. Incidences of fish mortalities of >50% were reported from two different culture ponds growing *P. hypophthalmus* in Bhimavaram region of Andhra Pradesh, India. The infected fish exhibited reddish lesions near the pectoral fin and the belly region. The moribund fishes collected for investigations were in emaciated condition, had swollen stomach, hemorrhagic belly and fins. Due to the accumulation of mucus, the gills were pale and viscous. The peritoneal cavity was distended with the presence of ascitic fluid and other organs were congested and pulpy (Fig. 1 and 2). A total of six diseased *P. hyopophthlmus,* weighing between 500 to 750 g, were collected for further studies. Fishes were sacrificed using an excess dose of the anesthetic MS222. Swab samples of liver, kidney and spleen were collected aseptically and streaked on to Luria Bertani (LB) agar plates (Hi Media, India) and incubated at 28 ^{0}C for 24 h. Individual colonies were isolated from the plates and purified on Brain Heart Infusion (BHI) agar (Hi Media, India). A total of seven

Fig. 1. Diseased *P. hypophthalmus* from the culture pond, showing clinical signs

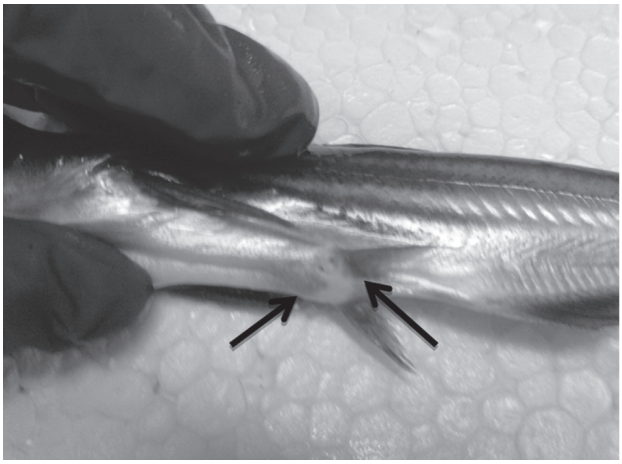

Fig. 2. *Pangasianodon hypophthalmus* after bacterial challenge (*Enterobacter cloacae*) showing clinical signs

isolates were selected on the basis of morphological characteristics. Bacteria were taken from the pure culture plates and inoculated into BHI broth (Hi Media, India) and incubated at 28 °C for 16 h. Subsequently, the culture was centrifuged for 15 min at 1800 *g*, the supernatant was discarded and the pellet was resuspended in 0.8% saline solution to an OD of 0.6 at 600 nm (2.46×10^7 cfu ml^{-1}). The bacterial suspensions were injected to seven sets of fishes, at the rate of 100-150 µl, each set comprising of 10 fish and the fish were examined for the development of clinical signs and mortality. The isolate, which produced clinical signs similar to the disease outbreak was subjected to further identification using biochemical tests and 16S rDNA sequencing.

For biochemical identification, KB002 Hiasssorted Biochemical Test kit for Gram negative rods (Hi Media, India) was used. For 16SrDNA sequencing, the genomic DNA was extracted from the bacteria using Qiaquick DNA extraction kit (Qiagen, USA) following manufacturer's instructions. The 16S ribosomal RNA gene was amplified with primers 27F (5' AGAGTTGATCATGGCTCAG 3') and 1498R (5' GGTTCACTTGTTACGACTT 3'). Amplification was performed in a thermal cycler (ESCO, USA) using the following programme: 1 cycle of 94 °C for 5 min and 35 Cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1.30 min and a final cycle of 72 °C for 10 min. The amplified products were checked on a 1% agarose gel. The amplified product was purified using Qiagen PCR purification kit (Qiagen, USA) and sequenced (Bangalore Genie, Bangalore, India). The 16S rRNA sequences were blast analysed with NCBI data base.

The outbreak of diseases is a major constraint in aquaculture systems, resulting in mortality and reduced yield. Though the information on bacterial diseases is sparse from India, studies from other Asian countries suggest that a number of bacterial diseases affect this species. Prominent among these are the bacillary necrosis of *Pangasius* (BNP) caused by *Edwardsiella icataluri*, and the motile aeromonad septicemia caused by *Aeromonas* spp,. mainly *Aeromonas hydrophila, A. sobria and A. caviae*) (Subagja *et al.,* 1999; Ferguson *et al.,* 2001; Crumlish *et al.,* 2002; Yuasa *et al.,* 2003). Our study was a preliminary investigation aimed at finding out the causative agent involved in the mortality of the *Pangasius* stocks reared in culture ponds in Bhimavaram, Andhra Pradesh. The isolates which produced similar clinical signs as in the case of disease outbreak formed small, spherical and elevated cream colour colonies on BHI agar plates and were Gram negative motile rods. Biochemical tests revealed that they were negative for H_2S production, phenylalanine deamination and lysine decarboxylase and positive for citrate utilisation and ornithine decarboxylase. The amplified 16S rRNA gene from this isolate was sequenced and BLAST analysed and the results revealed 99% homology with *Enterobacter cloacae* (GeneBank Accession Number: JN644526). The molecular sequence analysis and biochemical analysis confirmed that the isolated bacteria is *Enterobacter cloacae*. The involvement of *E. cloacae* in the mortality of the fish *Mugil cephalus* was earlier reported by Thillai Sekar *et al.* (2010). The causative strain MK produced a cationic factor identified as the putative virulence factor in fish. The results of our study indicated *E. cloacae* as the causative agent for infection and mortality of cultured *P. hypophthalmus* in Bhimavaram. Further studies are required to determine the prevalence of this bacterium in culture systems, its virulence factors responsible for infection in fish and the other biotic as well as abiotic factors that might predispose fish to infection by this opportunistic pathogen.

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