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Isolation and Identification of *Brucella Melitensis* in Diseased Nile Tilapia, *Oreochromis Niloticus* from a Commercial Fish Farm in Tamil Nadu, India

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Abstract

Brucellosis is a zoonotic bacterial disease affecting livestock with fewer reports in fishes. The present study reports the isolation and identification of *Brucella melitensis* from diseased Nile Tilapia, *Oreochromis niloticus* from a commercial fish farm. The diseased fish with clinical sings were collected and the organs *viz*., liver, kidney, spleen and intestine were aspectically dissected for isolation of bacterial pathogens. The bacteria isolate (H1/SRLAAH/2019) from the liver of the infected fish was characterised by the biochemical tests as *Brucella* sp. and confirmed by 16S rRNA gene sequencing analysis as *B.melitensis*. This is the first report on the isolation and identification of *B. melitensis* from *O. niloticus* in India.

Key words : Tilapia, zoonosis, *Brucella melitensis,* 16S rRNA gene sequencing

Brucellosis is a zoonotic disease caused by the bacterial pathogens of genus *Brucella* in mammals (Moreno *et al.,* 2002). *Brucella* spp. are facultative intracellular, gram-negative, cocco-

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bacilli, non-spore-forming, non-capsulated and non-motile bacteria (Seleem *et al.,* 2010; Fretin *et al.,* 2005). Nine *Brucella* sp., that are currently recognized to affect the terrestrial animals include *B. abortus, B. melitensis, B. suis, B. ovis, B. canis, B. neotomae, and B. microti* (Scholz *et al.,* 2008). However, *B. ceti, B. pinnipedialis* are known to infect the marine mammals with over 130 reported species as potential hosts of *Brucella* spp. (Hernandez-Mora *et al.,* 2013). Animal wastes are considered as the major source of contamination with *Brucella* spp. in water bodies. The major routes of transmission of Brucellosis from fish to humans are consumption of raw seafood and handling of infected fishes (Wael *et al.,* 2010; Hernandez-Mora *et al.,* 2013). The only available report on the *B.melitensis* infection in fish is from Nile catfish, *Clarias gariepinus,* Nile catfish collected from a water body contaminated with animal wastes in Egypt (Wael *et al.,* 2010). The aim of this work was to investigate the cause of the disease in a commercial fish farm undertaking culture of *O.niloticus* in Tamil Nadu, India.

Materials and Methods

Sample collection

A commercial fish farm with a total area of 1.5 ha, culturing Nile tilapia, *O.niloticus* in Tamil Nadu, India (12°49'31.2"N; 80°13'56.5"E) reported a disease outbreak with a mortality of 40-50% during June 2019. Diseased fish samples (n=20) (10 to 15cm, weighing 250 to 300g) with clinical symptoms discoloration of the skin, open ulcers on the body surface, fin and tail rot, sunken eyes and mortality ranging from 40-50% within a period of 7 days were collected in live condition and brought to the State Referral Laboratory for Aquatic Animal Health, TNJFU-Madhavaram Campus, Chennai. The fishes were aseptically dissected and swabs were collected from the liver, kidney, spleen and brain and subjected to bacterial isolation by streaking on to nutrient agar (Hi media) and incubated at 30 ± 2°C for 24–48 h. The predominant colonies from liver and spleen were streaked on the selective media for the isolation of the pathogens that are known to cause diseases in tilapia viz., Aeromonas isolation agar, Streptococcus isolation agar, HP6 Agar, XLD agar and Brucella selective medium base with Brucella selective supplement (Himedia, India) and incubated at appropriate temperatures and duration. Growth of the isolate was observed in the Brucella selective medium incubated under 5% CO_2 at 37°C for 3 days whereas no growth was observed in other selective media thereby showing the absence of other pathogens. Individual bacterial colonies from the liver, grown on Brucella media (H1/SRLAAH/2019) was sub-cultured and maintained on BHIA slant for further characterization by Gram staining, H_2S, catalase, oxidase, methyl red, indole and urease production.

Molecular characterization

For molecular confirmation by PCR, the genomic DNA was extracted from the isolate (H1/SRLAAH/2019) using QIAamp genomic DNA kit (Qiagen, Germany) following manufacturer's protocol. PCR was carried out in a thermal cycler (Biorad, USA) in a total volume of 25µl following the primers and protocols of Weisburg *et al.,*1991 targeting the 16S rRNA. The PCR amplicons were resolved by horizontal gel electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.8 mg/ml) at 100 V and visualized under UV illumination using a gel documentation system (Biorad, USA). The PCR amplified products were sequenced using commercial sequencing services (Eurofins, Bengaluru, India). The edited 16S rDNA gene sequence was compared with the sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) using the BLAST (Basic Local Alignment Search Tool) program (http://blast.ncbi.nlm.nih.gov) and submitted to the GenBank. The fixed tissues of the liver, kidney, spleen and intestine were processed for histopathology following standard methods (Roberts, 2012).

Results and Discussions

O.niloticus samples collected from the farm were observed to be weak and gasping at the water surface with symptoms of discoloration of the skin, open ulcers on the body surface, fin/tail rot and sunken eyes (Fig. 1). The microscopic observation of the wet mount squash of gills and skin (40x) showed the absence of parasite

infestation. The isolate (H1/SRLAAH/2019) from liver tissue grown in Brucella agar with selective supplements showed elevated circular colony with smooth margin, Gram-negative coccobacilli, positive for catalase, urease and oxidase and negative for H_2S production, methyl red and indole tests which are presumptive of *Brucella* sp. PCR amplification of 16S rRNA and sequencing confirmed the isolate as *Brucella melitensis* (Genbank Acc No. MT275734).

Fig.1. *Oreochromis niloticus* infected with Brucellosis

Brucellosis is an important zoonotic disease reported to cause chornic diseases in livestock (Scholz *et al.,* 2008), humans (Barua *et al.,* 2016; Lindahl, 2020) and marine mammals (Foster *et al.,* 2007). This bacterial pathogen is classified by the CDC as a category (B) pathogen that has potential for development as a bio-weapon (Seleem *et al.,* 2010). However, there is no report on this disease infecting freshwater farmed fish although *B.melitensis* presence has been confirmed in the wild in Nile catfish (*Clarias gariepinus*) from the delta region of the Nile river in Egypt contaminated with livestock wastes (Wael *et al.,* 2010). Catfish experimentally inoculated with *B.melitensis* biovar 3 showed skin lesions have been documented (Salem and Mohsen,1997). Brucellosis is considered a re-emerging zoonosis due to the emergence of novel Brucella strains that are establishing itself in new hosts and ecological niches (Thakur, 2012). Marine Brucellae have been identified in case of brucellosis in humans due to consumption of raw seafood (McDonald *et al.,* 2006; Sohn *et al.,* 2003). Many aspects of interaction between *Brucella* and its host remain unclear so far (Seleem *et al*, 2008). As *B.melitensis* has been isolated in this study from *O.niloticus,* which is one of the widely farmed and consumed fish, potential danger of the spread of this disease to humans through consumption of raw or improperly cooked infected fish and handling during farming operations have to be considered. Histopathological changes were observed in the kidney, liver and brain of the infected *O.niloticus.* Congestion of blood vessels in brain and liver, neutrophilis and mononuclear cell infiltration in kidney tissue due to bacterial infection were recorded (Fig.2a, b, c).

Summary

Tilapia is the second most farmed fish globally. Although many diseases in farmed tilapia have been reported, this study has documented the presence of *B.melitensis* in tilapia collected from

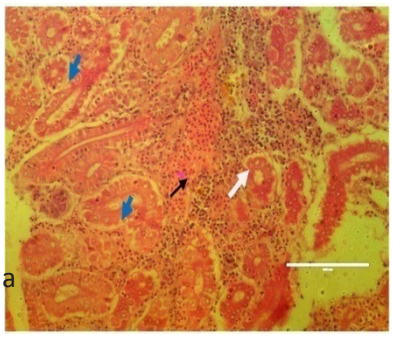

Fig 2.a. Kidney tissue of infected O.niloticus showing congestion, multifocal mild to moderate interstitial mononuclear cell infiltration with mild tubular epithelial cell degeneration and necrosis

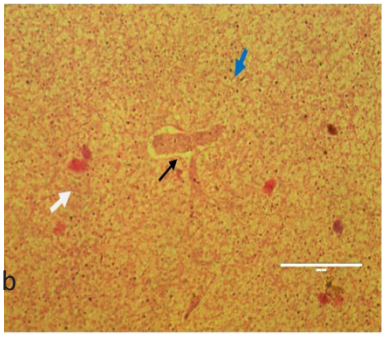

Fig 2.b. Infected Liver tissue of O.niloticus showing congestion, moderate degeneration and early necrosis of hepatocytes

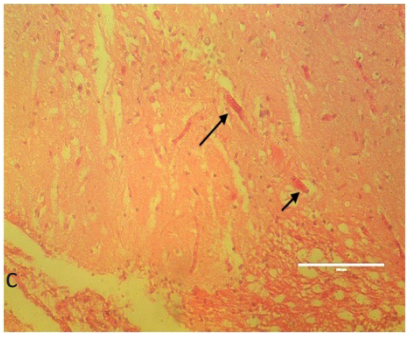

Fig 2.c. Brucella sp. infected Brain tissue of O.niloticus showing congestion of blood vessels.

a farm with disease outbreak. The results of their study highlight the importance of biosecurity in fish farms.

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