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# RAPID IDENTIFICATION OF PATHOGENIC STREPTOCOCCI ISOLATED FROM MORIBUND RED TILAPIA (*OREOCHROMIS* SPP.)

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Accurate and rapid identification of bacterial pathogens of fish is essential for the effective treatment and speedy control of infections. Massive mortalities in market-sized red tilapia (*Oreochromis* spp.) were noticed in mariculture concrete ponds in northern Egypt. Histopathological examination revealed marked congestion in the central vein of the liver with the presence of bacterial aggregates inside the lumen and in the vicinity of the central vein. A total of 12 isolates of streptococci were obtained from the moribund fish. This study documented the ability of the MicroSeq 500 16S bacterial sequencing method to accurately identify *Streptococcus agalactiae* and *S. dysgalactiae* mixed infections from moribund red tilapia that were difficult to be recognised by the commercial biochemical systems. The continuously decreasing cost of the sequencing technique should encourage its application in routine diagnostic procedures.

**Key words:** *Streptococcus agalactiae, S. dysgalactiae,* red tilapia, MicroSeq 500, histopathology

Tilapia culture had been practised in Egypt via traditional methods for thousands of years as depicted by paintings on the walls of Egyptian tombs (Bardach et al., 1972). Egypt is now the second largest tilapia producer in the world, after China (Eltholth et al., 2015). The scarcity of freshwater resources and the competition with agriculture and other urban activities oriented the effort towards mariculture (Moustafa et al., 2015). Red tilapia is a promising species for mariculture due to its salinity tolerance (Romana-Eguia and Eguia, 1999). However, the poor spawning performance of red tilapia and its vulnerability to infectious diseases are among the most significant constraints (Hulata et al., 1995).

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Streptococcosis is responsible for considerable losses in global tilapia production (Pretto-Giordano et al., 2010). This disease is caused by a variety of phenotypes belonging to several genera, such as *Streptococcus*, *Lactococcus* and *Enterococcus*. The most severe epizootics are caused by *S. agalactiae* and *S. iniae* (Chen et al., 2007). Recently, *S. dysgalactiae* has also been listed as an emerging fish pathogen (Abdelsalam et al., 2009*a*,*b*, 2010*a,b*, 2013, 2015). All these *Streptococcus* spp. are also significant zoonotic pathogens.

The identification of these pathogens using commercial biochemical systems has always resulted in misdiagnosis due to the variability of phenotypic traits among given isolates of the same species, and the inconsistency of biochemical results due to the modification in manual procedures adjusted for marine fish pathogens (Verner-Jeffreys et al., 2012). These phenotypic tests might also yield variable results depending on the culture conditions of the bacteria (Hopkins et al., 2001). In addition, 50% of *Streptococcus* spp. belonging to Lancefield groups B and C failed to be identified with the Rapid Strep system (Watts and Yancey, 1994). Therefore, comparison of the 16S rRNA sequences of investigated bacteria has high discriminatory power to resolve closely related species (Wagner et al., 2003). MicroSeq500 16S rRNA sequencing is an excellent commercial identification assay marketed for the rapid and accurate identification of bacterial pathogens (Woo et al., 2009) via sequencing the first 500 base pairs of the 16S rRNA gene. The present work was undertaken to investigate the aetiological agents of streptococcal septicaemia in cultured red tilapia in northern Egypt using Microseq 500 assay. The histopathological changes caused by natural streptococcal infections in red tilapia were also investigated in this study.

## Materials and methods

### *Sampling*

Massive losses in farmed red tilapia (160–250 g) were recorded in concrete ponds in northern Egypt during August 2015. Fish were stocked at densities of 40 fish/$m^3$. Sixty red tilapia and five cohabitant *Tilapia zilli* (invasive species) were randomly sampled from the investigated farm. Fish specimens were then preserved in isothermal boxes and transferred to the laboratory.

### *Bacteriological and biochemical examinations*

Loopfuls were obtained from the kidney, liver and brain under completely aseptic condition, then cultured on oxolinic acid–blood agar supplemented with 1.5% NaCl and incubated at 25 °C for 36 h for the selective isolation of streptococci according to Abdelsalam et al. (2013). Gram staining, haemolysis, oxidase and catalase tests were performed.

*MicroSeq500 16S rDNA sequencing*

Genomic DNA was extracted from the cultivated strains on tryptic soy agar (TSA) (Oxoid) supplemented with 1.5% NaCl using prepMan Ultra reagent as described by the manufacturer (Applied Biosystems, USA). A 500-bp to the 5' end segment of the 16S rRNA gene of bacterial strains was amplified using MicroSeq500 16S rDNA Bacterial Identification PCR kit (Applied Biosystems, USA). The PCR products were then purified using a PCR purification kit (Qiagen, Valencia, California). The sequencing reaction consisted of 13 µl of the MicroSeq 500 sequencing mix (containing 3.2 pmol of 005F and 531R primers), 4 µl of sterile distilled water, and 3 µl of purified amplified product. All sequencing analyses were performed in two directions. The sequencing data were analysed and compared with 16S rRNA gene sequences in the GenBank database using the blast program. The nucleotide sequences of the 16S rRNA of *Streptococcus* sp. strains were submitted to the DNA Data Bank of Japan. The detailed criteria were as follows: identification to the species level was defined as a 16S rRNA gene sequence similarity of ≥ 99% relative to the prototype strain sequence in GenBank, and identification to the genus level was defined as a 16S rRNA gene sequence similarity of ≥ 97% relative to the prototype strain sequence in GenBank. Failure to identify the isolate was defined as a 16S rRNA gene sequence similarity score below 97%. The phylogenetic analysis was carried out using MEGA version 5 (Tamura et al., 2011) and compared to related *Streptococcus* species (*S. equisimilis, S. iniae* and *S. parauberis*). A phylogenetic tree was constructed using the neighbour-joining method with a genetic distance calculated using the Kimura two-step algorithm (substitutions included transitions and transversions, the pattern among lineages assumed homogeneous, and the rate variation among sites uniform) with 1,000 bootstrap replicates.

*Histopathological examination*

Specimens from the liver, kidney, spleen and muscles of the infected red tilapia were fixed in 10% neutral buffered formalin, processed, then embedded in paraffin after dehydration and sectioned at 5 µm thickness using a microtome. The sections were stained with haematoxylin and eosin (HE) (Bancroft and Gamble, 2008).

## Results

*Clinical examination*

External haemorrhages, skin erosions, opacity and exophthalmia were recorded on the examined fish. Some fish demonstrated a prolapsed and haemorrhagic vent. Internally, the liver, spleen and kidney were congested with the presence of blood-tinged ascitic fluid. The average values measured for dissolved oxygen and un-ionised ammonia were 3 $mgl^{-1}$ and 1.05 $mgl^{-1}$, respectively.

*Identification of bacteria*

Gram-positive cocci arranged in chains were detected. A total number of 9 bacterial isolates were obtained from the samples. The sequencing of bacterial 16S rRNA genes using microseq 500 assay has identified the isolates as *S. agalactiae* (9 isolates) and *S. dysgalactiae* (3 isolates) (Table 1). The blast results of yielded sequences produced 100% homology with *S. agalactiae* and *S. dysgalactiae* in the GenBank database. *Streptococcus agalactiae* was detected in water (1 isolate), and also from invading *Tilapia zilli* (1 isolate). The generated phylogenetic tree grouped all *S. agalactiae* isolates in one cluster irrespective of their sources, and they were separated from *S. dysgalactiae* (Fig. 1). No other bacterial species were detected in any of the investigated samples.

**Table 1**

*Streptococcus* spp. isolated in this study

| No. | Isolate | Source | *Streptococcus* spp. | 16S rRNA Accession no. |
|---|---|---|---|---|
| 1 | dys150910 | Red tilapia | *S. dysgalactiae* | LC093434 |
| 2 | dys150911 | Red tilapia | *S. dysgalactiae* | LC093435 |
| 3 | dys150912 | Red tilapia | *S. dysgalactiae* | LC093436 |
| 4 | aga150914 | Red tilapia | *S. agalactiae* | LC093438 |
| 5 | aga150915 | Red tilapia | *S. agalactiae* | LC093439 |
| 6 | aga150916 | Red tilapia | *S. agalactiae* | LC093440 |
| 7 | aga150917 | Red tilapia | *S. agalactiae* | LC093441 |
| 8 | aga150918 | Red tilapia | *S. agalactiae* | LC093442 |
| 9 | aga150919 | Red tilapia | *S. agalactiae* | LC093443 |
| 10 | aga150920 | Red tilapia | *S. agalactiae* | LC093444 |
| 11 | aga150921 | Red tilapia | *S. agalactiae* | LC093445 |
| 12 | aga150922 | Red tilapia | *S. agalactiae* | LC093446 |
| 13 | aga150923 | *Tilapia zilli* | *S. agalactiae* | LC093447 |
| 14 | aga150926 | Water | *S. agalactiae* | LC093450 |

*Histopathological lesions*

Histopathological examination revealed hyperactivation of the melanomacrophage centres in the liver, kidney and spleen, which was a commonly detected finding. Marked congestion was observed in the central vein of the liver with the presence of bacterial colonies inside the lumen and in the vicinity of the central vein. Additionally, focal necrotic changes and fatty infiltrations of the hepatocytes were also noticed. Mononuclear cell infiltrations were noted between renal tubules. Moreover, infiltration by inflammatory cells between muscle bundles indicated mild myositis. Furthermore, degenerative changes were detected in the muscle bundles (Fig. 2).

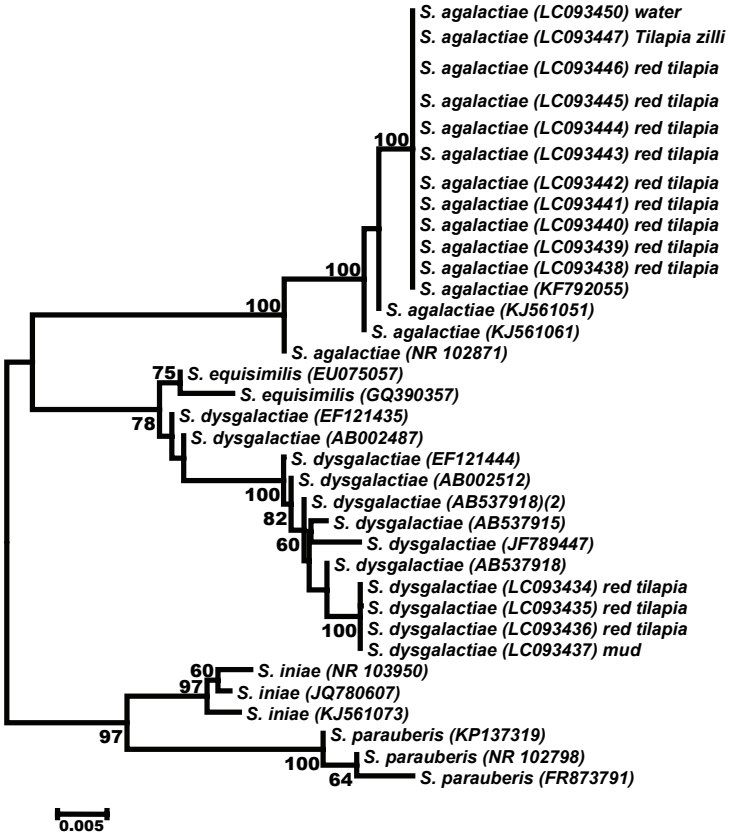
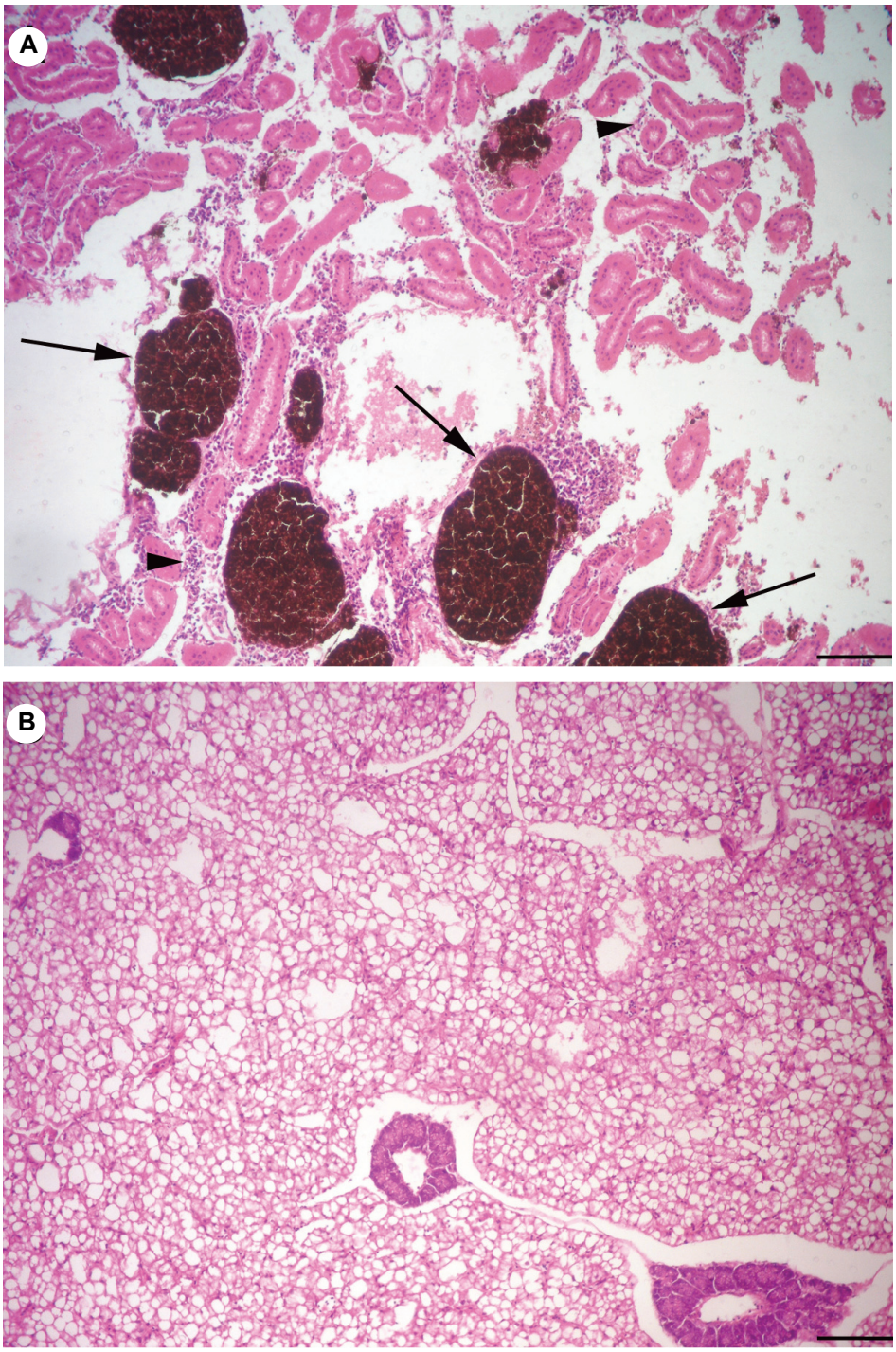



*Fig. 1.* Phylogenetic tree based on the 16S rDNA gene sequences of *Streptococcus dysgalactiae* and *S. agalactiae* recovered from moribund red tilapia

## Discussion

*Streptococcus agalactiae* was incriminated in the massive fish kill in the Kuwait bay during 2000–2001 (Evans et al., 2002). It was also isolated from the colossal red tilapia losses in Asia (Abuseliana et al., 2011). Recently *S. agalactiae* has also been incriminated in mass mortalities affecting seabream and seabass cultured in northern Egypt (Elgendy, 2013). Hernandez et al. (2009) recorded mortalities of similar intensity in the brood-stocks of red tilapia. The first epizootic outbreak caused by *S. dysgalactiae* was detected in amberjack *(Seriola dumerili)* and yellowtail *(S. quinqueradiata)* in Japan (Nomoto et al., 2004), with necrosis in the caudal peduncle area (Abdelsalam et al., 2013, 2015). The present study documented heavy losses in large-size red tilapia cultured in a marine aquaculture farm in Egypt. The recovered isolates were identified as *S. agalactiae* and *S. dysgalactiae*.


A
B

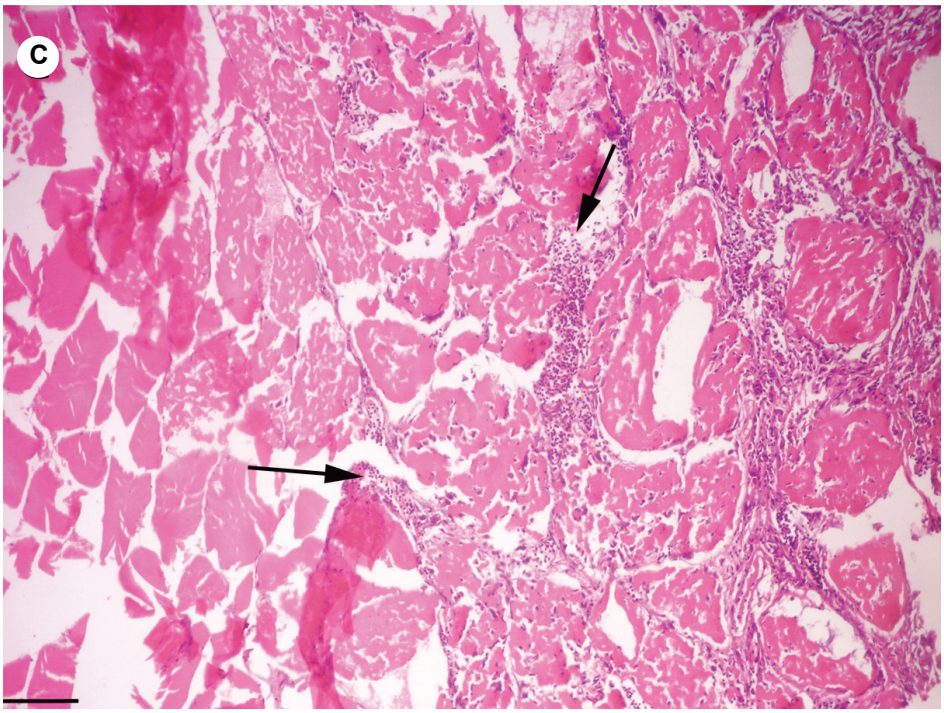



*Fig. 2.* (A) A microphotograph of the kidney showing mild infiltrations of mononuclear cells between the renal tubules (head arrows) and hyperactivation of the melanomacrophage centres (arrows). Haematoxylin and eosin (HE), scale bar = 50 µm; (B) A microphotograph of the liver showing fatty infiltrations in the hepatocytes with round regular vacuoles. HE, scale bar = 100 µm; (C) A microphotograph of the muscles showing moderate mononuclear cell infiltration between muscle bundles with degenerative changes (arrows). HE, scale bar = 100 µm

By sequencing the 16S rRNA gene of the retrieved isolates using microseq 500 assay, the identity of the recovered strains was confirmed as *S. agalactiae* and *S. dysgalactiae.* The blast results of the yielded sequence produced 100% homology with the *S. agalactiae* and *S. dysgalactiae* GenBank database. In addition, the generated phylogenetic tree confirmed their identities, and *S. dysgalactiae* isolates were well distinguished from those of *S. agalactiae*. This method has proven successful in human and veterinary clinical medicine. Results of the microseq 500 assay proved reliable for the identification and differentiation of *Streptococcus* spp. in fish. Microbiological analysis indicated the presence of streptococci in water and in *T. zilli*, highlighting the probable source of infections. Overcrowded conditions and high stocking densities are thought to be among the predisposing factors (Abdelsalam et al., 2013). In this study, the mean values recorded for dissolved oxygen and un-ionised ammonia in farming ponds were far from the optimum values. Prolonged exposure to un-ionised ammonia in water with low dissolved oxygen level predisposes tilapias to diseases including streptococcosis (Amal, 2011).

Various alterations were noticed in the histopathological sections. Hyperactivation of melanomacrophage centres in the haematopoietic organs was frequently detected. The hypertrophy of melanomacrophages is commonly linked with streptococcal infections (Fawzy et al., 2014). Their phagocytic functions as well as sequestration of cellular degradation products have been documented against fish pathogens (Agius and Roberts, 2003). Necrotic and degenerative changes were also detected in various tissues. These alterations are indicative of a systemic infection, which is supported by the isolation of bacteria from various fish tissues. Myositis of the skeletal muscles was observed, and this was also reported in the case of *S. iniae* infection (Lahav et al., 2004). The presence of bacterial aggregations suggestive of streptococci was observed in the histological sections, which was consistent with the results obtained by Zamri-Saad et al. (2010) in red tilapia naturally infected by *S. agalactiae*. This might also indicate the resistance of bacteria to fish defence mechanisms and their role in overwhelming the capacity of the immune system (Chen et al., 2007). The demonstrated histopathological lesions suggested the presence of acute infection and supported the assumption that the haematogenous route is the common way for *S. dysgalactiae* spread to induce systemic infection (Chang and Plumb, 1996).

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