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STREPTOCOCCUS INIAE OUTBREAKS IN BRAZILIAN NILE TILAPIA (*OREOCHROMIS NILOTICUS* L.) FARMS

H. C. P. Figueiredo*; L. Nobrega Netto; C. A. G. Leal; Ulisses P. Pereira; Glaúcia F. Mian.

AQUAVET- Laboratório de Doenças de Animais Aquáticos, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil.

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ABSTRACT

This is the first report of outbreaks of *Streptococcus iniae* in Nile tilapia farms in South America. Seven isolates were identified by biochemical, serological and molecular tests. Their 16S rRNA gene sequences showed 100% similarity with *S. iniae* ATCC 29178 and two distinct PFGE patterns were observed for Brazilian isolates.

Key words: Brazil, infection, MIC, PFGE, phylogenetic analysis, tilapia.

Streptococcosis is a major problem for fish production worldwide and it is associated with high economic losses. Currently, *Streptococcus iniae* (1), *Streptococcus agalactiae* (8), *Streptococcus dysgalactiae* (14), *Streptococcus phocae* (17), *Streptococcus parauberis* and *Streptococcus ictaluri* (2) have been described as pathogenic to fish.

Streptococcus iniae was first isolated from multifocal subcutaneous abscesses in captive Amazon freshwater dolphins, *Inia geoffrensis* (16). In fish, *S. iniae* infections are characterized by meningoencephalitis and septicemia that generally induce high morbidity and mortality rates. Disease outbreaks have been described in three distinct regions of the world: North America; the Middle East; and the Asia-Pacific (1). To date, only outbreaks of disease caused by *S. agalactiae* in Nile tilapia (*Oreochromis niloticus* L.) in commercial fish farms in Brazil were described (13). Thus, no description of *S. iniae* infection in fish was reported in the country until now.

The aim of this work was to describe outbreaks of *S. iniae* infection in Brazilian Nile tilapia farms, the sensibility of the isolates to florfenicol and to perform the genetic characterization of the strains.

Outbreaks of meningoencephalitis and septicemia were accompanied in two commercial Nile tilapia farms located in Paraná State, Brazil. These farms were located about 3 km apart on the same river. Fish (average weight of 800 g) were reared in cages under high stock densities (150 Kg m^{-3}). Inside the cages, the water temperature was approximately 30 °C and dissolved oxygen showed concentration higher than 4 mg L^{-1}. The main clinical signs observed were lethargy, erratic swimming, exophthalmia, skin darkness, and high mortality rates. Ten diseased fish from each farm were sampled, stored at 4 °C, and immediately transported to the laboratory for bacteriological analysis.

Swabs of brain and kidney tissue from each fish were sampled aseptically, streaked onto 5% sheep blood agar, and incubated at 28 °C for 72 h. The colonies obtained were tested by Gram stain, catalase and oxidase production and hemolysis. The isolates were further characterized phenotypically and

***Corresponding Author. Mailing address:** AQUAVET- Laboratory of Aquatic Animal Diseases, Av. Antônio Carlos 6627, Pampulha, Belo Horizonte, MG, Brazil. 30.123970.; Tel.: +55 31 34092126 Fax: +55 31 34092080.; E-mail: henrique@dmv.ufla.br

serologically using API20 STREP and Slidex Latex Agglutination kits (both from BioMerieux, France), respectively.

The isolates were grown on sheep blood agar supplemented with 5% sheep blood for 48 h at 30 °C. The colonies were collected, diluted in 180 µL of lysis solution (20 mg ml^{-1} lisozyme; 20 mM Tris-HCl, pH 8.0; 2 mM EDTA and 1.2% Triton®), and incubated at 37 °C for 1 h. Total bacterial DNA was then extracted using the commercial kit QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. To confirm the diagnosis, extracted DNA was used as a template in a *S. iniae*-specific PCR performed according to the procedure previously described by Mata *et al.* (12). In addition, 16S rRNA of three randomly selected strains (SI25-06, SI27-06 and SI29-06) were amplified by PCR with the universal primers C70 and B37 according to the method described by Fox *et al.* (10). After amplification, PCR products were purified using a Wizard PCR Preps kit (Promega) and sequenced. Sequencing reactions were performed using a BigDye™ Terminator Cycle sequencing kit and run on an ABI 3730XL genetic analyzer (both from Applied Biosystems).

The phylogenetic relationship of the three isolates was determined by comparative 16S rRNA gene sequence analysis. The sequences were aligned using CLUSTALW (23) with sequences of the following streptococcal species (available in the NCBI database): *Streptococcus iniae* ATCC 29178 (AF335572); *Streptococcus parauberis* (AY584477); *Streptococcus phocae* (AF235052); *Streptococcus agalactiae* (AB002479); *Streptococcus dysgalactiae* subsp. *dysgalactiae* (AB002485); *Streptococcus dysgalactiae* subsp. *equisimilis* (DQ232540); *Streptococcus ictaluri* (DQ462421); *Streptococcus didelphis* (AF176103); *Streptococcus porcinus* (AB002523); *Streptococcus castoreus* (AJ606047); and three fish strains of *S. iniae*, being two isolated from Nile tilapia, JW6 (GQ338314) and JW9 (GQ338315), and one from rainbow trout (*Oncorhyncus mykiss*) SF2 (FJ870987) . The genetic distances matrix was obtained using Kimura's two-parameter model (11) and an evolutionary tree was created using the neighbor joining method (18) with Mega4 (22). Bootstrap values from 1000 replicates are displayed as percentages.

Restriction enzyme *Sma*I digests of genomic DNA of all isolates were analyzed by pulsed field gel electrophoresis (PFGE) as previously described by Oliveira *et al.* (15). Gel image were analyzed using Gel ComparII software® (Applied Maths) to make dendograms of the genetic relationships among the strains. The Dice coefficient (95%) and unweighted pair group method with average linkages (UPGMA) was used for banding pattern and cluster analysis, respectively. The isolates that showed 100% similarity were considered indistinguishable and the isolates with similarity greater than 80% were considered clonally related (21).

Florfenicol susceptibility tests were conducted by broth microdilution and disk susceptibility methods according to the guidelines M42-A and M49-A, respectively, both established by the Clinical and Laboratory Standards Intitute (3, 4). The minimum inhibitory concentration (MIC) tests for florfenicol were performed using sterile dry microplates (Trek Diagnostic System) with the antibiotic concentration ranging from 0.06 to 64 µg mL^{-1}. *Escherichia coli* ATCC 25922 was used as quality control for plates and procedures performed each day. All strains were tested in duplicate. The MIC was defined as the lowest concentration of antibiotic that prevented visible bacterial growth. Disk diffusion tests were performed on Mueller–Hinton agar (Difco), and with disks containing 30 µg of florfenicol (Oxoid). *Escherichia coli* ATCC 25922 was used as quality control in all procedures. The diameters of the inhibition zones were measured with a ruler.

Gram-positive cocci were isolated from brain and kidney tissue of seven sick fish; five from farm A and two from farm B. These bacteria showed characteristic mucoid colonies with zones of β-haemolysis after 24 to 48 h incubation at 28 °C in ovine blood agar plates. Some colonies presented a double zone of haemolysis. None of the isolates could be serologically

typed to any Lancefield group and in the API 20 Strep test inconclusive results were obtained. All isolates showed positive reactions in the *S. iniae*-specific PCR assay, generating amplicons of 870 bp confirming the bacterial identification. Computational analysis of 16S rRNA sequences of *Streptococcus iniae* demonstrated that the Brazilian isolates are similar to each other and to reference strain ATCC 29178, showing a bootstrap percentage of 100%. Figure 1 presents the phylogenetic tree of *S. iniae* isolates from Brazil, other fish isolates, the reference strain, and closely related species of streptococci.

The PFGE analysis showed three different patterns (A_1, A_2, and B) among the studied strains. The seven Brazilian strains belonged to the same pulse type shared in two subtypes (A_1 and A_2) and *S. iniae* ATCC 29178 presented pattern B. The groups A_1 and A_2 were clonally related, with 88% similarity according to the Dice coefficient (Figure 2). The A_1 strains were isolated from both farms, whereas A_2 strains occurred only on farm B. Additionally, both Brazilian *S. iniae* clusters showed 36% similarity with pattern B of strain ATCC 29178 (Figure 2).

The distribution of the MIC values and the diameter of the inhibition zones of FLO against *Escherichia coli* ATCC 25922 ranged from 8 to 16 μg mL^{-1} and 24 to 30 mm, respectively. In both cases, these values were in agreement with the acceptable ranges (3, 4). The diameter of inhibition zone and MIC values of FLO against *S. iniae* ranged from 1 to 8 μg mL^{-1} and 28 to 32 mm, respectively. All strains were considered as susceptible to florfenicol by MIC and disk diffusion methods.

Currently, *S. iniae* is considered one of the most relevant streptococcal pathogen of cultured and wild fish (20, 5). While believing that this pathogen is distributed globally, before this study there is no report of its presence in Brazil, in contrast to other Gram-positive bacteria pathogenic to fish, such as *S. agalactiae* (13) and *Lactococcus garvieae* (9). Therefore, this study is the first report of disease outbreaks caused by *S. iniae* in Brazil.

In commercial fish farms, the main predisposing factors for *S. iniae* outbreaks are high stocking densities and high water temperatures; the disease usually occurs in adult fish (1, 19). Cages are the main culture system used for Nile tilapia production in Brazil. In this system, fish are reared under high stocking densities. Additionally, the outbreaks investigated in this study occurred during the summer and when fish reached the adult stage. These conditions were described as predisposing factors for outbreaks caused by *S. agalactiae* in Nile tilapia farms in Brazil (13), thus they seem to be common conditions associated with streptococcal infections in this fish species.

The seven strains presented to be clonally related in PFGE analysis. Considering the close proximity of the two farms, it would be expected that both outbreaks should be due to a single clone; however, two different PFGE types were observed. This epidemiological condition may be associated with the simultaneous introduction of these two genetic variants at the same time or with a possible recent divergence from a common ancestor of some strains during persistent infections in fish from these farms. The PFGE of the Brazilian strains of *S. iniae* displayed only 36% genetic similarity with the pattern of *S. iniae* ATCC 29178. In a previous report from China, Zhou *et al.* (24) used PFGE typing with the restriction enzyme *Sma*I and found a minimal similarity score of 68.4% between 32 Chinese strains of *S. iniae* with the reference strain ATCC 29178. This finding might suggest that Brazilian and Chinese strains constitute two genetic clusters of low relatedness, however, additional analysis are requested to clear this issue.

The main treatment applied in cases of *S. iniae* outbreaks in fish farms is the oral administration of antibiotics. In Brazil, only florfenicol is approved for use in tilapia farms. Previous studies presented that florfenicol (7) and erythromycin (6) are very efficient in the treatment of *S. iniae* infections in sunshine bass (*Morone chrysops* x *Morone saxatilis*) and barramundi (*Lates calcarifer*), respectively. However, there is no *in vitro*

data about florfenicol resistance pattern of fish isolates of *S. iniae*. Herein, we presented the first MIC values for *S. iniae* isolates against florfenicol.

The present work is the first report of phenotypic and genetic characterization of *S. iniae* isolated from outbreaks of meningoencephalitis and septicemia on Nile tilapia farms in Brazil. Moreover, two distinct genotypes of *S. iniae* are currently available in Brazil and all the strains were considered sensible to florfenicol.

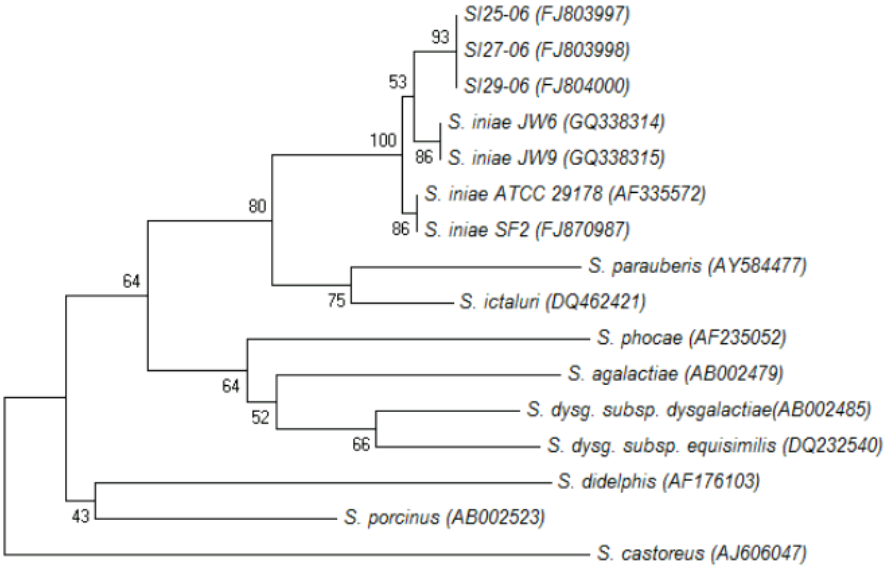

Figure 1. Neighbour-joining phylogenetic tree of 16S rRNA gene sequences of three Brazilian fish isolates (SI25-06, SI27-06 and SI29-06), other fish strains of *S. iniae* and the most closely related species of streptococci. Bootstrap percentages (based on 1000 replications) are shown at branch points

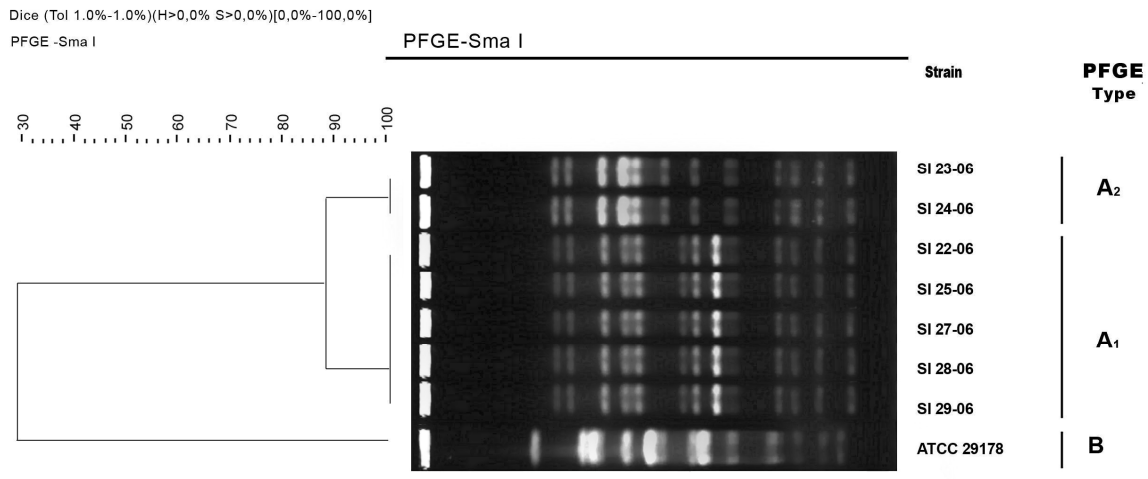

Figure 2. Dendogram constructed by similarity and clustering analyses using the Dice coefficient and UPGMA of digitized PFGE patterns for the seven isolates and *S. iniae* ATCC 29178 digested by *Sma* I. The code labels on the right of the figure represent the strains used in this study

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