Streptococcus iniae E *Streptococcus dysgalactiae* ASSOCIADOS À MENINGOENCEFALITE E SEPTICEMIA EM TILÁPIAS DO NILO NO BRASIL

LAMARTINE DA NÓBREGA NETTO

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para a obtenção do título de "Mestre".

Orientador

Prof. Dr. Henrique César Pereira Figueiredo

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LAVRAS
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A Deus, minha família, meus amigos e a todos que me apoiaram nessa caminhada.

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A piscicultura é um ramo recente do agronegócio brasileiro, porém, tem crescido substancialmente nos últimos anos. O principal peixe cultivado no Brasil é a tilápia, por apresentar grande aceitação no mercado consumidor e grande resistência a variações ambientais. Os principais motivos de prejuízos em piscicultura são as doenças infecciosas causadas por bactérias. Dentre estas, as estreptococoses são de grande relevância, por afetarem peixes de água doce e salgada em várias regiões do mundo. *S. iniae* tem sido apontada como a mais relevante causadora de doenças em peixes do mundo, sendo responsável por milhões de dólares em prejuízos. Atualmente 27 espécies de peixe já foram relatadas como susceptíveis a infecções por este *S. iniae*. As infecções levam a quadros de septicemia e meningoencefaite. A espécie *Streptococcus dysgalactiae* é um patógeno comum de animais de criação e seres humanos, porém, foi recentemente isolada a partir de surtos em peixes no Japão. Não existem relatos que associam ambas as espécies com doenças em peixes no Brasil. Portanto, o objetivo do presente trabalho é a identificação de *Streptococcus* sp. isolados a partir de surtos infecciosos em propriedades de tilápicultura, nos estados do Paraná e Ceará; bem como o estudo da variabilidade genética de isolados identificados como *S. iniae*, e o desafio de peixes frente à infecção experimental por um isolado identificado como *S. dysgalactiae*. Surtos característicos de estreptococoses foram acompanhados em propriedades de tilapicultura em dois estados do Brasil. Foram analisadas duas propriedades no Paraná, 2006, e uma no Ceará, 2007, ambas no verão. Exames bacteriológicos de cérebro, rim e abscessos foram realizados em peixes com sinais clínicos característicos. Os isolados foram submetidos à coloração de Gram e testes de catalase e oxidase. A identificação das espécies foi realizada fenotipicamente e geneticamente com o uso do Kit API 20 Strep, Slidex, PCR espécie-específica e seqüenciamento. O gene 16S rRNA de ambas as espécies foi amplificado e seqüenciado com a utilização de iniciadores universais. Isolados identificados como *S. dysgalactiae* tiveram o fragmento ISR 16S-23S rDNA também seqüenciado. Para acessar a relação genética entre os isolados identificados como *S. iniae* e a cepa de referência *S. iniae* ATCC 29178, PFGE foi realizado.

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Para testar a capacidade em causar doença de um dos isolados identificados como *S. dysgalactiae* foram realizados ensaios de infecção experimental. Em todas as fazendas estudadas, altas densidades de estocagem foram observadas, 150 Kg/m^3 e 300 Kg/m^3 no Paraná e Ceará, respectivamente. A temperatura da água encontrava-se por volta de 30 °C nos três surtos acompanhados. Os testes fenotípicos e moleculares confirmaram a identificação das espécies *S. iniae*, 07 isolados, e *S. dysgalactiae*, 10 isolados, como agentes etiológicos dos surtos ocorridos no Paraná e Ceará, respectivamente. Foi demonstrado, por PFGE, que dois genótipos distintos dos isolados, identificados como *S. iniae,* estavam presentes nos surtos analisados, e, um terceiro padrão foi observado para a amostra tipo *S. iniae* ATCC 29178. O isolado de *S. dysgalactiae* utilizado na infecção experimental mostrou-se capaz de provocar doença, causando sintomas discretos, porém, com reisolamento positivo de rim e cérebro.

ABSTRACT

NÓBREGA NETTO, Lamartine da. ***S. iniae*** **and** ***S. dysgalactiae*** **associated with meningoencephalitis and septicemia in Nile tilapia in Brazil**. 2009. 44p. Dissertation (Master in Agriculture Microbiology) – Universidade Federal de Lavras, Lavras, MG.*

Pisciculture is a relatively recent branch of agorbussines in Brazil, although, a large increase has been observed in recent decades. Tilapia is the mainly cultivate fish in Brazil, in view of the fact that this specie had a great acceptance in the consumer market and large resistance to environment variations. The major sources of fish production damage worldwide are streptococcosis. Outbreaks caused by *Streptococcus* stand out by affecting a broad range of hosts, in several region of the world. The specie *S. iniae* is commonly associated with aquatic animal disease. Nowadays it is pointed to as the main pathogen, causing millions of dollars in damage annually. To date, 27 fish species have been related as susceptible to *S. iniae* infection. Outbreaks in fish caused by this pathogen are characterized by septicemia and meningoencephalitis. Another specie of *Streptococcus* reported as pathogenic to fish is *Streptococcus dysgalactiae.* This specie is a common pathogen of domestic animals and humans, and, was recently isolated from outbreaks in cultured fish in Japan. This pathogen was related to septicemia and necrotic lesions on the caudal peduncle in the species *Seriola dumerili* and *Seriola quinqueradiata*. In Brazil both species have never been associated with fish infections before. The aim of this study was to identify *Streptococcus* sp. isolated from outbreaks in Nile tilapia farms in Paraná and Ceará states, Brazil. Beside this, access the genetic variability of identified *S. iniae* isolates, as well as testing the capacity of identified *S. dysgalactiae* isolate in causing disease in Nile tilapia, through experimental infection. Characteristically streptococcal outbreaks were accompanied in Nile tilapia farms in Brazil. Two proprieties in Paraná, 2006, and one in Ceará, 2007, were accompanied, both in the summer season. Bacteriological exams of kidney, brain and abscesses were carried out in the fish with characteristic clinical signs. Isolates were submitted to Gram-stain, catalase and oxidase tests. Bacterial identification was done phenotypically and genetically through API 20 Strep, Slidex, species-specific PCR and sequencing. The 16S rRNA gene of both species were amplified and sequenced with universal primers. The isolates with diagnostic for *S. dysgalactiae* were also

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submitted to sequencing of the 16S-23S rDNA intergenic spacer region. To test the capacity of these isolates to cause disease, experimental infection was carried out. To access the genetic variability of the isolates identified as *S. iniae* and with the reference strain *S. iniae* ATCC 29178, PFGE was realized. All studied farms have high stockages densities, 150 Kg/m^3 and 300 Kg/m^3 in Paraná and Ceará, respectively. In addition, the water temperature was around 30°C. The phenotypical and molecular tests confirmed the identification of *S. iniae*, 7 isolates, and *S. dysgalactiae*, 10 isolates, as the etiological agents of the outbreaks occurred in Paraná and Ceará states, respectively. The Brazilian *S. iniae* isolates had two distinct genotypes and a third pattern was observed with the type strain *S. iniae* ATCC 29178. Fish challenge showed that *S. dysgalactiae* isolate is able to cause disease, with discrete clinical signs, but with positive reisolation of kidney and brain.

INTRODUÇÃO

A aqüicultura é o setor agropecuário que mais cresce no mundo. A média "*per capita"* de consumo de produtos aqüícolas aumentou de 0,7 kg em 1970 para 32,4 Kg em 2004 (Food and Agriculture Organization of the United Nations - FAO, 2006). No Brasil, o aumento foi de 2%, no ano de 2004, perfazendo 17,8 % do abastecimento total de pescado no país, com a produção de 178.746 t (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais - IBAMA, 2004). Apesar de não estar entre os maiores produtores mundiais o país possui grande disponibilidade de águas e clima tropical favorável para ser o maior produtor mundial de organismos aquáticos (Fitzsimmons, 2000). Grande parte da demanda mundial por produtos de origem aqüícola é suprida pela pesca; porém, a produção de animais aquáticos cresce consideravelmente, a partir da década de 70 a aqüicultura apresentou 9,2% de crescimento comparado com apenas 1,4% da pesca extrativa (Instituto Brasileiro de Geografia e Estatística - IBGE, 2001). Tal fato pode ser justificado pelo esgotamento de recursos, já que as atividades pesqueiras extrativistas chegaram ao limite máximo sustentável nos anos 80. Desse modo, a produção aqüícola visa a atender essa janela formada entre a demanda e a disponibilidade de produtos do gênero.

As atividades de aqüicultura incluem a produção de peixes, moluscos, crustáceos e plantas aquáticas. Dentre essas a piscicultura detém valores bastante expressivos, chegando ao montante de 27 milhões de toneladas produzidas no ano de 2004, enquanto os setores de malacocultura e carcinocultura não excederam os valores de 15 e cinco milhões de toneladas, respectivamente (Food and Agriculture Organization of the United Nations - FAO, 2006). No Brasil, a tilápia é um dos principais peixes cultivados, devido à ampla resistência frente a variações ambientais e por possuírem grande aceitação comercial, tanto no

mercado interno quanto externo. A produção de tilápias encontra-se em ascensão, com média mundial de crescimento de 11,5% (El-Sayed, 1999). Um dos sistemas de cultivo preferenciais dos produtores no Brasil é o de tanques rede. Esse sistema tem sido freqüentemente adotado em função da facilidade de manejo e despesca, assim como baixos custos de implantação e aproveitamento de recursos hídricos já existentes em propriedades rurais (Carneiro et al., 1999).

Mesmo com todos esses atributos o censo agropecuário brasileiro ainda não incluiu as atividades de aqüicultura em sua contabilização. Sendo assim, visando a suprir tal necessidade, o Ministério da Ciência e Tecnologia e o Conselho Nacional de Desenvolvimento Científico e Tecnológico realizaram um levantamento em âmbito nacional das características, perspectivas e limitações da atividade aqüícola. Os resultados desse estudo mostraram as aptidões atuais e perspectivas de cada região do país, bem como os entraves para o aumento da produção dos plantéis. Dentre os entraves apontados, os aspectos sanitários da produção e a falta de estrutura para o diagnóstico das principais enfermidades infecciosas foram considerados de grande relevância.

As doenças infecciosas mais relevantes que acometem peixes de cultivo em todo mundo são causadas por vírus, bactérias, parasitas e fungos. As infecções bacterianas possuem grande relevância, já que atingem uma grande variedade de espécies de peixes de água salgada, doce ou estuarina. Dentre os principais agentes etiológicos bacterianos podem-se ressaltar *Flavobacterium columnare, Aeromonas hydrophila* e algumas espécies do gênero *Streptococcus*. As estreptococoses são consideradas as doenças mais proeminentes, principalmente por conduzirem a altos índices de morbidade e mortalidade em sistemas de cultivo de peixes em regiões tropicais (Evans et al., 2006).

Várias espécies de *Streptococcus* são associadas com infecções em peixes. Atualmente há relatos na literatura das espécies *S. iniae* (Agnew & Barnes, 2007), *S. agalactiae* (Robinson & Meyer, 1996; Figueiredo et al., 2006;

Michael et al., 2007), *S. dysgalactiae* (Hagiwara et al., 2009), *S. phocae* (Romalde et al., 2008), *S. parauberis* (Bercovier et al., 1997) e *S. ictaluri* (Shewmaker et al., 2007). A espécie *S. iniae* vem sendo, ao longo das últimas décadas, considerada um patógeno emergente, e, atualmente, é apontada como a principal espécie de *Streptococcus* causadora de septicemia e meningoencefalite em peixes mantidos em sistemas de cultivo (Shoemaker et al., 2001; Colorni et al., 2002). Estima-se que apenas essa espécie é responsável por aproximadamente 100 milhões de dólares por ano em prejuízos na indústria aqüícola em todo mundo (Shoemaker et al., 2001). A espécie *Streptococcus dysgalactiae* foi recentemente isolada a partir de lesões do pedúnculo caudal em *Seriola dumerili* e *Seriola quinqueradiata*, duas espécies de peixe de água salgada cultivadas no Japão (Nomoto et al., 2004).

S. iniae foi isolada e descrita pela primeira vez como causadora de abscessos subcutâneos em uma espécie amazônica de golfinho de água doce, *Inia geoffrensis* (Pier & Madin, 1976). Em peixes o primeiro relato foi no ano de 1958, no Japão; porém, o isolado só foi identificado décadas depois (Hoshina et al., 1958). Desde então *S. iniae* vem sendo relatado infectando um crescente espectro de hospedeiros que chega ao número de 27 espécies de peixes (Agnew & Barnes, 2007) em que se incluem também várias espécies de tilápia (Kitao et al., 1981; Eldar et al., 1994, 1995a,b; Browser et al., 1998; Shoemaker et al., 2001; Kivitt & Colorni, 2004). A infecção em peixes causada por *S. iniae* provoca quadros de septicemia e meningoencefalite, com altos índices de morbidade e mortalidade, chegando até a 100% de óbitos em animais adultos. Os principais sinais clínicos causados são natação errática, exoftalmia, escurecimento de córnea, escurecimento da pele, rigidez vertebral e pontos hemorrágicos nas inserções das nadadeiras (Bromage & Owens, 2002). Existe a perspectiva de distribuição mundial de *S. iniae*, mas até o presente momento, surtos foram relatados em três regiões distintas do mundo: América do Norte

(Perera et al., 1994; Stoffregen et al., 1996; Ferguson et al., 2000), Oriente Médio (Eldar et al., 1994; Yuasa et al., 1999) e Ásia Pacífica (Eldar et al., 1994; Stroffregen et al., 1996; Bromage & Owens, 2002; Nguyen et al., 2002; Shen et al., 2005).

Streptococcus dysgalactiae do grupo C de Lancefield são comumente relacionadas com infecções em animais domésticos e seres humanos (Efstratiou et al., 1994; Bert et al., 1997). A associação dessa espécie com infecção em peixes foi realizada recentemente no Japão. Infecções dessa natureza levam principalmente a quadros clínicos de lesões necróticas do pedúnculo caudal e septicemia, causando altos índices de mortalidade em *Seriola dumerili* e *Seriola quinqueradiata* (Nomoto et al., 2004; Hagiwara et al., 2009). Todavia, ainda não existem relatos em todo o mundo que associam esse patógeno a peixes de água doce. No Brasil, a única espécie de estreptococos relacionada com doença em peixes, até o presente momento, é *S. agalactiae*, relacionada com septicemia e meningoencefalite em tilápia (Salvador et al., 2005; Figuereido et al., 2006; Mian et al., 2008).

A identificação e a caracterização de bactérias patogênicas para peixes são de extrema importância para a aplicação de medidas sanitárias preventivas mais apropriadas ou para escolha de tratamentos mais eficazes. Alguns kits comerciais de testes bioquímicos são utilizados na identificação de *Streptococcus*, como por exemplo, API 20 Strep e API 32. O problema é que há limitações na utilização destes kits para identificação da espécie *S. iniae*. Isto porque esta espécie não se encontra listada no banco de dados dos softwares de identificação; além disso, os resultados de alguns testes bioquímicos obtidos com isolados de peixes geralmente são discordantes com padrões da cepa de referência, o que fatalmente resulta em identificações errôneas ou ilegíveis (Lau et al., 2003; Facklan et al., 2005).

Nas ultimas décadas, observou-se grande avanço em técnicas moleculares utilizadas na identificação de organismos procarióticos. Em acréscimo, um crescente montante de dados biológicos "*in silico"* vem sendo depositado em bancos públicos. Tais fatores aumentam consideravelmente a acurácia e a qualidade dos diagnósticos de doenças infecciosas. Um marcador genético comumente utilizado para análise de relações filogenéticas entre procariotos é o sequenciamento do gene 16S de rRNA. Este gene está presente em quase todas as eubactérias como um gene conservado, sendo assim utilizado como barômetro evolutivo (Janda & Abbott, 2007). Dois outros fatores que corroboram a utilização desse gene na caracterização de procariotos: (i) presença de regiões altamente conservadas em suas extremidades, o que permite o desenho de iniciadores universais para amplificar o fragmento em quase todas as eubactérias (Fox et al., 1995; Hassan et al., 2003; Clarrige III, 2004), e (ii) regiões internas com alto índice de variações, que são utilizadas para comparações taxonômicas (Tortoli, 2003; Ueda et al., 2003). Outra técnica comumente utilizada para identificação de procariotos é a PCR, espécies específica. Nessa técnica, iniciadores que possuem alvos específicos para cada espécie bacteriana são utilizados. Atualmente estão disponíveis iniciadores desenhados para identificação de *S. iniae* (Mata et al., 2004) e *S. dysgalactiae* (Hassan et al., 2003), o que economiza consideravelmente tempo e material para identificação desses patógenos na rotina laboratorial.

O objetivo do presente trabalho foi a identificação fenotípica e molecular de *Streptococcus* sp. isolados a partir de surtos infecciosos, ocorridos em sistemas de cultivo de tilápias do Nilo (*Oreochromis niloticus*), em dois estados do Brasil; Paraná e Ceará. Objetivou-se, também, a caracterização genética de isolados identificados como *S. iniae*, assim como a reprodução da doença, em ensaios de infecção experimental, para isolados identificados como *S. dysgalactiae.*

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CAPÍTULO 1

FIRST REPORT OF *Streptococcus iniae* OUTBREAKS IN NILE TILAPIA FARMS IN SOUTH AMERICA

O capítulo 1 será transcrito em formato de artigo e encaminhado para submissão do Periódico Científico **Veterinary Microbiology**

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ABSTRACT

The present work is the first report of outbreaks caused by *S. iniae* in South America, as well as the first genetic typing of these isolates. Outbreaks with characteristic streptococcocal signs in two Nile tilapia (*Oreochromis niloticus*) farms in Paraná state, Brazil, were followed. Bacteriological analysis of kidney and brain was carried out in all animals with characteristic symptoms of streptococosis. Seven isolates with characteristic *Streptococcus* profiles were analyzed with the commercial Kit API 20 Strep and the serological tests with Slidex. PCR and amplification with universal primers from 16S rRNA and species-specific PCR, with *lctO* target gene were realized for unequivocal identification. Sequences of the 16S rRNA gene were aligned, the phylogenetic tree was constructed utilizing sequences of related *Streptococcus* species from GenBank. PFGE was performed to access the genetic relationship between the isolates. All isolates showed biochemical patterns of *S. iniae* and were not typified to any Lancefield group. The seven isolates showed 100% of similarity in 16S rRNA sequence with the type strain *S. iniae* ATCC 29178. Two distinct patterns of PFGE were observed for Brazilian isolates and a third pattern was assigned to the type strain *S. iniae* ATCC 29178. The phenotypic and molecular characterization confirms that *S. iniae* is the etiological agent of outbreaks. In addition we demonstrated that two distinct genotypes of *S. iniae* are currently in Brazil, and these two genotypes are genetically distinct from the type strain *S. iniae* ATCC 29178.

Keywords: *Streptococcus iniae*, Nile tilapia, PFGE,16S rRNA, Brazil.

1 Introduction

Streptococcosis is a major problem for fish production worldwide, associated to high economic losses. Since the first report (Hoshima et al., 1958), several *Streptococcus* species have been associated with fish infections. Currently, *Streptococcus iniae* (Agnew & Barnes, 2007), *Streptococcus agalactiae* (Evans et al., 2002), *Streptococcus dysgalactiae* (Nomoto et al., 2006), *Streptococcus phocae* (Romalde, et al. 2008), *Streptococcus parauberis* (Bercovier et al., 1997) and *Streptococcus ictaluri* (Camus et al., 2008) have been described as pathogenic to fish.

S. iniae is a Gram-positive coccus that occurs in long chains under culture conditions. This bacterium is β-hemolitic, oxidase negative and catalase negative and does not react with sera against any Lancefield group (Pier et al., 1978). This species was first isolated from multifocus subcutaneous abscesses in captive Amazon freshwater dolphin, *Inia geoffrensis* (Pier & Madin, 1976). Nowadays, *S. iniae* is considered a major warm water fish pathogen, associated to outbreaks in 27 fish species (Agnew & Barnes, 2007). In fish, *S. iniae* infections are characterized by meningoencephalitis and septicemia that generally induce high morbidity and mortality rates. Erratic swimming, whirling, exophthalmia, dorsal rigity, anorexia and tachypnoea are the main clinical signs of illness (Bromage & Owens, 2002). Disease outbreaks have been described in tree distinct regions of the world, namely, North America (Canada, USA and Caribbean) (Perera et al., 1994; Stoffregen et al., 1996; Ferguson et al., 2000), Middle East (Bahrain and Israel) (Yuasa et al., 1999; Eldar et al., 1994) and Asia-Pacific (Australia, China, Japan, Singapore and Taiwan) (Eldar et al., 1994; Stroffregen et al., 1996; Bromage & Owens 2002; Nguyen et al., 2002; Shen et al., 2005). Outbreaks caused by *S. agalactiae* in Nile tilapia (Figueiredo et al., 2006; Mian et al., 2008) and *S. phocae* in Atlantic salmon (Romalde et al., 2007) were reported in commercial fish farms in Brazil and

Chile, respectively. However, in South America there is no description of *S. iniae* infection in fish.

Biochemical tests have been used for identification of *S. iniae*. Miniaturized diagnostic systems like API-20 Strep and API-32 are also utilized to perform phenotypic characterization of this bacterium. However, misidentification or unidentified readings are quite often obtained since this bacterial species is not included in the software's databases employed to interpret the results of this kits (Lau et al., 2003; Facklam et al., 2005). Therefore, some molecular tools have been applied to *S. iniae* identification, namely, species specific PCR with the lactate oxidase (*lctO*) gene target (Mata et al., 2004) and sequencing of 16S rRNA gene (Lau et al., 2003). The genetic variability of *S. iniae* is poorly understood and few reports describe, using pulsed-field gel electrophoresis (PFGE), diversity in field isolates according to the host species, virulence and geographic origin (Weinstein et al., 1997.; Facklam et al., 2005, Nawawi et al., 2008; Zhou et al., 2008).

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

2 Materials and methods

2.1 Fish farms

Outbreaks of meningoencephalitis and septicemia were accompanied in two commercial Nile tilapia farms located in Paraná State, Brazil. These two farms were closely located, about 3 km apart, on the same river. The fish were reared in cages under high stock densities (150 Kg/m³) and the water temperature, during the outbreaks, was approximately 30°C. The main clinical signs observed were lethargy, erratic swimming, exophthalmia and skin darkening, with high mortality rates. Both farms had records of fish showing

streptococcal disease signs in the past summer season, but no exams were done. Ten diseased fish from each farm were sampled, stored at 4°C and immediately transported to the laboratory for bacteriological analysis.

2.2 Bacterial isolation and phenotypic identification

For bacterial isolation, swabs of brain and kidney of each fish were sampled aseptically, streaked onto 5 % sheep blood agar and incubated at 28°C for 72 h. The colonies obtained were tested with Gram stain, catalase and oxidase production and hemolysis. The isolates were further characterized phenotypically and serologically using API20 STREP and Slidex Latex Agglutination kits (both from BioMerieux, France), respectively.

2.3 DNA Extraction, PCR and Sequencing

The isolates were grown onto blood agar supplemented with 5 % of sheep blood for 48 h at 30°C. The colonies were collected, diluted in 180 µL of lysis solution (20 mg/ml lisozyme; 20 mM Tris-HCl, pH 8.0; 2 mM EDTA and 1.2 % Triton®) and incubated at 37°C for one hour. Total bacterial DNA was then extracted using the commercial kit DNeasy (Qiagen, Germany) according to manufacturer's instructions. To confirm the diagnosis, extracted DNA was used as template in a *S. iniae*-specific PCR performed according to procedure previously described by Mata et al. (2004).

16S rRNA was amplified by PCR with the universal primers C70 (5´-AGAGTTTGATYMTGGC-3´) and B37 (5´-TACGGYTACCTTGTTACGA-3´) according to the method described by Fox et al. (1995) with some modifications. The PRC amplifications were performed in a 30 µl reaction volume containing 2 µl of chromosomal bacterial DNA (50-70 ng), 0.25 µM of both primers, 0.2 mM of each deoxynucleotide triphosphate and 2.5 U of Go Taq flexi DNA polymerase (Promega, USA) in 1 x reaction buffer. The amplification was

carried out with a pre-cycle denaturation step at 95°C for 5 min followed by 35 serial cycles at 94°C for 1 min, 55°C for 45 s and 72°C for 45 s, and a final extension step at 72°C for 10 min. After amplification, PCR products were purified using a Wizard PCR Preps kit (Promega, USA) and sequenced. Sequencing reactions were performed using a BigDye™ Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3730XL genetic analyzer (Applied Biosystems).

2.4 Sequencing analysis and phylogenetic tree construction

The phylogenetic relationship of the isolates was determined by comparative 16S rRNA gene sequence analysis. The sequences of the isolates were aligned in BioEdit using CLUSTALW (Thompson et al., 1994) with sequences of the following streptococcal species: *S. iniae* (accession number: AY762259), *S. dysgalactiae* subsp. *equisimilis* and *S. dysgalactiae* ATCC 43078 (accession number: DQ232540 and AB002485 respectively), *S. agalactiae* (accession number: AB002480), *S. pyogenes* (accession number: AB023575), *S. canis* (accession number: AB002483), *S. ictaluri* (accession number: DQ462421), *S. didelphis* (accession number: DQ303185), *S. uberis* (accession number: AB023573), *S. phocae* (accession number: AF235052) and *S. castoreus* (accession number: AJ606047), available in NCBI database. The genetic distances matrix was obtained by Kimura's two parameters model (Kimura, 1980) and evolutionary tree created by the neighbor joining method (Saitou & Nei, 1987) with Mega4 (Tamura et al., 2007). Bootstrap values from 1000 replicates were displayed as percentages.

2.5 PFGE

PFGE was performed as previously described (Teixeira et al., 1995; Oliveira et al., 2005). *Streptococcus agalactiae* was grown overnight in BHI

broth. The cells were harvested and washed two times with PIV solution (Tris-HCl 0.01 M, pH 8.0 and NaCl 1 M). The bacterial suspension was mixed with an equal volume of 2 % low-melting-point agarose (Sigma-aldrich®, USA) and pipeted into 20 µL plugs. Streptococcal cells in agarose plugs were lysed, the DNA digested with 12 U of *Sma* I restriction enzyme (Amersham Biosciences, UK) and submitted to PFGE with a program as follows: switch time of 1 to 30 s, 23 h, 120º angle, 11.3ºC and a voltage gradient of 6 V/cm in a CHEF DR III system (Bio-Rad Laboratories, USA). The lambda ladder PFGE marker (New England Biolabs, USA) was used as a DNA size marker. Gels were stained with ethidium bromide and photographed under UV light. Images were analyzed by Gel ComparII software® (Applied Maths, Belgium) to make dendograms of genetic relationship among the strains of different hosts. Briefly, bands were automatically assigned by the computer and were corrected manually after the original images were checked and evaluated visually. Only clearly resolved bands were counted. The Dice coefficient (95 %) was used to analyze the similarities of the banding patterns. The unweighted pair group method with average linkages (UPGMA) was used for cluster analysis. The isolates that showed 100 % of similarity were considered indistinguishable, and the isolates with similarity greater than 80 % were considered clonally related (Singh et al., 2006).

3 Results

In the year of 2006, outbreaks of septicemia and menigoencephalitis occurred during the summer in two Nile tilapia (*Oreochromis niloticus*) farms, located at Paraná State, Brazil. In this season the water temperature was around 30°C and fish usually are in pre-slaughter phase. The main clinical signs observed were exophthalmia, corneal opacity, skin darkening and erratic swimming. High mortality rates were verified on both farms.

Gram positive cocci were isolated from brain and kidney 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. All isolates could not be serologically typed to any Lancefield group. In the API 20 Strep test, the isolates presented positive reactions for pyrrolidonyl arylamidase, β-glucuronidase, alkaline phosphatase, leucine arylamidase, arginine dihydrolase, ribose, trealose, starch and glycogen and negative for catalase, hippurate hydrolysis, sorbitol, inulin and D-raffinose. In addition, molecular characterization was performed to confirm the diagnostic. All isolates showed positive reactions in *S. iniae*-specific PCR assay generating amplicons of 870 bp (data not shown).

Universal primers were used to amplify the 16S rRNA and produced fragments of approximately 1640 bp. Computational analysis of *S. iniae* sequences demonstrated that Brazilian isolates are similar to each other and to reference strain ATCC 29178 as well as to other streptococcal fish strains. Figure 1 presents the phylogenetic tree of *S. iniae* isolates from Brazil, reference strain and closely related species of streptococci.

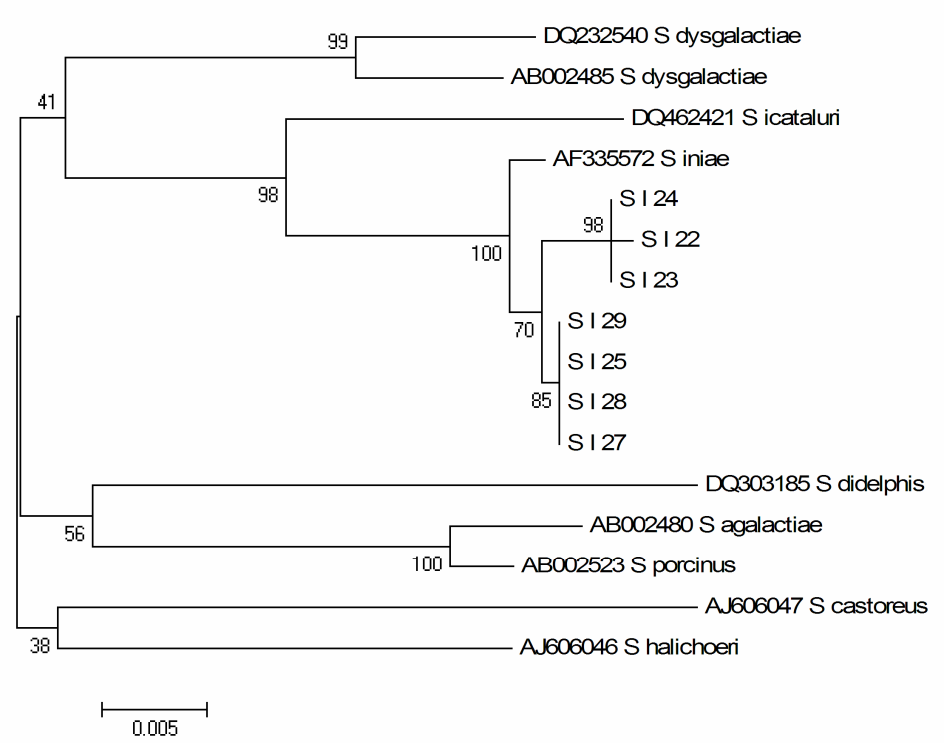

Figure 1 Neighbour-joining phylogenetic tree of 16S rRNA gene sequences of seven Brazilian isolates (SI22, SI23, SI24, SI25, SI27, SI28, and SI29) of *S. iniae* and the most closely related species of streptococci.

TABLE 1 *S. iniae* strains used in this study.

Strain	Host	Farm	PFGE Type	16S rRNA accession number ***
SI22	Nile tilapia*	A	A_1	-
SI23		A	A_2	-
SI24		A	A_2	-
SI25		A	A_1	-
SI27		B	A_1	-
SI28		B	A_1	-
SI29		A	A_1	-
ATCC 29178	Amazon dolphin**	-	B	AF335572

**Oreochromis niloticus* ***Inia geoffrensis* ***NCBI data bases

The PFGE analysis showed three different patterns (A_1, A_2 and B) among the studied strains. The seven Brazilian strains belonged to two different pulse-types, A_1 and A_2 and *S. iniae* ATCC 29178 presented the pattern B. Despite the distribution of seven isolates in two pulse-types, these groups were clonally related, with 88 % similarity according to DICE coefficient (Fig. 2). The A1 strains were isolated on farm A and B, while those of pattern A_2 occurred only on farm B. Additionally, both Brazilian *S. iniae* clusters showed 36% similarity with the pattern B, of the strain ATCC 29178 (Fig. 2).

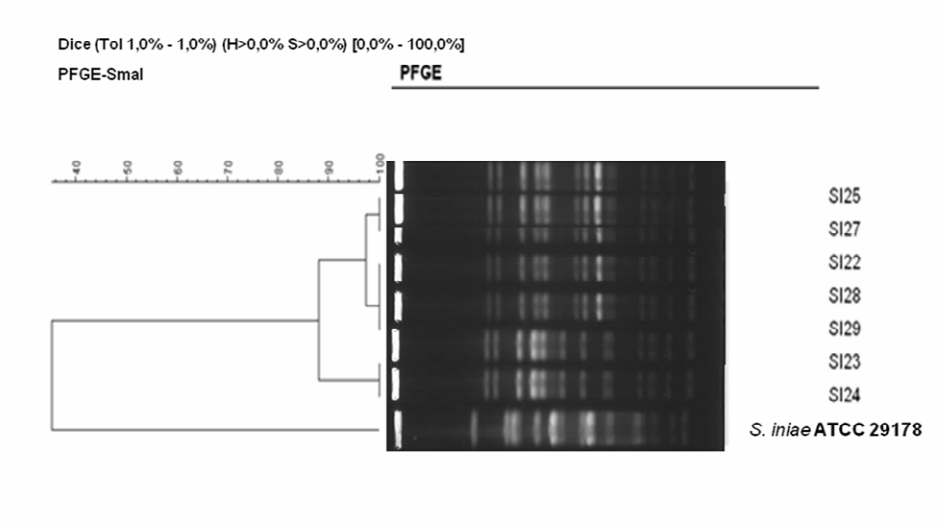

FIGURE 2 Dendogram constructed by similarity and clustering analyses using the Dice coefficient and UPGMA of digitized PFGE patterns for the seven isolates and *S. iniae* ATCC 29178 digested by *Sma* I. The code labels on the right of the figure represent the strains used in the present study.

4 Discussion

Currently, *S. iniae* is considered one of the most relevant streptococcal pathogen to cultured and wild fish (Shoemaker et al., 2001; Colorni et al., 2002), responsible for diseases in marine and freshwater species. Outbreaks caused by this bacterium are associated to high mortality rates with significant economic losses. *S. iniae* infection is believed to be the main problem for tilapia production in several countries worldwide (Kitao et al., 1981; Eldar et al., 1994; Eldar et al., 1995a, b; Bowser et al., 1998; Shoemaker et al., 2001; Kivitt & Colorni, 2004). Even though there is a perspective of a global distribution of this pathogen, there has been no report of disease in South America, in contrast to other streptococci fish pathogens, namely *S. agalactiae* (Figueiredo et al., 2006; Mian et al., 2008) and *S. phocae* (Romalde et al., 2008). This study is the first

report of outbreaks caused by *S. iniae* in South America. Seven isolates were identified and genetically characterized from sick fish of two Nile tilapias farms.

On commercial fish farms, the main predisposing factors for *S. iniae* outbreaks are high stocking densities and increasing water temperatures; the disease usually occurs in adult fish (Shoemaker et al., 2000; Agnew & Barnes, 2007). Cages are the main culture system adopted for Nile tilapia production in Brazil. In this system, fish are reared under high intensive husbandry and stocking densities. Additionally, the outbreaks investigated here occurred in summer and when fish reached adult stage. Several incorrect production features, as well as environmental characteristics can contribute to trigger the disease, acting as determinant factors for illness (Shoemaker et al., 2000; Bromage & Owens, 2002). These conditions were also described as predisposing factors for outbreaks caused by *S. agalactiae* in Nile tilapia farms in Brazil (Mian et al., 2008), seeming to be common conditions associated with streptococcal infections in this fish species.

The Brazilian Nile tilapia *S. iniae* isolates were beta-hemolytic and formed small colonies when growing in sheep blood agar. All strains were also not typed to any Lancefield group, as described for the *S. iniae* strain ATCC 29178 (Pier & Madin, 1976). All isolates presented the same phenotypic profile, similar to previous reports for *S. iniae* strains isolated from fish worldwide (Perera et al., 1994; Bromage et al., 1999; Nguyen & Kanai, 1999; Colorni et al., 2002). Species-specific PCR and 16S rRNA sequencing permitted the unequivocal species identification. The high degree of similarity in the 16S rRNA sequences observed among Brazilian isolates, suggests a clonal relation among them.

The seven strains were distributed in two clusters by the computational image analysis of PFGE, type A_1 and type A_2. These two PFGE types shared 88 % of similarity, thus the isolates were considered clonally related strains.

Considering the close location of the two farms it would be expected that both outbreaks should be due to only a single clone. However, two different PFGE types, although clonally related, were observed. This epidemiological condition may be associated to the simultaneous introduction of these two genetic variants at the same time or a possible recent divergence, from a common ancestor, of some strains during persistent infections in fish from these farms.

At PFGE Brazilian strains of *S. iniae* displayed only 36 % genetic similarity with the pattern of *S. iniae* ATCC 29178. In a previous report in China, Zhou et al. (2008), using PFGE typing with the restriction enzyme *Sma*I, found a minimal similarity score of 68.4 % between 32 Chinese strains of *S. iniae* with the reference strain ATCC 29178. This finding suggests that Brazilian and Chinese strains comprises two genetic clusters or low relatedness.

The present work is the first report in South America of phenotypic and genetic characterization of *S. iniae* isolated from outbreaks of meningoencephalitis and septicemia on Nile tilapia farms. Additionally, two distinct genotypes of *S. iniae* are currently present in Brazil.

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Competing interests statement

The authors declare no competing financial interests.

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CAPÍTULO 2

Streptococcus dysgalactiae as an agent of septicemia in the freshwater fish Nile tilapia.

O capítulo 2 será transcrito em formato de artigo e encaminhado para submissão do Periódico Científico **Veterinary Microbiology**

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ABSTRACT

Streptococcosis is a major problem in aquaculture worldwide, specially in tropical regions. Several species have been associated with fish diseases. *S. dysgalactiae* was recently isolated from necrotic lesions of caudal peduncle in cultured amberjack (*Seriola dumereli*) and yellowtail (*Seriola quinqueradiata*) in Japan, both from sea water. However, this bacterium have never been related to freshwater fish infections. This present work is the first association of *S. dysgalactiae* with infection in Nile tilapia (*Oreochromis niloticus*). An outbreak with characteristic streptococcocal signs on a Nile tilapia farm, in Ceará state, was investigated. Bacteriological analysis of kidney, brain and subcutaneous abscesses were carried out in ten fish with characteristic clinical signs. Isolates were tested by Gram, catalase and oxidase assays. Isolates with *Streptococcus* characteristic were analyzed biochemically with commercial Kit API 20 Strep and the serological tests with Slidex. *S. dysgalactiae*-species-specific PCR was done. Sequencing of 16S rRNA gene and 16S-23S rDNA ISR fragment were also carried out for unequivocal identification. Experimental infection with one representative isolate was done. The phenotypical and molecular assays confirmed that *S. dysgalactiae* was the etiological agent of the outbreak. The experimental infection could reproduce the disease. Light symptoms, with no abscesses in the caudal peduncle, were observed. The bacterium was effectively reisolated from brain and kidney.

Keywords: *Streptococcus dysgalactiae*, Nile tilapia, experimental infection, sequencing, 16S rRNA and 16S-23S ISR rDNA.

1 Introduction

Several streptococcal species, such as *Streptococcus iniae* (Agnew & Barnes, 2007), *Streptococcus agalactiae* (Evans et al., 2002), *Streptococcus dysgalactiae* (Nomoto et al., 2004), *Streptococcus phocae* (Romalde et al., 2007), *Streptococcus parauberis* (Bercovier et al., 1997) and *Streptococcus ictaluri* (Camus et al., 2008) have been related with outbreaks in tropical and temperate farmed fish. These bacteria are etiologic agents for marine and freshwater fish, causing high financial losses to aquaculture worldwide.

S. dysgalactiae is a Gram positive coccus, catalase and oxidase negative, alpha or beta hemolytic when growing in 5 % sheep blood agar and included in Lancefield's group C group, according to its capsular antigens. This bacterium is well recognized as an ethiological of mastitis in cattle (Aerestrup & Jesen, 1996; Wage et al., 1999) and pharyngites in humans (Fox et al., 1993; Williams, 2003). Recently, *S. dysgalactiae* was reported as a causative agent of illness in cultured amberjack, *Seriola dumerili,* and yellowtail, *Seriola quinqueradiata* in Japan (Nomoto et al., 2004). The disease in fish is characterized by focal necrosis in caudal peduncle and septicemia, with high mortality rates verified during outbreaks. Histopathologic analysis showed multiple granulomatous lesions in many organs, such as liver, kidney, heart and muscular tissue (Hagiwara et al., 2009). In contrast to other streptococcal fish pathogens, the reports of illness caused by *S. dysgalactiae* have been restricted to marine cultured fish in Japan, and, there is no previous description of infections attributed to this bacterium, neither in freshwater fish species nor in other countries.

The present work describes the isolation and characterization of *S. dysgalactiae* from diseased Nile tilapia presenting septicemia and the reproduction of the typical disease in experimental infection.

2 Material and methods

2.1 Bacterial isolation from diseased fish

One outbreak occurred in a Nile tilapia farm located at Ceará state, Brazil, was monitored in October of 2007. Ten fish showing typical clinical signs (lethargy, erratic swimming, exophthalmia and subcutaneous abscesses) were sampled, stored at 4°C and immediately transported to the laboratory. For bacterial isolation, swabs of subcutaneous abscesses of caudal peduncle, brain and kidney of each fish were sampled aseptically, streaked onto 5 % sheep blood agar and incubated at 28°C for 72 h. The colonies obtained were tested for Gram stain, catalase and oxidase production and hemolysis. The isolates were further phenotypically and serologically characterized using API20 STREP and Slidex Latex Agglutination kits (both from BioMerieux, France), respectively.

2.2 DNA Extraction, PCR and Sequencing

The isolates were grown onto Blood agar supplemented with 5 % of sheep blood for 48 h at 30°C. The colonies were collected, diluted in 180 µL of lyses solution (20 mg/ml lisozyme; 20 mM Tris-HCl, pH 8.0; 2 mM EDTA and 1.2% Triton®) and incubated at 37°C for one hour. Total bacterial DNA was then extracted using the commercial kit DNeasy (Qiagen, Germany) according manufacturer's instructions. To confirm the diagnostic, extracted DNA was used as template in a *S. dysgalactiae*-specific PCR performed according to previously described by Hassan et al. (2003).

Two random choosed strains (SD54-07 and SD64-07) were submitted to 16S rDNA and 16S-23S rDNA intergenic spacer region sequencing. 16S rRNA gene was amplified by PCR with the universal primers C70 (5´-AGAGTTTGATYMTGGC-3´) and B37 (5´-TACGGYTACCTTGTTACGA-3´) according to the method described by Fox et al. (1995) with some modifications. The PRC amplifications were performed in a 30 µl reaction volume containing 2

µl of chromosomal bacterial DNA (50-70 ng), 0.25 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate and 2.5 U of Go Taq flexi DNA polymerase (Promega, USA) in 1 x reaction buffer. The amplification was carried with a pre-cycle denaturation step at 95°C for 5 min followed by 35 serial cycles at 94°C for 1 min, 55°C for 45 s and 72°C for 45 s, and a final extension step at 72°C for 10 min. 16S-23S ISR rDNA was amplified following the protocol described by Fosman et al. (1997). After amplification, both PCR products, from 16S rRNA gene and 16S-23S rDNA ISR, were purified using a Wizard PCR Preps kit (Promega, USA) and sequenced. Sequencing reactions were performed using a BigDye™ Terminator Cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and run on an ABI 3730XL genetic analyzer (Applied Biosystems). Sequences were then compared to sequences from the NCBI database using the BLASTn algorithm. The limit fixed for identification of a bacterial species was 98 % nucleotide identity for both, 16S rRNA gene and 16S-23S rDNA intergenic spacer region.

2.3 Fish challenge

For fish challenge, Nile tilapia (*Oreochromis niloticus*) fingerlings with average weight of 40 g (± 5) were acquired from a commercial hatchery. They were submitted to bacteriological tests and showed to be free of bacterial infections and each experimental group comprised ten fingerlings. They were kept in a 57-L aquarium supplied with low flow-through dechlorinated tap water (0.5 L/h). Fish were maintained on a 12:12 h light/dark period at a water temperature of 32°C and were fed to satiation with VITAFISH 32% PB (Matsuda, GO, Brazil) twice a day.

The *S. dysgalactiae* strain SD 64-07 was thawed, streaked onto sheep blood agar and cultivated for 24 h at 32°C. One colony was sampled, inoculated into 150 mL of BHI and cultivated for 18 h at 32°C under low agitation (150

rpm). Fish were anesthetized by immersion in a bath containing 10 mg/L benzocaine. Two groups of ten fish were challenged by i.p. injection with 0.2 ml of the bacterial suspension at final concentration of 10^8 CFU/ml (1.2 OD_{600}). The third group was inoculated with 0.2 ml of sterile BHI (control). Challenged fish were monitored four times a day, during 15 days. Samples of brain and kidney were collected from all dead fish for reisolation of bacteria.

3 Results

3.1 Description of the natural disease

The outbreak occurred in the summer season of 2007, on a Nile tilapia farm located at Ceará state, Brazil. In this period the water temperature was approximately 30°C. The fish were reared in cages under high stock densities (300Kg/m³) and most of ill animals were in the pre-slaughter phase, with a weight near 1 Kg. The mortality observed was very high, reaching a total of 20 tons of fish. Clinical signs characteristic of streptococcosis, such as darkening of skin, lethargy, erratic swimming and exophthalmia were verified. Additionally, focal swelling in the region of caudal peduncle was also noted (Fig. 1), which, afterwards was shown to be a collection of pus accumulated in a cavity under the dermis, characterized as a subcutaneous abscess.

FIGURE 1 Subcutaneous abscesses in the region of caudal peduncle from a Nile tilapia infected with *Streptococcus dysgalactiae*.

3.2 Bacterial Identification

All ten fish had positive isolation of brain or kidney, one of these showed positive isolation of subcutaneous abscesses. Isolates obtained from Nile tilapia fish belonged to the Lancefiled group C and were phenotypicly identified as *Streptococcus dysgalactiae* using Api 20 Strep. The ten isolates exhibited the same phenotypic profile, with positive results for the beta-glucoronidase, leucine aminopeptidase, ribose and trealose tests, and negative results for the Voges-Proskauer test, hippurate hydrolysis, esculin, alpha-galactosidase, beta-galactosidase, arabinose, manitol, sorbitol, lactose, inulin, raffinose, starch and glycogen. Additionally, all isolates were beta-hemolytic on 5 % sheep blood agar. Species-specific PCR produced a characteristic PCR amplicon of 259 bp.

Additionally, amplification of the 16S rRNA and 16S-23S rDNA fragments from strains SD54-07 and SD64-07 yielded a product of approximately 1500 bp and 640 bp, respectively. Blast analysis of these product sequences revealed that all strains exhibited similarity of ≥98 % to previously reported sequences from *S. dysgalactiae* isolated from fish, confirming the phenotypical and serological identification.

TABLE 1 Strains of *Streptococcus dysgalactiae* isolated from Nile tilapia.

Strain	Organ/body site	Lancefield group	Identification by PCR	16S rRNA accession number**	16S-23S ISR accession number**
SD 52-07	Kidney	C	+	-	-
SD 54-07	Brain	C	+	S	S
SD 56-07	Brain	C	+	-	-
SD 57-07	Brain	C	+	-	-
SD 58-07	Kidney	C	+	-	-
SD 61-07	Abscesses	C	+	-	-
SD 62-07	Kidney	C	+	-	-
SD 64-07	Brain	C	+	S	S
SD 68-07	Kidney	C	+	-	-
SD 69-07	Brain	C	+	-	-

**Oreochromis niloticus*. **NCBI data bases. S – Submitted to GenBank.

3.3 Fish challenge

The disease was successfully reproduced in Nile tilapia fingerlings experimentally infected by intraperitoneal injection. First clinical signs were observed 24h after challenge. Typical signs were anorexia, lethargy, tachypnoea and skin darkness. Moribund fish began to die 48h post-infection and mortality was observed until 10 days post-challenge. Mortality rate observed was 65% and bacteria were reisolated from kidney and brain of all dead fish.

4 Discussion

In Brazil, Nile tilapia is the principal fish cultivated for commercial purposes. Similar to other freshwater species, Nile tilapia has been shown to be susceptible to streptococcosis, nowadays, one of the most important problems

for tilapia rearing worldwide. According to previous reports, *S. iniae* and *S. agalactiae* seem to be the most frequent pathogen of this germ involved in outbreaks of septicemia and meningoencephalitis in tilapia farms (Evans et al., 2002; Agnew & Barnes, 2007). However, recent description of novel *Streptococcus* specie (Shewmaker et al., 2007) and associations of previous unreported bacteria causing disease in fish are always possible.

In the summer of 2007, an outbreak of septicemia was monitored on one Nile tilapia farm located in northeast region of Brazil (Ceará state). Ill fish showed a focal swelling in the region of caudal peduncle together with clinical signs of exophthalmia and septicemia commonly verified in cases of streptococosis in the country. *S. dysgalactiae* were successfully isolated from diseased Nile tilapia. Up to now, this bacterium has just been reported to cause disease in amberjack, *Seriola dumerili,* and yellowtail, *Seriola quinqueradiata* in Japan (Nomoto et al., 2004). It is the first description of occurrence of *S. dysgalactiae* infection in freshwater and tropical fish species as well as outside Japan. This suggest that the bacteria can show a larger range of hosts similar to other species of the genus *Streptococcus*.

Cages are the main culture system adopted for Nile tilapia production in Brazil. Under this system, fish are reared under high intensive husbandry and stocking densities, as observed on the farmed monitored. These conditions were described as predisposing factors for outbreaks caused by *S. agalactiae* in Nile tilapia farms in Brazil (Mian et al., 2008). Similar to other streptococcal species, these conditions seem to be favorable for *S. dysgalactiae* infection.

The Brazilian isolates of *S. dysgalactiae* showed phenotypic and genetic patterns similar to fish strains previously isolated in Japan and positive results were obtained in species-specific PCR assay. Hassan et al. (2003) described the *S. dysgalactiae* species-specific PCR product as a single band with 259 bp, as observed in this study. Our isolates were hemolytic, in contrast to the Japanese

strain isolated from fish, which were non-hemolytic in 5 % sheep blood agar. However, non-hemolytic *S. dysgalactiae*, isolated from fish in Japan, can switch to beta-hemolytic after prolonged incubation (Nomoto et al., 2004). Although the hemolytic characterization has been utilized to distinguish *S. dysgalactiae* subsp. *dysgalactiae* to *S. dysgalactiae* subsp. *euiquimilis* (Vieira et al., 1998) this phenotypical feature can be confusing and results ambiguous (Facklam, 2002), which supports the idea that *Streptococcus dysgalactiae* identification of subspecies can not be carried out previously without molecular assays.

The 16S rRNA gene amplification generated a 1650 bp of fragment and both sequences, from the strains SD 54-07 and SD 64-07, Blast analysis showed a 99% of similarity with the sequences AB102730 and AB159678, from the diseased Japanese fish isolate strains (Nomoto et al., 2004), differing in just one base. The Brazilian isolates have also the same similarity with the 16S rRNA gene sequence EU075072 from the strain *S. dysgalactiae* subsp. *equisimilis* 18-39MP isolated from dogs in Australia, which suggests that 16S rRNA gene could not be the better molecular marker for differentiation of *S. dysgalactiae* at subspecies level. Alternatively, the gene *sodA* was recently described as a target gene for sequencing, and, seems to be useful for the differentiation of *S. dysgalactiae* strains isolated from fish to mammalian isolates (Nomoto et al., 2008). The 16S-23S rDNA ISR gene sequence had 100 % similarity with fish isolates previously described (Nomoto et al., 2006) and also differed from mammalian isolate sequences reported by Forsman et al. (1997) and Hassan et al. (2003), confirming the close relationship with the *S. dysgalactiae* strains isolated from fish. However more detailed studies were requested to elucidate this issue.

Clinical signs observed in the Nile tilapia fingerlings challenged with *S. dysgalactiae* were anorexia, lethargy, tachypnoea, and skin darkness. Different from other streptococcus species pathogenic to fish (Mian et al., 2008), *S. dysgalactiae* can cause systemic infection and reaches the central nervous

system (CNS) in experimental trials, but does not promote neither neurologic signs nor exophthalmia. In our in vivo experiment, all dead fish showed positive bacteria reisolation from brain and abscess lesions of caudal peduncle could not be induced. These results are in contrast with Nomoto et al. (2008) who reported no bacterial isolation from brain and the presence of necrotic lesions in the caudal peduncle in *Seriola dumerili* experimentally infected with this pathogen. This data suggests that with this bacteria, although showing similar field presentations, peculiar variations can occur according to the animal infected, maybe attributed to genetic variability of isolates from different fish species or a host-specific adaptation of the pathogen. These differences could be associated to particularities in the host-pathogen interaction. Additionally, previous reports have shown that Nile tilapia is very susceptible to develop brain infection and meningitis under streptococcal infections with *S. iniae* and *S. agalactiae* (Shoemaker et al., 2000; Mian et al., 2008). This fish specie seems to present an intrinsic susceptibility to diseases of CNS, verified by the capacity of different fish and mammal isolates of *S. agalactiae* and now *S. dysgalactiae* to infect this primordial organ. Such ability to reach the brain may be a specific feature of *S. dysgalactiae* when infecting Nile tilapia.

This is the first report that associated *S. dysgalactiae* with infection in freshwater tropical fish, as well as the first connection of this pathogen with CNS infection in fish.

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Competing interests statement

The authors declare no competing financial interests.

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6 CONCLUSÃO

O presente estudo demonstrou através dos testes bioquímicos e moleculares que as espécies *Streptococcus iniae* e *Streptococcus dysgalactiae* foram os agentes etiológicos dos surtos infecciosos ocorridos nas propriedades dos estados do Paraná e Ceará, respectivamente. A caracterização genética de isolados identificados como *S. iniae,* por PFGE, evidenciou a presença de dois genótipos presentes no Brasil. Estes se diferenciaram do padrão genotípico da cepa de referência *S.* iniae ATCC 29178. A infecção experimental, com isolado identificado como *S. dysgalactiae,* demonstrou a capacidade do patógeno em provocar doença em tilápias do Nilo incluindo a associação com quadros septicemia e encefalite.