

**GENOTIPAGEM DE *Streptococcus agalactiae*  
ISOLADOS DE SERES HUMANOS, BOVINOS  
E PEIXES E SEU POTENCIAL DE  
VIRULÊNCIA PARA TILÁPIA DO NILO**

**ULISSES DE PÁDUA PEREIRA**

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SERES HUMANOS, BOVINOS E PEIXES E SEU POTENCIAL  
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Dissertação apresentada à Universidade Federal de  
Lavras como parte das exigências do Curso de  
Mestrado em Ciências Veterinárias, para a  
obtenção do título de “Mestre”.

Orientador

Prof. Dr. Henrique César Pereira Figueiredo

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APROVADA em 18 de dezembro de 2008

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**Ao meu bom Deus por sempre me manter forte. A minha família  
e amigos que sempre estiveram muito presentes.**

**DEDICO.**

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## RESUMO

Pereira, Ulisses de Pádua. **Genotipagem de *Streptococcus agalactiae* isolados de seres humanos, bovinos e peixes e seu potencial de virulência para tilápia do Nilo.** 2008. 23p.\* Dissertação (Mestrado em Ciências Veterinárias) – Universidade Federal de Lavras, Lavras.\*

*Streptococcus agalactiae* (grupo B de Lancefield; GBS) é um importante patógeno para seres humanos, bovinos e peixes causando septicemia neonatal, mastite e meningo-encefalite respectivamente. O objetivo desse trabalho foi caracterizar por PFGE *S. agalactiae* isolados de peixes (n=27), bovinos (n=9) e seres humanos (n=11) e investigar a virulência das amostras de bovino, peixes e humanos para tilápias do Nilo (*Oreochromis niloticus*). As amostras de peixes foram isoladas de nove fazendas produtoras de tilápia do Nilo localizadas em seis estados brasileiros, as amostras de bovino foram provenientes de nove fazendas produtoras de leite e as amostras de seres humanos de isolamentos clínicos distintos. Os padrões de PFGE foram determinados por análise de dendograma e a virulência *in vivo* foi avaliada por infecção experimental (usando as vias intraperitoneal e imersão) em tilápias do Nilo. Entre as amostras de peixes cinco padrões diferentes pelo PFGE foram observados, incluindo a ocorrência de dois ou mais padrões na mesma fazenda. Vinte uma das vinte e sete amostras isoladas de peixe demonstraram o mesmo padrão genético (padrão predominante). Para as amostras de seres humanos e bovinos foi observada uma alta diversidade genética, mas não houve relação genética entre as amostras isoladas dos três hospedeiros. Em relação a infectividade para tilápias do Nilo duas amostras isoladas de bovinos foram capazes de infectar os peixes (por via intraperitoneal) e uma dessas amostras causou sinais clínicos de meningoencefalite. Todas as cinco amostras de seres humanos utilizadas para a infecção experimental foram capazes de infectar tilápias do Nilo e doença clínica foi causada por uma amostra (80427). Esse isolado foi capaz de infectar tilápia do Nilo em infecção experimental por imersão e induziu sinais clínicos. Oito amostras de *S. agalactiae* isoladas de peixe, pertencentes a diferentes padrões genéticos pelo PFGE, causaram alta mortalidade em tilápias do Nilo. Conclui-se que as amostras analisadas de três hospedeiros naturais desse patógeno não demonstraram relação genética, apesar de que, algumas amostras de bovino e seres humanos são capazes de infectar peixes e causar meningoencefalite. Nós sugerimos que essas amostras compartilhem alguns fatores de virulência e que ligação genética não é um pré requisito para *S. agalactiae* quebrar a barreira hospedeiro-específica.

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\*Orientador: Dr. Henrique César Pereira Figueiredo.



## ABSTRACT

Pereira, Ulisses de Pádua. **Genotyping of *S. agalactiae* strains isolated from human, bovine and fish and their virulence potential to Nile tilapia.** 2008. 23p. Dissertation (Master in Veterinary Science) – Federal University of Lavras, Lavras.\*

*Streptococcus agalactiae* (Lancefield group B; GBS) is an important pathogen to human, bovine and fish causing neonatal sepsis, mastitis and meningo-encephalitis, respectively. The objective of this study was to characterize by PFGE *S. agalactiae* isolated from fish (n=27), bovine (n=9) and human (n=11) and to investigate the virulence of bovine, fish and human strains to Nile tilapia (*Oreochromis niloticus*). The fish strains were isolated from nine Nile tilapia farms, the bovine strains from nine dairy cow farms and human strains from distinct clinical outcomes. The PFGE types were determined by dendrogram analyses and the *in vivo* virulence was evaluated by experimental infection (using i.p and immersion routes) in Nile tilapia. Among fish strains five different PFGE patterns were observed, including the occurrence of two or three profiles in the same farm. Twenty one of twenty seven strains isolated from fish showed the same genetic pattern (predominant pattern). To bovine and human strains high genetic diversity were observed, but no relations were established to the three host sources analyzed. Regarding the infectivity to Nile tilapia, three bovine strains were able to infect fish (by i.p.route) and two of those strains caused clinical signs of meningoencephalitis. All human strains (n= 5) submitted to experimental infection were able to infect Nile tilapia and clinical disease was induced by one strain (80427). This isolate was also able to infect Nile tilapia by immersion route and induce clinical signs. Eight fish strains of *S. agalactiae*, belonged to different PFGE types, caused high mortality in Nile tilapia. In conclusion the analyzed strains from the three natural hosts did not show genetic relatedness and, in spite of that, some bovine and human strains were able to infect fish and cause meningoencephalitis. We suggest that these strains share some virulence factors and that genetic linkage is not a prerequisite to *S. agalactiae* to cross the host-specific barrier.

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\* Advisor: Dr. Henrique César Pereira Figueiredo

## 1 INTRODUÇÃO

A produção mundial de pescado em 2002 foi de aproximadamente 133 milhões de toneladas. Desse total, 39,8 milhões de toneladas foram provenientes da aquicultura, cujo cultivo continental foi responsável por 60% e o marinho, por 40% (Food and Agriculture Organization of the United Nations - FAO, 2004).

A aquicultura é o setor do agronegócio que cresce mais rápido quando comparado a outras atividades relacionadas à produção de alimento de origem animal. Desde 1970, o crescimento mundial do setor é de 8,9% ao ano, significativamente maior que a pesca (1,2% a.a.) e que sistemas terrestres de produção de carne (2,8% a.a.) no mesmo período. No período de 2000 a 2002, o Brasil apresentou uma das dez maiores taxas de crescimento, com uma expansão de 18,1% na atividade (FAO, 2006).

Pode-se salientar que, hoje, a aquicultura é praticada em todos os Estados brasileiros e abrange, principalmente, as seguintes modalidades: piscicultura (criação de peixes), carcinicultura (camarões), ranicultura (rãs) e malacocultura (moluscos: ostras, mexilhões, escargot). Ainda, outras modalidades de produção aquática, como o cultivo de algas, são praticadas, mas em menor escala. Em 2002, a piscicultura respondeu por 67% da produção total da aquicultura, sendo a tilápia do Nilo (*Oreochromis niloticus*) o peixe mais cultivado no país (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais - Ibama, 2004; FAO, 2006).

No Brasil, a piscicultura está em sua fase de consolidação e expansão (Chammas, 1997). Apesar do franco crescimento da atividade no país, essa ainda se dá de forma desorganizada e problemática, apresentando problemas sanitários que favorecem o surgimento de doenças emergentes (bacterianas, parasitárias e por fungos), sendo as doenças bacterianas uma das principais (Perez, 1999), entre as quais as estreptococoses são, sem sombra de dúvida, o maior problema

encontrado na criação de tilápias em todo o mundo, sendo o *S. agalactiae* de grande importância (Suresh, 1998).

*S. agalactiae* é uma bactéria Gram positiva capaz de causar doença em diversos hospedeiros. Primeiramente isolado na glândula mamária de bovinos com mastite, *S. agalactiae* é uma das principais causas de meningite neonatal em seres humanos (Maione et al., 2005). Atualmente essa bactéria tem sido associada a casos de meningoencefalite e septicemia em peixes, sendo considerada um patógeno emergente para tilapicultura mundial. Ocasionalmente, também pode causar infecções em ratos, gatos, cães, hamsters, camelos e sapos (Spellerberg, 2000; Elliott et al., 1990).

Em peixes, surtos de estreptococose são responsáveis por grandes prejuízos econômicos para os produtores, pois podem causar mortalidades de até 90% do plantel e acometem, geralmente, animais na fase pré-abate, quando um volume de ração substancial já foi consumido, a qual é a parte mais onerosa no custo de produção. Esses patógenos podem ocorrer em peixes de ambientes estuarinos, marinhos e de água doce. Entre os peixes de água doce cultivados, o maior impacto econômico oriundo de infecções por *S. agalactiae* observado é em criações de tilápia do Nilo. Porém, essa bactéria tem sido isolada de diversas outras espécies de peixes em ambientes naturais e em pisciculturas comerciais (Evans et al., 2002).

A variabilidade genética de *S. agalactiae* isolados de humanos já é bem conhecida, tendo sido identificados clones com diferentes habilidades de virulência. Estudos demonstram que *S. agalactiae* isolados de casos de mastite bovina possuem padrão genético semelhante dentro de cada rebanho, havendo uma significativa variabilidade genética entre rebanhos (Baseggio et al, 1997). No entanto, pouco ainda se conhece sobre a variabilidade antigênica e genética de amostras de *S. agalactiae* isolados de peixes.

A alta incidência da infecção neonatal em seres humanos por *Streptococcus agalactiae* a partir da década de 70 chamou a atenção de médicos americanos, mas seus motivos não foram totalmente elucidados. Uma possível

explicação seria a infecção de humanos por *S. agalactiae* de origem animal (Jensen, 1985). Foi observado, em linhagens de *S. agalactiae* de origem bovina, o mesmo padrão genético de um clone de *S. agalactiae* hiperinvasivo para seres humanos (Bisharat et al., 2004). Esses resultados demonstram a possível transmissão horizontal da bactéria entre diferentes espécies de hospedeiros. Contudo, um estudo recente demonstrou que amostras *S. agalactiae* isoladas de mastite bovina não foram capazes de causar doença em tilápia do Nilo e “catfish” (*Ictalurus punctatus*) (Garcia et al., 2008). Esses resultados demonstram que tal fenômeno não está totalmente esclarecido para as relações de transmissão da bactéria entre diferentes hospedeiros, principalmente quando se trata de um hospedeiro de ambiente terrestre e outro, de ambiente aquático.

Existem poucos estudos sobre diversidade genética e filogenia de amostras de *S. agalactiae* isoladas de diferentes espécies de hospedeiro. Por conseguinte, o objetivo deste trabalho é analisar a diversidade genética de amostras de *S. agalactiae* de origem bovina, humana e de peixe e avaliar a capacidade que essas amostras têm de infectar e produzir doença clínica em tilápias do Nilo (*Oreochromis niloticus*).

**2 GENOTYPING OF *Streptococcus agalactiae* STRAINS ISOLATED FROM HUMAN, BOVINE AND FISH AND THEIR VIRULENCE POTENTIAL TO NILE TILAPIA**

(Preparado de acordo com as normas da revista “Veterinary Microbiology”)

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## ABSTRACT

*Streptococcus agalactiae* (Lancefield group B; GBS) is an important pathogen to human, bovine and fish causing neonatal sepsis, mastitis and meningo-encephalitis, respectively. The objective this study was to characterize by PFGE *S. agalactiae* isolated from fish (n=27), bovine (n=9) and human (n=11) and to investigate the virulence of bovine, fish and human strains to Nile tilapia (*Oreochromis niloticus*). The fish strains were isolated from nine Nile tilapia farms, the bovine strains from nine dairy cow farms and human strains from distinct clinical outcomes. The PFGE types were determined by dendrogram analyses and the *in vivo* virulence was evaluated by experimental infection (using i.p and immersion routes) in Nile tilapia. Among fish strains, five different PFGE patterns were observed, including the occurrence of two or three profiles in the same farm. Twenty one of twenty seven strains isolated from fish showed the same genetic pattern (predominant pattern). To bovine and human strains high genetic diversity were observed, but no relations were established to the three host sources analyzed. Regarding the infectivity to Nile tilapia, three bovine strains were able to infect fish (by i.p.route) and two of those strains caused clinical signs of meningoencephalitis. All human strains (n=5) submitted to experimental infection were able to infect Nile tilapia and clinical disease was induced by one strain (80427). This isolate was also able to infect Nile tilapia by immersion route and induce clinical signs. Eight fish strains of *S. agalactiae*, belonged to different PFGE types, caused high mortality in Nile tilapia. In conclusion the analyzed strains from the three natural hosts did not show genetic relatedness and, in spite of that, some bovine and human strains were able to infect fish and cause meningoencephalitis. We suggest that these strains share some virulence factors and that genetic linkage is not a prerequisite to *S. agalactiae* to cross the host-specific barrier.

**Key words:** *S. agalactiae*, fish, human, bovine, PFGE, virulence.

## INTRODUCTION

*Streptococcus agalactiae* (Lancefield group B; GBS) is an important pathogen frequently associated with diseases in bovine, human and fish causing mastitis, neonatal meningitis and meningoencephalitis in these hosts, respectively. (Evans et al., 2002; Mitchell et al., 2003; Oliveira et al., 2006; Mian et al., 2008).

To fish *S. agalactiae* has been considered an emerging pathogen associated to outbreaks with considerable morbidity and mortality in fish farms worldwide (Robinson & Meyer, 1966; Eldar et al., 1995; Garcia et al., 2008; Duremdez, et al., 2004; Mian, et al., 2008). Sporadically *S. agalactiae* is associated with cases of illness in many others hosts like chicken, camels, dogs, horses, cats, frogs, hamsters, mice, monkeys, emerald monitors and nutria (Elliot et al., 1990; Yildirim et al., 2002a; Yildirim et al., 2002b ; Hetzel et al., 2003; Johri et al., 2006; Garcia, et al. 2008).

The genetic diversity of *S. agalactiae* from human and bovine origins has been analyzed using a broad range of genomic techniques including ribotyping, RAPD, PFGE and MLST (Bassegio et al., 1997; Martinez et al., 2000; Oliveira et al., 2005; Bisharat et al., 2004; Honsa et al., 2008; Olivares-Fuster et al., 2008; van der Mee-Marquet et al., 2008). Previous studies about genetic relationships concluded that human and bovine strains belong to genetically distinct populations with limited interspecies transmission (Sukhnanand et al., 2005; Dogan et al., 2005) or common origin (Martinez et al., 2000). However, some reports describe isolates of bovine and human origins show the same genetic pattern (Bisharat et al., 2004; Bohnsack et al., 2004; Oliveira et al., 2006). Recently, a comparison of *S. agalactiae* strains from piscine, dolphin, human and bovine using MLST and molecular serotyping demonstrated that dolphin and piscine strains of Kuwait (ST-7, Ia) showed the same genetic pattern of a human strain associated to neonatal infection in Japan (Evans, et al., 2008), but there is no data about virulence of human strains to

fish, such as Nile tilapia, and few studies analyzing the putative virulence of bovine strains to fish (Garcia et al., 2008). Therefore, the possibility of *S. agalactiae* to cross the inter-species barrier and to cause disease in others host, such as fish, is still unclear.

The objective of this work was to study, by Pulsed-field Gel Electrophoresis (PFGE), the genetic relatedness of *S. agalactiae* strains isolated from human, bovine and fish and to investigate the virulence potential of those strains to Nile tilapia.

## **MATERIAL AND METHODS**

### **Bacterial strains**

A total of 47 *S. agalactiae* strains were selected for this study (table 1).

The fish strains (n=27) were isolated in the period of 2003-2007 from outbreaks of meningoencephalitis and septicemia in Nile tilapia characterized previously by Mian et al. (2008). Eight bovine isolates associated with clinical and sub-clinical mastitis were selected from eight dairy cattle farms in Minas Gerais State, Brazil. These isolates were recovered from milk of cows with bovine mastitis and identified by routine tests, including colony morphology, Gram stain, haemolysis on sheep blood 5% v/v agar, catalase, CAMP test, phenotypic profile in API 20 STREP (BioMerieux, France) and Lancefield group determination by Slidex Strepto-Kit (BioMerieux, France). Ten human strains of *S. agalactiae*, representative of different genetic patterns, previously characterized (Oliveira et al., 2005; Oliveira et al., 2006), were utilized to comparative genetic analysis with fish and bovine isolates and to infectivity studies in Nile tilapia.



## **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 0,01M, pH 8,0 and NaCl 1M). The bacterial suspension was mixed with an equal volume of 2% low-melting-point agarose (Sigma-aldrich<sup>®</sup>, USA) and pipeted into 20 µL plugs. Streptococcal cells in agarose plugs were lysed, the DNA digested with 12U of *Sma* I restriction enzyme (Amersham Biosciences, UK) and submitted to PFGE with a program as follows: switch time of 1 to 30s, 23h, 120° angle, 11.3°C 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<sup>®</sup> (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 analyse 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).

## **Experimental infection**

Five human isolates of *S. agalactiae* were selected for experimental infection. These isolates were previously studied by Oliveira et al., (2005) and show different serotypes and genetic patterns by PFGE. Eight fish strains and all nine bovine isolates were selected for experimental infection (table 1). The bacterial strains, stocked in freezer -70°C, were thawed, streaked onto 5% sheep

blood agar and incubated at 37°C for 24 h. After that, they were inoculated in BHI and incubated at 37°C for 24 h under low agitation. The bacterial suspension was then adjusted to an absorbance of 0.5 at 600 nm, corresponding to 10<sup>8</sup> CFU/mL from bovine, fish and human strains. Suspensions was serially diluted in 0.5 M phosphate-buffered saline, streaked onto blood agar and incubated at 37°C for 24 h for bacterial counting.

Nile tilapia fingerlings were acquired from a commercial hatchery and used for experimental infection with an average weight of 42.57±10.03 g. Before assays 10 fish were submitted to bacteriological analyses of brain and kidney to check the negative status to *S. agalactiae* infection. Each experimental group comprised eight animals kept in a 57-L aquarium supplied with flow-through dechlorinated tap water. Fish were maintained on a 12 h:12 h light/dark period at a water temperature of 28°C and were fed to satiation with VITAFISH 32% PB (Matsuda, GO, Brazil) twice a day. Before the assay procedure, fish were acclimated for a period of 10 days. Experimental infection was carried out as follows. Fish were anesthetized by immersion in a bath containing 10 mg/L benzocaine. Test fish were challenged by i.p. injection with 0.1 mL of *S. agalactiae* inoculum, corresponding to the dose of 10<sup>7</sup> CFU/ fish. Animals from control group were injected with 0.1 mL of sterile BHI. Challenged fish were monitored and fed twice a day for a period of 15 days. Samples of brain and kidney were collected from all dead fish to reisolate the bacteria, as well of the all remained fish at the end of the experiment. To verify the early colonization of kidney and brain by the tested bacterial strains two fish of each challenged group were sacrificed after 48h after challenge and samples of these organs were aseptically streaked in blood agar to bacterial recovery. The criteria considered to determine the ability of infection by a bacterial strain in Nile tilapia was the positive culture for *S. agalactiae* from the brain or kidney samples of at least one fish in each experimental group.

Experimental infection by immersion route was performed with one human strain (80427) and two bovine strains (812 and 87159). For the immersion trial, fish groups were anesthetized and then immersed in a 10-L bucket containing water inoculated with *S. agalactiae* at a final dose of  $10^7$  CFU/mL. After 15 min the fish were returned to aquaria with uncontaminated water. The control group was immersed in water containing sterile BHI. Fish were maintained under the same experimental conditions as described for i.p. infection and observed for a period of 15 days. The Ethics Committee for Animal Experiments of the Federal University of Lavras approved the protocols used in this study.

## **RESULTS**

### **PFGE**

The 27 *S. agalactiae* isolates of fish were distributed in five distinct PFGE types (A, B, C, D and E; Table 1; Figure 1). The pattern A was predominant in the fish strains analyzed, performing approximately 78%. Almost all strains belonged to the pattern variation A<sub>1</sub> and only one fish strain belonged to the pattern variation A<sub>2</sub>, considered clonally related to the pattern A<sub>1</sub>. These 21 strains were isolated from outbreaks in six different Brazilian States (Table 1). On the farm A three different types (A<sub>1</sub>, C and D) were observed, as well two types (A<sub>2</sub> and E) on the farm H.

All bovine strains showed different patterns by PFGE (Table 1). Comparison of the PFGE patterns of the strains isolated of fish and bovine demonstrated that these strains were genetically unrelated, with lack of a typical cluster formed according to the host origin (Fig. 1).

Comparison of the PFGE patterns of fish and human strains (Fig. 3) showed a low relatedness among them. The human strains were grouped in many genetic clusters, more related to themselves than with the different genetic patterns of the fish strains. The PFGE pattern predominant in the fish strains

(type A) demonstrated no clonal relation with *S. agalactiae* strains from human and bovine (Fig.1).

### **Experimental infection**

In the infection trial by i.p. route all fish strains of *S. agalactiae* were able to reach the brain and kidney 48h post-inoculation. Following the infection the mortality rates were 100% in all experimental groups, with positive bacterial recovery (table 2). Clinical signs of meningoencephalitis, such as lethargy, anorexia, erratic swimming and exophthalmia were induced by all fish strains tested.

In the infection trial, using *S. agalactiae* bovine strains tested, three (812, 1001 and 87159) were able to infect the challenged fish 48h post-inoculation, as verified by positive cultures from brain and kidney in all fish sampled (Table 2). At this time all fish were negative for any clinical signs of *S. agalactiae* infection. Following the experiment the bovine strain 812 caused clinical disease in one fish, three days post-inoculation. This animal showed the main clinical signs of disease, characterized by lethargy, anorexia, tail hemorrhages and erratic swimming. Five days post-inoculation the fish died and *S. agalactiae* was recovered from the brain and kidney. The bovine strain 87159 caused mortality of four fish, with deaths occurring between 72h and 96h post-inoculation (Table 2). The fish presented anorexia, lethargy and few hemorrhagic points in the caudal fins, but without neurological signs. The bovine strains 812, 1001 and 87159 belonged to different PFGE types (Table 1).

The five human strains tested were able to infect Nile tilapia, as verified by positive cultures from brain and kidney of sampled fish 48h post-inoculation (table 2). From those, only one *S. agalactiae* strain, 80427, caused classical signs of meningoencephalitis in fish. In this experimental group, four fish presented poor appetite, darkened skin, lethargy and erratic swimming. From those fish one was died in four days post- infection and the other three fish

recovered from disease and stopped with clinical signals seven days post-inoculation. However, from these three animals and from the all remained fish of this experimental group, *S. agalactiae* was recovered from the brain or kidney at the end of the experiment (15 days post-inoculation). For the human strains 90356 and 90221 no clinical signs were observed during the experimental period, but *S. agalactiae* was recovered from the brain or kidney in three and two animals, respectively. The strains 80381 and 84204 infected fish (with culture positive for the brain) 48 hours post-inoculation, but the six remaining fish from these two groups were negative for *S. agalactiae* isolation after 15 days of infection (table 2). Moreover, no clinical signs were observed.

The three human and bovine *S. agalactiae* strains (80427, 812 and 89759) that induced clinical signs in Nile tilapia and the fish strain SA 20-06 were then used in immersion trial to verify the ability of them to overcome the natural fish superficial defenses and establish systemic infection or clinical disease. The fish strain SA 20-06 induced disease and mortality in 37.5% of the exposed Nile tilapia. The clinical signs were observed early, 24 h p.i. For all diseased fish *S. agalactiae* was recovered from brain and kidney and the five remaining healthy fish were negative to bacterial isolation. The strains 812 and 87159 isolated from bovine were not able to infect fish and all animals from this experimental group were negative for *S. agalactiae* recovery at 48 p.i. as well as at the end of the experiment (table 2). For the human strain 80427 it was possible to isolate *S. agalactiae* from the brain and kidney of the two sampled fish 48h p.i., with absence of clinical signs. Within 72h one fish presented typical clinical signs of meningoencephalitis and died 30 h after. Positive cultures from brain and kidney were obtained. The three remaining fish of this group were healthy 15 days p.i and *S. agalactiae* was not recovered from brain and kidney cultures.

## DISCUSSION

The present study is the first description of PFGE characterization of *S. agalactiae* strains isolated from diseased fish using restriction enzyme *Sma* I. This valuable molecular tool has been previously used to determine PFGE types of fish isolates, however, with no success in achieve the digestion of chromosomal DNA of *S. agalactiae* using *Sma* I (Suanyuk et al., 2008). Our study shows that PFGE with restriction enzyme *Sma* I is an efficient molecular method and can be applied to genetic typing of *S. agalactiae* fish strains.

Five different genetic patterns were determined for the analyzed fish strains. The predominant PFGE pattern, A<sub>1</sub>, seems to be the main genetic type involved in the outbreaks of meningoencephalitis in Nile tilapia in Brazil. This epidemiological condition could be associated to a better adaptation or transmission of *S. agalactiae* of A<sub>1</sub> type or a spread of this PFGE type by commercial Brazilian tilapia hatcheries, which sell fingerlings to farms located in many Brazilian States.

*S. agalactiae* fish strains induced 100% of mortality by i.p. injection in Nile tilapia, regardless the different genetic types presented. In a previous report using the same fish strains we demonstrated that the LD50 of this pathogen is very low, as 90 bacterial cells, and that the outbreaks were associated to high water temperatures and accompanied by elevated mortality in different farms (Mian et. al., 2008). These conditions could be associated with any genetic type described here and demonstrates that these variants are able to induce high mortalities in farmed Nile tilapia. Also, the ability of strains from different PFGE types in induce mortality in infected fish suggest that these strains possesses same virulence factors, despite the genetic diversity presented.

Our study showed that there is large genetic diversity within fish strains and between strains from the different hosts evaluated. Different PFGE types could be observed in fish isolates from the same farm. Although the genetic variability verified in these strains classified in different clusters (A<sub>1</sub>, B, C, D

and E) were highly virulent to Nile tilapia in the infection trial. Independent of genetic pattern, fish strains were highly virulent to Nile Tilapia resulting in a 100% mortality rate. This data demonstrate that independently of the genetic cluster, the adaptation to the *S. agalactiae* isolates to fish was reached and suggests that strains from different hosts share virulence factors that enable the bacteria to colonize Nile tilapia and to cross the fish blood-brain barrier causing meningoencephalitis. Although the PFGE technique was capable of establishing different genetic types, probably this variability was not directly associated to virulence of the analyzed strains. Indeed, this genetic diversity should be associated to antigenic variations on the bacterial surface becoming an important parameter for the future development of vaccines against fish streptococcosis. Likewise, the PFGE will be a valuable tool to study the evolution dynamics of this pathogen in the next years and to monitor the epidemiology and spread of the different PFGE types in Nile tilapia farms.

Three bovine strains were able to infect Nile tilapia whereas two bovine strains, without genetic similarities with fish isolates, caused meningoencephalitis in fish with clinical signs of disease unlike shown by Garcia et al. (2008). In the same way, all five human strains were able to infect Nile Tilapia brain and kidney and one isolate caused clinical signs of disease and mortality. In the infection trials the strains from piscine origin induced a higher mortality in Nile tilapia than the strains from bovine and human origins. In the present study we didn't evaluate the possibility of the increase of virulence of bovine and human strains after serial passages in Nile tilapia. This is a phenomenon known to many bacterial pathogens and a previous work described that fish strains of *S. agalactiae* increased its virulence to tilapia after successive passages *in vivo* (Eldar et al., 1995). Although strains isolated of bovine and human were able to infect Nile tilapia by i.p. route, only the human strain 80427 was able to infect Nile tilapia by immersion route of experimental infection. The observed ability of infection using human strains suggests that these hosts might be reservoirs of *S. agalactiae* to fish.

The lack of previous association in the literature between genetic types of *S. agalactiae* and ability to cross the species-barrier might be associated to the low number of strains used to do the comparisons and the high degree of genetic diversity showed by this pathogen. Genetic studies in genomic scale have demonstrated that the core genome of *S. agalactiae* represent only approximately 80% of the whole genome of a strain and that, for each new genome strain sequenced, probably 33 new genes should be described (Tettelin et al., 2005). However, its estimate was done using only human strains of *S. agalactiae* and the use of fish strains could introduce a higher number of genes to the pan-genome of this pathogen.

Our study demonstrated that in spite of genetic disimilarities, *S. agalactiae* isolated from bovine and human can infect and cause clinical disease in Nile tilapia. We suggest that these strains share some virulence factors and that genetic linkage is not a prerequisite to *S. agalactiae* to cross the host-specific barrier.



**Table 1:** Epidemiological data for the *S. agalactiae* isolated from human, bovine and fish in the Brazil.

Host	Strain	Serotype	Disease/body site isolation	State	Farm	PFGE type
<b>Fish**</b>	SA 01-03	ND	meningoencephalitis	MG	A	A <sub>1</sub>
	SA 02-03	ND	meningoencephalitis	MG	A	A <sub>1</sub>
	SA 03-03	ND	meningoencephalitis	MG	A	C
	SA 04-03	ND	meningoencephalitis	MG	A	D
	SA 05-04	ND	meningoencephalitis	ES	B	A <sub>1</sub>
	SA 06-04	ND	meningoencephalitis	ES	B	A <sub>1</sub>
	SA 07-05	ND	meningoencephalitis	BA	C	B
	SA 08-05	ND	meningoencephalitis	BA	C	B
	SA 09-05	ND	meningoencephalitis	ES	D	A <sub>1</sub>
	SA 11-05	ND	meningoencephalitis	ES	D	A <sub>1</sub>
	SA 13-05	ND	meningoencephalitis	ES	D	A <sub>1</sub>
	SA 15-06	ND	meningoencephalitis	SP	E	A <sub>1</sub>
	SA 16-06	ND	meningoencephalitis	SP	E	A <sub>1</sub>
	SA17-06	ND	meningoencephalitis	PR	F	A <sub>1</sub>
	SA 18-06	ND	meningoencephalitis	SP	E	A <sub>1</sub>
	SA 19-06	ND	meningoencephalitis	SP	E	A <sub>1</sub>
	SA 20-06	ND	meningoencephalitis	PR	G	A <sub>1</sub>
	SA 26-06	ND	meningoencephalitis	PR	F	A <sub>1</sub>
	SA 33-06	ND	meningoencephalitis	SP	H	A <sub>1</sub>
	SA 34-06	ND	meningoencephalitis	SP	H	A <sub>1</sub>
SA 36-06	ND	meningoencephalitis	SP	H	A <sub>1</sub>	
SA 37-06	ND	meningoencephalitis	SP	H	A <sub>1</sub>	
SA 38-06	ND	meningoencephalitis	SP	H	A <sub>1</sub>	
SA 39-06	ND	meningoencephalitis	SP	H	A <sub>1</sub>	
SA 53-07	ND	meningoencephalitis	CE	I	E	
SA59-07	ND	meningoencephalitis	CE	I	A <sub>2</sub>	
SA66-07	ND	meningoencephalitis	CE	I	E	
<b>Cow</b>	160	ND	subclinical mastitis	MG	I	ND
	252	ND	subclinical mastitis	MG	II	H
	442	ND	clinical mastitis	MG	III	I
	477	ND	clinical mastitis	MG	IV	J
	812	ND	subclinical mastitis	MG	V	F
	1001	ND	subclinical mastitis	MG	VI	G
	1490	ND	subclinical mastitis	MG	VII	ND
	1640	ND	subclinical mastitis	MG	VIII	ND
	87159	ND	Clinical mastitis	SP	IX	K
<b>Human</b>	80427	V	cervix	RJ		K
	90188	II	urine	RJ		L
	85333	III	oropharinge	RJ		M
	88599	II	vagina	RJ		N
	83171	V	anal margin	SC		O <sub>1</sub>
	91285	III	oropharinge	RJ		P
	80381	V	oropharinge	RJ		O <sub>2</sub>
	90356	III	cerebrospinal fluid	RJ		Q
	84204	III	oropharinge	RJ		R
	90221	II	Placenta	RJ		S

\* Nile tilapia (*Oreochromis niloticus*).

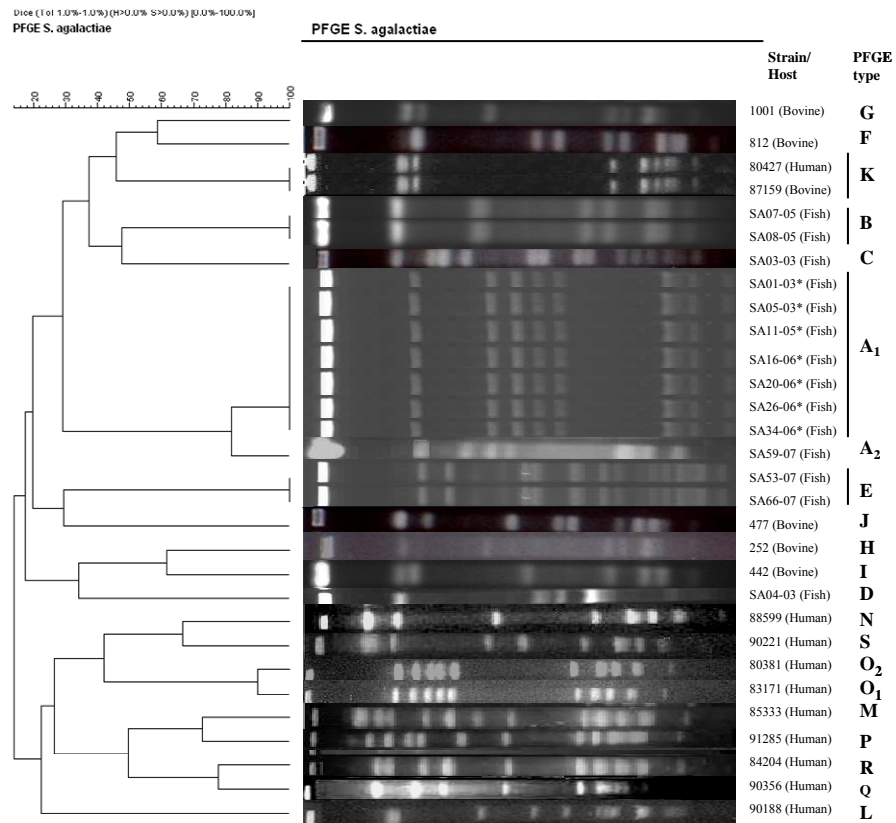
ND – not determined. (+)= positive and (-)= negative

**Table 2:** Results of experimental infection of *S. agalactiae* isolated from bovine, human and fish strains.

Strain	Host	Experimental Infection				
		Infection at 48h post-inoculation	Fish presenting clinical signs*	Mortality rate (%)*	% of recovery of <i>S. agalactiae</i> *	Immersion route
SA 01-03	Fish	+	+	100	100	ND
SA 03-03		+	+	100	100	ND
SA 04-03		+	+	100	100	ND
SA 05-04		+	+	100	100	ND
SA 08-05		+	+	100	100	ND
SA 16-06		+	+	100	100	ND
SA 20-06		+	+	100	100	+
SA 53-07		+	+	100	100	ND
160	Bovine	-	-	0	0	ND
252		-	-	0	0	ND
442		-	-	0	0	ND
477		-	-	0	0	ND
812		+	+	12,5	12,5	-
1001		+	-	0	0	ND
1490		-	-	0	0	ND
1640		-	-	0	0	ND
87159		+	+	50	50	-
80427		Human	+	+	12,5	100
80381	+		-	0	0	ND
90356	+		-	0	50	ND
84204	+		-	0	0	ND
90221	+		-	0	33,3	ND

ND – not determined. (+)= positive and (-)= negative

\* Data from fish inoculated by i.p. route.



**Figure 1:** Dendrogram constructed by similarity and clustering analyses using the Dice coefficient and UPGMA of the digitalized PFGE patterns for the five genetic patterns of *S. agalactiae* isolated from Nile tilapia, ten human genetic patterns and five bovine genetic patterns whose chromosomal DNAs were digested with *Sma* I. The codes on the right of the figure represent the the PFGE type of strains used in the present study (see table 1). \* Representative strains of predominant pattern A<sub>1</sub> to fish isolates.

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## **Competing interests statement**

The authors declare no competing financial interests.

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### 3 CONCLUSÃO

Apesar de aproximadamente 78% das amostras pertencerem a um mesmo padrão genético (padrão predominante) caracterizado pelo PFGE , a população de *Streptococcus agalactiae* isolados de peixes apresenta diversidade genética. Não houve similaridade genética entre as amostras de *S. agalactiae* isolados de ser humano, bovino e tilápia do Nilo.

Algumas amostras de *S. agalactiae* oriundas de seres humanos e bovinos podem causar doença em peixes mesmo possuindo padrões genéticos distintos. Amostras desse patógeno isolados de seres humanos foi capaz de infectar por imersão tilápias do Nilo, podendo o ser humano ser reservatório da doença para peixes e vice-versa.

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