

Evaluation on the Pathogenesis of *Streptococcus agalactiae* in Nile Tilapia (*Oreochromis niloticus*)

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ABSTRACT

The pathogenesis of a *Streptococcus agalactiae* was evaluated in a three-period experiment. Two groups of 40 fishes were intraperitoneally (i.p.) challenged in each experimental period with different infective doses of the pathogen. Doses varied from 1.0×10^6 to 1.5×10^8 CFU/fish. One group of 40 tilapia i.p. injected with tryptic soy broth (TSB) was used as a control group in each period. Mortalities varied from 67.5% in group 8 (infective dose 1.0×10^6 CFU/fish) to 90.0% in group 1 (infective dose 1.5×10^8 CFU/fish). Significant differences in mortalities were found only between group 8 and each of the other groups, except group 5 (infective dose 6.0×10^6 CFU/fish; mortality 75.0%). The highest mortality coefficients were observed in days 1-2 after inoculation (accumulated mortality 44.4%), and a second peak of mortality occurred at days 6-7. Challenged fishes from all the groups showed alterations in behaviour and similar clinical signs. These were anorexia, lethargy, erratic swimming, exophthalmia and ascites. Macroscopically, skin hemorrhage, splenomegaly, hepatomegaly with organ paleness and visceral adhesions were observed. *S. agalactiae* was re-isolated from all the fishes from the experimental groups submitted to bacteriological examination. The illness observed in tilapia naturally infected with *S. agalactiae* was experimentally reproduced in this study, and the clinical signs produced were similar to those reported from the natural infections.

Key words: *Streptococcus agalactiae*, Nile tilapia (*Oreochromis niloticus*), clinical signs, and death rate

INTRODUCTION

Aquaculture is among the fastest growing sectors in the food production industry, with an average annual growth of 8.9% (Bondad-Reantaso, et al. 2005). In the 1950s, the production of the fishes was approximately one million tons, and in 2004, it reached 59.4 million. The greatest average annual growth - of 21.3% - occurred in Latin America and in the Caribbean region, which were responsible for only 2.3% of the world production (FAO, 2006).

In 2004, Brazil produced 177,518 tons of fresh water fishes, from which 69,078 were tilapia. Brazil was classified as the 7th largest producer of tilapia in the world (FAO, 2006). According to statistics Ibama (2005), the state of Paraná produced 11,921 tons of tilapia in 2004. The growth in global aquaculture, associated to the globalization of commerce, introduction of new species and intensive handling systems, among others, have increased sanitary problems and the appearance of emergent diseases (Bondad-Reantaso et al., 2005). Among the bacterial

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diseases, streptococci have increased with the intensive farming of fresh water fishes. *Streptococcus* spp. is considered the main pathogen present in different fish species.

Streptococcus agalactiae is the most commonly found species in hot climate, being associated to different fresh water, marine and estuary fish species (Evans et al., 2002). First reports of streptococci in the fishes were from Hoshina et al. (1958), in Japan, in cultivated rainbow trout. Since then, infection from this pathogen has been reported in several other fish species (Inglis et al., 1993). *S. difficile* was first reported by Eldar et al. (1994), in Israel, as a non-hemolytic coccus causing septicemia and meningoencephalitis in tilapia (*Oreochromis* spp.) and rainbow trout (*Oncorhynchus mykiss*). Recent studies have shown that *S. difficile* belongs to Lancefield's B group, Ib type, with cellular protein electrophoretic profile indistinguishable from *S. agalactiae* (Vandamme et al., 1997). Kawamura et al. (2005) proposed a re-classification of *S. difficile* in order to unify the terminology, considering *S. difficile* and *S. agalactiae* synonyms. In Brazil, Salvador et al. (2005) isolated Lancefield's group B non-hemolytic *Streptococcus* spp. from tilapia cultivated in net-tanks and earthen ponds in the state of Paraná. Figueiredo et al. (2006) isolated *S. agalactiae* from tilapia cultivated in net-tanks in the states of Minas Gerais and Espírito Santo.

Eldar et al. (1995) reported the erratic swimming, decrease in appetite, lethargy, exophthalmia with intraocular hemorrhage and opaqueness of cornea, and ascites, in the fishes infected by *S. difficile*. Swimming in circles, uncoordinated movements, and dorsal rigidity are indicators of the compromising of central nervous system. Salvador et al. (2005) observed a high morbidity and mortality in *S. agalactiae* when tilapia were cultivated in net-tanks, which presented erratic and circular swimming, appetite decrease, uni- or bi-lateral exophthalmia and visceral cavity distension as main clinical signs. Main macroscopical alterations reported by Salvador et al. (2003) were injuries in the skin, fins, branchiae, opacity of the cornea, liquid in visceral cavity, liver and splenomegaly. The intensity of lesions and clinical signs in tilapia depends on factors related to *S. agalactiae* strain, infective dose, amount of water, temperature, biomass and also on handling (Chang and Plumb, 1996).

The objectives of the present study were to evaluate the mortality rate, clinical signs, and macroscopic injuries in Nile tilapia (*Oreochromis niloticus*) experimentally inoculated with a field sample of *S. agalactiae* in different concentrations.

MATERIALS AND METHODS

Fish

For this study, 360 Nile tilapia (*O. niloticus*) with an average weight of 50g (40 to 60g) were used, which were kindly provided by the Pisciculture Station, from the Animal and Vegetal Department, at Londrina State University. These fishes did not present any sign of infection neither by streptococci nor other pathogens. Before the beginning of each experiment, three fishes were randomly necropsied. Cranial kidney and brain were collected for the bacteriological examination, with the objective of verifying if the fishes were free from *S. agalactiae* and others.

Fish handling

Nine groups of the fishes were made, each with 40 animals. These groups were kept in 500L fiberglass containers with 400L water and renewal rate of 3L water per minute. The fishes were fed twice a day with extruded ration (FISH™ 30% gross protein, Cooperativa Integrada) in the ratio of 3% live weight per day. In these conditions, fishes were acclimatized during 10 days before the beginning of the experiments.

Water quality control evaluation

The containers were filled with semi-artesian well water, with continuous flow and aeration, and cleaning was performed by suction on a daily basis. During the experiment, average concentration of oxygen was 3.8 ± 0.5 mg/L, temperature $26.0 \pm 0.8^\circ\text{C}$, ammonia 0.05 mg/L and nitrites 0.25mg/L. Oxygen levels and temperature were measured daily with equipment model YSI 55 (Yellow Spring Instrument, Yellow Springs, OH, USA). Ammonia was measured quarterly by Berthelot reaction (Solaranzo, 1969) and nitrite was quantified by Griess reaction (Aminot and Chaussepied, 1983).

Preparation of inoculum

S. agalactiae (UEL 12) used as inoculum in the experiments was isolated from an outbreak

characterized by the elevated morbidity and mortality of tilapia in an intensive farming property using net-tanks in the Northern region of Paraná. The isolated strain was aliquoted and stored in liquid nitrogen until its use. One *S. agalactiae* aliquot was inoculated in TSB (Tryptic Soy Broth - Difco Laboratories, Sparks, MD) and incubated at 30°C for 48 h. In order to verify the purity of the inoculum, one aliquot from the culture was inoculated in Agar Columbia (Difco Laboratories, Sparks, MD) with an additional 5% sheep blood (ACS) and incubated at 30°C in aerophile atmosphere for 48 h.

For each period in the experiment, a culture with concentration of 1.5×10^9 CFU/mL standardized by spectrophotometries (Cintra 5) with wavelength of 540nm, optical density (OD) of 0.60, and counting in plates (CFU/ml) with TSA (Tryptic Soy Agar - Difco Laboratories, Sparks, MD) was prepared. Dilutions in TSB (Tryptic Soy Broth - Difco Laboratories, Sparks, MD) were performed from this original concentration for the obtainment of infective doses, confirmed by counting in TSA plates.

Experiment

The experiment was performed in three different periods. In each period, two infective doses of *S. agalactiae* were analyzed. Challenged fishes were intraperitoneally inoculated (i.p.) with 0.1 mL bacterial suspension, and those from the control groups with 0.1 mL i.p. sterile TSB. In the first period (April/2004), groups 1, 2 and 3 were inoculated with a bacterial suspension of 1.5×10^8 CFU/fish, 5.0×10^7 CFU/fish and TSB, respectively. In the second period (June/2004), groups 4, 5 and 6 were inoculated with the bacterial suspension of 2.0×10^7 CFU/fish, 6.0×10^6 CFU /fish and TSB, respectively. In the third period (August/2005), groups 7, 8, and 9 were inoculated with the bacterial suspension of 1.2×10^8 CFU/fish, 1.0×10^6 CFU/fish and TSB, respectively. After inoculation, the fishes were monitored twice a day, for a period of 12 days. Daily mortality and main clinical symptoms were recorded. Dead fishes were collected in sterile plastic bags for the necropsy, macroscopic injuries and bacteriological tests.

Collection and analyzes of samples

In order to collect the biological material, 12 tilapia presenting clinical signs were autopsied in each period. A total of 107 samples were

aseptically collected, being 36 from kidney, 32 from brain and the remaining from eye, liver, visceral liquid and heart. The samples were inoculated in ACS and the plates were incubated at 30°C during 72h. The morphology of the colonies and the absence of hemolysis were observed. Those colonies considered typical were submitted to Gram stain, catalase test, esculin, NaCl 6.5% and growth in Methylene Blue Agar. The identification of *S. agalactiae* was performed according to Evans et al. (2002). For the biochemical identification, Api 20 Strep (BioMérieux, France) was used, and for the classification in Lancefield group, Slidex strepto kit (BioMérieux, France) was used.

Statistics

Absolute and relative frequencies for daily mortality of tilapia per *S. agalactiae* concentration were verified. In order to compare the mortality rates in each concentration, Chi-Square test with 5% significance level was used.

RESULTS

Table 1 presents the mortality of the six inoculation experiments using different *S. agalactiae* infective doses. The mortality ranged from of 67.5 to 90.0% and the highest mortality was with 1.5×10^8 CFU/fish. There was no significant difference in the mortality among groups 1, 2, 4, 5 and 7. Group 8, which was inoculated with 1.0×10^6 CFU/fish, presented mortality of 67.5%, a significant difference when compared to the other groups, with the exception of group 5. In the three control groups, two of 120 (1.67%) fishes died.

The highest mortality coefficients occurred on the first and second day, 60 (30.3%) and 28 (14.1%), respectively, presenting an accumulated mortality of 44.4%. A second mortality peak occurred between the sixth and seventh day, with the death of 23 (11.6%) and 25 (12.6%) fishes, respectively. Until the seventh day, the accumulated mortality was 83.2%.

The fishes from the control groups fed normally and were active during the whole experiment. The fishes from all the groups inoculated with *S. agalactiae* presented alterations in behavior and similar clinical signs. On day 1 after the inoculation, an elevated mortality rate was observed, characterized by sudden death, while the

majority of the fishes were swimming lethargic at the bottom of the tank, suffering from alterations in skin color and anorexia. From day 2, a worsening of clinical signs was noticed, including erratic swimming, uni- and bi-lateral exophthalmia, opacity of the cornea, and stretching of visceral cavity. In 36 fishes autopsied, the main injuries were skin hemorrhage, ascites, softening of brain and liver, hepatomegaly and paleness of the organ, splenomegaly and

visceral adherence. In all the 107 samples submitted to bacteriological tests, *S. agalactiae* was isolated, presenting the same morphological, cultural, phenotypical and serological characteristics of the strain used for the experimental inoculation. *S. agalactiae* was re-isolated from the brain, kidney, liver, heart, eye and ascitic fluid. *S. agalactiae* was not isolated from the fishes in the control groups that died along the experiment.

Table 1 – Mortality of Nile tilapia (*Oreochromis niloticus*) observed during 12 days after inoculation, in three periods, with different infective doses of *Streptococcus agalactiae* isolated from tilapia naturally infected, Londrina, Brazil, 2005.

*Group	N Fish	Period	Inoculum UFC/fish	Total	%
1 ^a	40	April/2004	1.5 x 10 ⁸	36	90,0
2 ^a	40	April/2004	5.0 x 10 ⁷	35	87,5
3 ^c	40	April/2004	Control	0	0
4 ^a	40	June/2004	2.0 x 10 ⁷	35	87,5
5 ^{a,b}	40	June/2004	6.0 x 10 ⁶	30	75,0
6 ^c	40	June/2004	Control	1	2,5
7 ^a	40	August/2005	1.2 x 10 ⁸	35	87,5
8 ^b	40	August/2005	1.0 x 10 ⁶	27	67,5
9 ^c	40	August/2005	Control	1	2,5

*Different letters indicate significant difference among groups (P≤ 0.05)

DISCUSSION

Evans et al. (2002) had observed the onset of clinical signs within 24 h after the inoculation of *S. agalactiae* in the concentration of 1.0 x 10⁷ CFU/fish i.p. and accumulated mortality rate of 60% after seven days. Mortality rate of 30% was reported in the first 24 h after the inoculation of tilapia with *S. agalactiae* at 4.5 x 10⁶ and 5.5 x 10² CFU /fish, with mortality at the end of the experiment of 60 and 50%, respectively (Evans et al., 2004). Pasnik et al. (2005) observed mortality of 84 to 96% in the control groups with streptococcal vaccines challenged with *S. agalactiae* (1.89 x 10⁴ and 2.11 x 10⁴ CFU/fish), respectively. A comparison of the present results (Table 1) with those found in literature, show a sharp difference between the reported mortality coefficients. Several factors, such as the concentration of inoculum, *S. agalactiae* strain type, inoculation manner, observation period after

challenge, age and weight of fish, reactivation of pathogenicity, could induce these differences.

In this research, the *S. agalactiae* strain used was not reactivated for inoculation of the fishes in the different experimental groups, justifying the highest concentration of bacteria in the inoculum. However, there were no significant difference between the mortality coefficients from the fishes belonging to groups 1 and 7 (Table 1), performed with intervals of 16 months, using the same *S. agalactiae* strain, kept in liquid nitrogen. Eldar et al. (1995) observed that the virulence of an *S. difficile* strain isolated from the natural infection in tilapia increased after three passages in the fish. DL₅₀ lowered in the rate of five logarithms, from the initial concentration of 10⁷ to 10²/CFU. Rasheed and Plumb (1984) reactivated the virulence of non-hemolytic *Streptococcus* sp. belonging to Lancefield group B and observed mortality of 50% after 96 h from i.p. inoculation of 1.4 x 10⁴ CFU/fish (DL₅₀).

The inoculation route influences the mortality rate of experimentally inoculated fish. Perera et al. (1997) infected the tilapia with *S. iniae* through oral route (inoculation in the intestine through catheter), by immersing the fishes in the bacterial solution and through i.p. injection, and observed a mortality rate of 50, 34 and 95%, respectively. McNulty et al. (2003) have shown that *S. iniae* was able to invade the filamentous epithelium of the gill and cause septicemia. However, a low mortality rate (13.33%) was observed when the fishes were experimentally instilled in the gills with 5.0×10^5 CFU/fish.

Similar clinical signs to those observed in this study were reported by Salvador et al. (2003; 2005), in the Brazilian state of Parana, and also by Figueiredo et al. (2006) in the Brazilian states of Espírito Santo and Minas Gerais, in the tilapia cultivated in the earthen ponds and net-tanks. The authors reported lethargia, anorexia, erratic swimming, uni-or bilateral exophthamia, ascites and high mortality. Similar clinical signs were also observed in naturally infected tilapia in Israel and the USA (Eldar et al., 1995; Plumb, 1999). Clinical signs observed in the fishes experimentally inoculated with *S. difficile* and *S. agalactiae* described in literature were similar to those observed in natural infection (Eldar et al., 1995; Evans et al., 2002; Evans et al., 2004). Similar clinical signs were also observed in tilapia experimentally or naturally infected by *S. iniae* (Perera et al., 1994; Evans et al., 2000; Shelby et al., 2002). Hemorrhagic injuries in the skin, ascites, hepatomegaly and splenomegaly observed in the fishes inoculated in this experiment were compatible with those reported by Salvador et al. (2005) and Figueiredo et al. (2006) in the tilapia naturally infected in Brazil.

CONCLUSIONS

S. agalactiae strain isolated from the naturally infected tilapia reproduced the illness in the fishes intraperitoneally experimentally inoculated with different infective doses. Clinical signs and macroscopical injuries were similar to those described in natural the infection.

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RESUMO

A patogenicidade do *S. agalactiae* foi avaliada experimentalmente em três períodos. Dois grupos de 40 peixes foram inoculados intraperitonealmente (i.p.) em cada período com diferentes doses infectantes do patógeno. As doses variaram de $1,0 \times 10^6$ a $1,5 \times 10^8$ UFC/peixe. Como controle, um grupo de 40 peixes foi inoculado tryptic soy broth (TSB) via i.p. em cada período. Mortalidades variaram de 67,5% no grupo 8 (dose infectante $1,0 \times 10^6$ UFC/peixe) a 90,0% no grupo 1 (dose infectante $1,5 \times 10^8$ UFC/peixe). Diferença significativa de mortalidade foi observada somente entre o grupo 8 e os demais grupos, exceto com grupo 5 (dose infectante $6,0 \times 10^6$ UFC/peixe – 75,0% de mortalidade). Os maiores coeficientes de mortalidade foram observados no 1 e 2º dia após a inoculação (mortalidade acumulada de 44,4%), e o segundo pico de mortalidade ocorreu no 6 e 7º dia. Em todos os peixes inoculados foi observada alteração de comportamento e sinais clínicos semelhantes. Anorexia, letargia, natação errática, exoftalmia e ascite. Macroscopicamente, foi observada hemorragia na pele, esplenomegalia, hepatomegalia, palidez dos órgãos e aderências viscerais. *S. agalactiae* foi re-isolado dos peixes submetidos ao exame bacteriológico. A doença observada nas tilápias infectadas naturalmente com essa cepa de *S. agalactiae* foi experimentalmente reproduzida nesse trabalho, e os sinais clínicos foram similares à infecção natural.

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