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Histological findings of experimental *Streptococcus agalactiae* infecion in nile tilapias (*Oreochromis niloticus*)

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Abstract

Nile tilapias (*Oreochromis niloticus*) were intraperitoneally infected with a *Streptococcus agalactiae* suspension containing 10^5CFU/mL. The strain of *S. agalactiae* used to experimental infection was isolated from naturally infected tilapias from Paraná, south Brazil. Fishes were kept in laboratory aquaria with adequate limnologic parameters, being euthanized 3, 7, 14, 21 and 28 days post-inoculation. The isolate caused exophtalmia, ocular opacity, erratic swimming, lethargy and darkness of the skin 3, 7 and 14 post-inoculation. Histopathology revealed a septicemia with a severe mononuclear infiltrate in meninges, epicardium and eyes. Inflammation occurred mainly 3 and 7 days post-inoculation, and at 21 and 28 days was not observed. A significant association was verified between infection with *S. agalactiae* and the presence of melanomacrophages centers in the liver and spleen. *S. agalactiae* was reisolated from brain and kidney 3, 7 and 14 days post-inoculation.

Key Words: *Streptococcus agalactiae*, tilapia, histopathology, septicemia.

Introduction

Since the first report (9) streptococcal disease involving fish, streptococci have had increasing prominence as agents of fish disease with severe losses (10, 17, 8). A variety of marine fish and freshwater species, as tilapia, have been reported to be susceptible to *Streptococcus* spp. (19, 17). Streptococcal infections gained increased importance in many countries with intensive aquaculture (16, 14, 18).

In south Brazil, a disease related to streptococcal infection appeared for the first time in the summer of 2001. The affected fish were Nile tilapia, *Oreochromis niloticus*, in which morbidity and mortality was observed (13). The etiology of these deaths was a bacterial septicemia caused by *Streptococcus agalactiae* (14). In fish, isolations of *S. agalactiae* associated with natural outbreaks and experimental infectivity have been reported (19, 7, 4). However, little information is available describing the evolution of histological lesions in fish infected by *S. agalactiae*. This study concerns clinical signs, histological findings and pathogenicity of *S. agalactiae* Brazilian isolates from fish in experimentally infected Nile tilapia.

Material and Methods

The strain of *S. agalactiae* (UEL13) used in this study was isolated from a natural outbreak in Nile tilapia in Paraná, south Brazil. The bacteria were routinely grown on Agar Columbia (Difco Laboratories, Sparks, MD) with additional 5% ovine blood (ACS) under aerobic conditions at 30°C for 48 hours. Bacterial suspension used in this study was established in 10^5CFU/mL.

Before the inoculation juvenile Nile Tilapia (*O. niloticus*) of 80g weight (N = 60) were cultivated in Fish Hatchery Station at Universidade Estadual de Londrina, Brazil. The fish were acclimatized during 10 days in well-aerated water, continuous dechlorinated water change, at 25 ± 1 °C (600 L tanks). Daily feeding was carried out with extruded feed.

To verify the *S. agalactiae* free status of the fish, samples were obtained to bacterial culture. The bacterium was not isolate from randomly selected tilapia. Thirty fish (group A) were injected intraperitoneally with 0.1mL of bacterial suspension. Thirty fish (group B) received intraperitoneally 0.1 mL of a sterile saline solution.

Samples were collected 3, 7, 14, 21 and 28 days post-inoculation (pi). Aseptically collected tissues from the brain and anterior kidney were seeded in ACS and incubated at 30°C for 48 hours to confirm the presence of *S. agalactiae*. Samples from the brain, kidney, eye, liver, spleen, heart and gills were fixed in Bouin's solution. Routine histological methods were followed to obtain 5 µm sections, which were stained with hematoxylin and eosin (H&E) and Perodic Acid Schiff (PAS). The presence of melanomacrophages centers (MMP) in the liver and spleen was noted using a 10x lens objective. Direct impression smears from the anterior kidney were done in all animals and stained with Giemsa for 45 min for bacterial observation.

The Fisher's exact test was used to evaluate if the presence of MMP was associated with streptococcal infection. Significance was set at $p < 0.05$.

Results

Clinical signs and gross findings

Nile tilapia began to show clinical signs and morbidity 3 days after inoculation with *S. agalactiae*. Affected fish were anorexic, and showed signs of disorientation, circling listlessly at the water surface. Some of the animals had exophthalmia, and corneal opacity. Clinical signs decreased during the experiment, and at 21 days pi had disappeared. Bacteria were reisolated from the kidney and brain of necropsied fish mainly in the initial times of the experiment (Table 1). Macroscopic lesions were more severe at 3 and 7 days pi, and consisted of darkness of skin, enlarged spleen and epicardial opacity. No clinical signs or macroscopic lesions occurred in non-infected fish.

Histological findings

The *S. agalactiae* isolate (UEL 13) induced subacute to chronic inflammation in the epicardium, meninges and eye. Histological lesions were more severe at 3, 7 and 14 days pi. No histological lesions were observed at 28 days pi (Table 1). In the brain, the meninges of telencephalon and cerebellum were infiltrated with macrophages and lymphocytes (Fig. 1a), which in some cases were accompanied by hemorrhage and a mild infiltrate of eosinophils. Epicarditis was characterized by a severe infiltration of macrophages and lymphocytes (Fig. 1b), while in some animals macrophages also infiltrate the bulbus arteriosus. Inflammatory infiltrate in myocardium was rarely seen.

Eye lesions were characterized by a mild cellular infiltrate, composed of macrophages and eosinophils. These findings were observed in the choroid tissues and also in periorbital tissue involving both the optic nerve and the rectus muscles. The retina showed no lesions.

Hepatocytes were vacuolated and showed PAS positivity as expression of glycogen content. Melanomacrophages centers were characterized as groups of pigment-containing cells in spleen and liver. A significant association was verified between infection with *S. agalactiae* and the presence of melanomacrophages centers in the liver and spleen (Table 2). The spleen as well as the kidney showed congested vessels. Deposition of hialine droplets in tubular epithelia was observed in the distal kidney. Discrete macrophage infiltration was found in the anterior kidney. Extracellular cocci and phagocytized cocci were seen in direct impression smears in the fish slaughtered 3, 7 and 14 days pi. No lesions or bacteria were detected in non-inoculated fish.

Discussion

Isolation of *S. agalactiae* in Nile tilapias reared in hapas nets and earth nurseries was recently reported in Brazil (14, 8). The isolate used in this study produced natural disease and was also able to produce an experimental disease, supporting evidence of its pathogenicity. All the infected fish showed clinical signs similar with the signs described in other natural or experimental streptococcal infections (6, 13, 4, 8). However, in the present study clinical signs were not present all-over the experiment, which could be related to fish ability to resolve the infection. Shoemaker et al. (2000) also associated clinical signs with bacterial dose and fish density.

The histological findings detected in the brain tissue and eyes correspond to the gross aspects of loss of orientation, exophthalmia and corneal opacity. These findings substantiates that our isolate is neurotropic as is described for other non-haemolytic group B *S. agalactiae* (5, 19). Our data indicated that *S. agalactiae* caused a

Table 1 - Number of fish with bacteria recovery and histological lesions in experimental infection with *S. agalactiae*.

Days pi	Number of fish with bacteria reisolate		Number of fish with histological lesions		
	Brain (n 30)	Kidney (n 30)	Brain (n 30)	Heart (n 30)	Eye (n 30)
3 days	5/6	5/6	4/6	6/6	2/6
7 days	6/6	5/6	4/6	4/6	3/6
14 days	4/6	3/6	3/6	3/6	2/6
21 days	0/6	0/6	0/6	2/6	0/6
28 days	0/6	0/6	0/6	0/6	0/6
total	15/30	13/30	11/30	15/30	7/30

Table 2 - Number of fish with melanomacrophages centers in inoculated and non-inoculated animals.

Organ	Inoculated fish	Non-inoculated fish	P
Liver	17/30	6/30	0.007
Spleen	24/30	10/30	0.007

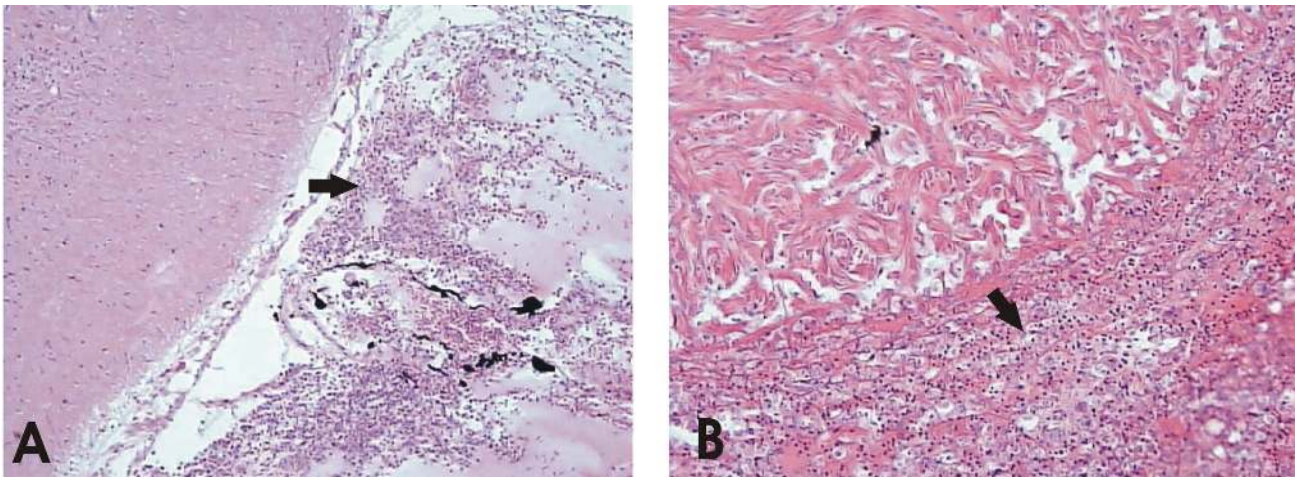

Figure 1 - A) Brain of tilapia infected with *Streptococcus agalactiae* with macrophage and lymphocyte infiltrate in the meninges (arrow). H.E., obj. 10x; B) Heart of a Nile tilapia infected with *Streptococcus agalactiae*. Macrophages and lymphocytes in the exudate in the epicardium (arrow). H.E., obj. 10x .

severe mononuclear exudate in the meninges, eyes and epicardium as a consequence of hematological dissemination. Various studies reported similar lesions as the main findings (3, 6, 12). Differently from other reports, we also observed an eosinophil infiltrate in the periorbital and choroid tissues. The reason for this kind of infiltrate remains uncertain.

Interestingly, the lesions and bacterial reisolation decreased over the time and, at the end of the experiment no lesions were observed. This fact explains the absence of clinical signs in the final periods of the experiment. Also, we can consider that a cellular effective immune response could be established in the later periods. The increase in melanomacrophages centers in the liver and spleen in the infected tilapias were possibly related with this response. It has been suggested that MMP may represent the primitive analogues of the germinal centers of lymph nodes of birds and mammals (1). Melanomacrophages are phagocytic, so the proliferation and hypertrophy of these cells may be interpreted as a relevant role in antigen trapping and presentation to lymphocytes, and sequestration of products of cellular degradation. In fact, Lyons et al. (2004) reported that an increase in size or frequency of MMP was associated with pathogen and parasite infections. The association between MMP and *Streptococcus* infection verified in our study suggests that analysis of melanomacrophages could be used as a biomarker for water quality.

S. *iniae* is a well established pathogen in aquaculture in North hemisphere countries (16). Bunch & Bejerano (1997) have observed that infections caused by *S. iniae* were predominant in tilapia raised in low temperatures (15°C to 16°C). However, *S. agalactiae* was isolated in hot weather regions, with average temperatures varying from 26°C to 28°C. High temperatures associated

to intensive breeding systems were considerate as stressing factors that contribute to streptococci disease (14, 15).

Our group was the first to isolate *S. agalactiae* from tilapias in Brazil, but the pathogenicity of these isolates remained uncertain. The results of the experimental inoculation showed that our local *S. agalactiae* isolate were pathogenic to tilapia, inducing a septicemia, which the main lesions are meningitis and epicarditis.

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