Streptococciosis on a red tilapia, *Oreochromis* sp., farm: a case study

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**Abstract**

A commercial red tilapia farm was diagnosed with *Streptococcus agalactiae* infection using histopathology, microbiology and immunohistochemistry. One hundred fish were randomly taken from different weight/age groups including broodstock, market fish (larger than 150 g), on-growing fish between 20 and 150 g, juveniles and larvae. Fish were clinically examined, anaesthetised and necropsied. Samples were taken from brain, liver, spleen, eyes and kidney for microbiology. All organs were processed for histopathology and an indirect immunoperoxidase test (IIP). Organs from wild fish and birds found in close proximity to the farm were also sampled for microbiology and IIP. The prevalence of lesions or infection found by IIP, histopathology and microbiology was 16%, 29% and 7% respectively. Clinical disease, lesions or infection were not seen in larvae or juveniles. By contrast, infection and disease were found in fish larger than 20 g, suggesting that the condition was linked to the intensive culture conditions of broodstock, on-growing and market fish. *S. agalactiae* was not found in wild fish, or in birds by microbiology and IIP.

*Keywords*: culture, diagnosis immunoperoxidase, histopathology, *Oreochromis* sp., *Streptococcus agalactiae*.

**Introduction**

Disease caused by streptococci has become a major problem in cultured freshwater and saltwater fish species worldwide. *Streptococcus* and *Enterococcus* spp. are commonly associated with mortality, whereas *Lactococcus* and *Vagococcus* spp. appear to be less pathogenic (Nieto, Devesa, Quiroga & Toranzo 1995). Initially reported in *Dolphin iniae* by Hoshina, Sano & Morimoto (1958), *S. iniae* and other species of streptococci have been described in numerous outbreaks of disease in several fish species including tilapia. Amongst the streptococci, *S. agalactiae* has a broad host range, infecting both terrestrial and aquatic animals. This bacterium causes neonatal meningitis and mastitis in humans and cattle, respectively. However, other animals such as mice, cats, dogs, hamsters, camels and frogs can also be infected (Evans, Klesius, Gilbert, Shoemaker, Al-Sarawi, Landsberg, Durendez, Al-Marzouk & Al-Zenki 2002). *Streptococcus agalactiae* has been isolated from numerous fish species in natural outbreaks of disease and has been shown to be pathogenic to several fish species in experimental trials using different routes of infection such as cohabitation, immersion, intraperitoneal and intra-muscular injections (Evans et al. 2002). The pathogenesis of the disease remains unknown. In the present study we used microbiological, histopathological and immunohistochemical tools in an attempt to understand the source and spread of disease induced by *S. agalactiae* in a red tilapia farm naturally infected by this bacterium.

**Materials and methods**

**Study farm**

The red tilapia farm selected for this study contained all life stages of fish, including broodstock, market fish (larger than 150 g), on-growing...
fish between 20 and 150 g, juveniles and larvae. In addition, the farm had been shown by the Veterinary Pathology Laboratory of the Universidad Nacional de Colombia to be infected with \textit{S. agalactiae} by clinical examination, histopathology, immunohistochemistry and microbiology. The farm was typical for the region in that it comprised earth ponds and netpens. A river fed a reservoir in which the on-growing and market fish were stocked in netpens at densities $> 40,000$ g m$^{-3}$. The water from this reservoir fed ponds where the broodstock, larvae and juveniles were stocked at densities of $< 1000$ g m$^{-2}$.

### Sampling

Using a prevalence of 50%, a confidence interval of 95%, and an accepted error of 10%, a sample size of 100 fish was determined (Martin, Meek & Willeberg 1987). A homogeneous sample consisting of twenty fish was taken from each of five defined groups A–E (Table 1).

### Microbiology

Fish were clinically examined, anaesthetised with tricaine methane sulphonate (MS222) and then necropsied. Brain, liver, spleen, eye and kidney were taken from groups A, B and C but only eye and brain samples from groups D and E. Gonad samples were also taken from group A. All samples were cultured in brain–heart infusion (BHI) agar enriched with 5% defibrinated ovine blood and incubated at 28 $^\circ$C for 36 h. Isolates were Gram-stained and subjected to biological tests such as haemolysis and to specific biochemical tests for \textit{S. agalactiae}, such as catalase, oxidase, growth in 6.5% NaCl, growth at 45 $^\circ$C, urea, arginine, and carbohydrate fermentation. Identification was confirmed by using the BBL Crystal system (BBL, Becton Dickinson Diagnostic Instrument System) according to the manufacturer’s instructions. PCR amplification and sequencing of the 16 s ribosomal ARN gene was also conducted. The results of sequencing were analyzed by the program bioinformatis BLAST NCBI (http://www.ncbi.nlm.nih.gov/blast).

### Histopathology and indirect immunoperoxidase test

Tissues were fixed in 3.7% buffered formaldehyde followed by routine processing to paraffin-wax embedding. Sections were stained with haematoxylin and eosin (H&E), and they were also processed for indirect immunoperoxidase test (IIP). All tissues from groups D and E were processed while only selected organs (brain, heart, kidney, gastrointestinal tract, spleen, eye, gill and gonad) were processed from the remaining groups. The IIP technique followed a protocol previously established in our laboratory using a rabbit polyclonal antisemur against \textit{S. agalactiae} (Pulido, Iregui, Figueroa & Klesius 2004). The rabbit polyclonal antisemur was used as a first antibody, and recombinant G protein conjugated with horseradish peroxidase (Sigma Chemical Co.) as a second antibody. The specificity of the IIP technique was assessed using pig muscle that had been infected with the following bacterial strains: \textit{Enterococcus faecalis ATCC 29272}, \textit{Streptococcus pneumoniae ATCC 49619} and \textit{Staphylococcus aureus}. Specificity was also confirmed in natural cases of disease in fish caused by \textit{Aeromonas hydrophila} and \textit{Mycobacterium} sp. and the brain from a pig suffering from meningoencephalitis caused by \textit{Streptococcus} sp.

### Environmental and wild fish and bird sampling

Twenty samples of water and mud from various locations across the farm were cultured in BHI agar with 0.025% sodium azide and incubated at 28 $^\circ$C for 14 days (Kitao, Aoki & Iwata 1974). Tissue samples from wild fish and bird species coming into direct contact with the farm were also processed by microbiology and IIP. These comprised 18 wild fish: two cuchas, \textit{Hypostomus plecostomus} (L.); one tolomba, \textit{Astyanax magdalenae} Eigenmann & Henn, two black tilapia, \textit{Oreochromis mossambicus} (Peters), three hooked bream, \textit{Caquetaia umbrifera} (Meek & Hildebrand), three nicuros, \textit{Pimelodus blochii} Valenciennes, two bocachicos, \textit{Prochilodus blochii} Valenciennes, and seven different bird species.

### Table 1 Groups of red tilapia and other fish and birds sampled

<table>
<thead>
<tr>
<th>Group</th>
<th>Types of fish</th>
<th>No. individual fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Broodstock (red tilapia)</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>&gt; 150 g (red tilapia)</td>
<td>20</td>
</tr>
<tr>
<td>C</td>
<td>20–150 g (red tilapia)</td>
<td>20</td>
</tr>
<tr>
<td>D</td>
<td>2–20 g (red tilapia)</td>
<td>20</td>
</tr>
<tr>
<td>E</td>
<td>&lt; 2 g (red tilapia)</td>
<td>20</td>
</tr>
</tbody>
</table>

Wild fish: 18

Wild birds: 7
magdalenae Steindachner, two caloches, Heigeman-
nias virence, one tota, Gephyrocharax melanocheir
Eienmann and two red tilapia (the latter were
escapees from the cages). Seven individuals of
different species of birds were also processed: two
vultures, Coragyps atratus, one dark heron, Ardea
herodiao, two chanas, Syringa sibilatrix and two
kingfishers Chlorocyrele amazona (Table 1).

Determination of the sensitivity and specificity
of tests
The on-line epidemiological program of the Uni-
versity of Montana (USA) http://www.afssa.fr/
interne/tags.htm was used for calculation of farm
real prevalence, sensitivity and specificity for each of
the techniques employed in this study.

Results
Clinical and macroscopic findings
Common clinical signs of disease in tilapia included
swimming in circles in lateral recumbence, and a
curved body shape, while gross lesions included
opacity of the eyes (unilateral or bilateral), exoph-
thalmia, and the presence of a white fibrinous
exudate covering the heart. These signs were more
evident in groups A, B and C.

Microbiology
Table 2 shows the microbiological results from
tilapia, native fish and bird species and from water
and mud samples. Streptococcus agalactiae was
isolated from only seven tilapia from groups A
and C.

Histopathology
Common histopathological lesions were grouped
into two characteristic patterns. The first consisted
of focal to multifocal, mild to severe granulomatous
inflammation. The centre of the granulomas com-
prised abundant cell detritus, enclosed by a thick
capsule of macrophages. Macrophages and melano-
macrophage centres were also found surrounding
the granulomas. The second pattern consisted of
multifocal, acute, necrotic, inflammatory lesions
involving leucocytes, macrophages, fibrin and
eosinophilic granular cells. Both kinds of lesion
were located mainly in brain and eyes. The
prevalence of lesions compatible with streptococco-
sis by this technique was 29%. For calculating the
real prevalence of infection on the farm, and the
sensitivity and specificity of histopathology, tissues
of 13 fish that had histopathological lesions com-
patible with streptococcosis, plus visible cocci, were
considered positive for infection. Affected fish came
from groups weighing more than 20 g, and from
the boodstock group. All fish with S. agalactiae
detected by both microbiology and IIP had path-
ological lesions.

Indirect immunoperoxidase test
The presence of coccus-shaped structures with
brown colour and/or a brown staining with a
diffuse distribution (corresponding to remnants or
antigens from bacterial destruction) was considered
to be positive for S. agalactiae (Figs 1 & 2).
Immunolabelling was found not only inside the
granulomas associated with cell detritus (Fig. 2),
but also within still viable macrophages (Fig. 1).
Immunolabelling associated with necrotic inflam-
matory reactions consisted of granular staining
(corresponding to coccus morphology) and diffuse
staining within or outside inflammatory cells, or
free in the interstitial space (Fig. 1). Figure 3 shows
the IIP negative control.

Pig muscle inoculated with Enterococcus faecalis
ATCC 29272, Streptococcus pneumoniae ATCC
49619 and Staphylococcus sp., pig brain infected
with Streptococcus sp., and diseased fish infected
with Aeromonas hydrophila and Mycobacterium sp.
were all negative by IIP (Fig. 3).

Table 2 Results of microbiological examination of different
groups and organs of red tilapia for Streptococcus agalactiae

<table>
<thead>
<tr>
<th>Group</th>
<th>Brain</th>
<th>Kidney</th>
<th>Eye</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Native fish</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Native birds</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mud</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

-, negative isolation; +, positive isolation.
Wild fish and bird species

No wild fish or birds showed positive labelling by IIP and all were negative by microbiology. However, two escaped red tilapia captured were positive for *S. agalactiae* by IIP (Table 3).

The real farm prevalence, and sensitivity and specificity of test

The real prevalence of the infection was determined to be 14.44% (Table 4).

Discussion

Despite widespread infection within broodstock and on-growing fish, no larvae or juveniles (2–20 g) showed clinical disease. This was surprising, given that young fish are generally found to be more susceptible to a whole range of bacterial diseases. The explanation may relate to the high stocking density of the on-growing fish predisposing them to infection with *S. agalactiae* and subsequent disease. Broodstock are also subjected to high stocking densities after spawning. A similar situation has been reported for tilapia infected by *S. iniae* (Shoemaker, Evans & Klesius 2000). In both of the affected groups of fish, feed quality and feeding practices were probably less than optimal compared to those in the young fish and the influence of this requires further investigation.

The very low mortality rates in the juvenile fish supports the finding that disease due to *S. agalactiae* was not present to any significant degree. Given that 20% of broodstock were infected it is surprising that *S. agalactiae* was not detected in larvae or juvenile fish derived from these parent fish, since fish of both groups spent at least 30 days with the broodstock before being moved to the netpens, and all fish thereafter shared a common source of water. One possibility is that the techniques used (especially the sample numbers) were not sufficiently sensitive or representative of these populations. In further unpublished studies we have assumed a lower prevalence for determining sampling (i.e. processing more fish per weight/age group) and added two more farms to the one in this study. Even with this expanded approach, however, we have been unable to detect the presence of bacteria or lesions in juvenile fish. It appears, therefore, that neither larvae nor juvenile fish are infected, suggesting that there was no vertical transmission from
parents to offspring, or if there was, the bacterium did not become established. If this is the case it suggests that infection-free populations of fish could be achieved, especially given the apparent lack of any significant reservoir of infection in wild fish or birds.

The prevalence of streptococcosis as determined by IIP was 16%, which generally agreed with the farm’s records of mortality. The specificity of the rabbit antiserum used in IIP was corroborated when no positive immunolabelling was seen with other phylogenetically related Gram-positive or non-related Gram-negative micro-organisms. The great percentage (more than double) of positive results by IIP, compared to microbiological isolates, indicates the higher sensitivity of IIP, and its advantage in diagnostic and epidemiological approaches.

Attempts to isolate the bacterium from mud and water samples were unsuccessful. This does not necessarily mean that the bacteria was not present in such environments, but rather that the culture medium used was possibly not the one most suitable for its detection. Future studies need to evaluate more appropriate culture media for *S. agalactiae*, as have been developed for *S. iniae* (Nguyen & Kanai 1999).

Table 3 Results of immunolabelling of *Streptococcus agalactiae* in red tilapia tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>Organ</th>
<th>Brain, heart</th>
<th>Brain, eye</th>
<th>Brain, gonad</th>
<th>Brain, eye, heart</th>
<th>GIT, CNS, eye, heart</th>
<th>Liver, eye, kidney, heart</th>
<th>Prevalence of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>–</td>
<td>3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>20</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>1</td>
<td>2</td>
<td>–</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Free red tilapia*</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2</td>
</tr>
</tbody>
</table>

The number of fish with positive staining in any tissue are shown.

*Escaped fish.

GIT, Gastrointestinal Tract; CNS, Central Nervous System.

Table 4 Sensitivity and specificity of each of the techniques used in this study for the detection of *Streptococcus agalactiae* and streptococcosis

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:</td>
<td>100</td>
<td>96.1</td>
</tr>
<tr>
<td>2:</td>
<td>30.8</td>
<td>96.1</td>
</tr>
<tr>
<td>3:</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The high specificity of the three techniques used in this study was reflected in the absence of infections with other Gram-|-|-| cocci. We have never seen infections with *Mycobacterium* in tilapia in Colombia. Microbiology showed lower sensitivity compared with IIP and histopathology, possibly...
because of a ‘viable but non-culturatable’ state of the micro-organisms, the result of environmental stress (McDouglas, Rice, Weichart & Kjelleberg 1998), or the number of bacteria in tissues was too low to be sampled using a bacteriological loop.

*Streptococcus agalactiae* had a predilection for some organs such as the brain, eyes and heart (71.2%, 43.7% and 37.1%, respectively) which were also positive by histopathology. It would seem that the brain is the primary target organ for *S. agalactiae* after it has reached the bloodstream. Paperna (1996) and Pulido, Iregvi, Figueroa & Klesius (2004) have also reported that the brain is most affected in clinical streptococcosis of tilapia as well as in those fish showing no nervous signs. 83% of IIP-positive fish had necrotic meningitis, suggesting bacterial entry via the bloodstream and, once established, spreading to different areas of the brain. Viable *S. agalactiae* were seen within macrophages, as previously demonstrated by Pulido *et al.* (2004) using electron microscopy. We suggest that bacteria reach different organs by transport within macrophages, i.e. the ‘Trojan horse’ phenomenon (Zlotkin, Chilmonczyk, Eyngor, Hurvitz, Ghittino & Eldar 2003).

Red tilapia seemed to be the only species susceptible to infection, and even then, only those fish in particular age/size groups. None of the wild fish species examined were infected. Thus the red tilapia is the only species sustaining *S. agalactiae* infection in the study region. This conclusion is supported by the IIP results and histopathology which demonstrated infection and lesions only in those red tilapia that had escaped from the netpens. Birds also do not seem to sustain the infection or act as vectors.

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**References**


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