Pathological Manifestations and Immune Responses of Serotypes Ia and III 

*Streptococcus agalactiae* Infections in Nile Tilapia (*Oreochromis niloticus*)

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Abstract

*Streptococcus agalactiae* (Group B Streptococcus, GBS) infections cause serious worldwide damage to fish farming. Here, we evaluated the pathological manifestations and immune responses of Nile tilapia (*Oreochromis niloticus*) infected with GBS serotype Ia (GBS-Ia) and GBS serotypes III (GBS-III). Experimental infection of fish with GBS by intraperitoneal injection indicated the severity of GBS-Ia infection as shown by the LD$_{50}$ of GBS-Ia and GBS-III, being $1.58 \times 10^6$ CFU/fish and $2.10 \times 10^8$ CFU/fish, respectively. The onset of disease and clinical observation revealed notable differences between the GBS-Ia and GBS-III infections. Histological finding showed severe changes of the liver, pancreas, kidney and brain of fish infected with GBS. In addition, significantly different haematological-immunological parameters were observed between GBS-Ia and GBS-III infected fish. Differences in the pathological manifestations and immune responses of GBS-Ia and GBS-III infections in tilapia are of practical use for fish pathologists who monitor tilapia mortality resulting from GBS infections.

Keywords: *Streptococcus agalactiae*; Tilapia; Pathology; Immune responses
Introduction

Streptococciosis is an emerging disease causing mass mortality in a variety of fish species. Recent reports demonstrate that streptococcal infections are occurring in Asia, where streptococci have been isolated from sea bream (Sparus auratus), wild mullet (Liza klunzingeri) and silver pomfret (Pampus argenteus) in Kuwait (Evans et al., 2002; Duremdez et al., 2004), and tilapia (Oreochromis spp.), Asian sea bass (Lates calcarifer) and golden pompano (Trachinotus blochii) in Thailand and Malaysia (Suanyuk et al., 2008, 2010; Zamri-Saad et al., 2010; Amal et al., 2012). Traditional classification of streptococci is usually based on the serogrouping of the carbohydrate antigens of the cell wall and haemolytic activity. Only Lancefield serogroup B corresponds to a single streptococcal species, Streptococcus agalactiae (Group B streptococcus, GBS) (Evans et al., 2002). GBS is an important pathogen affecting humans and animals, including aquatic species. (Rasheed et al., 1985; Martinez et al., 2000; Evans et al., 2002; Duremdez et al., 2004; Straková and Motlová, 2004; Ip et al., 2006). The main clinical signs of GBS infection in fish include erratic swimming, loss of appetite, lethargy, unilateral or bilateral exophthalmia, corneal opacity and visceral cavity distension (Salvador et al., 2005; Suanyuk et al., 2008). Based on the composition of the capsular polysaccharide, GBS can be sub-divided into ten serotypes (Ia, Ib and II to IX ) (Imperi et al., 2010). Among GBS isolates from infected tilapia, GBS-Ia, Ib and III are the most common serotypes (Evans et al., 2008; Suanyuk et al., 2008; Rodkhun et al., 2012). Two distinguishable GBS are known to infect tilapia cultured in Thailand. One of these belongs to serotype Ia and contains genes encoding proteins Cα (bca) and Cβ (bac), three insertions sequences (IS1381, IS861 and ISSag2) and the group II intron GBSi1. The other belongs to serotype III, contains bca, three insertion sequences (IS1381, ISSag1, ISSag2) and a tetracycline resistance gene (tetM) (Suanyuk et al., 2008). Despite
progress in diagnosis and genotyping of GBS isolated from infected fish, the pathological basis of the disease caused by different serotype of GBS has not been addressed. The aims of the present study were to define the pathological manifestation and immune responses of Nile tilapia (*Oreochromis niloticus*) infected with GBS-Ia and GBS-III.

2. Materials and methods

2.1 Fish

Healthy Nile tilapia (*Oreochromis niloticus*) with an average weight of 5 g were obtained from a commercial hatchery in Phatthalung province, southern Thailand. The fish were cultured to experimental size in three-tonne fiber glass tanks with aeration. During the cultivation and acclimatization period, they were fed daily to satiation with commercial feed and a sub-sample was cultured and determined to be free of pathogenic bacteria.

2.2 Bacteria

Bacterial strains used in this study were obtained from previous study (Suanyuk *et al.*, 2008). The GSB-Ia isolate (genotype *Ia-bca-bac-IS1381-IS861-GBSi1-ISSag2*) was isolated from diseased tilapia in Nakhon Si Thammarat province and GSB-III isolate (genotype *III-4-bca-IS1381-ISSag1-ISSag2-tetM-intTn*) was isolated from infected tilapia cultured in Songkhla province. A pure culture of the strain was kept at -70°C; to prepare for infecting fish, a sterile tryptic soy agar (TSA) was inoculated with a loop of the frozen culture and incubated at 30°C for 24-48 h. This plate was kept at 4°C and used for inoculation in all experiments; subculturing onto new plates was performed every week to keep the culture alive. Prior to use, the bacteria were inoculated intraperitoneally (i.p.) in Nile tilapia, and aseptically re-
isolated from the brain of a moribund fish to select for virulence. The bacterial colonies were confirmed to be GBS by standard biochemical methods as well as polymerase chain reaction (Suanyuk et al., 2008). The Lancefield group antigen and serotype of GBS isolates were confirmed by means of a SlideX Strepto Plus (bioMérieux, France) and by group B streptococci typing antisera (Denka Seiken Co. Ltd., Japan), respectively.

2.3 Experimental infectivity trials

Two hundred and seventy Nile tilapia with an average weight of 14.02±3.16 g were used in this study. The fish were kept in a separate 100 L fiber glass tank at 30.5±0.5°C and acclimated for a period of one week before infection. Overnight culture of either GBSTIa or GBSTIII in TSB was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. The cells were washed twice with PBS (pH7.4) and adjusted to the final concentration of approximately 10^2, 10^4, 10^6 and 10^8 CFU/ml. Viable bacterial count were determined using drop plating serial dilution technique on TSA. Experimental fish were anaesthetized with 40 mg/l clove oil (Coyle, 2004) and were given an i.p. injection with 0.1 ml of of each bacterial suspension. The experiment was conducted in triplicates using 10 fish per replication and dose level. A control group was similarly inoculated with sterile PBS. Daily mortality and clinical signs were recorded for 4 weeks and relative virulence of the GBSTIa and GBSTIII were assayed by determining the fifty percent lethal dose (LD50) using the probit analysis program developed by Luangthuvapranit (1988). Fresh tissue samples of all dead fish were collected and bacterial cells were isolated and identified to fulfill Koch’s postulates. Mortality was considered only if the GBS was reisolated from the infected organs and biochemically confirmed.

2.4 Histopathology
Infected tissues obtained from experimental infectivity trials were fixed in 10% buffered formalin and processed using standard histological techniques (Humason 1979). Histological sections were cut at 3-5 µm and stained with haematoxylin and eosin (H&E). Stained sections were examined under a light microscope. Control samples were examined in parallel, representing the same tissue type but from the control group of fish.

2.5 Haemato-immunological parameters

One hundred and eighty Nile tilapia with an average weight of 27.16±7.46 g were used in this study. Before infection the fish were acclimatized for 1 week in a 100 L fiber glass tank with dechlorinated water and aeration system, at 30.5±0.5°C. The fish were fed twice daily with a commercial feed. The experiments were conducted in triplicates with 20 fish per replication. Each group was i.p. injected with 0.1 mL of GBS-Ia or GBS-III (approximately 10^7 CFU/fish). A control group was similarly inoculated with sterile PBS (pH 7.4).

At 0, 1, 2, 3, 6 and 9 days post injection (dpi), the fish were euthanized with clove oil and blood was collected with heparinised syringe from caudal vein (six fish randomly from each treatment group). Red blood cell (RBC) and white blood cell (WBC) counts were measured using a method of Supamattaya (1995). Haemoglobin (Hb) level was determined colorimetrically by measuring the formation of cyanamet-haemoglobin according to Blaxhall and Daisley (1973). Haematocrit (Ht) was measured using a method of Larsen and Snieszko (1961). Total plasma protein was determined colorimetrically by the method of Lowry et al. (1951), see Supamattaya et al. (2000).
Plasma lysozyme activity was measured based on a turbidimetric assay with a microplate reader, according to Demers and Bayne (1997). Plasma (25 µl/well) was added to 175 µl of *Micrococcus lysodeikticus* (Sigma) suspension and the plate was read at a wavelength of 450 nm. Hen egg white lysozyme was used as an external standard. The rate of reduction in the absorbance of the samples was converted to lysozyme concentration (µg/ml) using a standard curve.

The respiratory burst of the leucocytes was quantified using the reduction of nitroblue tetrazolium to formazan as a measure of superoxide anion (O$_2^-$) production. The absorbance at 630 nm was measured spectrometrically in triplicate with a microplate reader (Model PowerWaveX, Biotek) using DMSO/KOH alone as a blank (Stasiak and Baumann, 1996).

Head kidney leucocytes were isolated under sterile conditions according to Chung and Secombes (1988). Leukocyte phagocytic activity and phagocytic index were measured following the method of Thuvander *et al*. (1987).

**2.6 Statistical analysis**

Blood and non-specific immune response data were analyzed by ANOVA followed by means comparisons using Duncan’s multiple-range test. A significant difference satisfies p<0.05.

**3. Results**

**3.1 Experimental infectivity trials**

Inoculation and reisolation of GBS-Ia and GBS-III from dead fish established the organisms as pathogenic for the fish and confirmed Koch’s postulates. The fish injected with GBS-Ia exhibited both early- and late-onset disease which mortality occurring continuously.
throughout the induction of disease experiment. Infected fish exhibited 10-60% mortality (Figure 1), with a 28 day-LD₅₀ of 1.576 × 10⁶ CFU/fish. Fish injected with GBS-III exhibited only early-onset disease, with high mortality only during the first week after infection. Infected fish showed 16 – 50% mortality, with a 28 day-LD₅₀ of 2.10×10⁸ CFU/fish (Figure 2).

Sixty eight moribund fish obtained from both GBS-Ia and GBS-III infection were observed clinically. Of these, 39 originated from GBS-Ia infection and 29 from GBS-III infection. No abnormal behavior was observed in the control fish injected with PBS. Clinical observation indicated that fish infected with GBS began to show clinical signs within 10 h post infection. Clinical manifestations due to experimental infection included lethargy, darkening of the skin, abnormal swimming, and some fish dieing without showing any prior clinical signs. After 2 dpi, infected fish showed circling listlessly at the water surface, redness of lip, and external haemorrhage. Corneal opacity and exophthalmia (uni- and bi-lateral exophthalmia) were observed during 3 and 5 dpi. Gross pathology study indicated that GBS-Ia produces higher virulence than GBS-III. Moreover, GBS-Ia infected fish presented 18% exophthalmia, 31-62% congestion and haemorrhage of the visceral organs and 77% congestion and haemorrhage of the brain, whereas the fish infected GBS-III showed 10%, 17-31% and 52% of exophthalmia, congestion and haemorrhage of the visceral organs and congestion and haemorrhage of the brain, respectively. Other disease signs of fish infected with GBS are shown in Table 1.

3.2 Histopathology

Histopathological changes in Nile tilapia infected by GBS were found in several organs including liver, pancreas, kidneys and brain. In GBS-Ia infected fish, the liver was
flooded with red blood cells along with hepatocytic vacuolization and necrosis (Figure 3).

The pancreas revealed degeneration of acinar cells and loss of zymogen granules (Figure 4).

Brain from infected fish showed meningitis associated with lymphocytic infiltration (Figure 5). There was shrinkage of the glomerulus associated with necrosis and degeneration of renal tubule in the kidney (Figure 6). Similar observations were found in GBS-III infected fish (Figures 3-6).

3.3 Haemato-immunological parameters

3.3.1 Blood parameters

Infected fish produce lower blood parameter values than the uninfected fish (Table 2). During 1-9 dpi, the GBS infected fish produced lower RBC than the uninfected fish. Similarly the Hb value of infected fish decreased significantly (p<0.05) during 1-3 dpi and returned to normal after 6 dpi. However, the WBC and plasma protein of fish infected fish GBS decreased during 1-3 dpi and increased after 6 dpi. In addition, at 9 dpi, the fish infected with GBS-Ia produced significantly (p<0.05) higher WBC and plasma protein than the GBS-III infected fish. No significant difference of the Ht value was observed between the infected and the control group (Table 2).

3.3.2 Lysozyme, respiratory burst and phagocytic activities

Infected fish exhibited lower immune responses than the uninfected fish (Figures 7-10). The lysozyme activity was decreased at 2 and 3 dpi, returning to normal value at 6 dpi, and increased at 9 dpi (Figure 7). On the other hand, the respiratory burst activity was increased at 3 and 6 dpi and returned to normal by 9 dpi (Figure 8). The phagocytic activity and phagocytic index were significantly (p<0.05) decreased at 1-3 and 1 dpi, respectively. However, six days after infection, phagocytic activity and phagocytic index were
significantly higher in the challenged groups than in the control group, and the enhanced phagocytic activity remained until day 9 of the experiment (Figures 9-10). No significant differences in the lysozyme activity, respiratory burst activity or phagocytic index were observed between the GBS-Ia and GBS-III infected groups, with the exception of phagocytic activity at 9 dpi: GBS-III treatment had a significantly (p<0.05) higher value than GBS-Ia treatment.

**Discussion**

GBS infections have a great negative impact on tilapia cultivation worldwide. Among GBS isolates from infected tilapia, GBS-Ia, Ib and III are the most common serotypes (Evans et al., 2008; Suanyuk et al., 2008; Rodkhun et al., 2012). In this study, the taxonomic status of GBS-Ia and GBS-III isolates, determined by using the methods described in Bergey’s Manual of Systematic Bacteriology, indicated that these isolates are biochemically and physiologically similar (data not shown). Experimental infection of tilapia demonstrated that GBS is pathogenic to fish. Clinical signs of fish infected with GBS were similar to those reported earlier in tilapia cultured in Thailand, Brazil and Malaysia (Suanyuk et al., 2008; Mian et al., 2009; Zamri-Saad et al., 2010; Abuseliana et al., 2011). However, while the clinical and histopathological findings revealing that the GBS-Ia and GBS-III infections of tilapia have common features, the onset of disease and clinical observation enabled us to differentiate between the serotypes based on symptoms. The hyperacute and acute disease following GBS-Ia infection, and mortality along with the differences in the LD$_{50}$, support the clinical and hemato-immunological data, demonstrating the severity of GBS-Ia infection. Among the human GBS isolates from the US, serotype Ia, III, and V are the most common isolates associated with early-onset disease (Lin et al., 1998). Studies on GBS virulence factors such as the IS1548 which has been reported as causative of neonatal meningitis and
endocarditis (Granlund et al., 2001; Safadi et al., 2010) and other GBS virulent related genes are needed to evaluate the pathogenesis of these GBS isolates.

GBS induced invasive systemic infection and led to haemato-immunological changes. Haematology values of infected Nile tilapia were different from those of the control group. Similar results have been reported by McNulty et al. (2003) for tilapia infected with S. iniae and Harikrishnan et al. (2003) for common carp injected with Aeromonas hydrophila. This result suggests that RBC and WBC counts can be used as indicators of bacterial infection. In this study, significantly elevated WBC was noted in the GBS-Ia infected group on 9-days post infection. The WBCs are normally lower in healthy fish and increase after infection with the pathogen (Jamalzaden et al., 2009).

The innate immune system is also of primary importance in combating infections in fish, including lysozyme and phagocytic cells (Magnadóttir, 2006). Lysozyme is one of the defensive factors against invasion by microorganisms. It cleaves the β-(1→4) linkages between N-acetylmuramic acid and N-Acetylglucosamine in the cell walls of Gram-positive bacteria, thus preventing them from invading (Salton and Ghysen, 1959; Yano, 1996). Increased lysozyme activity in the GBS infected group, in the present study, indicates that lysozyme is an important humoral component of the non-specific immune system.

Phagocytic cells play an important role in antibacterial defenses by production of toxic oxygen forms during a process called respiratory burst (Neumann et al., 2001). Significantly increased phagocytic activity, phagocytic index and respiratory burst of infected tilapia, from the present investigation, indicates bacterial pathogen killing activity by phagocytes and hence a better immunity (Kumar et al., 2011).
In summary, the data generated in the present study are of practical use for fish pathologists who determine tilapia mortality resulting from GBS infections. Despite the fact that a type-specific capsular polysaccharide – that prevents the activation of the complement system and inhibits opsonophagocytosis (Rubens et al., 1987; Chaffin et al., 2000) – is an indispensable GBS virulence determinant, further investigation of GBS virulence factor may provide additional useful insights into GBS induced pathogenesis. In addition, the application of effective vaccines against streptococcosis in fish, with different serotypes of GBS, could be investigated.

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tilapias (Oreochromis spp.) naturally infected by Streptococcus agalactiae.
Journal of Comparative Pathology. 143, 227 – 229.
1. **Table captions**

Table 1. Gross pathology of Nile tilapia infected with GBS-Ia and GBS-III. Values show number of fish exhibiting symptom per number of fish sampled (%).

<table>
<thead>
<tr>
<th>Pathology</th>
<th>GBS</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Serotype Ia</td>
</tr>
<tr>
<td>Lethargy</td>
<td>39/39 (100)</td>
</tr>
<tr>
<td>Darkening of the skin</td>
<td>11/39 (28)</td>
</tr>
<tr>
<td>Corneal opacity</td>
<td>6/39 (15)</td>
</tr>
<tr>
<td>Exophthalmia</td>
<td>7/39 (18)</td>
</tr>
<tr>
<td>External haemorrhage</td>
<td>10/39 (26)</td>
</tr>
<tr>
<td>Pale gills</td>
<td>25/39 (64)</td>
</tr>
<tr>
<td>Pale livers</td>
<td>29/39 (74)</td>
</tr>
<tr>
<td>Congestion of the liver</td>
<td>24/39 (62)</td>
</tr>
<tr>
<td>Liver haemorrhage</td>
<td>17/39 (44)</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>12/39 (31)</td>
</tr>
<tr>
<td>Accumulated fluid in the viscera</td>
<td>13/39 (33)</td>
</tr>
<tr>
<td>Congestion and haemorrhage of the brain</td>
<td>30/39 (77)</td>
</tr>
</tbody>
</table>
Table 2. Mean red blood cell count (RBC), white blood cell count (WBC), haematocrit (Ht), haemoglobin (Hb) and plasma protein at 1, 2, 3, 6 and 9 days post infection of Nile tilapia with GBS-Ia and GBS-III.

<table>
<thead>
<tr>
<th>Blood parameter</th>
<th>Treatment</th>
<th>Days after injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>RBC</strong> (×10^6 cell/ml)</td>
<td>Control</td>
<td>2.82 ± 0.52b</td>
</tr>
<tr>
<td></td>
<td>GBS-Ia</td>
<td>1.70 ± 0.25a</td>
</tr>
<tr>
<td></td>
<td>GBS-III</td>
<td>1.13 ± 0.37a</td>
</tr>
<tr>
<td><strong>WBC</strong> (×10^8 cell/ml)</td>
<td>Control</td>
<td>3.81 ± 1.21b</td>
</tr>
<tr>
<td></td>
<td>GBS-Ia</td>
<td>1.99±1.40a</td>
</tr>
<tr>
<td></td>
<td>GBS-III</td>
<td>1.89±1.28a</td>
</tr>
<tr>
<td><strong>Ht</strong> (%)</td>
<td>Control</td>
<td>25.54 ± 3.81ns</td>
</tr>
<tr>
<td></td>
<td>GBS-Ia</td>
<td>25.68 ± 2.50ns</td>
</tr>
<tr>
<td></td>
<td>GBS-III</td>
<td>20.95 ± 2.81ns</td>
</tr>
<tr>
<td><strong>Hb</strong> (g/dl)</td>
<td>Control</td>
<td>7.05 ± 0.85b</td>
</tr>
<tr>
<td></td>
<td>GBS-Ia</td>
<td>6.09 ± 0.34a</td>
</tr>
<tr>
<td></td>
<td>GBS-III</td>
<td>5.64 ± 0.96a</td>
</tr>
<tr>
<td><strong>Plasma protein</strong> (mg/ml)</td>
<td>Control</td>
<td>33.25 ± 8.18ns</td>
</tr>
<tr>
<td></td>
<td>GBS-Ia</td>
<td>26.89 ± 7.35ns</td>
</tr>
<tr>
<td></td>
<td>GBS-III</td>
<td>32.61 ± 7.08ns</td>
</tr>
</tbody>
</table>

*Values in the same column with different superscript differ significantly (p<0.05) Data expressed as Mean ± SD (N=6). †Initial fish (uninfected): RBC 2.46×10^9 ± 0.62×10^9 cell/ml, WBC 3.01×10^8 ± 0.62×10^8 cell/ml, Ht 25.03 ± 6.01%, Hb 6.65 ± 1.22 g/dl and plasma protein 32.70 ± 6.58 mg/ml.
Figure 1. Mean percent cumulative mortality of Nile tilapia challenged with different concentrations of GBS-Ia.
Figure 2. Mean percent cumulative mortality of Nile tilapia challenged with different concentrations of GBS-III.
Figure 3. Liver tissues of Nile tilapia infected with (a) GBS-Ia and (b) GBS-III showing haemorrhage (R), hepatocytic necrosis (N) and high degree of vacuolization (V) (H&E).
Figure 4. Pancreatic tissues (P) of Nile tilapia infected with (a) GBS-Ia and (b) GBS-III showing degeneration of acinar cells and loss of zymogen granules (H&E).
Figure 5. Brain tissues of Nile tilapia infected with (a) GBS-Ia and (b) GBS-III showing meningitis (M) and lymphocytic infiltration (L) (H&E).
Figure 6. Kidney tissues of Nile tilapia infected with (a) GBS-Ia and (b) GBS-III showing shrinkage of glomerulus (G) in the Bowman’s capsule and Lymphocytic infiltration (L) (H&E).
Figure 7. Plasma lysozyme activity of Nile tilapia at 1, 2, 3, 6 and 9 days post infection with GBS by i.p. injection. Each bar represents mean ± s.d. Different letters stand for statistically significant differences at p<0.05.
Figure 8. Respiratory burst activity of Nile tilapia at 1, 3, 6 and 9 days post infection with GBS by i.p. injection. Each bar represents mean ± s.d. Different letters stand for statistically significant differences at p<0.05.
Figure 9. Phagocytosis percentage (%) of Nile tilapia at 0, 1, 3, 6 and 9 days post infection with GBS by i.p. injection. Each bar represents mean ± s.d. Different letters stand for statistically significant differences at p<0.05.
Figure 10. Phagocytic index of Nile tilapia at 1, 3, 6 and 9 days post infection with GBS by i.p. injection. Each bar represents mean ± s.d. Different letters stand for statistically significant differences at p<0.05.