Songklanakarin J. Sci. Technol.
36 (5), 499-506, Sep. - Oct. 2014

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY
http://www.sjst.psu.ac.th

Original Article

Pathological manifestations and immune responses of serotypes Ia and III *Streptococcus agalactiae* infections in Nile tilapia (*Oreochromis niloticus*)

Atchariya Suwannasang[1,2], Machalin Dangwetngam[1], Akkarawit Issaro[1], Wutiporn Phromkunthong[1], and Naraid Suanyuk[1]*

[1] *Kidchakan Supamattaya Aquatic Animal Health Research Center, Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.*

[2] *Aquaculture Program, Faculty of Agricultural Technology, Phuket Rajabhat University, Mueang, Phuket, 83000 Thailand.*

Received: 25 April 2014; Accepted: 25 June 2014

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 1.58×10^6 CFU/fish and 2.10×10^8 CFU/fish, respectively. The onset of disease and clinical observation revealed notable differences between the GBS-Ia and GBS-III infections. Histological findings showed severe changes of the liver, pancreas, kidney and brain of fish infected with GBS. In addition, significantly different haemato-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

1. Introduction

Streptococcosis 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 from 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;

* Corresponding author.
Email address: naraid.s@psu.ac.th

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; Rodkhum *et al*., 2011). 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 (IS*1381*, IS*861* and IS*Sag2*) and the group II intron GBSi1. The other belongs to serotype III, contains *bca*, three insertion sequences (IS*1381*, IS*Sag1*, IS*Sag2*) and a tetracycline resistance gene (*tet*M) (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 GBS-Ia isolate (genotype Ia-*bca*-*bac*-IS*1381*-IS*861*-GBSi1-IS*Sag2*) was isolated from diseased tilapia in Nakhon Si Thammarat province and GBS-III isolate (genotype III-4-*bca*-IS*1381*-IS*Sag1*-IS*Sag2*-*tet*M-*int*Tn) 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 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 GBS-Ia or GBS-III in TSB was pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. The cells were washed twice with phosphate buffer saline (PBS, pH 7.4) 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 *et al.*, 2004) and were given an i.p. injection with 0.1 ml 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 GBS-Ia and GBS-III were assayed by determining the fifty percent lethal dose (LD_{50}) 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 the 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 the 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 ml 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 reduction rate in the absorbance of the samples was converted to lysozyme concentration (µg/ml) using a standard curve.

The respiratory burst activity of the leucocytes was determined using the reduction of nitroblue tetrazolium to formozan as a measure of superoxide anion (O_2^-) production. The absorbance at 630 nm was measured spectrophotometrically 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). Leucocyte 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 and mortality occurred continuously throughout the induction of disease experiment. Infected fish exhibited 10-60% mortality (Figure 1), with a 28 day-LD_{50} of 1.58×10^6 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-LD_{50} of 2.10×10^8 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 dying without showing any prior clinical signs. After 2 dpi, infected fish showed listless circling 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, 44-62% congestion and haemorrhage of the liver and 77% congestion and haemorrhage of the brain, whereas the fish infected with GBS-III showed 10%, 28-31% and 52% of exophthalmia, congestion and haemorrhage of the liver 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, kidney 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

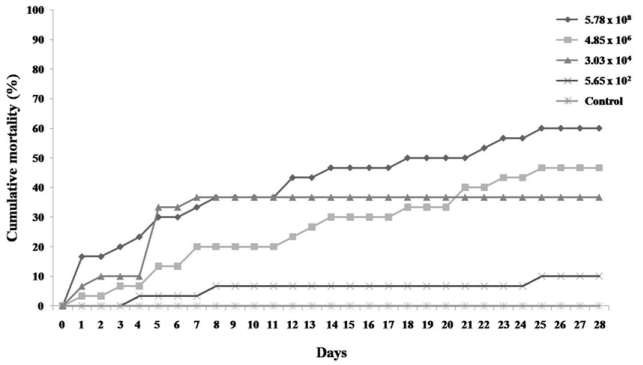

Figure 1. Mean percent cumulative mortality of Nile tilapia challenged with different concentrations of GBS- Ia.

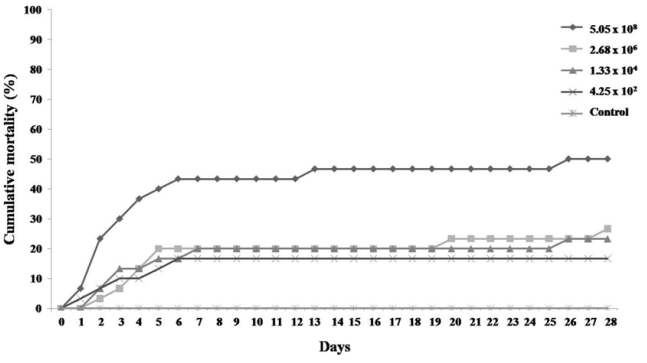

Figure 2. Mean percent cumulative mortality of Nile tilapia challenged with different concentrations of GBS-III.

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 (%).

Pathology	GBS	
	Serotype Ia	Serotype III
Lethargy	39/39 (100)	29/29 (100)
Darkening of the skin	11/39 (28)	5/29 (17)
Corneal opacity	6/39 (15)	4/29 (14)
Exophthalmia	7/39 (18)	3/29 (10)
External haemorrhage	10/39 (26)	5/29 (17)
Pale gills	25/39 (64)	17/29 (59)
Pale livers	29/39 (74)	20/29 (69)
Congestion of the liver	24/39 (62)	9/29 (31)
Liver haemorrhage	17/39 (44)	8/29 (28)
Splenomegaly	12/39 (31)	5/29 (17)
Accumulated fluid in the viscera	13/39 (33)	2/29 (7)
Congestion and haemorrhage of the brain	30/39 (77)	15/29 (52)

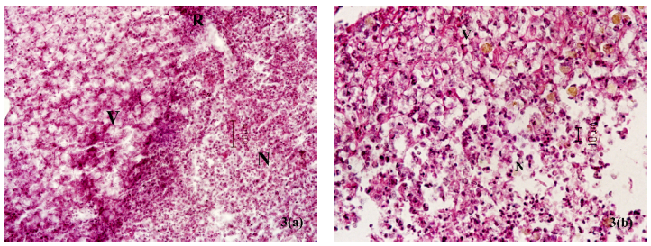

Figure 3. Liver tissues of Nile tilapia infected with (a) GBS-Ia and (b) GBS-III showing haemorrhage (R), hepatocytic necrosis (N) and high degree of vacuolization (V) (H&E).

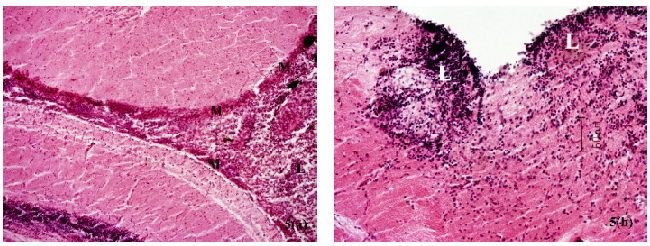

Figure 5. Brain tissues of Nile tilapia infected with (a) GBS-Ia and (b) GBS-III showing meningitis (M) and lymphocytic infiltration (L) (H&E).

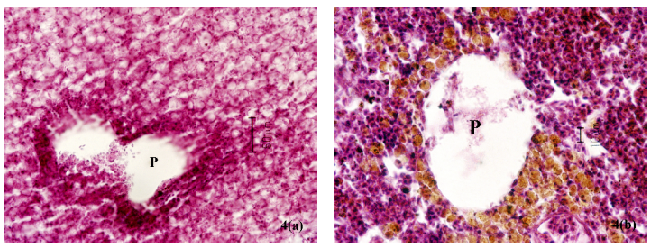

Figure 4. Pancreatic tissues (P) of Nile tilapia infected with (a) GBS-Ia and (b) GBS-III showing degeneration of acinar cells and loss of zymogen granules (H&E).

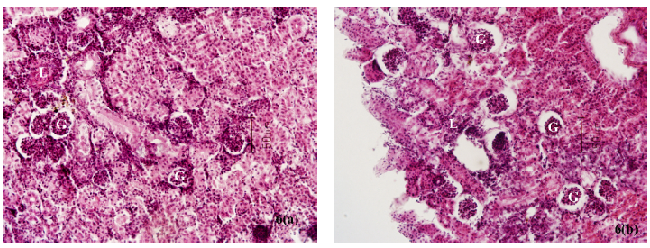

Figure 6. Kidney tissues of Nile tilapia infected with (a) GBS-Ia and (b) GBS-III showing shrinkage of glomerulus (G) in the Bowman's capsule and lymphocytic infiltration (L) (H&E).

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 produced 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

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[*].

Blood parameter	Treatment	Days after injection				
		1	2	3	6	9
RBC[**] ($\times 10^9$ cell/ml)	Control	2.82±0.52[b]	2.13±0.20[b]	2.23±0.17[b]	2.12±0.35[b]	2.17±0.28[b]
	GBS-Ia	1.70±0.25[a]	1.44±0.52[a]	1.07±0.38[a]	1.72±0.27[ab]	1.21±0.40[a]
	GBS-III	1.13±0.37[a]	1.54±0.25[a]	1.75±0.87[b]	1.47±0.48[a]	1.18±0.51[a]
WBC[**] ($\times 10^8$ cell/ml)	Control	3.81±1.21[b]	3.43±0.98[b]	3.59±1.02[b]	3.61±0.93[ns]	3.82±0.60[a]
	GBS-Ia	1.99±1.40[a]	1.04±0.44[a]	1.51±0.67[a]	4.35±1.21[ns]	6.23±1.15[b]
	GBS-III	1.89±1.28[a]	0.72±0.30[a]	2.25±0.36[a]	4.15±1.08[ns]	4.30±0.35[a]
Ht[**] (%)	Control	25.54±3.81[ns]	24.31±2.02[ns]	24.23±6.44[ns]	25.58±3.86[ns]	25.92±2.78[ns]
	GBS-Ia	25.68±2.50[ns]	20.00±3.35[ns]	13.49±4.70[ns]	23.99±1.62[ns]	24.58±3.43[ns]
	GBS-III	20.95±2.81[ns]	21.20±3.30[ns]	18.69±2.26[ns]	22.48±7.17[ns]	23.14±8.65[ns]
Hb[**] (g/dl)	Control	7.05±0.85[b]	6.94±0.92[b]	6.39±1.08[b]	6.11±0.85[ns]	6.09±1.51[ns]
	GBS-Ia	6.09±0.34[a]	5.16±0.92[a]	4.13±0.66[a]	5.99±0.74[ns]	5.35±1.06[ns]
	GBS-III	5.64±0.96[a]	5.78±0.27[a]	5.20±0.69[a]	5.18±1.07[ns]	5.13±1.60[ns]
Plasma protein[**] (mg/ml)	Control	33.25±8.18[ns]	33.68±4.38[ns]	32.82±3.72[ns]	32.50±3.74[a]	32.98±2.43[a]
	GBS-Ia	26.89±7.35[ns]	29.44±7.72[ns]	26.50±8.55[ns]	42.71±5.77[b]	44.49±2.72[b]
	GBS-III	32.61±7.08[ns]	35.19±4.46[ns]	28.57±5.39[ns]	40.81±7.26[b]	36.10±3.13[a]

[*] Values in the same column with different superscript differ significantly ($p<0.05$). Data expressed as Mean ± s.d. (N=6).
[**] Initial fish (uninfected): RBC $2.46\times10^9 \pm 0.62\times10^9$ cell/ml, WBC $3.01\times10^8 \pm 0.62\times10^8$ cell/ml, Ht 25.03 ± 6.01%, Hb 6.65 ± 1.22 g/dl and plasma protein 32.70 ± 6.58 mg/ml

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. 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, at 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.

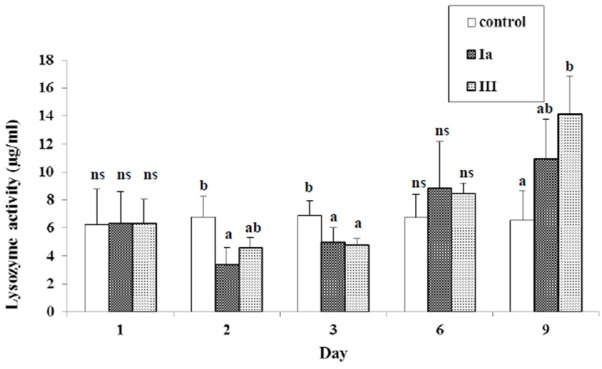

Figure 7. Plasma lysozyme activity of Nile tilapia at 1, 2, 3, 6 and 9 days post infection with GBS by i.p. injection. Each bar represents mean ± s.d. Different letters stand for statistically significant differences at $p<0.05$.

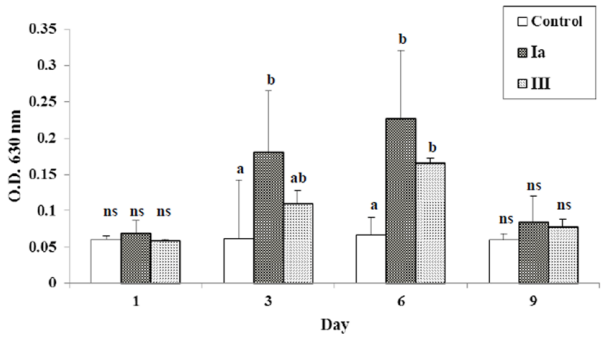

Figure 8. Respiratory burst activity of Nile tilapia at 1, 3, 6 and 9 days post infection with GBS by i.p. injection. Each bar represents mean ± s.d. Different letters stand for statistically significant differences at $p<0.05$.

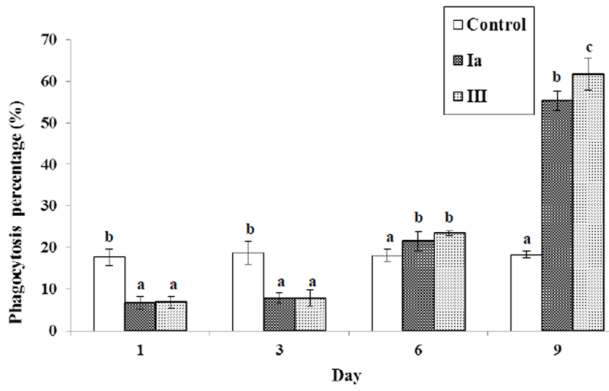

Figure 9. Phagocytic activity (%) of Nile tilapia at 1, 3, 6 and 9 days post infection with GBS by i.p. injection. Each bar represents mean ± s.d. Different letters stand for statistically significant differences at $p<0.05$.

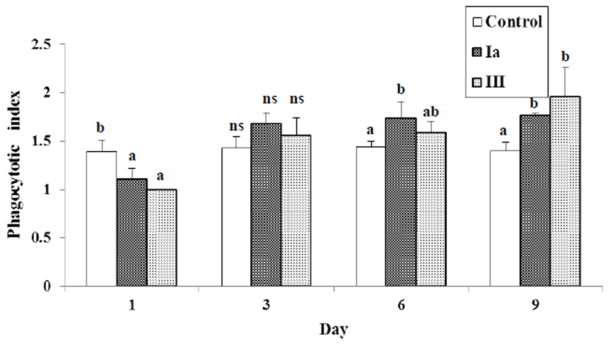

Figure 10. Phagocytic index of Nile tilapia at 1, 3, 6 and 9 days post infection with GBS by i.p. injection. Each bar represents mean ± s.d. Different letters stand for statistically significant differences at $p<0.05$.

4. 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; Rodkhum *et al*., 2011). 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 IS*1548* 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 Ghuysen, 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 anti-bacterial 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 opsono-phagocytosis (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.

Acknowledgements

This work was supported by the Higher Education Research Promotion and National Research University Project

of Thailand, Office of the Higher Education Commission. The authors thank Seppo J. Karrila and Alan F. Geater for editing the manuscript.

References

Abuseliana, A., Daud, H., Aziz, S.A., Bejo, S.K. and Alsaid, M. 2011. Pathogenicity of *Streptococcus agalactiae* isolated from a fish farm in Selangor to juvenile red tilapia (*Oreochromis* sp.). Journal of Animal and Veterinary Advances. 10, 914-919.

Amal, M.N.A., Zamri-Saad, M., Iftikhar, A.R., Siti-Zahrah, A., Aziel, S. and Fahmi, S. 2012. An outbreak of *Streptococcus agalactiae* infection in cage-cultured golden pompano, *Trachinotus blochii* (Lacépède), in Malaysia. Journal of Fish Diseases. 35, 849-852.

Blaxhall, P.C. and Daisley, K.W. 1973. Routine hematological methods for use with fish blood. Journal of Fish Biology. 5, 771-781.

Chaffin, D.O., Beres, S.B., Yim, H.H. and Rubens, C.E. 2000. The serotype of type Ia and III group B streptococci is determined by the polymerase gene within the polycistronic capsule operon. Journal of Bacteriology. 182, 4466-4477.

Chung, S. and Secombes, C.J. 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology. 88, 539-544.

Coyle, S.D., Durborow, R.M. and Tidwell, J.H. 2004. Anesthetics in Aquaculture. Publication No. 3900. Southern Regional Aquaculture Center.

Demers, N.E. and Bayne, C.J. 1997. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Development Comparative Immunology. 21, 363-373.

Duremdez, R., Al-Marzouk, A., Qasem, J.A., Al-Harbi, A. and Gharabally, H. 2004. Isolation of *Streptococcus agalactiae* from cultured silver pomfret, *Pampus argenteus* (Euphrasen), in Kuwait. Journal of Fish Diseases. 27, 307-310.

Evans, J.J., Bohnsack, J.F., Klesius, P.H., Whiting, A.A., Garcia, J.C., Shoemaker, C.A. and Takahashi, S. 2008. Phylogenetic relationships among *Streptococcus agalactiae* isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan. Journal of Medical Microbiology. 57, 1369-1376.

Evans, J.J., Klesius, P.H., Gilbert, P.M., Shoemaker, C.A., Al Sarawi, M.A., Landsberg, J., Duremdez, R., Al Markouk, A. and Al Kenzi, S. 2002. Characterization of β–haemolytic group B *Streptococcus agalactiae* in cultured sea bream, *Sparus auratus*, and wild mullet, *Liza klunzingeri* (Day), in Kuwait. Journal of Fish Diseases. 25, 505-513.

Granlund, M., Michel, F. and Norgren, M. 2001. Mutually exclusive distribution of *IS1548* and *GBSi1*, an active group II intron identified in human isolates of group B streptococci. Journal of Bacteriology. 183, 2560-2569.

Harikrishnan, R., Rani, N.M. and Balasundaram, C. 2003. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture. 221, 41-50.

Humason, G.L. 1979. Animal Tissue Techniques. W.H. Freeman and Company, San Francisco.

Imperi, M., Pataracchia, M., Alfarone, G., Baldassarri, L., Orefici, G. and Creti, R. 2010. A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of *Streptococcus agalactiae*. Journal of Microbiological Methods. 80, 212-214.

Ip, M., Cheuk, E.S.C., Tsui, M.H.Y., Kong, F., Leung, T.N. and Gillbert, G.L. 2006. Identification of a *Streptococcus agalactiae* serotype III subtype 4 clone in association with adult invasive disease in Hong Kong. Journal of Clinical Microbiology. 44, 4252-4254.

Jamalzaden, H.R., Keyvan, A., Ghomi, M.R. and Gherardi, F. 2009. Comparison of blood indices in healthy and fungal infected Caspian salmon (*Salmo trutta caspius*). African Journal of Biotechnology. 8, 319-322.

Kumar, N., Antony Jesu Prabhu, P., Pal, A.K., Remya, S., Aklakur, Md., Rana, R.S., Gupta, S., Raman, R.P. and Jadhao, S.B. 2011. Anti-oxidative and immunohematological status of Tilapia (*Oreochromis mossambicus*) during acute toxicity test of endosulfan. Pesticide Biochemistry and Physiology. 99, 45-52.

Larsen, H.M. and Snieszko, S.F. 1961. Modification of the microhaematocrit technique with trout blood. Transactions of the American Fisheries Society. 90, 139-142.

Lin, F.Y., Clemens, J.D., Azimi, P.H., Regan, J.A., Weisman, L.E., Philips, J.B., Rhoads, G.G., Clark, P., Brenner, R.A. and Ferrieri, P. 1998. Capsular polysaccharide types of group B streptococcal isolates from neonates with early-onset systemic infection. The Journal of Infectious Diseases. 177, 790-792.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. Journal Biological Chemistry. 193, 265-275.

Luangthuvapranit, C. 1988. Methodology for using a microcomputer to evaluate toxicity in fish. Journal of Science and Technology. 3, 25-38.

Magnadóttir, B. 2006. Innate immunity of fish (overview). Fish and Shellfish Immunology. 20, 137-151.

Martinez, G., Harel, J., Higgins, R., Lacouture, S., Daignault, D. and Gottschalk, M. 2000. Characterization of *Streptococcus agalactiae* isolates of bovine and human origin by randomly amplified polymorphic DNA analysis. Journal of Clinical Microbiology. 38,

71-78.

McNulty, S.T., Klesius, P.H., Shoemaker, C.A. and Evans, J.J. 2003. Hematological changes in Nile tilapia (*Oreochromis niloticus*) infected with *Streptococcus iniae* by nare inoculation. Journal of the World Aquaculture Society. 34, 418-422.

Mian, G.F., Godoy, D.T., Leal, C.A.G., Yuhara, T.Y., Costa, G.M. and Figueiredo, H.C.P. 2009. Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. Veterinary Microbiology. 136, 180-183.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J. and Belosevic, M. 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. Developmental and Comparative Immunology. 25, 807-825.

Rasheed, V., Limsuwan, C. and Plumb, J.A. 1985. Histopathology of bullminnows, *Fundulus grandis* Baird and Girard, infected with a non-haemolytic group B *Streptococcus* sp. Journal of Fish Diseases. 8, 65-74.

Rodkhum, C., Kayansamruaj, P. and Pirarat, N. 2011. Effect of water temperature on susceptibility to *Streptococcus agalactiae* serotype Ia infection in Nile tilapia (*Oreochromis niloticus*). Thai Journal of Veterinary Medicine. 41, 309-314.

Rubens, C.E., Wessels, M.R., Heggen, L.M. and Kasper, D.L. 1987. Transposon mutagenesis of type III group B *Streptococcus*: Correlation of capsule expression with virulence. Proceedings of the National Academy of Sciences of the United States of America. 84, 7208-7212.

Safadi, R.A., Amor, S., Hery-Arnaud, G., Spellerberg, B., Lanotte, P., Mereghetti, L., Gannier, F., Quentin, R. and Rosenau, A. 2010. Enhanced expression of *lmb* gene encoding laminin binding protein in *Streptococcus agalactiae* strains harboring IS*1548* intergenic region. PLoS One. 5, e10794.

Salton, M.R.J. and Ghuysen, J.M. 1959. The structure of di- and tetra-saccharides released from cell walls by lysozyme and *Streptomyces* F_1 enzyme and the $\beta(1\rightarrow4)$ *N*-acetylhexosaminidase activity of these enzymes. Biochimica et Biophysica Acta. 36, 552-554.

Salvador, R., Müller, E.E., Freitas, J.C., Leonhardt, J.H., Pretto-Giordano, L.G. and Dias, J.A. 2005. Isolation and characterization of *Streptococcus* spp. group B in Nile tilapia (*Oreochromis niloticus*) reared in hapas nets and earth nurseries in the northern region of Parana State, Brazil. Ciência Rural. 35, 1374-1378.

Stasiak, A.S. and Baumann, C.P. 1996. Neutrophil activity as a potential bioindicator for contaminant analysis. Fish and Shellfish Immunology. 6, 537-539.

Straková, L. and Motlová, J. 2004. Active surveillance of early onset disease due to group B streptococci in newborns. Indian Journal of Medical Research. 119 (Suppl.), 205-207.

Suanyuk, N., Kong, F., Ko, D., Gilbert, G.L. and Supamattaya, K. 2008. Occurrence of rare genotypes of *Streptococcus agalactiae* in cultured red tilapia *Oreochromis* sp. and Nile tilapia *O. niloticus* in Thailand – Relationship to human isolates? Aquaculture. 284, 35-40.

Suanyuk, N., Sukkasame, N., Tanmark, N., Yoshida, T., Itami, T., Thune, R.L., Tantikitti, C. and Supamattaya, K. 2010. *Streptococcus iniae* infection in cultured Asian sea bass (*Lates calcarifer*) and red tilapia (*Oreochromis* sp.) in southern Thailand. Songklanakarin Journal of Science and Technology. 32, 341-348.

Supamattaya, K. 1995 Laboratory Manual for fish Disease and Parasites. Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University.

Supamattaya, K., Ruangsri, J., Kiriratnikom, S. and Songsrichan, N. 2000. The immune system in black tiger shrimp, *Penaeus monodon*, Fabricius: IV. Normal immuno - physiological values in black tiger shrimp, *Penaeus monodon*, Fabricius. Songklanakarin Journal of Science and Technology. 22(suppl.), 597-603.

Thuvander, A., Norrgren, L. and Fossum, C. 1987 Phagocytic cells in blood from rainbow trout (*Salmo gairdneri* Richardson) characterized by flow cytometry and electronmicroscopy. Journal of Fish Biology. 31, 197-208.

Yano, T. 1996. The nonspecific immune system: humoral defense. In: The Fish Immune System: Organism, Pathogen, and Environment, Iwama, G., Nakanishi, T. (Eds.), Academic Press, San Diego, pp. 105-157.

Zamri-Saad, M., Amal, M.N.A. and Siti-Zahrah, A. 2010. Pathological changes in red tilapias (*Oreochromis* spp.) naturally infected by *Streptococcus agalactiae*. Journal of Comparative Pathology. 143, 227-229.