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Original Article

Epizootiology, pathogenicity and haemato-immunology associated with *Streptococcus agalactiae* serotype Ib infection in climbing perch (*Anabas testudineus*)

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Abstract

The present study report on epizootiology, pathogenicity and haemato-immunological aspects of *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) serotype Ib (GBS-Ib) affecting climbing perch (*Anabas testudineus*) in southern Thailand. Study on growth of GBS-Ib under various conditions indicated that this bacterium grew from 25 to 35°C, pH 5 to 9 and 0.5 to 4.0% NaCl. Climbing perch inoculated by intraperitoneally injected GBS-Ib at concentrations of 10^1 to 10^4 CFU/mL exhibited 60.00-96.67% mortality, with 60 h-LD_{50} and 72 h-LD_{50} at $4.5x10^2$ CFU/mL and $6.3x10^1$ CFU/mL inoculums, respectively. Study of the haemato-immunological changes in the fish infected with GBS-Ib revealed declines of haematocrit, haemoglobin, serum protein, red blood cells, and respiratory burst activity. On the other hand, white blood cells, lysozyme activity, phagocytic index, and average beads per cell, were significantly increased in the infected fish. Study on the effect of environmental factors on the susceptibility of fish to GBS-Ib infection indicated that high water temperature and low dissolved oxygen levels proved to increase mortality of climbing perch. These findings indicate that GBS-Ib is a pathogenic bacterium responsible for fish mortality in Thailand.

Keywords: climbing perch, *Streptococcus agalactiae*, epizootiology, pathogenicity, immunology

1. Introduction

Climbing perch (*Anabas testudineus*) (Perciformes: Anabantidae) is an extremely hardy brown or dark greenish-brown fish and an economic fish species consumed in Thailand, Malaysia and the Philippines (Chotipuntu & Avakul, 2010). This omnivorous fish exhibits obligatory air-gulping behavior, which enables survival out of water for several days (Hitchcock, 2008; Sarkar *et al.*, 2005). However, the intensive cultivation of climbing perch under stressful conditions may lead to serious outbreaks of infectious diseases in the fish farms. At present, motile *Aeromonas* septicemia (Hossain, Rashid, & Sayed, 2011), tail and fin rot disease (Rahman, Ferdowsy, Kashem, & Foysal, 2010), and streptococcosis (Klingklib & Suanyuk, 2017) have been reported as serious problems in climbing perch farming.

Streptococcosis causes significant losses to the fish farmers, particularly in intensive culture systems. The major causative agents of streptococcosis in fish are *Streptococcus iniae*, *S. dysgalactiae*, *S. parauberis* and *S. agalactiae* (Group B *Streptococcus*, GBS). In Thailand, GBS and *S. iniae* have been reported as causative agents of streptococcosis in fish (Suanyuk, Kong, Ko, Gilbert, & Supamattaya 2008; Suanyuk *et al.*, 2010), while GBS is among the species most commonly causing serious damage to farmed fish. Based on GBS isolated from infected fish, two distinguishable GBS serotypes Ia (GBS-Ia) and III (GBS-III) are known to infect the cultured

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fish in Thailand (Dangwetngam, Suanyuk, Kong, & Phromkunthong, 2016; Rodkhum, Kayansamruaj, & Pirarat, 2011; Suanyuk *et al.*, 2008). However, recent reports indicate the emergence of GBS serotype Ib (GBS-Ib) (synonym: *S. difficilis*) in climbing perch and in Günther's walking catfish (*Clarias macrocephalus*) that are polycultured in Nakhon Si Thammarat province of southern Thailand (Klingklib & Suanyuk, 2017).

GBS-Ib has been reported as an important pathogen affecting humans and aquatic animals (Bowater *et al.*, 2012; Delannoy *et al.*, 2013; Martins *et al.*, 2007). While the phenotypic and genotypic characteristics of GBS-Ib isolated from infected climbing perch have already been evaluated, the effects of GBS-Ib infection in the fish have not been well characterized. Therefore, the purpose of the present study was to assess some aspects of epizootiology, pathogenicity and haemato-immunology in climbing perch exposed to GBS-Ib infection.

2. Materials and Methods

2.1 Bacteria

GBS-Ib isolate PSU-KSAAHRC-ST298 (GBS-Ib-298), originally isolated from a natural outbreak of the disease in climbing perch cultured in earthen ponds of Nakhon Si Thammarat province in southern Thailand, was chosen for all experiments in the present study. Prior to use, the bacterium was confirmed to be GBS serogroup B and serotype Ib by polymerase chain reaction (Martinez, Harel, & Gottschalk, 2001), standard biochemical methods, as well as by using a Slidex Strepto Plus (bioMérieux, France) and group B streptococci typing antisera (Denka Seiken Co. Ltd., Japan) using the method described by Klingklib and Suanyuk (2017). Unless otherwise stated, GBS-Ib-298 was routinely grown at 30 °C on tryptic soy agar (TSA: Difco) or in tryptic soy broth (TSB: Difco). Stock cultures were kept at -80 ºC in TSB suspension containing 15 % glycerol.

2.2 Experimental fish

Healthy climbing perch with an average weight of approximately 3 g/fish, were obtained from commercial fish hatcheries in southern Thailand. Fish cultivation was done following the protocol in Suwannasang, Dangwetngam, Issaro, Phromkunthong, and Suanyuk (2014). All procedures were performed under anesthesia to minimize suffering. Briefly, the fish were cultured to the experimental size in three-ton fiberglass tanks with aeration system, at water temperature 26-28 °C. Water in the tank was changed every 2 days at 80 % rate. During the rearing period the fish were fed *ad libitum* twice daily with commercial fish feed. A sample of the experimental fish was examined to be free of pathogenic bacteria prior to use in the study trial.

2.3 Optimal growth of GBS-Ib at various conditions

GBS-Ib-298, cultured on TSA for 24 h at 30 °C before transferred to 100 mL of TSB and further incubated for 24 h at 30 °C, was used as a bacterial starter culture. For the growth tests, 0.5 mL of starter culture was inoculated into 50 mL TSB in 125 mL flasks and incubated under various conditions following the method described by Al-Harbi (1994) and Cheng and Chen (1999). Temperature tests were conducted from 25 to 45 °C at pH 7.2 and 0.5 % NaCl. pH tests were carried out from pH 3 to 11 at temperature 30 °C and 0.5 % NaCl. Salinity tests were conducted from 0.5 to 8% at pH 7.2 and 30 °C. Overall, there were 23 tests (5 temperature tests, 9 pH tests and 9 NaCl tests). Each test was conducted in triplicate and bacterial growth was monitored by measuring absorbance at 600 nm using a UV-1201 spectrophotometer (Shimadzu Corporation, Japan) at 0, 6, 12, 24, 48, 72, 96 and 120 h incubation.

2.4 Infectivity trials

Virulence of GBS-Ib-298 in the climbing perch with an average weight of 20.30±1.19 g/fish was evaluated by determining the median lethal dose (LD_{50}), by using a method modified from Suanyuk, Kangheae, Khongpradit, and Supamattaya (2005). Briefly, the fish were acclimatized for 1 week in fifteen 120x50x45 cm glass tanks containing 150 L dechlorinated water, with an aeration system, at water temperature 26-28 °C and 10 fish/tank density. The experiment was conducted in triplicate using ten fish per replication (i.e. 30 fish per concentration). Experimental climbing perch were injected intraperitoneally with 0.1 ml of a bacterial suspension prepared using optimal growth conditions determined from the growth study. Briefly, the GBS-Ib-298 cultured in TSB at 30 °C for 48 h was harvested by centrifugation at 10,000 rpm for 10 min at 4 °C and resuspended in phosphate buffered saline (PBS; pH 7.4) to achieve final concentrations in the range 10^1 - 10^4 colony forming units (CFU)/mL. The viable bacteria were counted by serial dilution with drop plating onto TSA, and incubating at 30 °C for 24-48 h. The control group was similarly injected with PBS. Clinical signs and mortality of infected fish were recorded for 14 days. To confirm the cause of death, tissue samples of newly dead fish i.e., liver, kidney and brain were aseptically collected and streaked onto TSA and incubated at 30 °C for 24-48 h. The bacterial cultures were identified and confirmed to be GBS-Ib by API20STREP and group B streptococci typing antisera.

2.5 Haemato-immunological changes of climbing perch infected with GBS-Ib

One hundred and fifty climbing perch with an average weight of 40.20±7.18 g/fish were used in this study. Before infection, the fish were acclimatized for 1 week in a 120x50x45 cm glass tanks containing 150 L dechlorinated water with an aeration system and 26-28 ºC water temperature. The experiments were conducted in triplicate with 25 fish per replication. Each group was intraperitoneally injected with 0.1 mL of GBS-Ib-298 at $4.80x10^2$ CFU/mL concentration. The control group was similarly inoculated with sterile PBS (pH 7.4).

At days 0, 1, 2, 3, 5 and 7 post-injection, sampled fish were euthanized with clove oil and blood was collected from caudal vein (two fish randomly from each replication). Haematocrit, haemoglobin, total serum protein, and red blood cell and white blood cell counts, were determined as described

by Suwannasang *et al.* (2014). The respiratory burst activity of the leucocytes was determined using the reduction of nitroblue tetrazolium to formazan as a measurement of superoxide anion (O_2^-) production (Stasiak & Baumann, 1996). Lysozyme activity was measured in a turbidimetric assay with a microplate reader, according to Demers and Bayne (1997).

Head kidney leucocytes were isolated under sterile conditions following Chung and Secombes (1988). Leucocyte phagocytic activity, phagocytic index and average number of the beads per cell were measured following methods of Thuvander, Norrgren, and Fossum (1987) and Rengpipat, Rukpratanporn, Piyatiratitivorakul, and Menasaveta (2000).

2.6 Effect of temperature and dissolved oxygen (DO) on mortality of climbing perch infected with GBS-Ib

Climbing perch with an average weight of 46.0±0.24 g/fish were acclimated to combinations of temperature and DO in a 3x3 factorial experiment (three temperatures: 28.97±0.12, 31.85±0.34 and 34.07±0.45 °C; and three DO levels: 1.73±0.21, 3.77±0.14 and 5.52±0.21 mg/L) for 1 week prior to challenge with GBS-Ib-298. The experiment was conducted in triplicate in 60x45x45 cm fiber glass tanks containing 60 L dechlorinated water using 10 fish/tank. Temperatures above ambient were maintained with aquarium heaters and the DO was maintained by adjusting the air flow or by replacement of DO with N_2 which was regulated using pressure gauge (Small, Kopf, Watts, & Howitt, 2014). DO and temperature in the test chamber was measured three times daily by using a handheld oxygen meter (Oxi 330i, Germany). Unchallenged control fish were maintained at the highest temperature and lowest DO. Water quality was controlled by daily exchanges of 20% freshwater and other water quality parameters i.e. ammonia, alkalinity, and pH were measured and recorded using standard methods (American Public Health Association (APHA), American Water Works Association (AWWA), & Water Environment Federation (WEF), 1998; Boyd & Tucker, 1992). After 1 week exposure, experimental climbing perch were injected intraperitoneally with 0.1 ml of GBS-Ib-298 at $3.05x10^2$ CFU/mL. Clinical signs and mortality of infected fish were recorded for 14 days. Tissue samples (especially brain) of newly dead fish were collected and bacteria were re-isolated on blood agar to confirm GBS-Ib as the cause of death.

2.7 Statistical analysis

Data was reported as means±standard deviations. Percentage data sets were subjected to arcsine transformation prior to analysis of variance. Significant differences in growth of GBS-Ib-298 under various conditions and cumulative mortality of climbing perch after infection were analyzed using one way ANOVA. The haemato-immunological values were analyzed using independent samples t-test. The effect of temperature and DO on mortality of climbing perch infected with GBS-Ib-298 were analyzed using both two-way and one-way ANOVA. Differences between treatments were analyzed using Duncan's multiple range test and were considered significant at $p<0.05$.

3. Results

3.1 Optimal growth of GBS-Ib at various conditions

Growth of GBS-Ib-298 in TSB was observed at temperature ranging from 25 to 35°C. (Figure 1a), pH 5 to 9 (Figure 1b) and 0.5 to 4.0% NaCl (Figure 1c).

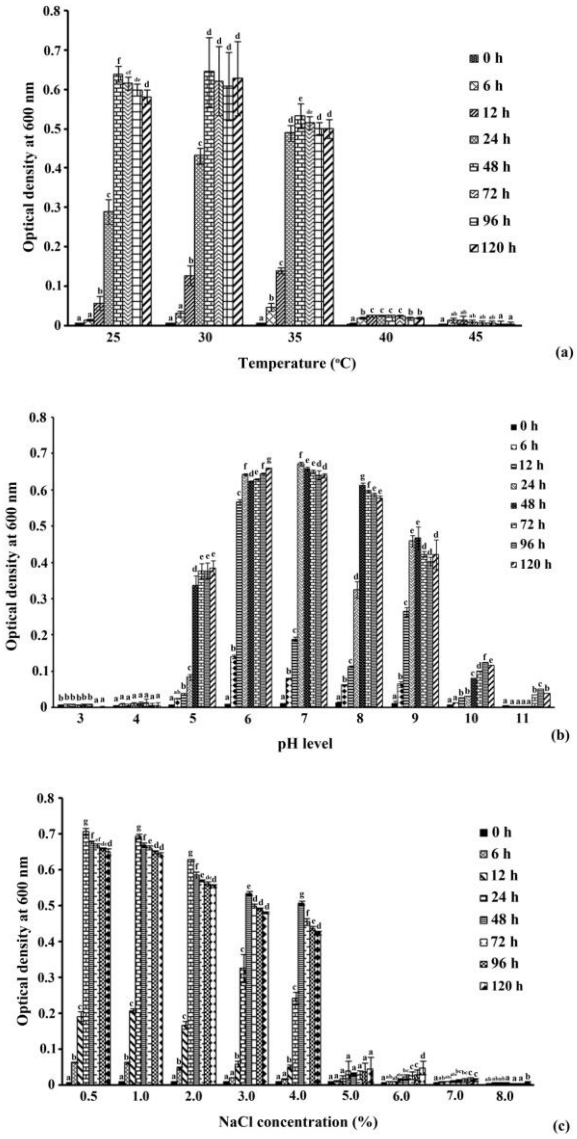

Figure 1. Effect of temperature (a) pH (b) and salinity (c) on growth of GBS-Ib-298 in TSB medium. Bacterial numbers were determined by optical density at 600 nm at 0, 6, 12, 24, 48, 72, 96 and 120 h. Different letters within each temperature, pH or NaCl concentration indicate a statistically significant difference ($p<0.05$).

3.2 Infectivity trials

No mortality was observed in the climbing perch intraperitoneally injected with sterile PBS. However, the climbing perch injected with GBS-Ib-298 exhibited early-onset disease with high mortality occurring continuously

during days 2-9 after inoculation. The climbing perch inoculated by intraperitoneally injected with GBS-Ib-298 at concentrations $6.10x10^4$ or $6.00x10^3$ CFU/mL exhibited 96.67 % mortality, while the fish injected with bacterial concentrations $9.00x10^2$ and $5.00x10^1$ CFU/mL exhibited 86.67 and 60.00 % mortalities within 14 days, respectively (Figure 2). The experimental infection of climbing perch with GBS-Ib-298 resulted in 60 h-LD_{50} and 72 h LD_{50} at $4.5x10^2$ CFU/mL and $6.3x10^1$ CFU/mL, respectively. The infected fish displayed lethargy, darkening of skin pigment, eye opacity, serpentine movement, and haemorrhage from anus. GBS-Ib recovered from deceased fish was examined bacteriologically and serologically.

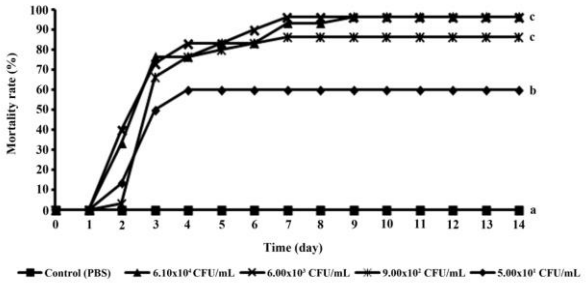

Figure 2. Mortality of climbing perch intraperitoneally injected with various concentrations of GBS-Ib-298. Values with different letters are significantly different ($p<0.05$).

3.3 Haemato-immunological changes of climbing perch infected with GBS-Ib

The climbing perch infected with GBS-Ib-298 exhibited significant haemato-immunological changes ($p<0.05$) in comparison to the uninfected fish. Most blood parameters, with the exception of white blood cell count, lysozyme, phagocytic index, and average beads per cell, decreased with the infection. Briefly, the infected climbing perch exhibited lower haematocrit during days 3-7 post infection. Similar results were observed for serum protein and red blood cell levels, in that the values were reduced during days 1 and 5-7 post infection. Increased haemoglobin was observed on the first day post infection but then returned to normal on days 2-3 and further decreased during days 5-7 post infection. Declining NBT was observed during days 1-3 and returned to normal on days 5-7 post infection. On the other hand, the lysozyme activity increased significantly during days 2-7 post infection. Similarly, white blood cell count of the climbing perch infected with GBS-Ib-298 increased significantly on day 5 post infection, corresponding to phagocytic index and average number of the bead per cell, which increased significantly on days 1, 2, 3 and 7 and on days 1-3 post infection, respectively. No significant difference was observed in phagocytic activity between control and infected fish (Table 1).

3.4 Effect of temperature and dissolved oxygen (DO) on mortality of climbing perch infected with GBS-Ib

Climbing perch cultured at various temperatures and DO concentrations showed variable behavior. Under normal conditions (Temperature of 28.97±0.12 °C, DO of 5.52±0.21 mg/L), fish swam and fed normally. Abnormal behaviors, such as lethargy, loss of appetite and swimming at the water surface or at the bottom of aquarium was observed at higher temperature and lower DO. After injection with GBS-Ib-298, susceptibility was both temperature and DO independent. Statistical analysis is shown in Table 2. The greatest mortality (96.67±5.77%) was observed in fish maintained in water of highest temperature and lowest DO while the lowest mortality (56.67±5.77%) was observed in fish maintained in water of lowest temperature and highest DO (Table 2). Clinical signs due to experimental infection were observed, including loss of appetite, lethargy, bottom swimming, meningitis, anal haemorrhage. Some fish died, however, without showing any clinical signs. GBS-Ib was recovered from deceased fish examined bacteriologically and serologically. No mortalities were observed in the unchallenged control group.

4. Discussion

Understanding the dynamic aquatic environment and its role in fish health is an important baseline to management of infectious diseases. In this study, GBS-Ib-298 exhibited a tremendous range of tolerance in terms of growth at various temperatures, pH and salinities. Survival outside of the host under various environmental conditions leading to an increased probability of contact and infection with sensitive hosts. Similar optimal growth conditions were reported from *S. iniae* isolated from diseased tilapia cultured in Saudi Arabia and the US (Al-Harbi, 1994; Perera, Johnson, & Lewis, 1997) and *Enterococcus*-like bacterium from giant freshwater prawn (*Macrobrachium rosenbergii*) cultured in Taiwan (Cheng & Chen, 1999).

GBS-Ib has been reported as a pathogen causing disease in wild and cultured fish species, including the wild giant Queensland grouper (*Epinephelus lanceolatus*) (Bowater *et al.*, 2012), tilapia (*Oreochromis* sp.) (Delannoy *et al.*, 2013; Li *et al.*, 2013), rosy barb (*Puntius conchonius*) and golden ram (*Mikrogeophagus ramirezi*) (Delannoy *et al.*, 2013). In Thailand, GBS-Ib has been reported first in the climbing perch and Günther's walking catfish polycultured in southern Thailand (Klingklib & Suanyuk, 2017). The present study confirmed the pathology and mortality of climbing perch associated with GBS-Ib infection and in order to fulfil Koch's postulates and confirmed the previous finding that GBS-Ib was causative agent responsible for climbing perch mortality. Clinical signs of fish infected with GBS-Ib include lethargy, exophthalmia, corneal opacity, ascites, haemorrhage and erratic swimming. In this study, climbing perch infected with GBS-Ib-298 exhibited similar disease signs to those reported by Klingklib and Suanyuk (2017) and to those reported in tilapia cultured in Brazil and Malaysia (Abuseliana, Daud, Aziz, Bejo, & Alsaid, 2011; Mian *et al.*, 2009).

Study of the pathogenicity of GBS-Ib-298 in climbing perch indicated that the infected fish tend to die within the first week of infection, while less mortality was observed in the second week of observation. This is similar to tilapia infected with GBS-III, which has been reported previously (Suwannasang *et al.*, 2014). The acute disease progression following GBS-Ib infection and the high mortality indicate that GBS-Ib is a fish pathogenic bacterium.

Table 1. Mean haemato-immunological parameters on days 0, 1, 2, 3, 5 and 7 post infection, in climbing perch infected with GBS-Ib-298.

Parameter		Time (day)					
		0	1	2	3	5	7
Haematocrit (%)	Control	53.09±4.26^{a}	54.66±2.99^{a}	52.08±3.80^{a}	52.61±2.64^{a}	54.37±2.92^{a}	52.51±3.21^{a}
	Treatment	53.46±3.74^{a}	57.51±3.33^{a}	54.96±1.99^{a}	46.05±2.05^{b}	45.66±3.49^{b}	40.06±1.25^{b}
Haemoglobin (g/dL)	Control	15.52±1.41^{a}	15.91±4.39^{a}	16.78±2.15^{a}	15.28±1.18^{a}	16.13±1.52^{a}	15.28±2.10^{a}
	Treatment	16.20±1.82^{a}	21.55±2.26^{b}	16.33±1.02^{a}	15.85±1.85^{a}	13.05±1.58^{b}	12.25±0.82^{b}
Serum protein (mg/mL)	Control	100.00±12.24^{a}	100.12±8.65^{a}	91.08±21.35^{a}	104.80±13.48^{a}	108.33±7.34^{a}	104.40±7.85^{a}
	Treatment	100.28±17.48^{a}	86.04±6.23^{b}	88.89±16.33^{a}	95.73±9.87^{a}	94.44±8.94^{b}	90.00±6.77^{b}
Red blood cell	Control	5.02±0.48^{a}	5.20±0.36^{a}	5.18±0.78^{a}	5.20±0.39^{a}	5.12±0.31^{a}	4.80±0.42^{a}
($\times10^{9}$cell/mL)	Treatment	5.05±0.16^{a}	5.60±0.40^{a}	5.14±0.70^{a}	4.89±0.63^{a}	3.73±0.47^{b}	3.99±0.33^{b}
White blood cell	Control	2.94±0.53^{a}	2.50±0.66^{a}	2.39±0.30^{a}	2.47±0.87^{a}	2.92±0.37^{a}	2.85±0.31^{a}
($\times10^{7}$cell/mL)	Treatment	2.94±0.53^{a}	2.44±0.69^{a}	1.84±0.70^{a}	2.11±1.22^{a}	3.89±0.08^{b}	4.58±1.36^{a}
NBT reduction (OD)	Control	0.015±0.006^{a}	0.020±0.003^{a}	0.025±0.004^{a}	0.044±0.002^{a}	0.032±0.007^{a}	0.037±0.006^{a}
	Treatment	0.016±0.009^{a}	0.016±0.001^{b}	0.017±0.004^{b}	0.036±0.006^{b}	0.033±0.07^{a}	0.037±0.007^{a}
Lysozyme activity	Control	13.37±9.90^{a}	13.82±4.00^{a}	13.45±2.61^{a}	14.91±3.73^{a}	14.76±3.40^{a}	14.48±2.93^{a}
(µg/mL)	Treatment	13.51±3.42^{a}	14.63±3.03^{a}	19.09±2.82^{b}	24.31±4.11^{b}	35.93±2.39^{b}	32.04±5.15^{b}
Phagocytic activity (%)	Control	12.83±1.03^{a}	12.00±0.00^{a}	14.00±0.76^{a}	10.67±3.06^{a}	11.75±2.47^{a}	11.67±1.04^{a}
	Treatment	12.50±1.00^{a}	17.83±3.40^{a}	14.67±0.29^{a}	16.00±2.83^{a}	15.83±5.39^{a}	16.50±3.04^{a}
Phagocytic index	Control	2.16±0.33^{a}	2.14±0.45^{a}	2.35±0.46^{a}	1.72±0.70^{a}	1.75±0.77^{a}	1.87±0.32^{a}
	Treatment	2.17±0.02^{a}	6.06±0.02^{b}	4.55±0.28^{b}	5.84±2.01^{b}	3.67±2.27^{a}	3.75±1.07^{b}
Average bead per cell	Control	1.24±0.04^{a}	1.26±0.13^{a}	1.21±0.09^{a}	1.32±0.05^{a}	1.33±0.18^{a}	1.37±0.13^{a}
(bead/cell)	Treatment	1.28±0.11^{a}	1.65±019^{b}	2.11±0.05^{b}	1.94±0.11^{b}	1.40±0.10^{a}	1.38±0.21^{a}

Different letters within each time indicate a statistically significant difference ($p<0.05$)

Table 2. Effects of temperature and DO on mortality of climbing perch infected with GBS-Ib-298.

DO (mg/L)	Temperature (◦C)	Mortality (%)
1.73±0.21	28.97±0.12	86.67±5.77
	31.85±0.34	95.00±7.07
	34.07±0.45	96.67±5.77
3.77±0.14	28.97±0.12	76.67±15.28
	31.85±0.34	80.00±17.32
	34.07±0.45	80.00±10.00
5.52±0.21	28.97±0.12	56.67±5.77
	31.85±0.34	83.33±11.55
	34.07±0.45	83.33±5.77
Pooled mean square error		120.59
Mean of main effects		
DO (mg/L)	1.73±0.21	92.50±7.07^{b}
	3.77±0.14	78.89±12.69^{a}
	5.52±0.21	75.55±16.66^{a}
Temperature (◦C)	28.97±0.12	73.33±15.81^{x}
	31.85±0.34	86.25±14.07^{y}
	34.07±0.45	86.67±10.00^{y}
ANOVA: P value		
Temperature		0.032
DO		0.004
Temperature*DO		0.665

Other water parameters during experiment were as following: Ammonia was 0.35±0.42 mg/L, Alkalinity was 17.64±11.50 mg/L and pH was 6.58±0.59. Values with different letters are significantly different ($p<0.05$).

The results on haemato-immunological parameters of fish infected with GBS-Ib-298 support the study on pathogenicity. Most blood parameters i.e., haematocrit, haemoglobin, serum protein, red blood cell count and respiratory burst activity decreased after infection. Reduction of haematocrit, haemoglobin and red blood cell could be due to haemodilution, haemorrhage and red blood cell haemolysis (Harbell, Hodgins, & Schiewe, 1979) which resulted in an alteration of the ability of the blood to transport essential oxygen, thus decreased overall health of the fish (McNulty, Klesius, & Shoemaker, 2003). Similar results have been reported in tilapia intraperitoneally infected with GBS, in that the haemato-immunological parameters i.e., haematocrit, haemoglobin, protein, and red blood cell decreased during infection (Alsaid *et al.*, 2014; Suwannasang *et al.*, 2014). Although the time profiles of blood parameters reported by Suwannasang *et al.* (2014) differ from the present study, we suspect that this is due to different fish species and different concentrations of inoculums used. In this current study, however, increased non-specific immunity, i.e., lysozyme activity, white blood cell, phagocytic index, and average beads per cell, were observed in the fish infected with GBS-Ib-298. This finding supports the fact that innate immunity is one of the major modes of action of fish respond to a pathogen. Lysozyme is important in the defense against invasions by microorganisms. It cleaves the β(1→4) linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the Gram-positive bacterial cell wall (Salton & Ghuysen, 1959; Yano, 1996). Increased lysozyme activity in the GBS-Ib-298 infected climbing perch, in the present study, indicates that lysozyme is an important humoral component in combating infections caused by GBS-Ib in fish. Phagocytosis is the process where phagocytic cells internalize, kill and digest invading microorganisms (Secombes, 1996). Significantly increased white blood cell, phagocytic index, and average beads per cell in the infected fish of the present investigation, indicate pathogen killing activity by phagocytes as part of the innate immune system contributing to survival. White blood cells play an important role in fish cell-mediated immune response, which usually increase after infection. In this study, increased white blood cell counts after day 5 of bacterial infection are in agreement with the studies in tilapia intraperitoneally infected with GBS (Alsaid *et al.*, 2014;

Suwannasang *et al.*, 2014). However, low white blood cell on days 2 and 3 after infection observed in this study may be explained by the leucocyte migration to the site of infection as well as destruction by bacteria (Balfry, Shariff, & Iwama, 1997; Harbell *et al.*, 1979; Larmas, Santos, Bruno, Toranzo, & Anadon, 1994; Martins, Xu, Shoemaker, & Klesius, 2011).

In this study, high mortality of climbing perch infected with GBS-Ib-298 was observed in fish exposed to low DO with high temperature. The ability of GBS-Ib-298 to growth at 25-35°C in TSB and abnormal behavior of climbing perch observed at high temperature and low DO supports the high level of mortality of fish at high temperature and low DO. Several studies have focused on thermal and DO influence on susceptibility of fish to bacterial infection. Increased susceptibility to infection at low DO or high temperature conditions is reported in several aquatic animals including channel catfish (*Ictalurus punctatus*) experimentally infected with *Edwardsiella ictaluri* (Mqolomba & Plumb, 1992), Nile tilapia experimentally infected with GBS-Ia (Rodkhum *et al.*, 2011) and giant freshwater prawn experimentally infected with *Enterococcus*-like bacterium (Cheng & Chen, 1998). Previously, Farkas, Fodor, Kitajka, and Halver (2001) reported that lipid components of membranes respond immediately to the change of temperature. In order to maintain the structure and function of membrane under this condition, fish must maintain proper membrane fluidity in the new thermal environment. Nevertheless, it is important to understand the stress responses of fish to the change of DO levels. During hypoxic stress condition, metabolic adjustments may include reduction of energy metabolism, increase of the energy supply by shifting to anaerobic glycolysis, maintenance of oxygen supply to critical organs, and expression of stress-related proteins (Dunn & Hochachka, 1986; Hochachka & Somero, 1984). Alteration of the ability to maintain membrane fluidity and metabolic adjustment of fish under the change of temperature and DO conditions may lead to stress and susceptibility to infection.

To date, the preventive measures of streptococcosis in fish are based on good aquaculture farm management as well as vaccination program. Unfortunately, there is no effective vaccine against streptococcosis caused by GBS-Ib available to immunize climbing perch. Therefore, application of GBS-Ib vaccine in climbing perch need to be investigated.

5. Conclusions

The present study reported the epizootiology, pathogenicity and mortality of climbing perch associated with GBS-Ib infection. This bacterium grew from 25 to 35°C, pH 5 to 9 and 0.5 to 4.0% NaCl. Climbing perch intraperitoneally injected with GBS-Ib exhibited 60.00-96.67 % mortality, with 60 h-LD_{50} and 72-h LD_{50} at $4.5x10^2$ CFU/mL and $6.3x10^1$ CFU/mL, respectively. Infected fish revealed declines of haematocrit, haemoglobin, serum protein, red blood cells, and respiratory burst activity. On the other hand, white blood cells, lysozyme activity, phagocytic index, and average beads per cell, were significantly increased in the infected fish. Study on the effect of environmental factors on the susceptibility of fish to GBS-Ib infection indicated that high water temperature and low DO levels proved to increase mortality of climbing perch. The ability of GBS-Ib to survive in various environments, combined with their high pathogenicity and reduction of fish immunity indicate that this is a pathogenic bacterium responsible for fish mortality in Thailand.

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