อิทธิพลของอุณหภูมิน้ำต่อโรคสเตรปโตคอคโคซิสซึ่งเกิดจากเชื้อสเตรปโตคอคคัส อกาแลคติเอ้ในปลานิล (โอรีโอโครมิส เอสพี)

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INFLUENCE OF WATER TEMPERATURE TO STREPTOCOCCOSIS CAUSED BY STREPTOCOCCUS AGALACTIAE IN TILAPIA (OREOCHROMIS SP.)

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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พัฒนพล ขยันสำรวจ : อิทธิพลของอุณหภูมิน้ำต่อโรคสเตรปโตคอคโคซิสซึ่งเกิดจากเชื้อสเตรปโตคอคคัส อกาแลคติเอ้ในปลานิล (โอรีโอโครมิส เอสพี). (INFLUENCE OF WATER TEMPERATURE TO STREPTOCOCCOSIS CAUSED BY STREPTOCOCCUS AGALACTIAE IN TILAPIA (OREOCHROMIS SP.)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร. นพดล พิฆารัตน์, 164 หน้า.

โรคสเตรปโตคอคโคซิสซึ่งเกิดจากการติดเชื้อแบคทีเรียสเตรปโตคอคคัส อกาแลคติเอ้ถือเป็นอุปสรรคสำคัญต่ออุตสาหกรรมการผลิตสัตว์น้ำทั่วโลก เนื่องจากก่อให้เกิดอัตราการตายสูงได้ถึงร้อยละ 70 ภายในระเวลาเพียง 1-2 สัปดาห์ ปัจจุบันมีรายงานการระบาดของโรคจากสัตว์น้ำหลากหลายชนิดทั้งน้ำจืดและน้ำเค็มซึ่งปลานิลจัดเป็นหนึ่งในสัตว์น้ำที่มีความไวต่อโรคค่อนข้างสูง อย่างไรก็ดีความรุนแรงของโรคติดเชื้อในสัตว์น้ำนั้นขึ้นอยู่กับปัจจัยแวดล้อมหลายชนิด อาทิเช่น ขนาดปลา ความหนาแน่นในการเลี้ยง คุณภาพน้ำ และอุณหภูมิ ซึ่งจากรายงานการศึกษาจำนวนมากพบว่าการเปลี่ยนแปลงของสภาพอากาศโดยเฉพาะอุณหภูมิที่เพิ่มสูงขึ้นนั้นมีความสัมพันธ์ต่อการเกิดการระบาดของโรคอย่างมีนัยสำคัญ ด้วยเหตุนี้เป้าหมายหลักของวิทยานิพนธ์ฉบับนี้จึงมุ่งศึกษาถึงผลกระทบจากการเปลี่ยนแปลงอุณหภูมิที่มีต่อโรคติดเชื้อสเตรปโตคอคคัสและการตอบสนองทางสรีระของเชื้อแบคทีเรียและปลานิล

เชื้อสเตรปโตคอคคัส อกาแลคติเอ้ที่ใช้ในการศึกษาได้จากการเพาะแยกเชื้อจากปลานิล ตัวอย่างน้ำและดินจากแหล่งเพาะเลี้ยงในประเทศไทยตั้งแต่ปี พ.ศ. 2552-2555 ทั้งจากในช่วงที่มีภาวการณ์ระบาดของโรคและในสภาพการเลี้ยงปกติ โดยอัตราการเพาะแยกเชื้อสเตรปโตคอคคัสจากสิ่งแวดล้อมในแต่ละเดือน (น้ำและดิน) พบได้ตั้งแต่ที่ร้อยละ 13-67 และสามารถเพาะแยกเชื้อสเตรปโตคอคคัสอกาแลคติเอ้ที่มีได้เป็นจำนวน 60 ไอโซเลท (ยืนยันผลการวินิจฉัยด้วยวิธีพีซีอาร์ที่มีความจำเพาะต่อเชื้อ) ซึ่งทั้งหมดมีความสามารถในการย่อยสลายเม็ดลือดแดงแบบสมบูรณ์ (เบต้าฮีโมไลซิส) เมื่อทำการจำแนกคุณลักษณะของเชื้อด้วยวิธีทางอณูชีววิทยาพบว่าเชื้อ 59 ไอโซเลทจัดอยู่ในซีโรไทป์ Ia ส่วนอีกหนึ่งไปโซเลทจัดอยู่ในซีโรไทป์ III ซึ่งเมื่อพิจารณาร่วมกับผลการตรวจสอบลำดับนิวคลีโอไทด์ของยีน infB การตรวจสอบหาไวรูเลนซ์ยีนด้วยวิธีพีซีอาร์และวิธี RAPD ก็พบว่ามีความหลากหลายทางพันธุกรรมในกลุ่มเชื้อเสตรปโตคอคคัส อกาแลคติเอ้ที่เพาะแยกได้ในการศึกษานี้ ซึ่งความหลากหลายดังกล่าวมีแนวโน้มเกี่ยวพันกับสถานที่ที่ทำการเก็บตัวอย่างเชื้อ และลักษณะทางพันธุกรรมของเชื้อที่เพาะแยกได้จากปลานิลและสิ่งแวดล้อมจากแหล่งเพาะเลี้ยงนั้นมีความแตกต่างจากเชื้อที่เพาะแยกจากมนุษย์และโคนมอย่างมีนัยสำคัญ จากการทดสอบความสามารถในการก่อโรคของเชื้อด้วยวิธีการฉีดเชื้อเข้าช่องท้องของปลานิลพบว่าเชื้อจากปลานิลและสิ่งแวดล้อมสามารถก่อโรคได้โดยมีค่า LD50 เท่ากับ 6.25-7.56 log CFU (เชื้อที่มีความรุนแรงในการก่อโรคสูงสุดจะนำไปใช้ในการทดลองขั้นถัดไป)

การเปรียบเทียบคุณสมบัติในการก่อโรคของเชื้อในสภาพอุณหภูมิ 28 และ 35 องศาเซลเซียส พบว่าที่อุณหภูมิสูงเชื้อจะสามารถเพิ่มจำนวนได้อย่างรวดเร็ว สามารถย่อยสลายเม็ดเลือดแดงได้ดีกว่า สร้างแคปซูลได้ในจำนวนมากกว่าและสามารถมีชีวิตอยู่ได้ในเลือดปลานิลได้ดีกว่า การศึกษาการเปลี่ยนแปลงของการแสดงออกของยีนด้วยวิธีเรียลไทม์พีซีอาร์ก็ให้ผลที่สอดคล้องกัน โดยในภาวะอุณหภูมิสูงนั้นเชื้อจะมีการแสดงออกของยีนที่มีความเกี่ยวข้องกับการก่อโรคเพิ่มขึ้นเป็นจำนวน 9 ยีน (จากที่ทำการศึกษาทั้งสิ้น 13 ยีน) ซึ่งมียีนจำนวนสามยีนอันประกอบด้วย cylE (เบต้าฮีโมไลซิน), cfb (CAMP factor) และ PI-2b (พิลไล) ที่มีการแสดงออกเพิ่มขึ้นในปริมาณถึง 15-40 เท่าเมื่อเทียบกับภาวะอุณหภูมิต่ำ

จากการทดสอบนำปลานิลที่ฉีดเชื้อเข้าช่องท้องมาแยกเลี้ยงในสภาพที่มีอุณหภูมิน้ำที่ 28 และ 35 องศาเซลเซียส พบว่าในสภาพอุณหภูมิสูงนั้นปลานิลมีอัตราการตายสูงถึงร้อยละ 85 (ในขณะที่อุณหภูมิต่ำพบเพียงร้อยละ 45) และยังสามารถเพาะแยกเชื้อจากสมองของปลาป่วยได้มากเป็นจำนวนถึง 1000 เท่าเมื่อเทียบกับอุณหภูมิต่ำ นอกจากนี้ยังตรวจพบการเพิ่มขึ้นของการแสดงออกของยีนที่มีความเกี่ยวข้องกับการอักเสบ (COX-2, IL-1β และ TNF-α) นับตั้งแต่เวลา 6 ชั่วโมงจนถึง 96 ชั่วโมงหลังจากติดเชื้อ โดยมีการแสดงออกสูงถึงมากกว่า 40 เท่า ซึ่งแสดงให้เห็นว่าปลามีการอักเสบอย่างรุนแรงหากอยู่ในสภาพที่มีอุณหภูมิสูง อย่างไรก็ดีการทดสอบการแสดงออกทางภูมิคุ้มกันอื่นๆ อาทิเช่น จำนวนเม็ดเลือดขาว ความสามารถในการกำจัดเชื้อแบคทีเรียของซีรัม และการแสดงออกของยีน TGF-β นั้นไม่พบว่ามีความแตกต่างแต่อย่างใด โดยสรุปการศึกษานี้แสดงให้เห็นว่าโรคสเตรปโตคอคคัสในปลานิลอันเกิดจากการติดเชื้อสเตรปโตคอคคัส อกาแลคติเอ้มีความรุนแรงเพิ่มขึ้นในสภาวะอุณหภูมิสูง ซึ่งสภาพดังกล่าวก่อให้เกิดการอักเสบอย่างรุนแรงในปลาและอาจเป็นสาเหตุให้เกิดการตายอย่างเฉียบพลัน

ภาควิชา พยาธิวิทยา ลายมือชื่อนิสิต .....

สาขาวิชา พยาธิชีววิทยาทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2556 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS: MOLECULAR CHARACTERISTIC / STREPTOCOCCUS AGALACTIAE / TEMPERATURE / TILAPIA / VIRULENCE

PATTANAPON KAYANSAMRUAJ: INFLUENCE OF WATER TEMPERATURE TO STREPTOCOCCOSIS CAUSED BY STREPTOCOCCUS AGALACTIAE IN TILAPIA (OREOCHROMIS SP.). ADVISOR: ASST. PROF. DR. CHANNARONG RODKHUM, CO-ADVISOR: ASSOC. PROF. DR. NOPADON PIRARAT, 164 pp.

Streptococcus agalactiae is one of the most important pathogen affecting aquaculture business worldwide. Various warm-water fish species were reported to be infected by S. agalactiae but one of the most vulnerable species is tilapia (Oreochromis sp.). Presently, tilapia farming has been recognized as the most valuable fresh-water cultured fish industry in Thailand. Streptococcosis associated with S. agalactiae infection can produce high mortalities up to 70% according to acute septicemia condition within only short period as 1-2 weeks. However, the susceptibility to the infection can be affected by several factors such as fish size, stocking density, pH and temperature. Currently, the information obtained from both field and experimental studies were mentioning on the closely relationship between an increasing of temperature and occurrence of streptococcosis and its virulence. Generally, shifting of water temperature not only affect to the fish physiology but also to the pathogenic organisms. Therefore, the main objective of this study is to elucidate the effect of temperature to the tilapia and S. agalactiae by studying their physiological responses in parallel and observing the outcome of disease in different temperature condition.

Streptococcus were obtained from the fish and environmental samples (such as mud and pond water) in tilapia farms in Thailand during 2009 to 2012. Samples were collected during both disease outbreak and disease-calming situation. The relative percent recovery of streptococcus from environmental samples was 13-67%.

Totally 60 isolates of streptococcus were found and all of them were identified as β-hemolytic S. agalactiae using standard biochemical assays and species-specific PCR. Molecular serotyping of S. agalactiae revealed that 59 isolates belong to type Ia while only 1 isolates were type III. Additional genotypic analyses (infB allelic assay, virulence genes profiling and RAPD) of S. agalactiae suggested a large diversity among S. agalactiae strains which is tend to be geographically dependent. Comparison of genotypic characteristic with other strains of S. agalactiae isolated from mammalian host showed distinct genetic of fish-originated streptococcus apart from human and bovine strains. Experimental infection via intraperitoneal injection of S. agalactiae to Nile tilapia revealed that both fish and environmental strains were pathogenic, with 6.25-7.56 log CFU LD50. Due to this LD50 analysis, we select for the highest virulence strain to conduct the experiment in the next step.

An increasing of cultured temperature of S. agalactiae tremendously enhances in vitro pathogenicity as expected. Comparison between S. agalactiae cultured in 35 ^{0}C and 28 ^{0}C difference temperature suggested that the bacterium can growth faster, producing more hemolysin, surface-capsular polysaccharide and tolerate to tilapia whole blood when high temperature condition was applied. The expression analysis of putative virulence genes by qPCR also revealed the concordance result as up-regulation of 9 genes (from 13 genes included in the test) in 35 ^{0}C condition comparing with 28 ^{0}C. Among these virulence genes, three were exhibited massive up-regulation with more than 15 to 40 folds increased. Theses 3 genes comprised of cylE, cfb and PI-2b which encoded for β-hemolysin/cytolysin, CAMP factor and pili backbone, respectively.

Susceptibility of S. agalactiae infected Nile tilapia reared in hot (35 ^{0}C) and normal (28 ^{0}C) water temperature were determined from experimental infection analysis. The accumulated mortalities over 14 days period was 85% and 45% for hot and normal condition, respectively. Higher numbers of streptococcus were enumerated from the brain of infected tilapia kept in hot condition with 1000 times higher than normal condition. Abundant up-regulation of inflammatory related genes (COX-2, IL-1β and TNF-α) had been detected since 6 hpi until 96 hpi, which indicated for massive inflammation of the fish in high temperature condition. In conclusion, our study suggested that the increasing of S. agalactiae pathogenicity due to the high temperature condition can leads to massive inflammatory response of infected Nile tilapia and producing acute mortality.

Department:	Veterinary Pathology	Student's Signature	----------
Field of Study:	Veterinary Pathobiology	Advisor's Signature	----------
Academic Year:	2013	Co-Advisor's Signature	----------

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Significance and rationale of research

Streptococcus sp. is the cause of streptococcosis in fishes. Up to this date, streptococcosis is recognized as major infectious disease producing significant economic loss in aquaculture industries globally. Various species of the fish were reported as susceptible host for streptococci including striped bass, mullet and tilapia. In Thailand, tilapia is regarded as one of the most important aquaculture with the highest production so far and the two species of streptococcus, *S. iniae* and *S. agalactiae,* have been reported to be associated with streptococcosis outbreaks in the farms (Suanyuk et al., 2005; Maisak et al., 2008; DOF, 2010).

The conventional serological characterization of *S. agalactiae* (also called 'Group B streptococcus') can classified the bacterium as 10 serotypes due to antigenic property of capsular polysaccharide (Slotved et al., 2007). Distribution of *S. agalactiae* serotypes in particular location is considered as one of the most important epidemiological data since several publications indicating *S. agalactiae* capsule as the major protective antigen but, unfortunately, the cross-reactivity of host antibody against heterologous serotypes was very limited (Paoletti et al., 1994; Lindahl et al., 2005). Therefore, the surveillance of *S. agalactiae* serotypes could be helpful in order to design the protection policy against streptococcsis such as vaccination (Lindahl et al., 2005). Furthermore, the information about serotypes distribution had been applied for designing of the composition of polyvalent vaccine against human *S. agalactiae* (Paoletti et al., 1994; Lindahl et al., 2005; Edwards, 2008). In the case of fish *S. agalactiae*, serotype Ia, Ib and III have been reported to be isolated from diseased fish and dolphin but only type Ia and III were isolated from in Thailand (Suanyuk et al., 2008). Presently, only a few studies about genetic variation of *S. agalactiae* in Thailand were available. Thus, it should be the first priority to investigate the genetic variation of *S. agalactiae* inhabited in Thailand.

Although massive mortality associated with *S. agalactiae* has been recognized as the major obstacle for fish farming for years, the pathogenesis of streptococcosis in fish is not well understood. Nowadays, almost information had relied on the

previous studies conducted in mammalian models. However, the pathogenic mechanisms of infectious disease in aquatic animal, including streptococcosis, generally considered as far more complicated than the mammals because the environmental factors always play extremely critical roles in diseases development in aquatic animal (Marcogliese, 2008). Likewise, for streptococcosis, several scientific evidences indicated that the occurrence, frequency and severity of disease were largely associated with inappropriate environments, especially high water temperature (Shoemaker et al., 2000; Bromage and Owens, 2009; Mian et al., 2009). Host stressful respond upon the harsh environments was prompted to be major predisposing cause of streptococcosis in fish farms (Yanong and Francis-Floyd, 2010). Additionally, water temperature not only affects the host, but also the pathogen. The increasing of temperature can directly induces the virulence of streptococci which was demonstrated in recent study that the up-regulation of several virulence genes of *S. agalactiae* was occurred in 40 °C condition comparing with 37 °C (Mereghetti et al., 2008b). Nevertheless, the environmental conditions affecting disease occurrence are entirely different between human and aquatic animal. Therefore, the information referred from human *S. agalactiae* might not be enough to explain how streptococcosis emerged in fish farms.

Since the previous publications mentioned about the close relationship between streptococcosis outbreaks and the high temperature (Mian et al., 2009; Rodkhum et al., 2011; Amal et al., 2013b), the objectives of this study were aimed to investigate on the responses of *S. agalactiae* and tilapia upon an increasing of water temperature.

The results acquired from could be the important information especially in the aspect of epidemiology and pathogenesis of *S. agalactiae* in tilapia which may provide a new lead to the novel protection strategiy against for streptococcosis in tilapia farms in Thailand.

Objectives of study

The main objectives of this study are to investigate the intraspecies variation of *S. agalactiae* isolated from tilapia farms in Thailand and to investigate the effect of temperature to the occurrence and pathogenicity of streptococcosis associated with *S. agalactiae* infection in tilapia. To accomplish our objectives, three stages of the study had been addressed.

1. Characterization of phenotype and genotype of *S. agalactiae* isolated from tilapia farms in Thailand.
2. Determination of the effects of water temperature to virulence of *S. agalactiae*.
3. Determination of the effects of water temperature to immune parameters of tilapia infected with *S. agalactiae*.

Chapter 1: Literature review – infection of fish pathogen *S. agalactiae* and general immunology in fish

1. Tilapia

Tilapia is a common name of the freshwater food-fish which belongs to the family *Cichlidae*. The name 'Tilapia' was originated from native African word '*thiape'* which means fish (Chapman, 1992). Tilapias are tropical fish, native to Africa and the Middle East. They are classified into three important generas, i.e. *Tilapia, Sarotherodon* and *Oreochromis,* according to their reproductive behavior, but only the genus *Oreochromis* has been developed as aquacultured animal and *Oreochromis niloticus* (Nile tilapia) is one of the most popular species which has been employed as a food sources for communities by aquaculture industries worldwide (Popma and Masser, 1999). To date, tilapia is regarded as one of the top-three highest production of global freshwater aquaculture (Popma and Masser, 1999; FAO, 2012). Nile tilapia was introduced to Thailand in 1965 from Japan (FAO, 2010) and, since then, it has been emerged as the major freshwater aquaculture in this country. Presently, the production of tilapia (including both Nile and hybrid tilapia) in Thailand had increased annually from 259,700 tonnes in 2000 to 485,100 tonnes in 2008, the highest among freshwater aquacultured fishes in Thailand (DOF, 2010).

Tilapia have many attractive characteristics, making them suitable for farming. They are omnivorous - eating wide variety of natural food organisms as well as artificial food. They can tolerate poor water qualities including high water temperature, high water salinity, low dissolved oxygen and high concentration of ammonia, and also barely tolerate the stress resulting from high stocking density (Siddiqui et al., 1989; Popma and Masser, 1999). They grow and attain market size rapidly in warm-water temperature such as in tropical country. Finally, they are palatable (FAO, 2006). Moreover, tilapia are more resistant to diseases associated with bacterial, virus or parasite infestation comparing with other cultured fish species (Popma and Masser, 1999). Due to palatability, tilapia markets all over the world have expanded in recent years. Tilapia culture has become the most important

aquaculture crop in many countries including Thailand. In Thailand, culturing system for juvenile tilapia can be divided roughly due to the culturing habitat as earthen pond and river floating-cage. Each particular culturing system has different advantages and disadvantages concerning with cost benefit, disease resistance and production capacity. Nowadays, tilapia often raise in the highly intensive culturing system in order to maximize the production with the limited unit surface area (Popma and Masser, 1999). Recently, the male-monosex culture is replacing the conventional polysex culture since the growth performance of male tilapia is better than female for double (Rakocy and McGinty, 1989; Popma and Masser, 1999). However, several strategies, i.e. intensive culture, monosex culture or feeding with high-protein food, using to promote the production have detrimental consequences as it can increase the susceptibility of the fish to several infectious pathogens. It is a critical challenge for the intended workers and technicians in tilapia culture business in order to maintain the maximum production in parallel with the healthiness of animal.

Figure 1 Red tilapia (*Oreochrois* sp.)

2. Basic immunology of the fish

Fish is the animal which life-long exposed to the free-living microorganisms (both non-pathogenic and pathogenic) inhabited in their natural habitat. The immune system of the fish plays a role in keeping of the homeostasis of animals' body by preventing colonization of the surrounded pathogens. Generally, the physiological mechanism of immune response in the fish is similar to higher vertebrates, despites some different in lymphoid organs and the functional proteins (Ellis, 2012). In contrast to mammalian species, disease resistance of the fish rely largely on innate immune system due to the limited functionality of acquired immunity (Aoki et al., 2008; Uribe et al., 2011). The innate immune system of the fish is highly developed and more advance than mammalian animal in some aspects such as the large variety of complement isoforms are presented in the fish (Plouffe et al., 2005). Anti-pathogenic strategies of innate immunity of the fish begin with the intact mucosal/epithelial barrier at the skin, gill and alimentary tract. The mucus released in epithelial surface contains myriad humoral substance such as lectins, complement proteins, antimicrobial peptide (AMP) and IgM, which involved in blocking of pathogenic entries. Humoral compartments of innate immunity also found in all kind of body fluid including plasma and mucus which play the important roles in systemic protection against invading pathogens. The infection in fish will induce the releasing of proinflammatory cytokines, mostly are TNF-α and IL-1β, then subsequently followed by the recruitment and activation of phagocytic cells. The major phagocytes in fish are macrophages and neutrophils, similar to higher vertebrate. Another cells involved in cellular innate immune response exclusively found in fish is called nonspecific cytotoxic cells (NCC). The NCC is responsible for the elimination of bacterial and viral infected cells using phagocytosis independent mechanisms which could refer to NK cells in mammal.

Even fish innate immunity is extensively sophisticated, on the other hand, the biological function of acquired immunity is less developed. Acquired immune response of the fish has limited repertoire of immunoglobulin (Ig) while proliferation and maturation of lymphocyte are rather slow (Aoki et al., 2008; Uribe et al., 2011). The primary Ig found in the fish is IgM. The other Ig that can be found in the lesser

extent is IgD and IgZ/T (Kaattari et al., 2009). Both type of lymphocytes (B- and T-cells) are present in the fish including the subset of T lymphocyte such as Th1, Th2 and cytotoxic T-cell. The existence of regulatory T-lymphocytes (T_{reg}) in the fish has been proposed since several transcription factors and cytokine produced from T_{reg} (FoxP3, IL-10 and TGF-ß) were discovered recently, though an attempt to isolates T_{reg} cells is still unsuccessful (Secombes, 2008).

3. Streptococcis in fish

The opportunistic bacterial infection in aquatic animal normally caused by Gram negative bacteria such as *Aeromonas* sp., *Flavobacterium* sp., *Edwardsiella* sp (Yanong and Francis-Floyd, 2010). The Gram positive pathogens in terrestrial mammalian animal such as *staphylococcus* sp., *listeria* sp., *clostridium* sp. and *bacillus* sp. are hardly associated with massive mortality in aquatic animal. However, the Gram positive *Streptococcus* sp. is an exception since it has been proved to be the cause of heavy mortality in aquacultures worldwide (Amal and Zamri-Saad, 2011). Generally, the word 'streptococcosis' represents to a disease causing by the infection of streptococcus or related bacteria (Yanong and Francis-Floyd, 2010). Numerous species of bacteria have been reported as the cause of streptococcosis (table 1), for the example *S. dysgalactiae, S. focae, Lactococcus garviae, Enterococcus* sp.., *Vagococcus* sp., however, the most important species responsible for warm-water streptococcosis are *S. inae* and *S. agalactiae* (Evans et al., 2006; Yanong and Francis-Floyd, 2010). *Streptococcus iniae* was reported firstly in captive Amazon fresh water dolphin in 1976 and, since then, this bacteria have been isolated so far from many marine and fresh water fish including ayu, barramundi, coho salmon, European seabass, grey mullet, grouper, rainbow trout, red drum, snapper, silver bream, tilapia, and yellowtail (Eldar et al., 1995; Zlotkin et al., 1998; Bromage et al., 1999; Ferguson et al., 2000; Bromage and Owens, 2002; Abuseliana et al., 2010). For *S. agalactiae*, it was originated nearly a century ago from mastitis cow (Martinez et al., 2001). During a long period of dynamic evolutional history, *S. agalactiae* has been recognized as a wide-host range pathogen because of its capability to infect and cause sickness in several host species including human and fishes. The fish

originated *S. agalactiae* had been isolated for first time from rainbow trout in Japan since 1966 (Hoshina et al., 1958).

Table 1. 1 Causative agents of streptococcosis in fish

Bacterial species	First isolation	Location	References
Streptococcus agalactiae	Rainbow trout (*Oncorhynchus mykiss*); 1958	Japan	(Hoshina et al., 1958)
Streptococcus iniae	Amazon freshwater dolphin (*Inia geoffrensis*); 1976	USA	(Pier and Madin, 1976)
Streptococcus dysgalactiae	Cultured amberjack (*Seriola dumerili*)	Japan	(Nomoto et al., 2004)
Streptococcus parauberis	Cultured turbot (*Scophthalmus maximus*); 1994	Spain	(Toranzo et al., 1994)
Streptococcus ictaluri	Channel Catfish (*Ictalurus punctatus*)	USA	(Shewmaker et al., 2007)
Streptococcus phocae	Atlantic Salmon (*Salmo salar*); 2005	Scotland	(Gibello et al., 2005)
Lactococcus garviae	eel (*Anguilla japonica*) and yellow tail (*Seriola quinqueradiata*); 1974	Japan	(Kusuda et al., 1991)
Vagococcus	Rainbow trout	USA	(Wallbanks et al.,

Bacterial species	First isolation	Location	References
salmoninarum	(*Oncorhynchus mykiss*); 1968		1990)

Infection of streptococci can cause severe acute septicemia condition which may brought about 50% (or higher) of cumulative mortality within 3-5 days. However, consecutively loss of animals over a several weeks may occur in the case of chronic infection (Yanong and Francis-Floyd, 2010). Normally, streptococci can maintain their viability in the environments as opportunistic pathogen without causing disease. To gain a successful infection, the pathogen needs more than one pre-disposing factors to take an advantage above the natural defense mechanisms of the fish. The dynamic interplay between host immunity and bacterial pathogenicity will lose its balance when the environments have changed into an inappropriate range. Several environmental factors, such as high salinity and alkalinity (pH>8), low dissolved oxygen concentration, poor water quality (such as high ammonia or nitrite concentration), high stocking densities; have been involved with the development of diseases (Chang and Plumb, 1996; Shoemaker et al., 2000; Yanong and Francis-Floyd, 2010). However, considered from numerous scientific evidences, water temperature is believed to be the most potent factors contributed with the prevalence and severity of streptococcosis (Bromage and Owens, 2009; Rodkhum et al., 2011). The optimal temperature (29 to 31 ^{o}C) is keeping tilapia to maintain their best physiological and reproductive performances thus, vice versa, the immunological response of the fish cannot efficiently operate in the inappropriate condition which consequently increases the susceptibility of the animal to the opportunistic pathogen (Popma and Masser, 1999). According to previous publications, the mortality caused by *S.iniae* infection was highest when water temperature attained 25-28 ^{o}C, while the predisposing condition for *S. agalactiae*was about 31 ^{o}C (Bromage and Owens, 2009; Rodkhum et al., 2011).

The most common clinical signs of streptococcosis are exopthalmia (also known as 'Pop-eye') and erratic swimming, while the others such as darken skin, haemorrhages at the gill plate, loss of appetite, spine displacement, haemorrhages in the eye, corneal opacity, and hemorrhage at the base of the fins and in the opercula, are occasionally found (Salvador et al., 2005; Yanong and Francis-Floyd, 2010). The infected fish usually exhibit the plenty of internal lesions such as blood-tinged fluid in the body cavity, enlarged spleen, liver, kidney and endocarditis. Nevertheless, the diagnoses of streptococcosis is rather tentative since the bacterial sepsis caused by other pathogens could produce closely similar signs and lesions (Austin and Austin, 2007).

In septicemia condition, streptococcal infected fish are dying from vital organ failures (Neely et al., 2002; Yanong and Francis-Floyd, 2010). Streptococcus have an extraordinary capability to invade hosts' blood brain barrier (BBB) in numerous manners. Translocation of bacterium through BBB into the brain tissue can be accomplished without damaging the endothelial cells due to the 'transcytosis' and 'Trojan horse' (using host macrophages as a carrier) mechanisms or passing directly by destroying the host endothelial cells (Zlotkin et al., 2003; Maisey et al., 2008a). Brain parenchymal tissues of diseased fish are subsequently damaged by the streptococcal producing cytolytic enzymes or could be damaged from inflammatory response of the host itself, which, finally, leads to the hemorrhagic meningoencephalitic state (Neely et al., 2002; Miller and Neely, 2004).

The issue of streptococcal transmission in aquatic animal has been investigated for a long period of time and, in the recent study, it was hypothesized that the dissemination of bacteria mostly rely on horizontal transfer from the carrier host (Amal and Zamri-Saad, 2011). The pathogenic streptococci can be introduced in to fish farms via the import of carrier fish into the farms (Yanong and Francis-Floyd, 2010). Additionally, transmission of streptococcus from wild- to captive-animal is also concerned to be the important cause of disease outbreak in the farms as it had been reported in the cultured seabream in Kuwait (Evans et al., 2002). This infected fish can excrete viable streptococcus into the environments and the infectivity of bacteria is continuing for weeks in the nutrient-deprived water (Nguyen et al., 2002).

The healthy fish will get infected directly via the mucosal surface and the susceptibility to the infection will be elevated if the fish have wounds or abrasive epidermis (Xu et al., 2007). Furthermore, the zoonotic potential of fish originated streptococcus is dramatically concerned since the cases of human infection by fish pathogens have been reported periodically (Weinstein et al., 1997; Novotny et al., 2004). The workers in fish farms and food processing facilities are recognized as greatest risk-population whom might get infected from streptococcal-contaminated carcass via the cut and puncture wound that could happen during the handling process (Novotny et al., 2004). The infected patients were subsequently have cellulitis lesion, however, in some cases, the lethal septicemia conditions (such as endocarditis, septic arthritis) could be found, albeit this severe illness are rarely occur and it mostly found only in the immunocompromised cases such as elder, diabetes and cancerous patient (Weinstein et al., 1997; Novotny et al., 2004). On the other hand, the reverse zoonotic infection of streptococcus was also considered to be possible and might be responsible for streptococcosis outbreaks in tilapia farms (Evans et al., 2009).

4. Taxonomy and characteristic of *Streptococcus agalactiae*

Streptococcus agalactiae is a Gram positive, encapsulated, cocci, non-spore forming and β-hemolysis bacteria with 0.5-1 μm in diameter. They grow in pair or chain like any other streptococci. For the biochemical characteristics, the bacterium gives negative result for catalase, oxidase and motility test and usually positive for CAMP test (Ross, 1984). The phenotypic/biochemical characteristics of *S. agalactiae* and other related bacteria are demonstrated in Table 1.2. *Streptococcus agalactiae* might be classified as 'fastidious bacteria' because they grow very well in micro-aerophilic condition with limited carbohydrate concentration. Without supporting reagents such as blood, serum or cerebrospinal fluid in culturing media, the proliferation of *S. agalactiae* is poor (Hardie and Whiley, 2009).

Table 1. 2 Phenotypic characteristics of fish originated *S. agalactiae* and related bacteria

Test	*S. agalactiae*		Other fish streptococcus		
	(Duremdez et al., 2004)	(Abuseliana et al., 2010)	*S. iniae* (Bromage et al., 1999)	*S. parauberis* (Domeénech et al., 1996)	*L. garvieae* (Vendrell et al., 2006)
Gram's stain	+, cocci	+, cocci	+, cocci	+, cocci	+, cocci
Haemolysis	β	β	β	α	β
Catalase	-	-	-	-	-
Oxidase	-	-	-	-	-
Motility	-	nr	-	+	-
O/F	nr	nr	nr	nr	F
Voges-Proskauer	-	nr	nr	nr	+
Starch hydrolysis	-	nr	+	-	-
Growth in 6.5% NaCl	-	-	-	-	+
Esculin	-	-	+	+	+
Hippurate	+	nr	-	v	-
Carbohydrate utilization					
Trehalose	+	+	+	+	+
Raffinose	-	-	-	-	-

Test	*S. agalactiae*		Other fish streptococcus		
	(Duremdez et al., 2004)	(Abuseliana et al., 2010)	*S. iniae* (Bromage et al., 1999)	*S. parauberis* (Domeénech et al., 1996)	*L. garvieae* (Vendrell et al., 2006)
Mannitol	-	-	+	+	+
Lactose	-	-	-	+	+
Galactose	nr	nr	nr	nr	+
Salicin	nr	nr	nr	nr	nr
Sucrose	+	+	+	nr	v

nr = not reported, v = variable

Streptococcus agalactiae has been called as a 'wide-host range pathogen' owing to its ability to produce disease in a variety of mammalian and aquatic animal species. Neonatal meningitis in human, mastitis in dairy cows, and meningoencephalitis in fish were recognized as important life threatening and economically important disease caused by *S. agalactiae* (Hardie and Whiley, 2009).

Fish originated *S. agalactiae* can be divided roughly into biotype I and II due to their hemolytic appearance on blood containing medium. Biotype II is non-hemolysis while biotype I is ß-hemolysis. In the past, *S. agalactiae* biotype II was specified as *S. difficilis* but their scientific nomenclature had been changed to *S. agalactiae* afterwards because of the similarities of their genotypic characteristics (Kawamura et al., 2005).

According to the antigenic properties of the capsular polysaccharide, ten serotypes of *S. agalactiae* were classified as serotype Ia, Ib, II to X (Lindahl et al., 2005; Slotved et al., 2007). Capsular typing was proposed as the standard system for the identification *S. agalactiae* in sub-species level since the capsular serotypes were particularly related with the pathogenicity of streptococci (Lindahl et al., 2005). For the example, the serious invasive streptococcosis were often produced by serotype

Ia, III and V infection, while serotype VI and VII were mostly found as normal flora (Lindahl et al., 2005). In addition, the serotypes distribution is accounted as the highly important epidemiological data because it practically useful in designing the appropriate protection policy, including vaccination, against *S. agalactiae* in each particular region (Heath, 2011).

5. Virulence factors of *Streptococcus agalactiae*

5.1. Capsule

Polysaccharide is one of the potent antigens produced by broad-range of Gram negative and Gram positive bacteria. Microorganisms can generate polysaccharide as a surface-coverage substance attached to their cell-wall or released in to surrounded environment which called 'capsular and extracellular polysaccharide' respectively. This antigen is composed of the repeat of various monosaccharide joined together in complicated fashion making diversity in its antigenic property. In general, bacterial polysaccharide has numerous biological functions as a survival factor by preventing desiccation and keeping morphological structures of bacterial cell, enhancing the host-adherence activity and impedes host immune responses (Roberts, 1996).

Streptococcus agalactiae and other streptococcus can produce antiphagocytic capsules which play an important role in disease pathogenesis (Yoshida et al., 1996). Capsular polysaccharide (CPS) of *S. agalactiae* is comprised of the repetitive units of polysaccharide and sialic acid presenting as a terminal sugar. Sialic acid consisted in the polysaccharide side chain distinguishes *S. agalactiae* CPS from other streptococcal capsular polysaccharides (Cieslewicz et al., 2005). The antigenic property of *S. agalactiae* CPS divided this bacterium into 10 capsular serotypes (Ia, Ib, II to IX) according to the variations of monosaccharide arrangement (Slotved et al., 2007). Some monosaccharide components, such as glucose, galactose and N-acetylmuramic acid, are conserved in all *S. agalactiae* serotypes while N-acetylglucosamine and rhamnose can be found specifically in the repetitive units of serotype VI and VIII (Cieslewicz et al., 2005). The carbohydrate polymerase activity of the streptococci has a potent role in the construction of CPS as it can determine the polysaccharide linkage positions and, in turn, generate diversification in streptococci

serotypes (Yamamoto et al., 1999; Cieslewicz et al., 2005). In serotype Ia, a type-specific CPS is constructed from the repeat of pentasaccharide 4)-[α-D-Neu*p*NAc-(2→3)-β-D-Galp-(1→4)-β-D-Glc*p*NAc-(1→3)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1 (Yamamoto et al., 1999). The CPS structure of serotype Ia is nearly identical to serotype III with only exception at the position of glycosidic linkage (Cieslewicz et al., 2005). The structural diagram of polysaccharide repeat units of serotype Ia and the others are demonstrated in figure 2.

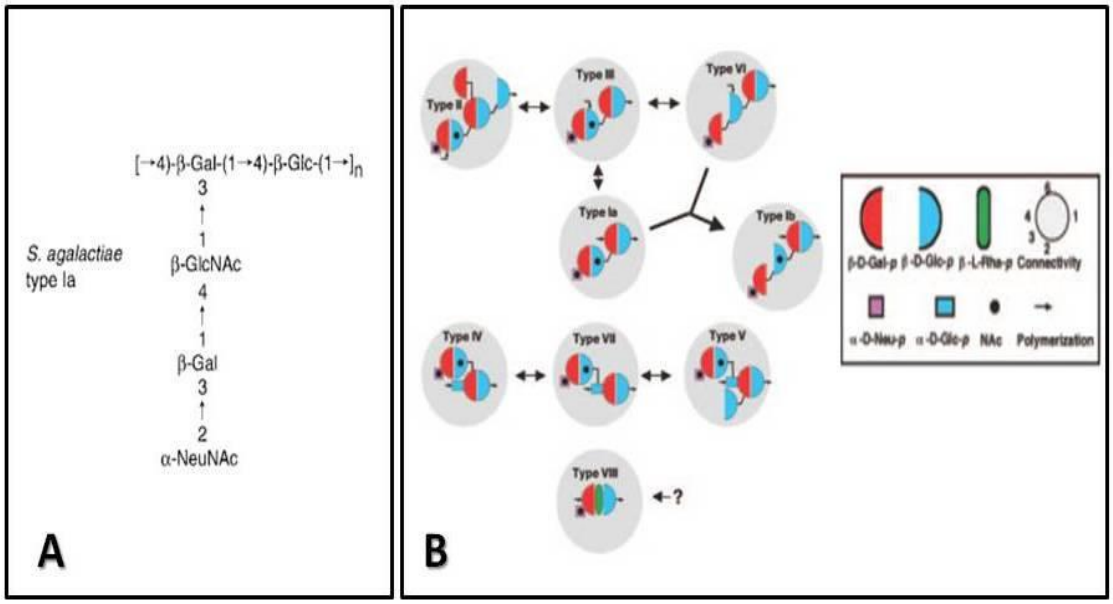


Figure 2 Schematic diagram of polysaccharide repeat unit structure of *Streptococcus agalactiae* serotype Ia (A) and other serotype (B). Glc, glucose; Gal, galactose; GlcNAc, N-acetylglucosamine; NeuNAc, N_acetylneuraminic acid. (Yamamoto et al., 1999; Cieslewicz et al., 2005)

The molecular characterization of 26 kbp CPS operon of *S. agalactiae* indicated that 16 genes (*cpsA-L, neuA-D*) are involved in CPS biosynthesis. Each gene has unique function in CPS production which can be divided roughly as sialic acid synthesis, pentasaccharide repeat unit formation, oligosaccharide transportation and polymerization. The mRNA unit of CPS operon is transcribed as single polycistronic messenger started from *cpsA* and ended at *neuA*. Comparison of the entire CPS operon between *S. agalactiae* serotype Ia and III suggesting the similarity in CPS genes sequence with only exception for *cpsH* encoding CPS polymerase (Chaffin et al., 2000). For other serotypes, the dissimilarity within CPS operon appeared in *cpsG* and *cpsK* which encoded for gactotransferase and sialyl transferease, respectively (Yamamoto et al., 1999; Cieslewicz et al., 2005).

Since the genetic loci of CPS operon in all 9 serotypes are flanked by conserved regions, the concept of capsular serotype switching among *S. agalactiae* population via homologous recombination was mentioned (Cieslewicz et al., 2005). The recombination of some CPS loci segments between two or more *S. agalactiae* serotypes was concerned as it may drive the arisen of new serotypes (Cieslewicz et al., 2005). Homologous recombination is believed to be the evasion strategies employed by streptococcus upon the CPS-specific immunity of the host (Cieslewicz et al., 2005; Martins et al., 2010). However, the recent evidence suggested that the capsular switching mechanism is rarely occurred since only 2% of 483 isolates of *S. agalactiae* were proved to have the evidences of CPS switching (Martins et al., 2010). It was noticed that capsular switching in the field may rather appear only by the exchange of the entire-CPS locus than the recombination of some genes. Moreover, it is still difficult for *S. agalactiae* to obtain the external DNA fragments since this bacterium is, unlike *S. pneumoniae*, not the natural competence (Nesin et al., 1998; Martins et al., 2010).

Capsular polysaccharide of *S. agalactiae* has an important role in immune evasion mechanism of the pathogen. The terminal $\alpha 2 \rightarrow 3$ sialic acid of CPS is identical to the sugar epitope that widely displayed on mammalian cells, hence *S. agalactiae* is less capable to trigger host immunity using this molecular mimicry

(Doran and Nizet, 2004). In addition, the strong antigenic proteins presenting on cell surface of *S. agalactiae,* such asteichoic acid and peptidoglycan, are hidden under the CPS envelope and hardly detected by host defence mechanisms. The sialylated CPS can reduces opsonophagocytosis by suppressing complement (C_3) composition at bacterial cells surface and promoting survival within the host circulatory system. Mutation of CPS related genes can enhance bacterial susceptibility to mouse leukocytes and reduce the bacterial virulence for 100 times comparing with *S. agalactiae* wild-type strain (Wessels et al., 1989). However, despite the pivotal roles of CPS in host immune evasions, the over-expression of CPS can leads to the unsuccessful infection. As described above that CPS can conceals surface antigenic proteins of bacteria. Simultaneously, some surface protein related to bacterial adhesion ability, such as adhesin, is also hidden under bacterial capsule cloak. The recent publication indicated that several streptococcal species can decrease the CPS production when they come to contact with host epithelial cells which associated to the enhancing of bacterial adhesion and invasion abilities (Hammerschmidt et al., 2005; Locke et al., 2007). The regulation of CPS expression level was believed to be associated with the dynamic change of bacterial microenvironments, however the in-depth detail of this mechanism is still being suspicious.

5.2. β-hemolysin/cytolysin

Most of clinical isolates of *S. agalactiae* own hemolysis activity which is apparently seen as clear zone around bacterium colony grown on blood containing agar. β-hemolysin/cytolysin (βH/C) is the surface-associated toxin responsible for hemolytic phenotype of *S. agalactiae*. Many publications indicated that βH/C plays a key role in several steps of pathogenesis, i.e. bacterial invasion, immune evasion, and dissemination of bacteria in the hosts' circulation (Maisey et al., 2008a).

Stdying of non-hemolytic strains from *S. agalactiae* mutants revealed that the *cyl* operon is responsible for βH/C production (Spellerberg et al., 1999; Pritzlaff et al., 2001). The *cyl* operon composed by 13 ORF encoding proteins responsible for toxin processing and transportation. There 13 ORF and their encoded protein are *cylA-B* (ABC transporter), *acpC* (acyl carrier protein), *cylD, G, Z* (fatty acid synthesis) and *cylE*

(βH/C structural protein) (Pritzlaff et al., 2001). Analysis of nucleotide sequences of *cylE* had revealed that the βH/C of *S. agalactiae* is a novel toxin since no homologous from other streptococcus was found in GenBank database (Pritzlaff et al., 2001).

As mentioned above, βH/C is toxic to various types of eukaryotic cells. The toxin is capable to form the membrane-associated pore at the host cells and use this activity to damage the epithelial/endothelial barrier, then invade into deep tissue layer (Maisey et al., 2008a). More importantly, the bacterium can takes an advantage upon phagocytic escape using the βH/C activity to destroy phagocytic cells by triggering pore-forming lysis and apoptotic partways (Maisey et al., 2008a).

Additionally, the production of streptococcus orange carotenoid pigments also largely linked to *cyl* operon which is observed from non-pigmented appeareance of *cyl* mutant strain of *S. agalatiae* (Spellerberg et al., 2000). Carotenoid pigments have the oxidative free-radical scavenging activity, which is similar to pigment produced by *Staphylococcus aurues* (Dahl et al., 1989). According to previous study, *S. agalactiae* can extent the viability duration within the host macrophages even it cannot produce catalase enzyme (Cornacchione et al., 1998). Comparing to wild type strain, the non-pigmented mutant strain of *S. agalactiae* was more susceptible to several types of reactive oxygen species (ROS) and easily diminished from the mice which suggesting the importance of *cyl* operon as immune evasion factor (Liu et al., 2004).

5.3. Hyaluronate lyase

Hyaluronate lyase is the releasing enzyme which regarded as bacterial-distribution related factors because of its abilities to degrade the host hyaluronan and chondroitin sulfate. This enzyme also present in other Gram positive pathogens such as *S. pneumonia*, *S. aureus* and *Clostridium perfringen* (Li and Jedrzejas, 2001). Study of hyaluronase activity in large population of *S. agalactiae* revealed that 76% of bacteria have hyaluronan degradation capability (Gunther et al., 1996).

The hyaluronate lyase encoding gene (*hylB*) of *S. agalactiae* was cloned and characterized for the first time in 1994 (Lin et al., 1994). Formerly, hyaluronate lyase had been misunderstood as a neuraminidase because of the inaccuracy of the detection methods (Pritchard and Lin, 1993). The genetic basis of *hylB* is composed of 3.3 kbp nucleotide which encoded for 110 kDa of mature protein. However, an insertion of 0.98 kbp of insertional sequence (IS1548) in the coding region of *hylB* may appears in some bacterial strains which terminate the production of hyaluronase (Yildirim et al., 2002).

Streptococcus hyaluronase can cleaves 1,4-glycosidic bond between the N-acetyl-ß-D-glucosamine and D-glucoronic acid residue in hyaluronan and produces the mixture of oligonucleotide fragments and disaccharide unit (Gase et al., 1998; Li and Jedrzejas, 2001). The destruction of hyaluronan matrix makes the host cells become visible to various streptococcal toxin and leading to the invasion of bacteria to deep tissue layer. The invasion ability of *S. agalactiae* had been proved to be related with the production of hyaluronidase in the dose-dependent manner (Musser et al., 1989).

5.4. CAMP factor

Other than ß-hemolysin, the most well-known pathogenic determinant secreted by *S. agalactiae* is CAMP factor. *Streptococcus agalactiae* has an extraordinary phenotype as it can produces an arrow-shape hemolysis zone on blood containing agar in the area between streptococci colony growing adjacent to *S. aureus,* which has been designated as 'CAMP reaction' (Christie et al., 1944). This reaction has prompted to be a rapid method for presumptive diagnosis of group B streptococcus. Complete hemolysis caused by CAMP reaction is derived from hydrolysis of sphingomyelin in erythrocyte membrane by ß-toxin from *S. aureus* (sphingomyelinase) cooperate with CAMP factor secreted from *S. agalactiae*. Mechanism of erytheolysis due to CAMP factor consists of 3 phases started from the binding of CAMP factor to glycosylphosphatidylinositol (GPI)-anchored protein receptor on cell membrane, the binding capacity of CAMP factor is enhanced by sphingomelin lysis of erythrocyte (Lang et al., 2007). Afterward, CAMP factor will

insert into cell membrane to form the oligomeric-pore followed by leaking of intracellular small molecules and increasing of intracellular osmotic pressure which makes erythrocytic bursts eventually (Lang and Palmer, 2003).

CAMP factor is extracellular 25 kDa protein encoded from *cfb* gene (Schneewind et al., 1988). CAMP factor is confirmed as a pathogenic factor since the injection of CAMP factor together with sub-lethal dose of *S. agalactiae* had influenced fatality in mice (Jurgens et al., 1987). Additionally, CAMP factor also has alternative immune modulatory function as it can bind non-specifically with the Fc portion of mammalian IgM and IgG (Jurgens et al., 1987).

5.5. Pilus

The pathogenesis of invasive bacterial pathogen always started from colonization and adherence to host tissue. In this initially critical step, many Gram negative bacteria possess their constituent's pili, a hair-like filamentous structure extended from bacterial surface, which plays a pivotal role in bacterial colonization. Bacterial pili is the member of the "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) family due to their ability to bind with host extracellular matrix (Telford et al., 2006). Up to date, the pilus-like structures were also found in Gram positive bacteria such as *Corynebacterium diphtheria*, *C. renale* and many species of streptococcus including *S. agalactiae*.

Gene determinant responsible for pilus production in *S. agalactiae* was firstly discovered during the screening of multiple genomes of bacteria to identified the universal vaccine candidate antigen (Lauer et al., 2005). The novel pilus operon is composed of 3 genes encoded for the structural component of pilus, two of them encoded for class C sortase enzyme that operate pilus anchoring in bacterial cell wall and the stand-alone transcription regulator. The cluster of pilus-associated genes has been called 'pilus-island' and shortly after the first described about it, another pilus-island has been discovered (Rosini et al., 2006). Two pilus-islands were annotated as PI-1, PI-2 (due to chronological discovery), while the latter island is divided into 2 different alleles designated as PI-2a and PI-2b. All pilus-islands are flanked by highly conserved repeat sequences which suggested that these genomic

regions can be transferred among *S. agalactiae* strains via horizontal transmission. Study of the distribution of pilus-islands possessed among 289 human clinical isolates of *S. agalactiae* was found that PI-1, PI-2a and PI-2b were appeared in 72, 73 and 27% of bacterial population, respectively (Margarit et al., 2009).

One of the major different between Gram negative and Gram positive pilus is about the mechanism of pilus construction. In Gram positive pilus, the construction is started from sec-dependent secretions of the pilus subunit proteins to bacterial surface membrane and the three pilus subunits will be formed together by covalent linkage which facilitated by catalytic activity of pilus-specific class C sortase followed by the anchoring of the assembled pilus into cell wall peptidoglycan using house-keeping sortase of the bacteria (Telford et al., 2006). In the case of *S. agalactiae* pilus, one of the three pilus subunit (PilB) is build up as a backbone structure enhancing pilus integrity while the other two are accessory proteins, i.e. PilA and PilC, linking alongside pilus backbone by covalent bond (Dramsi et al., 2006). Moreover, PilA protein contains a von Willebrand adhesion domain (VWA) which essential for pilus adhesion, therefore this PilA is usually displayed on the tip of pilus (Konto-Ghiorghi et al., 2009).

The biological roles of pilus protein in the pathogenesis Gram positive pathogen has not been thoroughly understood yet. It is well-known that Gram negative bacterial pilus plays an important role in adhesion ability, hence the pilus of Gram positive should rather have the similar functions (Telford et al., 2006). Study of the pilus-absence strains of *S. agalactiae* revealed that both *bona fide* and accessory pilus subunits were involved with bacterial pathogenicity (Maisey et al., 2007; Maisey et al., 2008b). As we described above, a PilA accessory protein is involved with adhesion of bacteria to host epithelial and endothelial cells, nevertheless a PilB backbone protein also indirectly correlated with adhesion since PilB can promote pili's architecture integrity which support the PilA exhibiting on the tip of pilus (Maisey et al., 2007; Konto-Ghiorghi et al., 2009). Moreover, the recent publication also revealed that *S. agalactiae* pilus was contributed to translocalization through epithelial barrier using paracellular route and promote the distribution of bacteria to circulatory system (Pezzicoli et al., 2008). *Streptococcus agalactiae* could quietly co-

colonized with other resident bacteria at epithelial niche using its pilus activity and, later on, gain access to deep tissue layer when the production of host mucus layer is reduced (Pezzicoli et al., 2008). Furthermore, pilus backbone was postulated as anti-cationic antimicrobial peptide (cAMP) which may participate in the intracellular survival within host phagocyte (Maisey et al., 2008b; Papasergi et al., 2011).

5.6. C5a peptidase

Complement component 5a (C5a) is a fragment protein released from the activation of complement cascade which act as chemoattractant and promoting opsonophagocytosis. However, *S. agalactiae* can subverts C5a cascade using a surface-anchored serine protease C5a peptidase (C5a-ase) to cleave the His67 and Lys68 active site of C5a (Bohnsack et al., 1991).

C5a-ase activity of *S. agalactiae* had been described since two decades ago. Noticed from remarkable feature of *S. agalactiae* infection that usually associated with poor localization of neutrophil at the site of infection, the authors had founded that this pathogen can degrade C5a and inhibit chemoattractive response (Hill et al., 1988). Afterward, the biological activity of C5a-ase is widely-studied which confirms the role of C5a-ase in the anti-phagocytic clearance and invasion factor using the fibronectin-binding ability (Bohnsack et al., 1991; Takahashi et al., 1995; Bohnsack et al., 1997; Cheng et al., 2002). The molecular structure of C5a-ase contains putative integrin binding domain that can bind to integrin located on host epithelial surface to stabilize conformational change of C5a-ase structure which required to expose C5a docking site (Brown et al., 2005).

Genetic determinant of C5s-ase, *ScpB*, present in a large composite transposon which also contains a laminin-binding protein encoded gene (*lmb*) and a putative protein encoded ORF2 (Franken et al., 2001). This transposon was inferred to be received from the related streptococcus species since its nucleotide sequence is likely identical to putative transposon of *S. pyogenes.* Most *S. agalactiae* strains isolated from human patients carried this transposon, while it hardly found in some bovine originated strains (Franken et al., 2001). In addition, the other recent studies

reported that C5a-ase function was carried out in a host-dependent manner (Bohnsack et al., 1993; Gleich-Theurer et al., 2009).

5.7. Fibrinogen binding protein

Fibrinogen binding protein is one of the MSCRAMMs family that specifically bind to fibrinogen (Fg), a extracellular matrix that can be vastly found in blood and plasma. *Streptococcus agalactiae* carries two of the fibrinogen binding protein, i.e. Fbs A and FbsB. The first Fg-binding protein, FbsA, is a surface anchored protein which found commonly among *S. agalactiae* strains (Schubert et al., 2002). The molecular basis of FbsA contains many repetitive units that varied in repeat numbers among the different *S. agalactiae* strains (Schubert et al., 2002). The number of repetitive unit in FbsA was hypothesized to be related with Fg-binding ability since the Fg-binding site is located in those repeat regions (Schubert et al., 2002; Pietrocola et al., 2006). Several studies had demonstrated the roles of FbsA in the pathogenesis of *S. agalactiae* infection and founded that the Fg-binding ability of FbsA is involved with host epithelial cells adhesion and resistant to opsonophagocytic killing due to its anti-complement feature (Schubert et al., 2002; Schubert et al., 2004; Jonsson et al., 2005). Furthermore, FbsA is also contributed to the platelet aggregation leading to thromboemboli production which subsequently induces neonatal endocarditis in human cases (Pietrocola et al., 2005). While Fg-binding activity of FbsA was directly facilitate epithelial cells attachment, another Fb-binding protein of *S. agalactiae*, FbsB, is insignificantly promote host cell attachment despite their Fg-binding activity (Gutekunst et al., 2004; Devi and Ponnuraj, 2010). The FbsB is, instead, promoting invasion into epithelial cell by triggering endocytosis upon FgsB-Fg/surface ligand complex (Gutekunst et al., 2004).

Since there are many of virulence factors of *S. agalactiae* have been identified so far, only some of them that more likely to be the major important virulence factors are mentioned in detail. The brief information of other putative virulence factors of *S. agalactiae* could be found in Table 1.3.

Table 1. 3 Some virulence factors of *Streptococcus agalactiae* and their biological function

Virulence factor	Mode of action	Genetic basis	References
Immune evasion factors			
Superoxide dismutase (SodA)	Detoxifies singlet oxygen and superoxide	*sodA*	(Poyart et al., 2001b)
Serine protease (CspA)	Cleavage of host fibrinogen and chemokine	*cspA*	(Bryan and Shelver, 2009)
Alanylation of Lipotechoic acid	Decreases net negative charge on cell surface, repels host antimicrobial peptide (AMPs)	*dltA–D*	(Poyart et al., 2003)
GAPDH	Induce host IL-10 production	*gapC*	(Madureira et al., 2007)
Host cell adherence and invasion			
Laminin-binding protein (Lmb)	Binding to host laminin, promote adhesion and invasion	*lmb*	(Tenenbaum et al., 2007)
Serine-rich repeat proteins (Srr)	Adhesion with host keratin	*srr*	(van Sorge et al., 2009)
Immunogenic bacterial adhesin (BibA)	Promotes adherence of bacteria to host cells and binds complement regulatory protein C4bp	*bibA*	(Santi et al., 2007)

Virulence factor	Mode of action	Genetic basis	References
C protein (α and β components)	Facilitates bacterial adherence to epithelial cells, IgA bindings	bca	(Gravekamp et al., 1998)

6. Virulence regulation of *Streptococcus agalactiae*

All living organisms have encountered with the dynamic change of surrounded environments without exception. Unlike multicellular organisms, prokaryotes cannot buffer themselves against the environmental changes (Krell et al., 2010). Alternatively, the bacteria has to modulate the expression of several proteins involved with metabolism, nutrients acquisition and other survival factors in response to any particular environmental condition. In the case of invasive bacterial pathogens which have to confront with the dramatically change of micro-environments wihin host niches along the pathogenic process, the successful of infection is largely rely on the proper expression of virulence genes in correspondent circumstances. However, the up-regulation of required virulence factors upon the micro-environmental fluctuations is insufficient to survive thoroughly inside the host body, but the silencing of unnecessary virulence factors also needed. For the example, the capsular polysaccharide of *Streptococcus* sp. can enhance phagocytic resistance of the bacteria which is necessarily important to the viability inside host body but, on the other hand, the capsule can inhibit adhesion and invasion ability of streptococcus. Hence, the up-regulation of capsule-production genes at the initial colonization process may result as unsuccessful infection. Moreover, inappropriate expression of virulence factors also resulted as a wasteful loss of valuable metabolic energy of microorganisms (Krell et al., 2010).

The proper and instant responses of bacteria to critical change of micro-environments are largely account for the highly efficient environmental sensing system and rapid regulation of the correspondent genes. In living organisms, The biological mechanisms for sensing of external signals and regulate genes expression

generally called ‘Signal transduction system’ aka STS (Groisman and Mouslim, 2006). The most common STS found among prokaryote is ‘Two-component system’ (TCS) and the genes encoded for TCS components carried by proteobacteria genomes are approximately 52 genes (Krell et al., 2010). Bacterial TCS is comprised of two functional units, 1) membrane-associated histidine kinase (HK) and 2) a cognate-response regulator (RR) (Rajagopal, 2009). Regulation of TCS’s subordinated genes expression is initiated by sensing of external stimulatory signals via HK which can induce autophosphorylation at His residue, followed by transphosphorylation to the Asp residue of the cognate RR. At last, this phosphorylated RR will be served as a transcription regulator using their DNA/protein binding activity to modulate transcription level (Figure 3).

Bacterial cells are unavoidably exposed with broad range of environmental conditions including various physical (pH, temperature etc.) chemical (oxygen, nutrient, heavy metal etc.) and biological (host immune defenses) states, therefore the bacteria must expand their sensory systems to cope with all kind of environmental circumstances. Until now, several types of HK families have already been identified. The HK contains three major domains, 1) periplasmic sensor domain 2) cytosolic autokinase domain which linked together with 3) transmembrane region. The His residue containing cytosolic domain of HK generally acts as functional domain through their autokinase activity (Figure 3). Interestingly, despite that vast numbers of HK families, the functional activity of HK is still highly specific to their coupled cognate RR without significant cross-talk (Wadhams and Armitage, 2004).

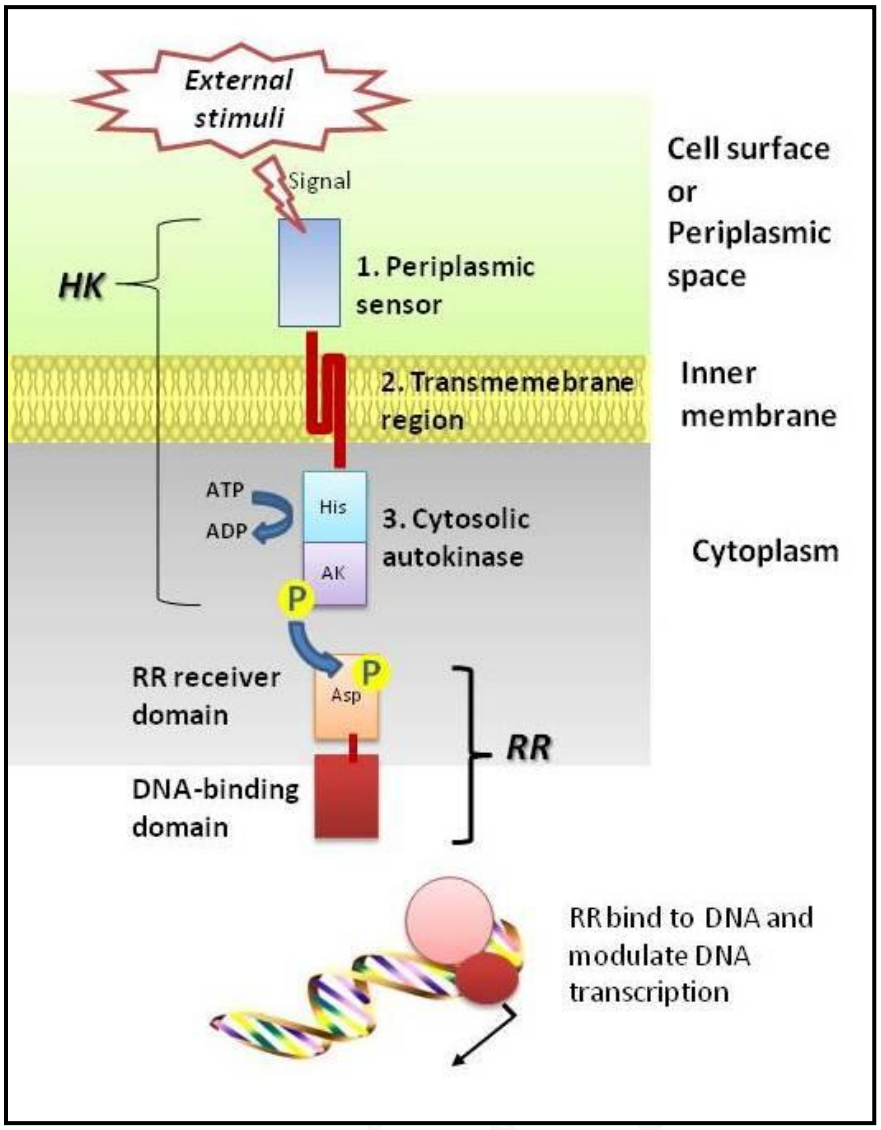


Figure 3 The constituents of two-component regulatory system (TCS) and their functional mechanism. AK, autokinase domain; HK, membrane-associated histidine kinase; RR, cognate response regulator.

Streptococcus agalactiae undergoes myriad of host niches during pathogenic process. In the case of human streptococcosis, switching from the colonization state inside genital tract of female carriers into the invasive state by infiltration to host bloodstream brought out an instant change of microenvironments. The successful infection of the highly virulent *S. agalactiae* suggested that the bacteria can efficiently adapt themselves to the dynamic fluctuation of micro-environments. Just like any other protobacteria, *S. agalactiae* also use TCS as a key factor in order to manipulate expression of various virulence and survival factors at the proper time/place they are needed (Rajagopal, 2009). Investigation of *S. agalactiae* type V and III genomes revealed that at least 17-20 TCS are possessed in these bacteria (Glaser et al., 2002; Tettelin et al., 2002). Unfortunately, the molecular mechanisms and functional activities of the most of TCS determinants and their under-controlled genes still lack of investigation. To date, only CovR/S, DltR/S, RgfC/A and CiaR/H are the TCS of *S. agalactiae* that have been proved to be involved with bacterial virulence. Among them, CovR/S was prompted to be a major transcriptional regulatory system of *S. agalactiae* because more than 139 genes (7% approximately) are governed by it (Lamy et al., 2004). The absence of CovR/S function is related to the increasing of bacterial adhesion and hemolysis activity while survival ability in human blood is decreased instead (Lamy et al., 2004).

Additionally, some stand-alone regulators can also be found in *S. agalactiae* which also helpful in operating genes expression, though their responses usually limited only to the change of cytosolic environments (Rajagopal, 2009). The example of some important regulatory systems available on *S. agalactiae* and their governed virulence genes are summarized in Table 1.4.

Table 1. 4 Some important TCS of *S. agalactiae* associated with bacterial virulence

Regulatory system		External stimulatory signal	Operated virulence protein	References
TCS	CovR/S	Growth phase, pH, Mg^{2+} (in *S. pyogenes*)	***Stimulated***: ß-hemolysin, CAMP, CpsA-D, NeuA-D, FbsA, BibA, CspA, Pili, Surface anchored gene ***Inhibited***: ß-hemolysin, CAMP, Cyl(pigment), ScpB	(Lamy et al., 2004; Jiang et al., 2005; Santi et al., 2009)
	DltR/S	Unknown	***Stimulated***: DltA-D	(Poyart et al., 2001a)
	RgfC/A	Growth phase	***Inhibited***: ScpB	(Spellerberg et al., 2002)
	CiaR/H	Unknown	Unknown	(Rajagopal, 2009)
Stand-alone regulator	MtaR	Unknown	***Stimulated***: CspA ***Inhibited***: FbsB	(Bryan et al., 2008)
	Rga	Unknown	***Stimulated***: PiliA, Srr, arginine deiminase components ***Inhibited***: FbsA	(Samen et al., 2011; Dramsi et al., 2012)
	RogB	Growth phase	***Stimulated***: Pili, FbsA ***Inhibited***: CpsA-L	(Gutekunst et al., 2003)

Regulatory system	External stimulatory signal	Operated virulence protein	References
RovS	Unknown	***Stimulated***: ß-hemolysin, Cyl (pigment), SodA, RogB ***Inhibited***: FbsA, Cyl (pigment)	(Samen et al., 2006)

Streptococcus agalactiae employed the regulatory system to overcome host defense mechanisms for the successful infection. Several publications indicated that transcriptomic and proteomic profiles of *S. agalactiae* were often change during pathogenic process (Johri et al., 2007; Mereghetti et al., 2008a; Yang et al., 2010). To make it more obvious about the relationship between the expression level and the outcome of disease, the pathogenic process of streptococcosis (in mammal) could be divided into 2 steps which are 1) colonization and 2) invasion. Generally, dissimilarities of micro-environments between these two states are pH, temperature, and oxygen concentration which the bacteria are exposed with. In the initial colonization state, bacteria are encountered with low pH (4-5 pH), low temperature (30-32 ℃) and micro-aerophilic condition in lower urogenital/reproductive tract of human carriers (Johri et al., 2003; Johri et al., 2007; Mereghetti et al., 2008b; Santi et al., 2009). However, those micro-environments are completely different during invasive state (neutral pH, 40 ℃ temperature in fever state, high oxygen concentration and exposure to serum/blood). Briefly, the factor associated with metabolic process and adhesion usually up-regulated during initial colonization state, afterwards, factors involved with bacterial replication, invasion and immune evasion are expressed in the followed invasive state, while some unnecessary protein are down-regulated. According to previous publications, *S. agalactiae* was well-adapted to fit with various conditions which can promote their viability in both colonized and invasive states (Yamamoto et al., 2005; Mereghetti et al., 2008a; Mereghetti et al.,

2008b). The brief information about the responsive of *S. agalactiae* to various stimulatory factors is summarized in Table 1.5.

Table 1. 5 Adaptation of *Streptococcus agalactiae* in response to external and internal stimuli.

Stimulatory signal		Bacterial adaptation	References
Temperature	40 °C	Increasing of hemolytic activity Up-regulation of: purine/pyrimidine and iron acquisition protein, LPXTG surface associated protein, DNase protein, pathogenicity island	(Mereghetti et al., 2008b)
	30 °C	Up-regulation of: stress protein, cell wall associated, cellular process and metabolism associated	
pH	pH 7	Up-regulation of: hemolysin, bibA (pathogenicity protein), C5a peptidase, pilus	(Santi et al., 2009)
	pH 5.5	Up-regulation of: ABC transporter, cellular and energy metabolism	
Oxygen concentration	Aerophilic	Increasing of bacterial invasion to epithelium, increasing of bacterial pathogenicity Up-regulation of: virulent factor (PBP2b, CPS, Dlt, GAPDH, iron-binding protein)	(Johri et al., 2003; Johri et al., 2007)

Stimulatory signal		Bacterial adaptation	References
	microaerophilic	Bacterial invasiveness were sub-optimum	
Invasive associated condition	neutral pH, high O_2, high nutrient	Maximum growth rate Up-regulation of: C protein-ß antigen, protein liked to resistance to oxidative stress	(Yang et al., 2010)
	low pH, low O2 and low nutrient	Slower growth rate Up-regulation of: stress protein, cellular metabolism protein	
Bacterial growth phase	Stationary phase	Up-regulation of: virulence factor (hemolysin, CAMP, CovR/S, adhesin), carbohydrate utilization protein, stress protein	(Sitkiewicz and Musser, 2009)
	Log phase	Up-regulation of: Cellular and metabolic processed gene	
Exposed to human serum		Up-regulation of: C5a peptidase protein	(Gleich-Theurer et al., 2009)
Exposed to human blood		Up-regulation of: virulent factor (fbsA, bibA, enolase, GAPDH and streptokinase like protein), carbohydrate utilization factor Down-regulation of: cellular process and cellular metabolism related	(Mereghetti et al., 2008a)

Stimulatory signal	Bacterial adaptation factor	References
Exposed to heam and quinine	Bacterium undergoes respiration metabolism which associated to bacterial growth and virulence	(Yamamoto et al., 2005)

Chapter 2: Identification and characterization of *S. agalactiae* recovered from tilapia and their culturing environments

Abstract

Streptococcus sp. were recovered from diseased tilapias in Thailand during 2009-2012 (*n* = 35), and were also continually collected from environmental samples (sediment, water) from tilapia farms for nine months in 2011 (*n* = 25). The relative percent recovery of streptococci from environmental samples was 13-67%. All streptococcal isolates were identified as *S. agalactiae* by species-specific PCR. In molecular characterization assays, four genotypic categories comprised of [I] molecular serotypes [II] *infB* allele [III] virulence genes profiling patterns (*cylE, hylB, scpB, lmb, cspA, dltA, fbsA, fbsB, bibA, gap* and pili-backbone encoded genes) and [IV] random amplified polymorphic DNA (RAPD) fingerprinting patterns, were used to describe the genotypic diversity. There was only 1 isolate identified as molecular serotype III, while the others were serotype Ia. Most serotype Ia isolates had an identical *infB* allele and virulence genes profiling patterns, but a large diversity in these strains was established by RAPD analysis with that diversity tending to be geographically dependent. Experimental infection of Nile tilapia revealed that serotype III isolate was non-pathogenic in the fish, while serotype Ia (both fish and environmental isolates) were pathogenic, with a median lethal dose of 6.25 to 7.56 $\log_{10}$ colony forming units. In conclusion, *S. agalactiae* isolated from tilapia farms in Thailand showed large genetic diversity which was associated with geographical origin of bacteria.

1. Introduction

Streptococcus agalactiae is one of the most serious pathogenic bacteria producing the massive mortalities in several aquaculture species. Streptococcosis has become the major obstacle of global aquaculture-industries for decades (Yanong and Francis-Floyd, 2010). Many species of the marine and fresh water fish have been reported to be susceptible to streptococcosis such as rainbow trout (*Oncorhynchus mykiss*), hybrid striped bass (*Morone saxatilis* X *M. chrysops*), channel catfish (*Ictalurus punctatus*), wild mullet (*Liza klunzingeri*), and Nile tilapia (*Oreochromis niloticus*) (Eldar and Ghittino, 1999; Shoemaker et al., 2001; Evans et al., 2002; Suanyuk et al., 2005). The specific clinical signs and lesions associated with bacterial sepsis, such as emaciation, exophthalmos, skin hemorrhage and anal protrusion could be found among the infected fish, while the pathognomonic changes such as erratic swimming and meningoenchephalitis may occur in severely acute infection. The cumulative mortality caused by streptococcosis may higher than 50% within 3-4 days in case of severely acute infection. Occasionally, the gradual loss of the fish over several weeks period may take place in case of chronic infection (Yanong and Francis-Floyd, 2010).

Characterization of *S. agalactiae* can be conducted using several phenotypic and genotypic systems. The most basic categorization system for *S. agalactiae* isolated based on the hemolytic appearance of streptococcal colony on blood containing medium and *S. agalactiae* isolated from fish has been separated into 2 different biotypes, i.e. β-hemolysis biotype I and γ-hemolysis biotype II (Vandamme et al., 1997; Kawamura et al., 2005). Both biotypes had responsible for the massive outbreaks in many fish species, however the global endemic areas for each biotype was dissimilar (Lindahl et al., 2005). According to the antigenic properties of capsular polysaccharide, *S. agalactiae* can be divided into 10 capsular serotypes (Ia, Ib, II to IX). The distribution of each serotype was found to be associated with the geographical origin (Lindahl et al., 2005). In the case of aquatic animals, several evidences had indicated that serotype Ia, Ib and III were the cause of streptococcosis outbreak in fish farms (Vandamme et al., 1997; Jafar et al., 2008; Amal et al., 2013a). In Thailand, *S. agalactiae* serotype Ia and III had been isolated from diseased red tilapia and sea bass at the southern part of the country (Suanyuk et al., 2010).

Additionally, the intraserotype diversity of *S. agalactiae* has been manifested using several molecular techniques such as pulse-field gel electrophoresis (PFGE) and multi locus sequence typing (MLST), random amplified polymorphic DNA (RAPD) and virulence genes profiling pattern (Evans et al., 2008; Pereira et al., 2010; Amal et al., 2013a; Godoy et al., 2013). Unfortunately, only a few information of genetic diversity of *S. agalactiae* isolated in Thailand is available so far.

Streptococcosis outbreaks associated with seasonal variation in aquaculture farms have been reported to be related to another species of streptococcus, i.e. *S. iniae* (Bromage and Owens, 2009). Despite several harshly environmental conditions causing stressful responses such as low dissolved oxygen, high concentrations of nitrite, and high stocking density, the high water temperature appears to be the most important factor contributing to the susceptibility of the fish to streptococcosis (Perera et al., 1997; Ndong et al., 2007; Mian et al., 2009; Rodkhum et al., 2011). However, it is difficult to clarify a relationship between disease occurrences and seasonal variations, since the surveillance information about streptococcosis outbreaks over long duration periods is still limited.

In the current study, the presence of *S. agalactiae* in the clinical and environmental samples collected from tilapia culturing sites during 2009 to 2011 were examined, and molecular characteristics and virulence genes profiles of streptococci were also investigated.

2. Materials and methods

2.1. Sample collection and isolation of bacteria from diseased fish

The samples were collected from tilapia farms located in 6 provinces, i.e. Ayutthaya Nakhon Pathom, Phetchaburi, Prachinburi, Ratchaburi and Suphanburi, during disease outbreak (Accumulated mortalities was more than 30%) since 2009. Moribund and freshly dead fish were selected, preserved on ice and send to laboratory within 6 hours. The bacteria were directly isolated from internal organs of the fish (kidney, liver, spleen and brain) using streptococcal selective media which composed of 10 mg/l of colistin (Sigma, Germany) and 5 mg/l of oxolinic acid (Sigma, Germany) in tryptic soy agar (TSA; Difco, USA) plus 5% sheep blood based (Nguyen and Kanai, 1999). Cultured condition for bacteria was 32 ^{0}C for 24 hours. Three (3) colonies appeared on agar were randomly selected for the species identification.

2.2. Sample collection and isolation of bacteria from farming environments

Isolation of streptococcus from environmental samples was conducted in three juvenile tilapia farms in Nakhon Pathom province between Januarys to September 2011 on a monthly basis. All three farms acquired fingerlings tilapia from the same streptococcus-free hatchery (diagnosed by cultured-based method) located in the same province. Tilapia farms were located approximately 10 km apart from each other and no mammalian livestock were presented in nearby areas, which minimized the possibility of contamination by streptococci from mammalian origin.

For sample collection procedures, at least 3 earthen pond containing 3-5 months old tilapia were randomly selected from each farm to collect water and sediment samples. Water samples were drawn from a depth of 50 centimetres at 3 meters far from the shore and the sediments were also collected from the same spot. Two sampling spots were chosen for each sampled pond. One hundred (100) milliliters of water and 50 g of sediment were collected in sterile glass bottle and plastic bag, respectively. The samples were preserved on-ice and transported to laboratory within 6 hours. In addition, the water from irrigation canals supplying water to the farms was also collected in the same manner. Water quality parameters

including salinity, alkalinity, dissolved oxygen, hardness, pH, ammonia, nitrite and temperature were recorded at the same time that the samples were taken.

To isolate streptococci, the environmental samples were enriched by tryptic soy broth (Difco, USA) containing 10 mg/l of colistin sulfate and 5 mg/l of oxolinic acid at 32 °C for 48 hours followed by the sub-culturing of bacterial suspensions at 32 °C for 24 hours using streptococcus selective agar. Three (3) colonies of bacteria appears on medium were randomly selected for species identification.

2.3. Identification of *S. agalactiae*

Standard biochemical assays were employed to identified species of bacteria and the results were interpreted according to Bergey's Manual of Systematic Bacteriology (Hardie and Whiley, 2009). The nucleic acid of the bacteria was extracted by standard phenol-chloroform method (Ausubel et al., 2003). The bacteria were confirmed as *S. agalactiae* using species-specific duplex PCR method (Rodkhum et al., 2012). The detail of primers used for this PCR reaction is showed in Table 2.1 and the PCR thermocycling reaction is one cycle of initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, and subsequently extension by one cycle of 72 °C for 7 min. Herein, *S. agalactiae* strain A909 (serotype Ia) was included as a positive control for PCR assays.

Table 2. 1 Detail of primer using for *Streptococcus agalactiae* identification.

Target gene	Size of amplicon	Primer name	Oligonucleotide primer sequences	Reference
16S rRNA/IMOD	220 bp	F1 IMOD	5'-GAGTTTGATCATGGCTCAG-3' 5'-ACCAACATGTGTTAATTACTC-3'	(Martinez et al., 2001)

2.4. Phenotypic characterization of *S. agalactiae*

Phenotypic characteristics of *S. agalactiae* were determined from biochemical test results and the utilization of the trehalose, lactose, sucrose, mannitol, raffinose, salicin and galactose in cystine trypticase agar (Difco, USA). In addition, the details for each biochemical test are described in Appendices of this thesis. clinical strains isolated from mastitis cows (9 isolates; *B01-B09*), and strains of human origin purchased from the Department of Medical Science, Thailand (6 isolates; *H01-H06*) also included in these assays to compare with *S. agalactiae* fish isolates.

2.5. Genotypic characterization of *S. agalactiae*

2.5.1. Molecular serotyping

Multiplex PCR had been operated to classified *S. agalactiae* molecular serotype. Nine teen primers set were applied in the multiplex PCR reaction and their detail are presented in table 2.2 The composition of multiplex PCR reaction contained 2mM $MgCl_2$, 200 µM of dNTP, 400 nM of cpsI-Ia-6-7-F and cpsI-7-9-F primers, 250 nM of other primers and 0.3 U of HotMaster® Taq DNA Polymerase (5 Prime, USA). The PCR thermocycle reaction started with one cycle of 95 ℃ for 5 min, followed by 15 cycles of 95 ℃ for 1 min, 54 ℃ for 1 min, and 72 ℃ for 2 min and then by additional 25 cycles of 95 ℃ for 1 min, 56 ℃ for 1 min, and 72 ℃ for 2 min and a final extension of 72 ℃ for 10 min. PCR products were separated by electrophoresis on

1.5% agarose gel containing 0.2 μg/ml of ethidium bromide and visualized under UV light.

Table 2. 2 Sequence of nine teen primers used in multiplex PCR for identification of *Streptococcus agalactiae* molecular serotype (Imperi et al., 2010).

Primer	Sequence (5' → 3')
cpsI-Ia-6-7-F	GAATTGATAACTTTTGTGGATTGCGATGA
cpsI-6-R	CAATTCTGTCGGACTATCCTGATG
cpsI-7-R	TGTCGCTTCCACACTGAGTGTTGA
cpsL-F	CAATCCTAAGTATTTTCGGTTCATT
cpsL-R	TAGGAACATGTTCATTAACATAGC
cpsG-F	ACATGAACAGCAGTTCAACCGT
cpsG-R	ATGCTCTCCAAACTGTTCTTGT
cpsG-2-3-6-R	TCCATCTACATCTTCAATCCAAGC
cpsN-5-F	ATGCAACCAAGTGATTATCATGTA
cpsN-5-R	CTCTTCACTCTTTAGTGTAGGTAT
cpsJ-8-F	TATTTGGGAGGTAATCAAGAGACA
cpsJ-8-R	GTTTGGAGCATTCAAGATAACTCT
cpsJ-2-4-F	CATTTATTGATTCAGACGATTACATTGA
cpsJ-2-R	CCTCTTTCTCTAAAATATTCCAACC
cpsJ-4-R	CCTCAGGATATTTACGAATTCTGTA
cpsI-7-9-F	CTGTAATTGGAGGAATGTGGATCG
cpsI-9-R	AATCATCTTCATAATTTATCTCCCATT
cpsJ-Ib-F	GCAATTCTTAACAGAATATTCAGTTG
cpsJ-Ib-R	GCGTTTCTTTATCACATACTCTTG

2.5.2. Virulence genes profiling

The virulence genes which were possessed in *S. agalactiae* genome were identified using standard PCR technique. In this study, several primer pairs listed in table 2.3 were newly designed from conserved region of the putative virulence genes of S. *agalactiae* serotype Ia (A909, GD201008-001), serotype III (NEM316) and serotype V (2603V/R) using primer-BLAST designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Specificity of the primers had been tested by conventional PCR using strain A909 and another human isolate (H04, serotype Ia), then the PCR products were subjected for sequencing. Strain A909 was also applied as positive control in the virulence genes identification experiment. The detail of each primer pairs are listed in Table 2.3 The PCR parameter are comprised of 95 ℃ for 5 min, followed by 30 cycles of 95 ℃ for 1 min, 52-56 ℃ for 1 min (depend on melting temperature of primer), and 72 ℃ for 2 min, and the additional extension step by one cycle of 72 ℃ for 10 min. Amplification products were separated by electrophoresis on 1.5% agarose gel containing 0.2 μg/ml of ethidium bromide and visualized under UV light.

Table 2. 3 Detail of primer pairs using in PCR for virulence genes identification of *Streptococcus agalactiae.*

Target gene	**Primer name**	**Oligonucleotide primer sequences (5' → 3')**	**Size of amplicons**
β-hemolysin/ cytolysin (*cylE*)	cylE_F	TTCTCCTCCTGGCAAAGCCAGC	124 bp
	cylE_R	CGCCTCCTCCGATGATGCTTG	
CAMP factor (*cfb*)	cfb_F	AACTGTCTCAGGGTTGGCACGC	158 bp
	cfb_R	AAGCCCAGCAAATGGCTCAAAAGC	
Capsule biosynthesis regulatory protein (*cpsX*)	cpsX_F	GCTTGTGACTCGACAGTGAAGCG	109 bp
	cpsX_R	CCTCACAGCTCCAAACGTCCG	
Superoxide dismutase (*sodA*)	soda_F	TCACAGCAGCAGCAACAGGACG	155 bp
	soda_R	AGCATGCTCCCATACATCAAGCCC	
C5a peptidase (*scpB*)	scpB_F	TGAGCCTCAGGCATCGCACC	109 bp
	scpB_R	CCGCTGTCGATCAAGAGCACGG	
Serine protease (*cspA*)	cspA_F	GGTCGCGATAGAGTTTCTTCCGC	104 bp
	cspA_R	AACGCCTGGGGCTGATTTGGC	
Fibrinogen-binding protein A (*fbsA*)	fbsA_F	GTCACCTTGACTAGAGTGATTATT	85 bp
	fbsA_R	CCAAGTAGGTCAACTTATAGGGA	
Fibrinogen-binding protein B (*fbsB*)	fbsB_F	TCTGTCCAACAGCCGGCTCC	144 bp
	fbsB_R	TTCCGCAGTTGTTACACCGGC	

Target gene	**Primer name**	**Oligonucleotide primer sequences (5' → 3')**	**Size of ampli cons**
Laminin-binding protein (*lmb*)	lmb_F	TGGCGAGGAGAGGGCTCTTG	105 bp
	lmb_R	ATTCGTGACGCAACACACGGC	
Immunogenic bacterial adhesin (*bibA*)	bibA_F	AACCAGAAGCCAAGCCAGCAACC	127 bp
	bibA_R	AGTGGACTTGCGGCTTCACCC	
Hyaluronate lyase (*hylB*)	hylB_F	TCTAGTCGATATGGGGCGCGT	136 bp
	hylB_R	ACCGTCAGCATAGAAGCCTTCAGC	
D-alanylation ligase(*dltA*)	dltA_F	GTTTTTGGTAGGGCAAACAGGGTGC	100 bp
	dltA_R	CGCAAATGTTGGCTCAACCGCC	
GAPDH (*gap*)	gap_F	AGACCGATAGCTTTTGCAGCACC	100 bp
	gap_R	GATCCTTGACGGACCACACCG	
Pili-1 backbone (*gbs80*)	PI_F	AACAATAGTGGCGGGGTCAACTG	102 bp
	PI_R	TTTCGCTGGGCGTTCTTGTGAC	
Pili-2a backone (*SAG1407*)	PI2a_F	CACGTGTCGCATCTTTTTGGTTGC	128 bp
	PI2a_R	AACACTTGCTCCAGCAGGATTTGC	
Pili-2b backbone (*SAN1518*)	PI2b_F	AGGAGATGGAGCCACTGATACGAC	175 bp
	PI2b_R	ACGACGACGAGCAACAAGCAC	

2.5.3. *infB* allele sequencing

DNA sequences of translation initiation factor IF2 encoded gene were analyzed in order to identify the *infB* allele type of *S. agalactiae.* The central variable region of *infB* will be amplified with primers 5'-TACTGAGGGCATGACCGTTGC-3' and 5'-GACACCCGCAGCTTTAGAGTGAT-3' as described elsewhere (Hedegaard et al., 2000). Amplification products were purified using Nucleospin® Extract II (Macherey-Nagel, Germany) and sequencing were carried out using the BigDye Terminator™ (QIAGEN, USA).). The sequences of *infB* allele A-S found out in GenBank database were aligned and compared with nucleotide sequences obtained in this study using BioEdit version 7.0.5.2 (http://www.mbio.ncsu.edu/BioEdit/).

2.5.4. Random amplified polymorphic DNA (RAPD)

The genetic relationship of *S. agalactiae* isolates was examined using RAPD analysis. To generate RAPD fingerprint, three type of primer (AP42, OPS11 and OPS16) were used in the preliminary test in order to select only one that gave the reproducible result with better discrimination (Chatellier et al., 1997). The RAPD reaction was performed in 25 µl reaction mixture containing 100 µM of each dNTP, 0.2 µM primer, 2.5 mM $MgCl_2$ and 0.5 Unit of *Taq* DNA polymerase. The amplification condition comprised of initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min, and subsequently extension by one cycle of 72 °C for 10 min. The thermocycling was performed in Life Express Thermal Cycler® (Bioer, Japan). The amplification products were separated by electrophoresis on 1.5 % agarose gel. Analysis of RAPD pattern was carried out by GelAnalyzer 2010 software (http://www.gelanalyzer.com/). RAPD fingerprinting patterns of bacterial isolates were compared, then the similarity matrix were calculated using DICE coefficient. Finally, the dendrogram was constructed based on similarity matrix using web based-program using UPGMA method (http://genomes.urv.es/UPGMA/) (Reguant and Bordons, 2003).

2.6. Median lethal dose (LD_{50}) analysis

The median lethal doses (LD_{50}) of six *S. agalactiae* isolates (ENC03, ENC10, ENC24, FNB12, FNB17 and FPhA01) from different molecular clusters were determined. Bacterium was grown overnight in tryptic soy broth (TSB). Bacterial cells were harvested, washed twice with 0.85% saline, and suspended in sterile PBS. The concentration of bacterial suspension was evaluated using spectrophotometry and adjusted to 10^8 CFU/ml (OD_{600}=0.6), followed by ten-fold serial dilutions. Five groups of 30 to 40 g Nile tilapia (six fish per group) were injected intraperitoneally with 0.1 ml of bacterial suspension (10^8, 10^7,10^6, 10^5 or 10^4 CFU). Accumulated mortality of the fish was observed until 7 days post injection and the LD_{50} was calculated by Probit analysis (Finney and Stevens, 1948). Totally, one hundred-eighty (180) tilapia were used in this inoculation study

2.7. Data analysis

The relationship between the month that samples were collected and the RPI was analysed using the chi-square test in the SPSS 17.5 software package (IBM).

3. Results

3.1. Isolation of streptococcus from diseased fish

During the outbreak, the cumulative mortality in fish farms were about 20-50% and the mortality were prolonged for 1 to 3 weeks period. Some irregular signs were noticed, such as lowering feed intake, darkening skin, hemorrhage at skin and base of fin, anal protrusion and erratic swimming. Totally, sixty two (62) fish from 11 farms were collected and 56.4% (35/62) fish carried streptococci. Result of streptococcal isolation categorized by time and location that samples were collected is demonstrated in Table 2.4.

Table 2. 4 Result of streptococcus isolation from samples collected from clinical and environmental samples.

Type of samples	Year	Geographical origin (number of farms)	Numbers of sample	Streptococcus positive samples
Diseased tilapia	2009	Suphanburi (1)	1	1
		Phetchaburi (1)	4	4
		Prachinburi (1)	2	2
		Ayutthaya (1)	2	2
	2009-2011	Nakhon Pathom (6)	51	24
	2012	Ratchaburi (1)	2	2
		Total	*62*	*35 (56.4%)*
Environmental samples	2011	Nakhon Pathom (3)	134 ponds	25 (18.65%)
Total			***196***	***60***

3.2.Isolation of streptococcus from environmental samples

No streptococcus was found in and fingerling from hatchery and the water obtained from irrigation canal indicating no streptococci from the external sources had been introduced to tilapia farms. While the isolations of streptococci from water and sediment samples collected from 3 tilapia farms were success. Streptococci had been recovered from 18.65% (25/134) of the samples we collected (figure 4). In detail, the RPI of streptococci isolates ranged from about 12-65% in each month except June when it was 0%. The water qualities in the fish pond were quite stable during nine-month period of study, i.e. pH (7.5-8.0), salinity (0-0.4%), DO (5-10 ppm), alkalinity (120-200 ppm), ammonia (0-0.2 ppm) and nitrite (0-0.04 ppm). Temperature was the only water quality that found to have variation and the average temperature was higher than 32 ^{o}C in April, July and August. In addition, the RPI of streptococci seems to have no relationship with the season or water temperature (P = 0.101). The RPI of streptococci and water qualities are compiled in figure 5 and 6.

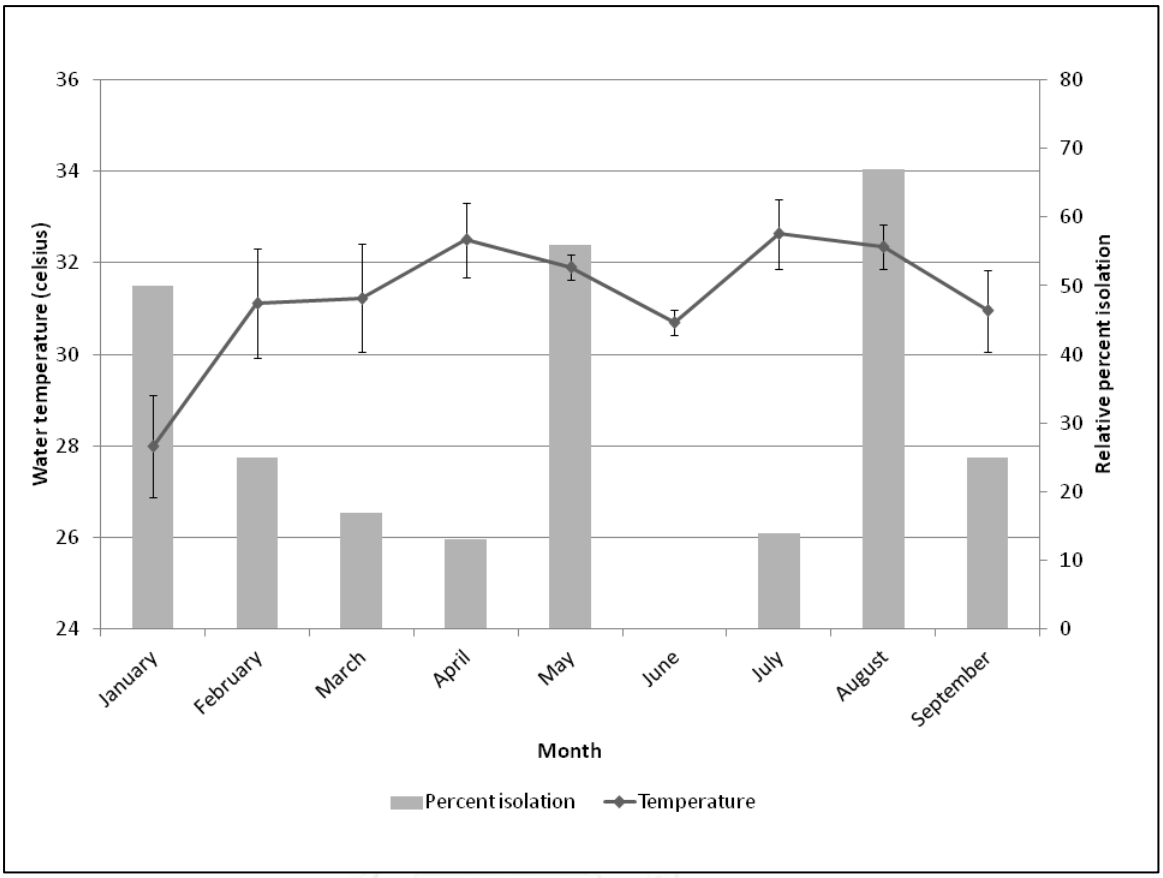


Figure 4 The relative percent isolation of *S. agalactiae* from environmental samples collected from three commercial tilapia farms in Nakhon Pathom province. Sediment and water from fish ponds were collected and bacteria were isolated over nine months (January-September 2011).

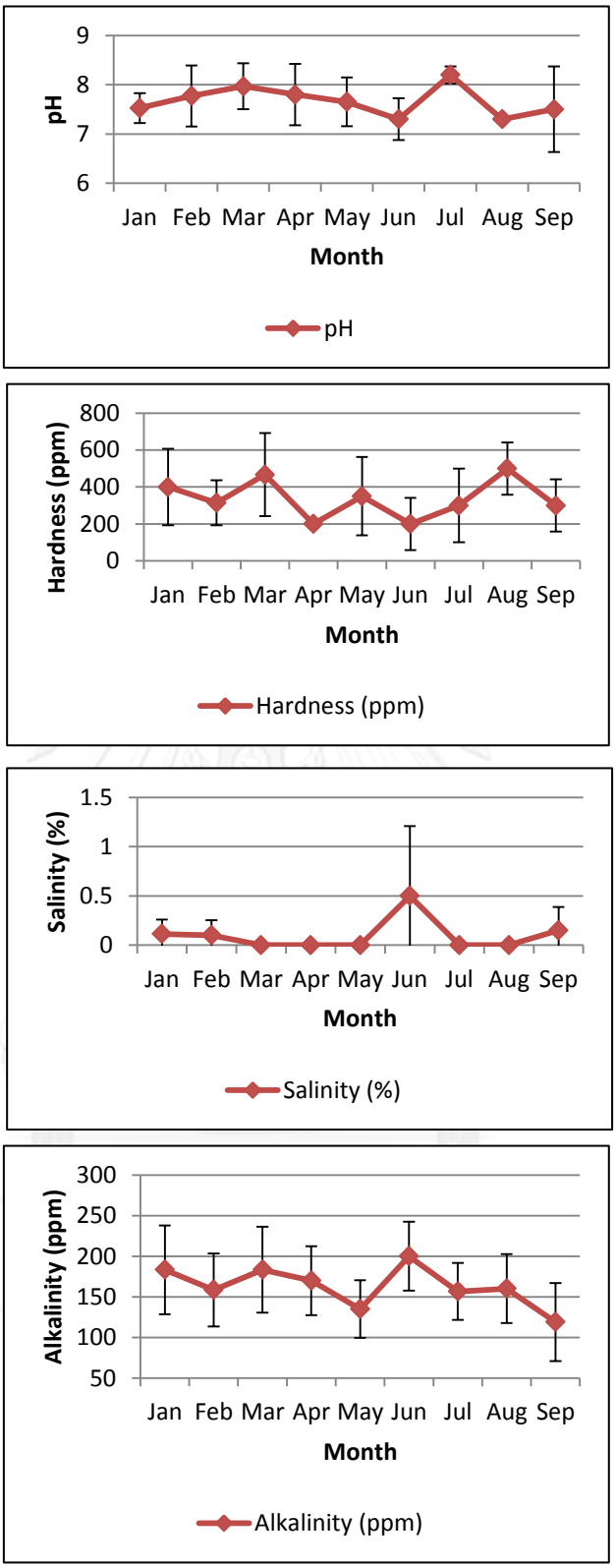
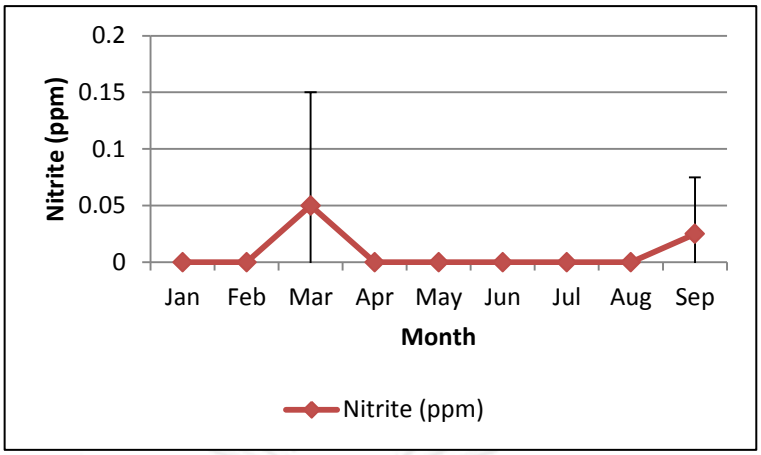
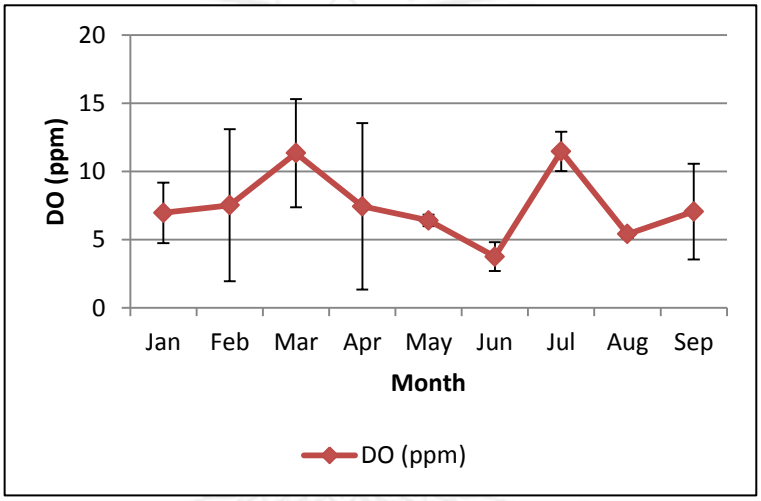


Figure 5 Water qualities in tilapia ponds. Samples were collected between Januarys to September 2011.





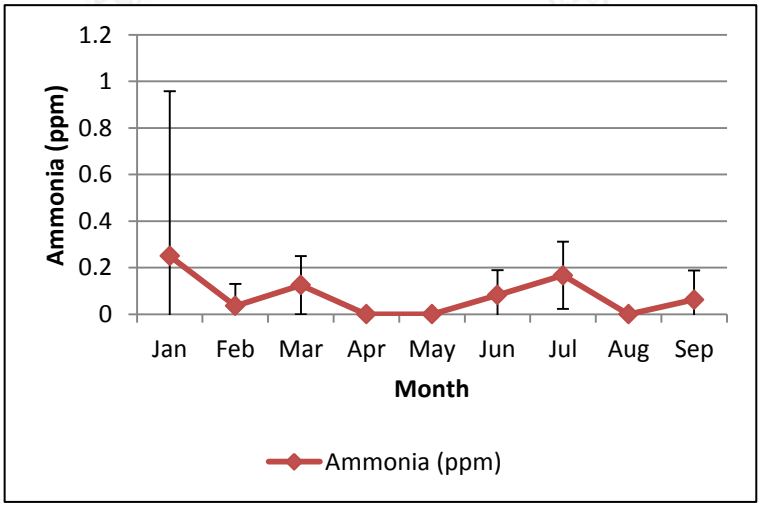


Figure 6 Water qualities in tilapia ponds. Samples were collected between Januarys to September 2011.

3.3. Identification and characterization of *Streptococcus agalactiae*

All streptococci isolated from clinical (35 isolates) and environmental samples (25 isolates) were identified as β-hemolytic *S. agalactiae* based on the conventional biochemical properties and species specific PCR. The macroscopic and microscopic appearance of *S. agalactiae* colony on TSA medium and the PCR results are presented in figure 7 and figure 8, respectively. Some environmental samples were contaminated by Gram-positive with α-hemolytic appearance but the biochemical assays indicating as Enterococci.

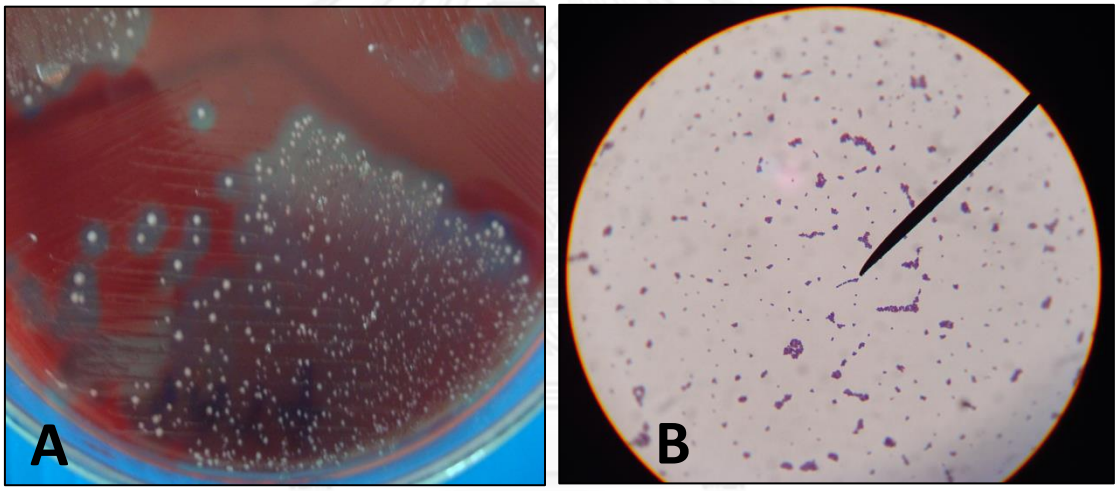


Figure 7 *Streptococcus agalactiae* colonies on TSA containing 5% sheep blood (A). Microscopic appearance of *S. agalactiae* at 100X magnification power (B).

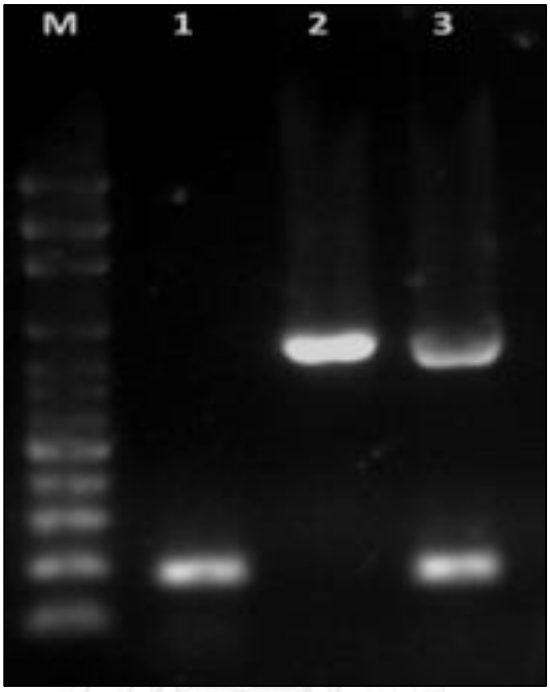


Figure 8 Species specific PCR for detection of *S. agalactiae* using 16S rRNAF1/IMOD primers and *S. iniae* using *lctO* specific primers. Lane M: 100 bp DNA marker, lane 1: fish originated *S. agalactiae* DNA, lane 2: *S. iniae* ATCC 29178 DNA and lane 4: mixture of *S. agalactiae* and *S. iniae* DNA.

The biochemical test results of catalase, oxidase, motility, O/F, Voges-Proskauer, CAMP, starch, aesculin and hippurate hydrolysis assays were identical among streptococci isolates obtained from environments, fish, bovine and human (Table 2.5). The streptococci isolates did not grow in a medium containing 6.5% NaCl except for the 7 isolates of environmental streptococci. Almost every isolates also shared common carbohydrate utilization pattern, but except for lactose utilization which the positive result was found only in human streptococci.

Table 2. 5 Phenotypic characteristics of *S. agalactiae* isolated in this study.

Test	Percent positive			
	Environmental strains (n=25)	Tilapia strains (n=33)	Bovine strains (n=9)	Human strains (n=6)
Gram stain	+,cocci	+, cocci	+, cocci	+, cocci
Haemolysis	ß	ß	ß	ß
Catalase	0%	0%	0%	0%
Oxidase	0%	0%	0%	0%
Motility	0%	0%	0%	0%
Oxidation/Fermentation of glucose (O/F)	100% F	100% F	100% F	100% F
Voges-Proskauer	0%	0%	0%	0%
Starch hydrolysis	0%	0%	0%	0%
CAMP	100%	100%	100%	100%
Growth in 6.5% NaCl	28%	0%	0%	0%
Esculin	0%	0%	0%	0%
Hippurate	100%	100%	100%	100%
Carbohydrate utilize				
Trehalose	100%	100%	100%	100%
Raffinose	8%	0%	0%	0%
Mannitol	12%	0%	0%	0%

Test	Percent positive			
	Environmental strains (n=25)	Tilapia strains (n=33)	Bovine strains (n=9)	Human strains (n=6)
Lactose	4%	0%	100%	0%
Galactose	100%	100%	100%	100%
Salicin	96%	100%	100%	100%
Sucrose	96%	100%	100%	100%

nr = not reported, v = variable

The molecular serotyping indicated that most of fish and environmental isolates belong to serotype Ia while only 2 isolates (from clinical sample and environmental samples collected from Ratchaburi and Nakhon Pathom province) were serotype III. For bovine isolates, all of them (9 isolates) were also categorized as serotype Ia while serotype Ia, Ib and III were found upon human isolates. The result of multiplex PCR for identification of *S. agalactiae* molecular serotypes is presented in figure 9.

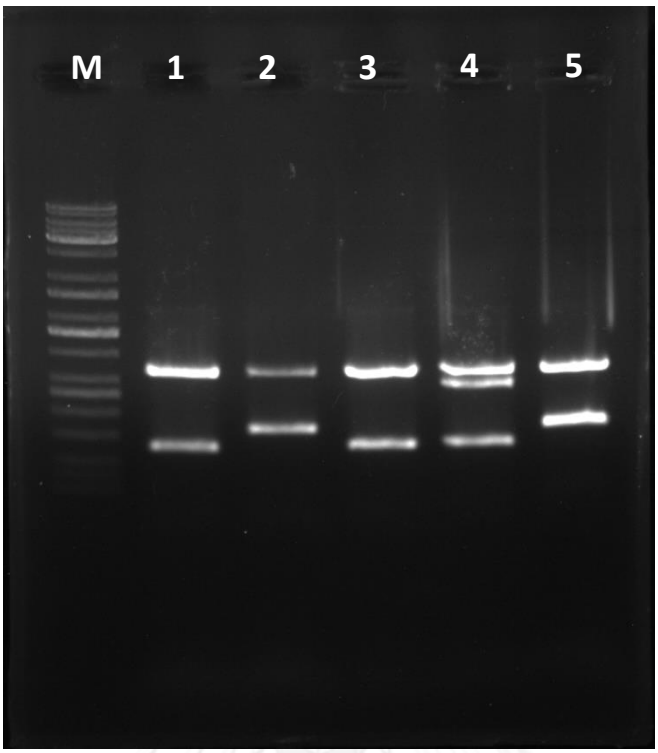


Figure 9 The electrophoresis picture of multiplex PCR for identification of *S. agalactiae* molecular serotype. Lane 1: *S. agalactiae* fish isolate serotype Ia, lane 2: *S. agalactiae* environmental isolate serotype III, lane 3-5: *agalactiae* human isolates serotype Ia, Ib and III, respectively. M: universal DNA ladder.

Clustering of streptococci isolates using the nucleotide sequences of the central variable region of the *infB* gene can identified four *infB* allele patterns. BLAST analysis of the *infB* sequences revealed that our streptococci isolates were categorized as alleles A, D, I and a newly identified allele, the sequence of which is different from all *infB* sequences currently in the GenBank database. This allele was named the '*infB* allele T'(GenBank accession no. JQ762635).

The preliminary testing of virulence-genes primers specificity with *S. agalactiae* A909 and H04 could generate all expected-PCR products except PI-2a. Since the Pilus island type-2 of *S. agalactiae* can carry either by PI-2a or PI-2b gene (Rosini et al., 2006), therefore the PI-2a cannot be found in the 'PI-2b-positive isolates' (i.e. *S. agalactiae* A909 and H04). In addition, the nucleotide sequence alignment of the PCR products using BLAST program showed 98-100% identity with

the target-virulence genes of *S. agalactiae* A909, GD201008-001, NEM316 and 2603V/R.

Virulence genes profile of *S. agalactiae* had been monitored and 10 profiling patterns were found among our bacterial collection. For fish/environmental isolates, PI-2a was completely absence and *scpB* and *lmb* were found in 3 isolates. On the other hand, the *bibA, cspA*, PI1, PI-2b were found from 58 isolates and the *cylE, hylB, bibA, gap, fbsA,* and *fbsB* were possessed in every fish/environmental isolates (figure 11). %).

Three (3) candidate primers (AP42, OPS11 and OPS16) were screened by preliminary RAPD analysis of six *S. agalactiae* strains. According to the result, OPS11 was the most suitable primer for this assay due to the better discrimination achieved with this primer as showed in figure 10.

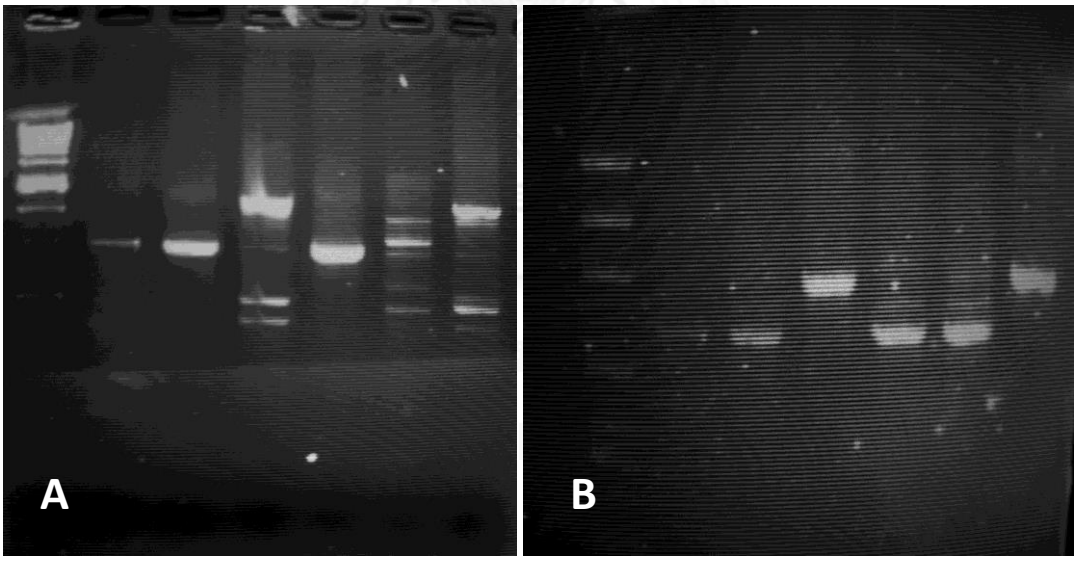


Figure 10 RAPD fingerprints generated from OPS11 primer (A) and P2 primer (B). Lane 1-2: DNA of *S. agalactiae* fish isolates, lane 3-4: DNA from *S. agalactiae* environmental isolates, lane 5: DNA of *S. agalactiae* human isolate and lane 6: DNA of *S. agalactiae* bovine isolate.

By using OPS11 primer, 42 different RAPD finger printing patterns were generated among 75 isolates (including environmental, fish, bovine and human isolates). The similarity matrix obtained by those RAPD fingerprinting patterns comparison was used to construct the dendrogram, which can roughly divided *S. agalactiae* strains into 6 clusters (I, II, III, IV, human and bovine). Notably, there was only 1 isolate (FNB06) that cannot categorize into any RAPD cluster. Most of fish and environmental originated strains belong to cluster I-IV. The members of RAPD cluster I composed of 13 bacterial isolates collected from several geographical regions (Ayutthaya, Nakhon Pathom, Phetchaburi and Prachinburi) Cluster II was the largest group that made up from 38 isolates which mostly obtained from Nakhon Pathom. Cluster III and IV were the very small group containing only 2 and 3 isolates, respectively. Interestingly, RAPD analysis of human/bovine isolates showed little relatedness with fish/environmental isolates (figure 11).

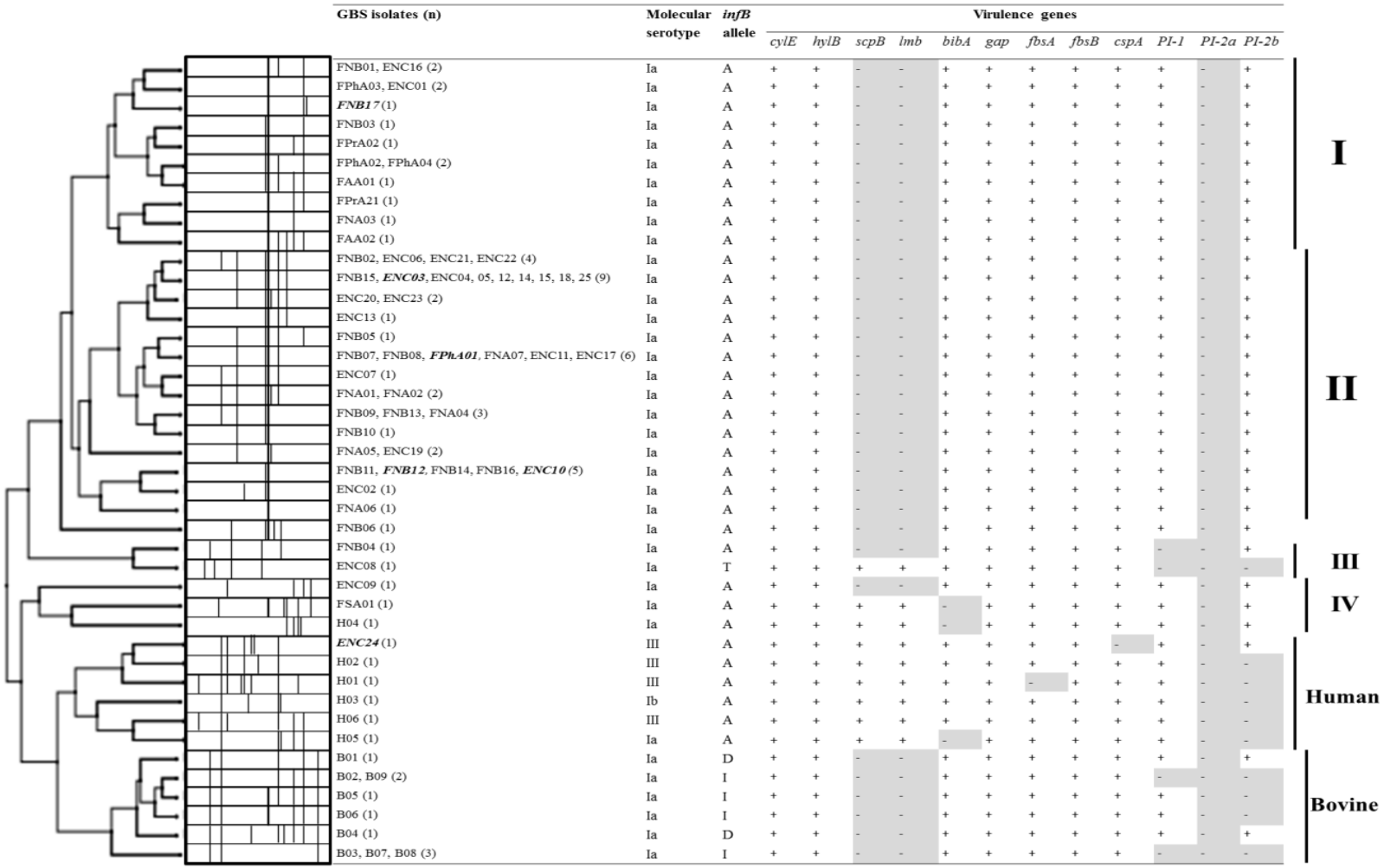


Figure 11 Compilation of molecular characterization tests. The results of the molecular serotyping, *inf* allele identification and virulence genes profiling are also included in the table adjacent to the dendrogram generated from RAPD fingerprints. Bacterial codes wrote in Bold and Italic was selected for LD_{50} analysis.

3.4. Median lethal dose

All isolates, except ENC24 (serotype III), can produce mortality in experimental Nile tilapia. The FPhA01 isolate had the lowest LD_{50} and produced clinical signs (darkened skin, exophthalmos and erratic swimming) and mortality after only 1 day post inoculation. Streptococci can be re-isolated from the brain of the fish that die in this experiment confirming that streptococci was the cause of death. Results of LD_{50} assays and details about the six *S. agalactiae* isolates used in this experiment are concluded in table 2.6.

Table 2. 6 Result of LD_{50} of six *S. agalactiae* isolates collected from environmental and fish samples.

Isolates	Molecular serotype	Source/Place (province)/Date of isolation	LD_{50} (log CFU)
ENC03	Ia	Environment/Nakhon Pathom/Jan 2011	7.12
ENC10	Ia	Environment/Nakhon Pathom/May 2011	7.56
ENC24	III	Environment/Nakhon Pathom/Sep 2011	>8 (not virulent in tilapia)
FNB12	Ia	Tilapia/Nakhon Pathom /July 2010	6.30
FNB17	Ia	Tilapia/Nakhon Pathom/July 2010	6.87
FPhA01	Ia	Tilapia/Phetchaburi /2009	6.25

4. Discussion

This study described the distribution of streptococcus among diseased tilapia and the environments in the farms. Among all of those samples, *S. agalactiae* was the only pathogenic streptococci that could be successfully isolated. This result was consistent with several previous epidemiological studies emphasizing *S. agalactiae* as the most important bacteria responsible for streptococcosis outbreaks in Thailand (Maisak et al., 2008; Suanyuk et al., 2008).

In this study, only ß-hemolytic *S. agalactiae* were recovered while the non-hemolytic *S. agalactiae* isolate could not be isolated from any sample. Until now, the non-hemolytic *S. agalactiae* has never been isolated from streptococcosis-associated fish in Thailand. The non-hemolytic *S. agalactiae* has been reported as major pathogenic streptococci in Brazil, Auatralia, Costa Rica and Vietnam, but the ß-hemolytic *S. agalactiae* is frequently found in UK, Malaysia and Thailand (Suanyuk et al., 2008; Yanong and Francis-Floyd, 2010; Amal et al., 2013a). For the other species of streptococci, *S. iniae* and *Lactococcus garvieae* were reported as the causative agents of streptococcosis in Red tilapia and Asian sea bass (*Lates calcarifer*) but the occurrence was very rear and limited only in the southern part of Thailand (Suanyuk et al., 2010). The dissimilarity of biochemical characteristics among *S. agalactiae* isolates was mostly rely on the variation of carbohydrate assimilation properties, while the other biochemical assays still gave the identical results. In order to identify ß-hemolytic *S. agalactiae*, we strongly recommended conventional biochemical assays especially for CAMP and hippurate hydrolysis test since these methods can confer rapidly and prominently positive result. However, it should be mentioned that non-hemolysis *S. agalactiae* (biotype II) deliver negative-result for both hippurate and CAMP tests, hence the diagnostic process of *S. agalactiae* should be interpret carefully (Eldar et al., 1994).

The prevalence of *S. agalactiae*-positive samples collected from the diseased fish was 56.4% while the common Gram negative bacteria such as *Aeromonas* sp. were predominated in most *S. agalactiae*-negative samples instead. The clinical signs presented among streptococci-infected fish were not quite specific and the

pathognomonic neurological signs of streptococcosis (erratic swimming) could not be observed in any case (Hernandez et al., 2009), hence it was possible to misinterpret streptococcosis as the septicemia-associated disease caused by other bacteria such as Aeromonadaceae, Enterobacteriaceae. Nevertheless, the prevalence of *S. agalactiae* described by this study was similar to the results obtained from the previous surveillance study conducted in Malaysia which reported that *S. agalactiae* in cage-cultured tilapia was vary between 0-35% (Amal et al., 2013b). Since we only collect the fish samples during the diseases outbreak, therefore it is possible to gain a very high prevalence rate of streptococci.

According to previous publications, the recovery rate of streptococcus from water samples had large variation as its occurrence may higher than 50% during streptococcosis outbreak or may absolutely absence during non-diseased incidence (Nguyen et al., 2002; Bromage and Owens, 2009). In our study, *S. agalactiae* were deposited at least for 18.65% in tilapia culturing ponds during non-disease situation. According to the result of the LD_{50} assays, both the environmental isolated streptococci and those collected from diseased fish can cause mortality in experimental fish suggesting that the pathogenic streptococci could be inhabited in tilapia farms in the opportunistic fashion.

In this study, the molecular serotypes analysis indicated that serotype Ia was the majority of the fish originated *S. agalactiae* in Thailand which is agreeing with the result from the recent publication (Suanyuk et al., 2008). In case of aquatic animals, the nonhemolytic-serotype Ib was often reported in the South America and Israel but never been isolated in Thailand (Vandamme et al., 1997). It is very crucial to have the information of serotype distribution in particular area because the cross-immunity against different serotypes was extremely limited (Lindahl et al., 2005). This epidemiological data, in turn, could be helpful in order to design the appropriate protection policy especially for the vaccination against streptococcosis in particular region.

Allele typing of translation initiation factor IF2 gene (*infB*) showed only minor dissimilarity among environmental/fish isolates which largely diverged from

human/bovine isolates. Comparison of central variable region of *infB* was regarded as a useful tool for studying the relationship between streptococcal strains due to their correlation with an evolutionary tree generated by 16S rRNA sequences and, presently, a total of 19 *infB* allele sequences have been available in GenBank database. In the current study, a novel *infB* allele was identified in 1 isolate of *S. agalactiae* and designated as 'allele T' (Hedegaard et al., 2000; Sorensen et al., 2010).

The virulence genes profiling analysis employed in the current study was based on the identification of 12 postulated virulence genes using conventional PCR. The roles of these 12 virulence genes in disease pathogenesis have been demonstrated in mammalian model (Doran and Nizet, 2004; Maisey et al., 2008a). However, their involvement to the pathogenesis in aquatic animals has never been investigated yet. The 12 postulated virulence genes included in the test can be categorized as being associated with 1) bacterial adhesion and colonization (*lmb, dltA, bibA, fbsA, fbsB,* PI-1*,* PI-2), 2) bacterial invasion (*hylE, cspA, gap*), 3) immune evasion (*cylE, cpsA, scpB*) and 4) toxin production (*cylE*). According to our results, the diversity of virulence genes profiling patterns was influenced by the variation of *scpB, lmb,* PI-1 and PI-2 (figure 11). The horizontal gene transfer was prompted to be the crucial cause that responsible for the variation of virulence genes profiling pattern since *scpB, lmb,* PI-1 and PI-2 were possessed in mobile genetic element (MGE) which could randomly leave or inserted into bacterial chromosome (Franken et al., 2001; Rosini et al., 2006). According to a prior publications, identification of MGEs-related fragment such as group II introns, virulence genes and several insertion sequences in streptococcal genomes were proposed to be useful for characterizing *S. agalactiae* genetic traits (Kong et al., 2003; Godoy et al., 2013). In this study, the absence of *scpB/lmb* from 95% of environmental/fish isolates is consistent with the previous publications indicating the deletion of *scpB/lmb* in the majority of fish (70%) and bovine strains (80%) (Franken et al., 2001; Rosinski-Chupin et al., 2013). Intriguingly, despite the absence of *scpB* and *lmb,* experimental challenge of the fish with *S. agalactiae* still be successful suggesting that *scpB* encoded-C5a peptidase and *lmb* encoded-laminin-binding proteins might not involve with the pathogenesis

of streptococcus in the fish. Of note, the absence of some chromosomal-dependent virulence genes in the few bacterial isolates indicated that none-horizontal transfer may involve in the deletion and we trusted that the genetic recombination among several *S. agalactiae* via bacterial temperate phage may responsible for the losing of some genetic content (Domelier et al., 2009; Rosinski-Chupin et al., 2013). The depletion of chromosomal content is regarded as one of the long-lasting mechanisms that bacteria used to adapt itself to new host tropism (designated as 'reductive evolution') which may define host specialization of *S. agalactiae* fish strains (Rosinski-Chupin et al., 2013).

According to the constructed dendrogram, the fish/environmental isolates of *S. agalactiae* were divided into 4 distinct molecular clusters (I-IV; figure 11) with high genetic variation and, interestingly, they seem to be related with geographical origins. For the example, most of the samples collected from Nakhon Pathom province were apparently belonged to cluster II while the others were the members of cluster I, III and IV. Our finding was consistent with the recent epidemiological study indicating the relationship between genotypic traits and geographical origins (Amal et al., 2013a). Herein, it should be mentioned that the different *S. agalactiae* genotype hold the immense variation of immunological property despite the sharing of the same capsular serotype. The genotypic diversity among *S. agalactiae* found in Thailand emphasizes the difficulty of the vaccine application as it previously described in the literature that protective immunity against different genotype, but the same serotype, of *S. agalactiae* was very limited (Chen et al., 2012). Furthermore, the little relationship between fish/environmental and human/bovine strains (due to the generated dendrogram) was consistent to the recent epidemiological studies using MLST and PFGE technique suggesting that *S. agalactiae* fish strains had developed their own distinct genetic lineage (Evans et al., 2008; Pereira et al., 2010). According to literatures, MLST assay can categorized*S. agalactiae* fish strains into 2 main groups (Evans et al., 2008; Delannoy et al., 2013). The first group referred to *S. agalactiae* belonging to clonal complex (CC) 7 and the other was classified as sequence type (ST) 246, 257, 258, 259, 260, 261 and 283. Within CC7, the ST7 strains are considered as the important fish pathogenic *S. agalactiae* which had

been proposed to be evolutionarily diverged from pathogenic human and bovine ST7 strains (Rosinski-Chupin et al., 2013). Since the experiments in the current study did not include MLST assay, defining of the ST of *S. agalactiae* isolates is hardly imply. Nevertheless, the results obtained in this study convinced that environmental/fish *S. agalactiae* were most resemble to fish ST7 because of the similarities in biochemical characteristics (ß-hemolysis, positive in CAMP, hippurate hydrolysis and carbohydrate utilization properties), serotype (Ia) and the virulence gene profile, while the other fish STs were mostly reported to be non-hemolytic serotype Ib and CAMP negative. (Evans et al., 2008; Delannoy et al., 2013). Besides, ST7 was identified recently from *S. agalactiae* collected from diseased tilapia in Thailand (Suanyuk et al., 2008).

In summary, this study emphasizes that ß-hemolytic serotype Ia *S. agalactiae* was the most important cause of warm-water streptococcosis of tilapia in Thailand. The pathogenic *S. agalactiae* can be inhabited opportunistically in farming environments and the diversity in molecular characteristics had occurred in geographical-dependent manner.

Chapter 3: Influence of temperatures to the pathogenicity of *S. agalactiae*

Abstract

Temperature has been recognized as one of most important factors affecting aquatic animals' health status and increasing of water temperature was reported to be related with high severity of streptococcosis in fish. In this study, the *in vitro* and *in vivo* pathogenicity of *S. agalactiae* in different temperatures were determined in order to describe the relationship between disease and the temperature. The results indicated that, at 35 and 37 ^{0}C, the bacterium growth can enter exponential-phase within 3 hours which was significantly faster than 28 ^{0}C. The hemolysis activity and anti-whole blood killing activity also showed that *S. agalactiae* grew at 35 ^{0}C can lyse erythrocytes and prolonged their viability in tilapia whole-blood more efficiently than those grew at 28 ^{0}C. The microscopic pictures also indicated that, at 35 ^{0}C, the number of capsule-covered streptococcal cells was much higher. Extensive up-regulation of *cylE* (ß-hemolysin/cytolysin), *cfb* (CAMP factor) and PI-2b (pilus type 2b-backbone) were detected from 35 ^{0}C cultured streptococcus comparing with 28 ^{0}C. The experimentally infected tilapia rearing at 35 ^{0}C condition had 85% accumulated mortality while only 45% was found in the fish rearing at 28 ^{0}C. In conclusion, this experiment indicated that increasing of temperature can influences the pathogenicity of fish originated *S. agalactiae*.

1. **Introduction**

Streptococcus agalactiae is currently recognized as one of the most severe pathogens in aquaculture industry. Nowadays, the massive outbreaks of streptococcosis associated with *S. agalactiae* have been reported in every continent but mostly clustered in tropical countries in South America and South-East Asia including Thailand (Suanyuk et al., 2005).

According to chapter 2 of this thesis, our study demonstrated that the viable *S. agalactiae* is commonly existed in the farming environments such as water and pond sediment. However, the inhabited *S. agalactiae* may acts as opportunistic pathogen and inhabited calmly in the environments until the homeostasis losing their balance and disease outbreak can occur eventually. In aquatic animals, the 'stressful condition' is the predisposing factor for the development of disease, which can be introduced by the rapid and extreme change of the environments and making the animal more vulnerable to the infectious organisms. Several environmental factors such as stocking density, pH, salinity, dissolved oxygen and water qualities have been reported to be associated with streptococcosis occurrence in the farms, but the most critically important factor is believed to be the 'water temperature' (Perera et al., 1997; Bromage and Owens, 2009; Suanyuk, 2009; Yanong and Francis-Floyd, 2010; Amal and Zamri-Saad, 2011). The outbreaks of disease caused by *S. agalactiae* infection were mostly occurred in warm condition, which can refer to the synonym of this disease as 'warm-water streptococcosis' (Yanong and Francis-Floyd, 2010; Amal and Zamri-Saad, 2011). Many epidemiological evidences confirmed the close relationship between the water temperature and prevalence of streptococcosis, however the critical temperature for treptococcosis outbreak might be different depends on the species of the fish and the geographical locations (Bromage and Owens, 2009; Mian et al., 2009). In some particular temperature, the bacterium can efficiently replicate and produces virulent-associated proteins (Paterson et al., 2006). In the case of *S. agalactiae*, the detailed information of physiological responses of *S. agalactiae* upon the various temperatures is still needed to be elucidated.

Therefore, the objective of this study is to examine the effects of temperature to the pathogenicity of *S. agalactiae* and their relationship with the disease occurrences in the fields.

2. Materials and methods

2.1. Bacterial strain

Streptococcus agalactiae FNB12 serotype Ia which was isolated from diseased tilapia from Nakhon Pathom province in central region of Thailand since 2010 had been used in this study. The identification procedure and their molecular characteristics already described in Chapter 2. This isolate was selected because of the high pathogenicity (LD_{50} = 10^6, approximately) comparing with the other isolates in our bacterial collection. The bacterium was preserved in 75% glycerol at -80 ^{0}C until use.

2.2. Fish

Fingerling Nile tilapia (*Oreochromis niloticus*) were purchased from commercial tilapia farm and had been raised in our facility until gaining 30-50 g body weight. At least 1 % of the fingerlings were randomly sampling for bacterial isolation to confirm that the fish were free from streptococcus. The experimental tilapia were then randomly selected and maintained in 60 l PVC tanks under natural light and feeding daily with 3% body weight of commercial diet. The water qualities were steady at 5.0-6.0 ppm of dissolved oxygen (DO) and pH 7.5-8.0 throughout the experiment.

2.3. Study of *S. agalactiae* responses to various temperature conditions

Bacterium from glycerol stock was sub-cultured on tryptic soy agar (TSA) with 5% sheep blood at 37 ^{0}C overnight. Streptococcal colony growth on agar surface had been applied for the downstream processes as described below.

2.3.1. Bacterial growing ability

Single colony of *S. agalactiae* on TSA medium was transferred to tryptic soy broth (TSB) and incubated at 37 ^{0}C overnight. After incubation, the bacterial mixture was aliquot to newly sterile TSB with 1:10 dilution in a 10 ml of final volume and incubated again at 25, 32, 35 and 37 ^{0}C. Bacterial growth had been determine through the turbidity of bacterial mixture which measured by OD_{600} spectrophotometer for 8 hours. In addition, all experiments were conducted in triplicate.

2.3.2. Bacterial hemolysis activity

Hemolysis activity of *S. agalactiae* was analyzed in both qualitative and quantitative manner. The hemolysis of bacterium grew on TSA blood medium was observed and interpreted using descriptive analysis. For quantitative assay, the hemolysis activity was evaluated by the method modified from Santi and colleagues (Santi et al., 2009). Shortly, streptococcal colony was sub-cultured in 5 ml of TSB at 28 and 35 ^{0}C until attained exponential-phase. Then, approximately 10^{8} CFU (OD_{600}=0.6) were washed twice with phosphate-buffered saline (PBS) and suspended in 1 ml of PBS containing 0.2% glucose. The bacterial solution was then mixed with equal volume of 1% sheep blood in PBS/glucose buffer followed by incubation at 30 ^{0}C for 1 hour. Bacterial cells, erythrocytes and other contents were then separated from supernatant by centrifugation at 3000 Xg for 3 minutes. The amount hemoglobin which released from erythrocytes was determined by OD_{420} spectrophotometry. Additionally, the PBS/glucose buffer containing 0.1% SDS was used instead of the bacterial suspension and applied as hemolysis-positive control (because 100% of erythrocytes were hydrolyzed by SDS). On the other hand, the PBS/glucose buffer (without bacterial cells) was used as a negative control. The experiments were performed in triplicate.

2.3.3. Bacterial viability in tilapia whole blood

The viability of *S. agalactiae* in tilapia whole blood was examined in order to demonstrate the resistance of the bacterium to phagocytosis and antimicrobial substance from the fish. The experimental procedure was employed according to the study of Locke and colleagues (Locke et al., 2007). Briefly, streptococcus was grown in TSB at 28 and 35 ^{0}C until exponential-phase and then washing of the bacterial cells twice by sterile PBS. Whole blood was freshly drew from caudal vain of Nile tilapia using heparinized tuberculin and 300 µl blood was then mixed with 100-1000 CFU of streptococcal cells followed by an incubation at 30 ℃ for 1 hour. After incubation, the reaction was stopped immediately by cooling on-ice. The viable bacterial cells were then enumerated by standard plate count technique using TSA blood medium. The relative survival of streptococcus was presented as percentage

which calculated as the enumerated CFU divided by numbers of initial inoculum. As usual, all experiments were performed in triplicate.

2.3.4. Bacterial capsule production

The production of bacterial capsule was examined and analyzed descriptively. Bacteria was grown in TSB at 28 and 35 ^{0}C until attained exponential-phase before subjected to capsular staining process called 'Anthony's stain' (Anthony, 1931). The detail of staining procedure can be found in the Appendices of this thesis. To determine the capsular production, at least 3 microscopic filed of 1000X magnifying power were observed and all experiments were conducted in triplicate.

2.3.5. Expression of putative virulence factors

Streptococcus agalactiae was cultured at 25 and 35 ^{0}C in TSB until reaching exponential- and stationary-phase (determined by OD_{600}) then dissolved in RNAlater® reagent (Invitrogen, USA) immediately. Total RNA of bacteria was isolated using Invitrap® spin universal RNA mini kit according to manufacturer's instruction (Stratec, Germany). Quality and concentration of RNA were evaluated by the absorbance at 260/280 nm. One micrograms of total RNA samples were converted to first-strand cDNA using Omniscript® RT kit (QIAGEN, USA) according to the manufacturer's instruction. The cDNA synthesis reaction also carried out in the absence of reverse transcriptase to use as a control to determine whether genomic DNA contaminated in RNA samples. All of cDNA samples were preserved at -20 °C refrigerator until performing quantitative PCR.

Quantitative RT-PCR (qRT-PCR) for analysis of genes expression were conducted in Real-Time PCR ABI Prism 7500® platform (Applied Biosystems, USA). Thirteen putative virulence related genes (*cfb, cylE, cpsX, sodA, dltA, cspA, bibA, gap, hylB, fbsA, fbsB*, PI-1 and PI-2b) while *gyrA* was chosen to be an endogenous normalizer gene. Specific primers for each particular gene are already mentioned in chapter 2 (Table 2.2). Each qRT-PCR reaction contained 1 µl of cDNA, 0.2 µM of each forward and reverse primer, 1X Evagreen real-time-PCR master mix E4 (GeneON, Germany) and deionized water up to 20 µl of the final volume. PCR condition

comprised of an initial denaturation at 95 °C for 3 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s and extension with fluorescence acquisition at 72°C for 45 s. The relative expression of virulence genes in the 35 ^{0}C grew *S. agalactiae* were then analyzed by ΔΔCt method using 28 ^{0}C grew bacteria as a calibrator sample.

2.4. Pathogenicity in Nile tilapia

One hundred and eighty (180) tilapia were used in this pathogenicity assay. In brief, the *in vivo* passage *of S. agalactiae* had been conducted once before starting this experiment. The prepared bacterium was then cultured in TSB at 28 and 35 ^{0}C until reaching exponential-phase followed by washing bacterial cells twice and re-suspended with sterile PBS. The LD_{50} dose of *S. agalactiae* (4 X 10^{6} CFU) was challenged to the fish by intraperitoneal injection. The negative controls were challenged by sterile PBS instead of bacteria. The fish were divided into 4 groups comprised of 15 fish per group which reared in separated 6o l glass tank. The water temperature in the tanks had been controlled by 900W electrical heaters and the temperature was examined twice per day. Those 4 experimental groups were 1) streptococcus challenged group reared at 28 ^{0}C, 2) streptococcus challenged group reared at 35 ^{0}C, 3) control group reared at 28 ^{0}C and 4) control group reared at 35 ^{0}C. The clinical signs and mortalities of the fish had been observed for 14 days after infection. Dead fish were rapidly removed from the tank to conducted bacterial isolation in order to confirm whether *S. agalactiae* was the cause of death. All experiments were conducted in triplicate.

2.5. Statistical analysis

Analysis were conducted using one-way ANOVA statistic followed by the LSD multiple comparison in SPSS 17.0 program (IBM). In this assay, any percentage numbers were transformed to a rational numbers using arc-sine transformation prior subjected to statistical analysis.

3. Results

3.1. Bacterial growth ability

Colonies of *S. agalactiae* on TSA blood was much smaller in 28 ^{0}C growth bacteria (pin-point size) comparing with 35 ^{0}C growth (2-3 mm size) as showed in figure 12. The growing capacity of bacterium in TSB was extremely fast in high temperature condition since *S. agalactiae* reaching exponential-phase within 3 hours but it took more than 6 hours in normal temperature condition (figure 13). Moreover, the bacterial concentration in TSB at late-stationary phase (end stage of growing) was also much higher when culture at 37, 35 and 32 ^{0}C comparing with 28 ^{0}C growth condition.

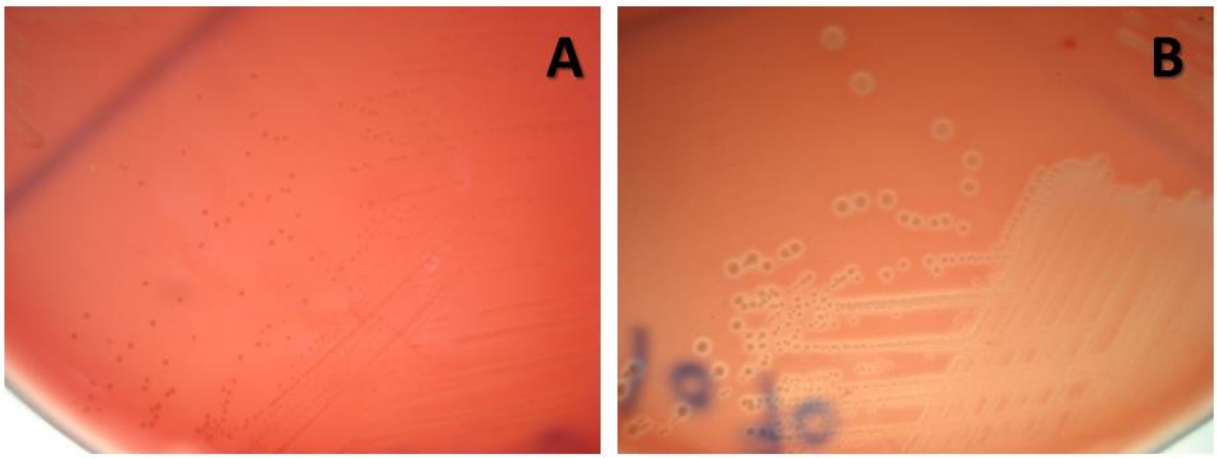


Figure 12 Phenotypic appearance of *S. agalactiae* growth on TSA containing 5% sheep blood at 28^{0}C (A) and 35 ^{0}C (B).

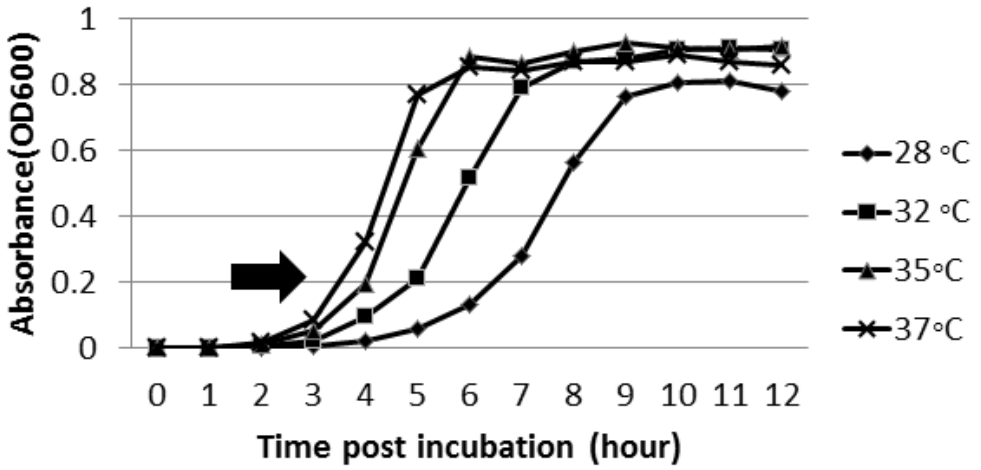


Figure 13 Growth curves of *S. agalactiae* cultured in TSB at 28, 32, 35 and 37 ^{0}C conditions. Growing capacity of bacteria was determined from the of OD_{600} spectrophotometry. The arrow represents the specific time-point that the bacteria reaching early-exponential phase.

3.2. Bacterial hemolysis activity

Hemolysis activity of *S. agalactiae* was higher at 35 ^{0}C comparing with 28 ^{0}C growing temperature. The hemolysis zone around the bacterial colonies was much larger at high temperature condition (figure 12). In broth culture, the hemolysis activity between high and low growing temperatures was also significantly different ($P<0.05$). While the bacteria grew at 35 ^{0}C can almost hemolyze 100% of RBC in the reaction mixture, only little of RBC was damaged by 28 ^{0}C growth bacteria (figure 14).

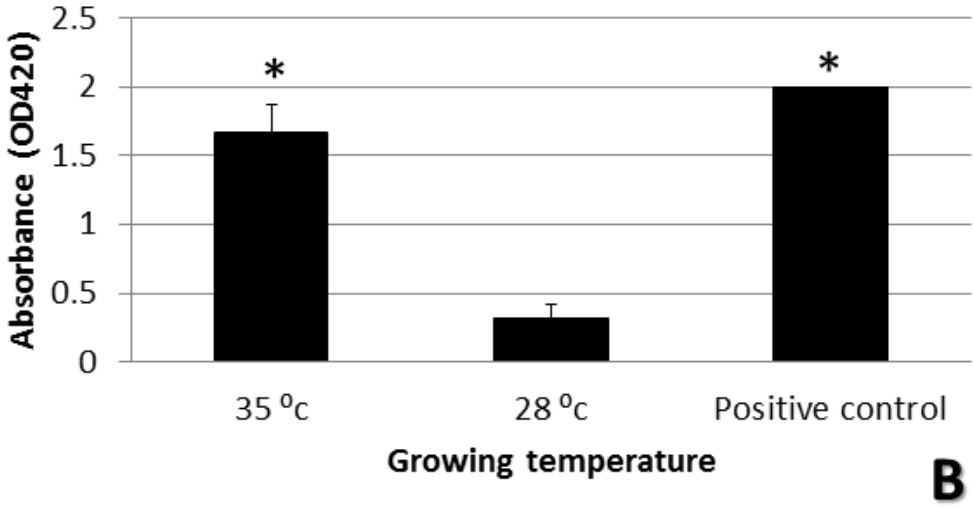


Figure 14 The hemolysis activity of *S. agalactiae* grew in 28 °C and 35 °C. Sheep RBC was co-incubated with the bacteria and the hemoglobin released from lytic RBC was determined by OD_{420} spectrophotometry. In positive control, the buffer contain 1%SDS was used instead of bacterial cells to lyse 1005 of RBC. Asterisk represents to a statistical difference ($P<0.05$).

3.3. Bacterial viability in tilapia whole blood

The survival ability of 28 °C and 35 °C grew *S. agalactiae* in Nile tilapia whole blood were remarkably different ($p<0.05$). In high temperature, the relative percent survival of streptococci was 97.4% approximately, while only 1.9% was found in low temperature growth streptococci (figure 15).

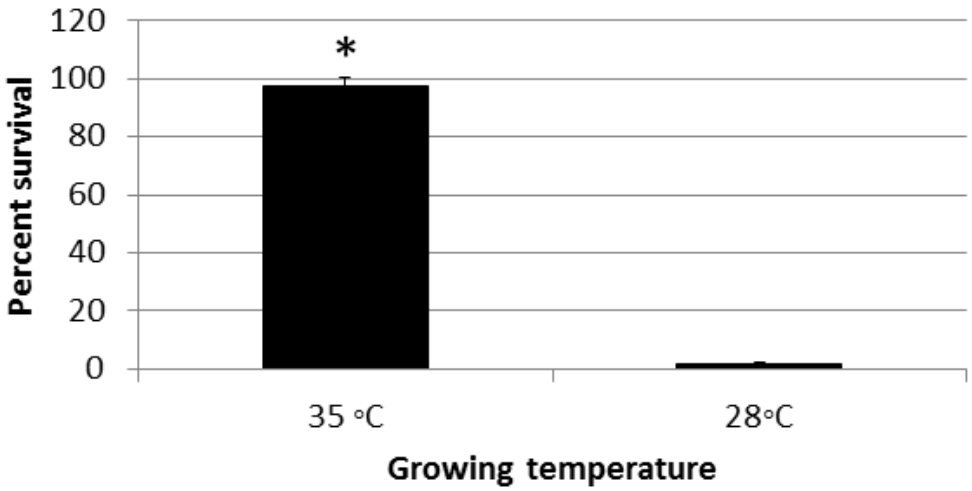


Figure 15 Relative percent survival of *S. agalactiae* after incubated with Nile tilapia whole blood for 1 hour. Bacteria were cultured in TSB at 28 ^{0}C and 35 ^{0}C until reaching exponential-phase prior co-incubation with freshly prepared fish blood. Asterisk represents to a statistical difference (P<0.05).

3.4. Bacterial capsule production

Screening of capsule staining *S. agalactiae* revealed that the capsular polysaccharide of bacteria was increasingly upon high growing temperature. It was obviously seen under microscope that more than 80% of the 35 ^{0}C growth bacteria were covered by the thick capsule layer, while the 28 ^{0}C growth bacteria had no capsule at all (figure 16).

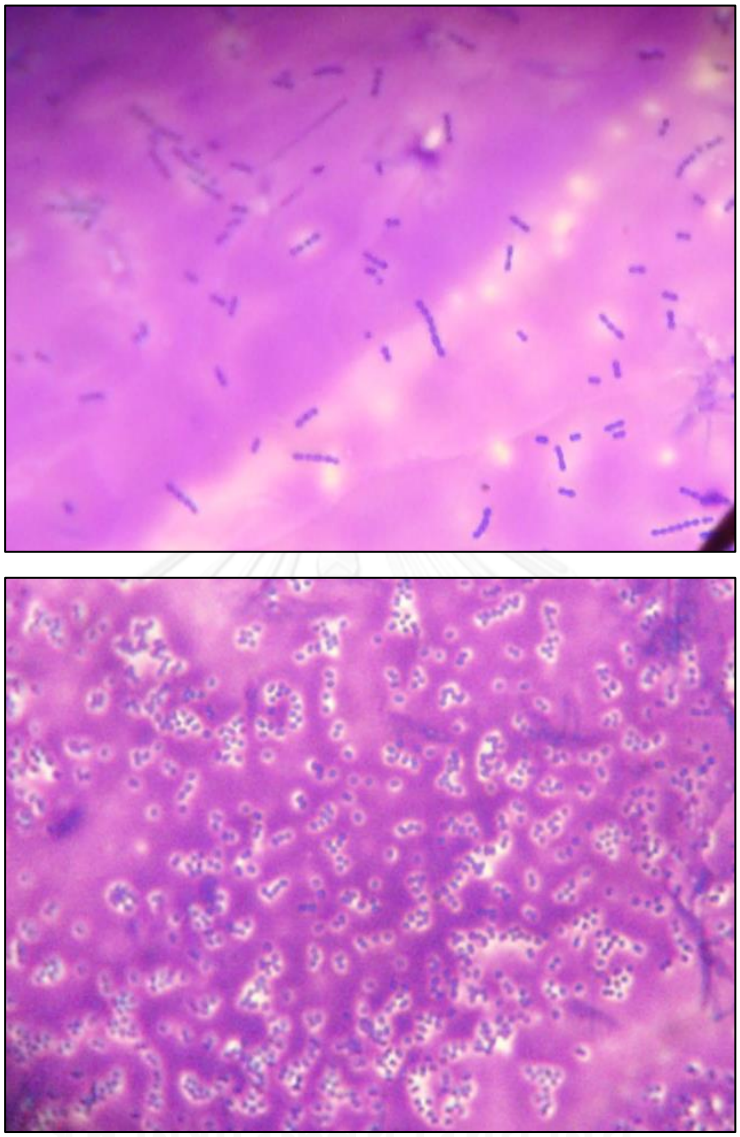
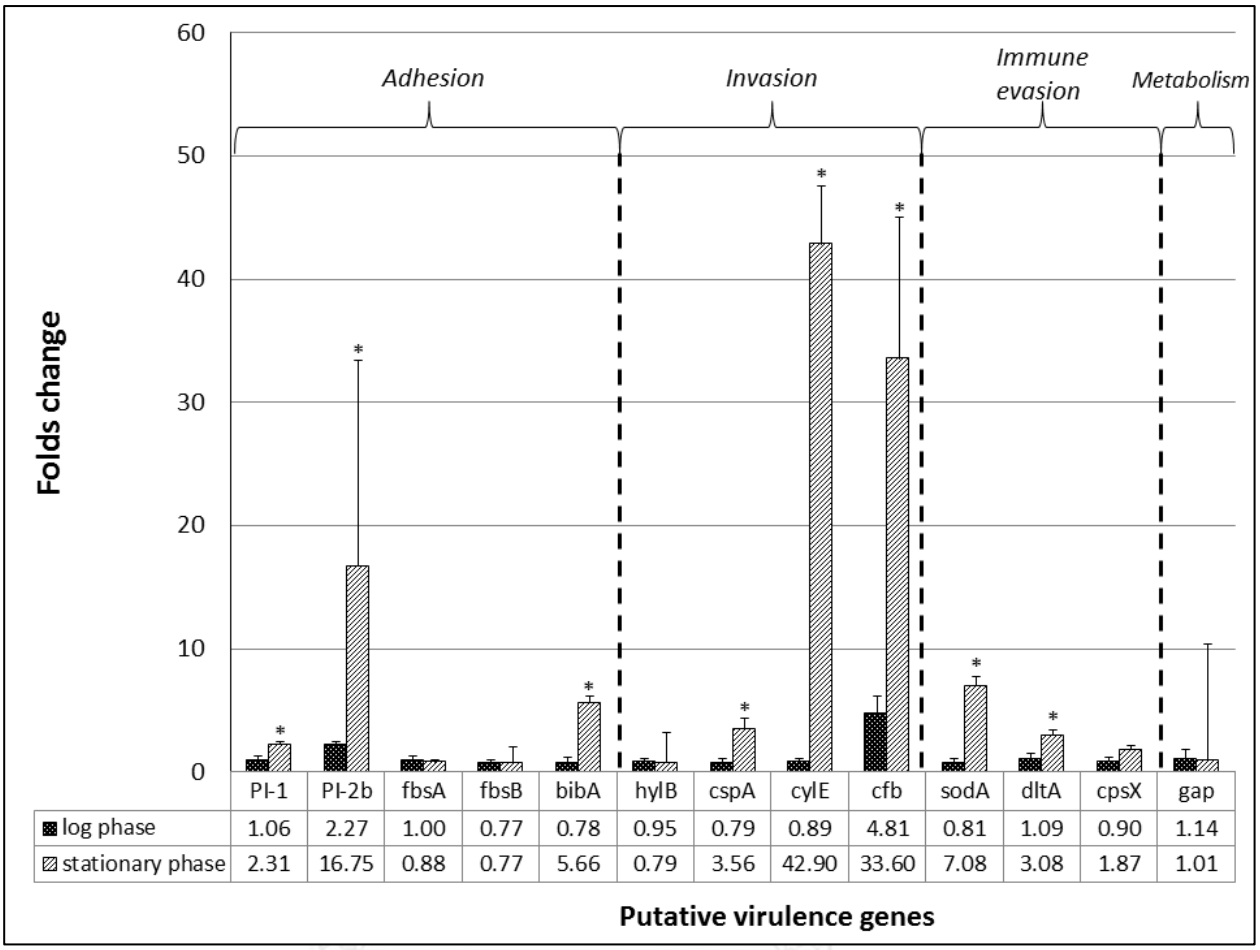


Figure 16 Anthony's staining of *S. agalactiae* grew at 28 ^{0}C (top) and 35 ^{0}C (bottom). The photos were captured under 1000X microscopic field. The white area (clear zone) around bacterial cells that grew in 35 ^{0}C was the surface covered capsular polysaccharide.

3.5. Virulence genes expression of *S. agalactiae*

In exponential-phase of 35 ^{o}C grew *S. agalactiae*, the up-regulation of putative virulence genes, comparing with 28 ^{o}C grew bacteria, had been detected from only 2 genes, i.e. *cfb* (encoded for CAMP factor) and PI-2b (encoded for pilus-2b backbone), The up-regulation was apparently observed during the stationary-phase which found that *cyl*E (ß-hemolysin/cytolysin) was up-regulated for 42 folds, followed by *cfb* and PI-2b at 33 and 16 folds, respectively. The up-regulation of other virulence genes (*cpsX,sodA, dltA, cspA*, and PI-1) were varied from 1.8 to 7 folds approximately. While only 4 genes (*hylB, fbsA, fbsB* and *gap*) had stable expression level (figure 17). The expression level of stationary-phase streptococci grew at 28 ^{o}C was insignificantly changed comparing with those 28 ^{o}C growth exponential-phase bacteria (data not showed).



	PI-1	PI-2b	fbsA	fbsB	bibA	hylB	cspA	cylE	cfb	sodA	dltA	cpsX	gap
log phase	1.06	2.27	1.00	0.77	0.78	0.95	0.79	0.89	4.81	0.81	1.09	0.90	1.14
stationary phase	2.31	16.75	0.88	0.77	5.66	0.79	3.56	42.90	33.60	7.08	3.08	1.87	1.01

Figure 17 Expression of putative virulence genes of *S. agalactiae* in response to an increasing of temperature. The expression values of log and stationary-phase of streptococcus grew at 35 ^{0}C were calculated in relation with the bacteria grew at 28 ^{0}C by ΔΔCt method. The involvement of virulence genes and pathogenic mechanism is briefly stated above the bar charts. Asterisk represents to a statistical difference (P<0.05).

3.6. Pathogenicity in Nile tilapia

The accumulated mortalities of the *S. agalactiae* challenged fish which reared at 28 and 35 ^{0}C conditions were 45% and 85%, respectively (figure 18). The mortality was started since 2 dpi followed by sharply increased at 3-5 dpi and stop at 7 dpi. The remarkably clinical signs, such as neurological sign (erratic swimming) and bi-ocular exophthalmos, and the internal lesions, such as swollen spleen, hemorrhage at kidney and brain, had been found in most of the moribund fish. Streptococci had been re-isolated from the brain of every dead animal which can confirm that streptococcosis was the cause of mortalities. No death had occurred in the control groups reared in both 28 and 35 ^{0}C through 14 days of experiment.

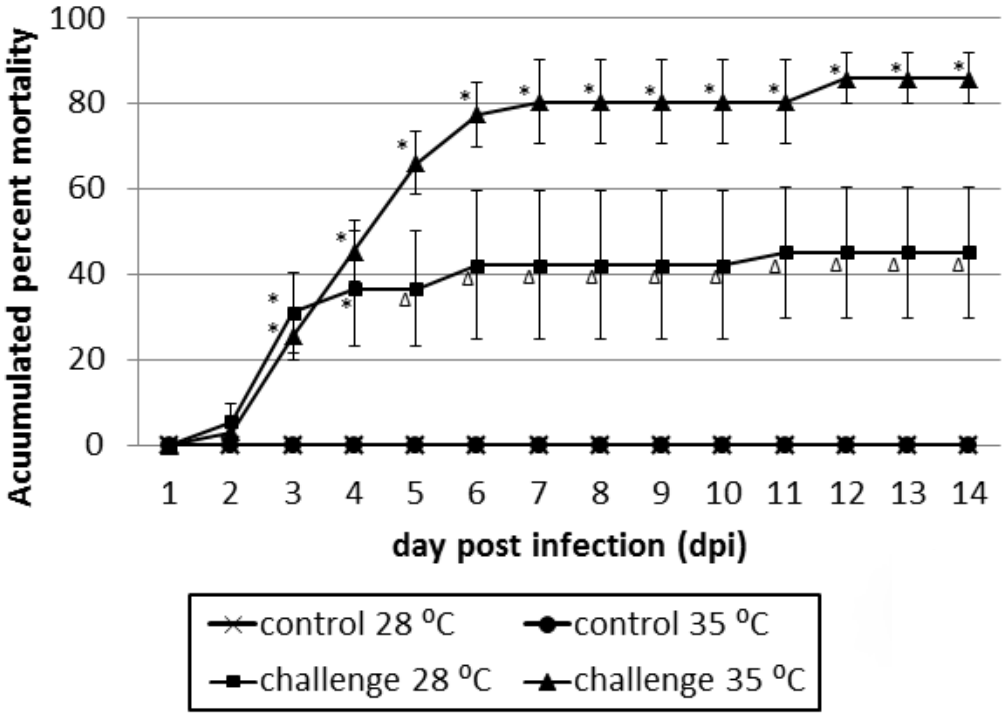


Figure 18 Accumulated mortalities of Nile tilapia kept in different water temperature conditions. The symbols (* and Δ) represent the significant difference ($P<0.05$).

4. Discussion

In aquatic animal, water temperature is widely recognized as one of the most important factors affecting the severity of disease (Le Morvan et al., 1998). According to several publications, the prevalence and severity of diseases caused by pathogenic bacteria, protozoa and parasite, such as *Pseudomonas* sp. *Aeromonas* sp. *Flavobacterium* sp. *Gyrodactylus* sp. *Dactylogyrus* sp. *Ichthyophthirius* sp. *Costia* sp., have been reported to be associated with environmental temperature changes (Meyer, 1978; Karvonen et al., 2010). Change of water temperature directly affects the physiological responses of poikilothermic animals and, simultaneously, has an impact on aquatic microorganisms as well.

In this study, the optimum temperature supporting *S. agalactiae* growth was 35-37 ^{0}C while the growing capacity was much lower at 25 ^{0}C condition. Our results are consistent with several publications suggesting that the warm-water temperature (32 ^{0}C or higher) significantly induce the replication of *S. agalactiae* (Perera et al., 1997; Zhou et al., 2008; Suanyuk, 2009; Rodkhum et al., 2011). In the inappropriate temperatures, the growth of bacteria was slowly preceded due to the improper work of replication-related enzymes and molecules (Ratkowsky et al., 1982; Ratkowsky et al., 2005). Since the *S. agalactiae* ancestors were originated in mammalian host, i.e. bovine and human, and theirs optimal temperature was 35-38 ^{0}C (resemblance with body temperature of the host), hence it is reasonable to believed that some important phenotypes such as the 'normal physiological temperatures' still be inherited from their predecessors (Stephanie, 2012).

The other *in vitro* virulence properties of the *S. agalactiae* were also enhanced upon high temperature. The hemolysis activity of *S. agalactiae* in broth and agar medium was extremely high at 35 ^{0}C growing temperature. The RBC destruction ability of *S. agalactiae* mainly relies on the functionality of ß-hemolysin/cytolysin (Pritzlaff et al., 2001). Here in, the vast up-regulation of ß-hemolysin/cytolysin encoding gene (*cylE*) was also found out in 35 ^{0}C grew streptococci as well (figure 17). These results were agreed with the previous mammalian-based study indicating the elevation of hemolysis activity and *cylE*

expression level in 40 ^{0}C comparing with 30 ^{0}C (Mereghetti et al., 2008b). According to several publications, ß-hemolysin/cytolysin was the potent cell-lysis toxin which generated cell destruction through the pore-forming activity (Nizet, 2002; Doran and Nizet, 2004; Ge et al., 2009). Additionally, this toxin was also has alternative function in carotenoid-pigment construction which significantly related with the anti-oxidant activity and intracellular survival of bacteria (Liu et al., 2004; Sagar et al., 2013). The enhancement of *S. agalactiae* virulence *in vitro* was also apparently seen in its viability in tilapia whole blood. Our result indicated that *S. agalactiae* grew at 35 ^{0}C, comparing with 28 ^{0}C, can extend their viability in tilapia whole blood. The prolonged survival in the blood was directly related with the resistant capability of streptococci to the host antimicrobial peptide and phagocytosis (Doran and Nizet, 2004; Maisey et al., 2008a). Several virulence factors have been reported to be related with viability of streptococci in blood, such as ß-hemolysin/cytolysin, C5a-peptidase, serine protease, superoxide dismutase, BibA surface protein, penicillin binding protein and capsular polysaccharide (Doran and Nizet, 2004; Maisey et al., 2008a). According to the capsular staining technique, our study also indicating an enhancement of capsule production in 35 ^{0}C culturing condition which may responsible for the prolonged survival of streptococci in blood. The common feature of capsular polysaccharide is to protect the bacterium from phagocytosis by hiding bacterial cell surface-antigen underneath the capsule (Locke et al., 2007; Lowe et al., 2007).

Quantitative RT-PCR analysis of exponential-phase *S. agalactiae* showed insignificant change in the expression level of putative virulence genes from 28 ^{0}C and 35 ^{0}C except for *cfb* and PI-2b. A PI-2b represents the gene encoding the structural component of streptococcus pili type-2 (Rosini et al., 2006). The roles of streptococcal pili in the biofilm formation, adherence and translocation through host epithelial and endothelial cells has been documented in several recent studies (Maisey et al., 2007; Pezzicoli et al., 2008; Konto-Ghiorghi et al., 2009). Thus, an enhancing of pili expression may involve with the successful of the localization and dissemination during the early step of infection. The up-regulation of *cfb* (encoding CAMP factor) may help promoting cellular destruction and invasion into the

circulatory system of the host via its pore-forming activity (Lang and Palmer, 2003). In this study, the massive up-regulation of virulence genes was observed at the time that streptococci entered stationary-phase. The highest up-regulation had been detected from *cylE* (ß-hemolysin/cytolysin) followed by *cfb* (CAMP factor), PI-2b 1 (Pili-2b backbone), *sodA* (superoxide dismutase), *dltA* (D-alanylation ligase), *bibA* (Immunogenic bacterial adhesin), PI-1 (Pili-1 backbone) and *cpsX* (capsule biosynthesis regulatory protein), respectively. This result suggested that the virulence genes up-regulation involved in every step of pathogenic process (i.e. adhesion, invasion and immune evasion). Variation of the expression profiles of virulence genes correspondence to the high-growing temperature was reported earlier in recent publication which found that several putative virulence genes involved with construction of hemolysins, DNase, pathogenicity-island and surface anchored proteins were greatly up-regulated in stationary-phase of *S. agalactiae* grew in 40 ^{0}C comparing with those cultured at 30 ^{0}C (Mereghetti et al., 2008b). It might be taken into account that the dissimilarity of virulence genes expression profile between hot and normal temperatures is a representative of the conversion of streptococci from opportunistic state into highly virulent state.

In present study, the experimental infection of tilapia revealed that high mortality had been founded in high water temperature condition. The increase of disease severity has been reported earlier in several environmental and epidemiological studies which mentioned that the critical temperatures for streptococcosis outbreak in fish farms were above 30 or 33 ^{0}C (Bromage and Owens, 2009; Mian et al., 2009; Rodkhum et al., 2011; Amal et al., 2013b). In this experiment, the accumulated mortality of tilapia kept in 28 ^{0}C was 45 %, and increased to 85 % in 35 ^{0}C. Mortality had been observed since 2 dpi and stopped after 3 dpi in the 28 ^{0}C group. On the other hand, the mortality of the 35 ^{0}C challenged group was still continuously appeared until 7 dpi. The difference of disease susceptibility between 28 and 35 ^{0}C condition may be associated with both host and pathogenic determinants. According to our *in vitro* study, the increase of streptococcus pathogenicity upon high temperature condition was demonstrated. For the host responses, the variations of temperatures was proposed to have the direct influences

on host physiological response, especially immunity (Le Morvan et al., 1998). The issue of host responses is described thoroughly in the next chapter of this thesis.

In conclusion, this experiment exhibited the effects of temperature to the virulence characteristics of *S. agalactiae*. The results clearly indicated that *S. agalactiae* has more virulence in warm-temperature condition (35 ^{0}C), comparing with the normal temperature (28 ^{0}C), and also produce higher mortality in tilapia.

Chapter 4: Immunological responses of Nile tilapia (*Oreochromis niloticus*) reared in different temperature conditions to *S. agalactiae* infection

Abstract

Fluctuation of water temperature directly affects not only to the inhabited microorganisms but also to the physiological properties of the aquatic animal. The immune responses can be influenced by temperature which is largely associated with the disease outcome. The objective of this study is to determine the effect of high water temperature to the immune responses of *S. agalactiae* infected Nile tilapia. Two hundred and fifty (250) Nile tilapia of 30-50 g weight were divided in to 4 groups comprised of, 1) streptococcus-challenged group raised at 28 ^{0}C, 2) streptococcus-challenged group raised at 35 ^{0}C, 3) control group (challenged with PBS) group raised at 28 ^{0}C and 4) control group raised at 35 ^{0}C. The accumulated mortality were higher in the fish reared at 35 ^{0}C comparing with 28 ^{0}C and the numbers of viable streptococci resided in brain tissue were approximately 10^{7} and 10^{4}, respectively. Total leukocyte numbers had decreased since 24 hpi in the streptococcus-challenged tilapia comparing with non-challenged fish. However, the serum bactericidal (against *S. agalactiae*) activity among experimental groups showed no particular trend. Quantitative RT-PCR assay of immune-related genes in the spleen of tilapia indicating the high variation of TGF-β expression. While the up-regulation of pro-inflammatory cytokines (COX-2, IL-1β and TNF-α) were found in challenged 28 ^{0}C fish (10-20 folds) and extremely high in challenged 35 ^{0}C fish (40-50 folds). In summary, this experiment indicated that high water temperature involved with massive inflammation in *S. agalactiae* infected Nile tilapia which may leads to acute mortality of the fish.

1. Introduction

Nile tilapia (*Oreocheomis niloticus*) is becoming the most important fresh-water cultured species in Thailand. Presently, the production of tilapia (including Nile tilapia, red tilapia and hybrid tilapia) has shared the highest economic values among fresh-water fishery industry, while the production capacity and consumption are still increasing (DOF, 2010; FAO, 2012). The tilapia farming systems have been developed into the very-high density production in order to response to the expanding of consumers' demand. However, the animal cultured in this highly intensive environment may undergo the stressful condition which, as a consequence, is increased disease susceptibility (Barton, 2002). In addition, the highly intensive farming also increases the direct-contact between the animal which enhance the pathogen transmission in the production sites. Among the infectious pathogens, *S. agalactiae* has been recognized as a crucial cause of warm-water streptococcus accounting for massive mortalities involved in aquaculture farms worldwide (Yanong and Francis-Floyd, 2010).

The host/pathogen imbalance triggered by rapid environmental changes was known as a truly important reason for disease occurrences (Plumb, 1999). In the case of streptococcosis, the high temperature was proposed to be the predisposing cause of disease outbreak and also reported to be associated with the increase of disease severity (Bromage and Owens, 2009; Mian et al., 2009; Amal et al., 2013b). According to our recent publication, tilapia was more vulnerable to *S. agalactiae* when water temperature was above 32 ^{0}C (Rodkhum et al., 2011)

Increasing of the temperature significantly affects the physiological responses of both host and pathogen living in a common habitat. In poikilothermic animals, the optimal temperature supporting the healthiness and growing efficiency may differ depends on species of the fish. In the extreme temperature conditions (too hot or too cold), stress will be induced and several physiological systems, including immune-defense, will inefficiently operate (Barton, 2002). Suppression of both innate and acquire immunity associated with heat-induced stress has been reported in several fish species, such as Atlantic cod, Atlantic halibut, Sockeye salmon, and

Chinook salmon (Le Morvan et al., 1998; Alcorn et al., 2002; Langston et al., 2002; Perez-Casanova et al., 2008). The deterioration of in leukocyte and lymphocyte numbers, respiratory burst, phagocytic activity and immunoglobulin concentration, could be found shortly after fish exposed to high-temperature conditions (Langston et al., 2002; Ndong et al., 2007). More importantly, the increase of the average temperature lately due to the global warming situation leads to the flourishing of some bacterial and parasitic diseases like never before (Karvonen et al., 2010). In Thailand, the average temperature is also increasing annually which may, more or less, resulted in the heighten prevalence and severity of warm-water associated diseases including streptococcosis (Suksuwan, 2009). Therefore, the objective of this study is to evaluate the immune responses of *S. agalactiae* infected Nile tilapia in the different water temperature condition to gather more knowledge about the disease pathogenesis.

2. Materials and methods

2.1. Bacterial strain

Streptococcus agalactiae FNB12 serotype Ia was isolated from diseased tilapia during streptococcosis outbreak in tilapia farms in central region of Thailand since 2010 and had been used in chapter 3 of this thesis. The LD_{50} of this bacterium at 28 ^{0}C condition was equivalently with $6.30X10^{6}$ CFU in tilapia weighted 30-50 g. Streptococci was preserved in 75% glycerol at -80 ^{0}C until use. An *in vivo* passage in tilapia was conducted prior the beginning of the experiment to maintain pathogenicity level of streptococci.

2.2. Fish

Two hundred and fifty (250) Nile tilapia (*Oreochromis niloticus*) weighted 30-50 g were employed in this experiment. All experimental fish had been quarantined in three-ton aerated PVC tank for at least 2 weeks prior experiment and 5% of the fish were randomly selected for bacterial isolation in order to confirm whether the fish was streptococcus-free.

2.3. Challenging of tilapia

Streptococcus agalactiae was cultured in TSB at 37 ^{0}C until reaching late exponential-phase (approximately 6-8 hours). Bacterium was washed twice and suspended in sterile PBS. The concentration of bacterial mixture was adjusted using spectrophotometry (OD_{600} at 0.6 is equivalent with 10^{8} CFU, approximately). All experimental fish were divided into 4 groups consisted of 35 fish per group. Each group had been raised separately in 90 l acrylic glass tank with 5.0-6.0 dissolved oxygen, 6.5-7.0 pH, 12/12 natural light cycle and 28 or 35 ^{0}C water temperature controlled by electrical heater. All 4 experimental groups were comprised of 1) Streptococcus-challenged group raised in 28 ^{0}C, 2) Streptococcus-challenged group raised in 35 ^{0}C, 3) control group (challenged with PBS) group raised in 28 ^{0}C and 4) control group raised in 35 ^{0}C. To challenge the animal, Nile tilapia was restraint by wet towel and challenge by intraperitoneal injection of $4 X 10^{6}$ CFU in 0.1 ml volume

using 26 G tuberculin syringe. For the control groups, the fish were injected by equal volume of sterile PBS instead of bacteria. The fish were return to their tank immediately after injection process.

2.4. Evaluation of immune responses of streptococcus-challenged fish

After challenging of the animal, three fish were randomly selected from the tanks at 6, 12, 24, 48, 72 and 96 hours post injection (hpi). The fish were euthanized using 50 ppm of clove oil followed by collecting of samples immediately after animal were passed out. Tilapia blood was drawn from caudal vein by heparinized syringe and leukocyte count had been performed immediately using Natt & Herrick's staining procedure with standard haemocytometer (Campbell, 1995). Serum were separated from whole blood by centrifugation at 3,000Xg for 15 minutes followed by filtering with 0.4 µm filter and preserved at -20 ^{0}C (Sigma-Aldrich, USA). Spleen and brain were aseptically removed and rinsed with sterile PBS. Spleen were kept in RNAlater® reagent (Invitrogen, USA) and stored at -80 ^{0}C for using in gene expression analysis. Enumerations of viable streptococci inhabited in the brain were carried out using standard plate count technique.

2.4.1. Serum bactericidal activity

Bactericidal activity of Nile tilapia serum against *S. agalactiae* was determined using method modified from Pirarat and colleagues (2011). Briefly, one thousand (1000) CFU of exponential-phased *S. agalactiae* in 100 µl volumes was mixed with the equal volume of tilapia serum followed by incubation at 30 ^{0}C for 1 hour. Then, the bacterial mixture was cooled immediately on-ice to stop the reaction. The number of viable streptococci was then enumerated by standard plate count technique. The experiments were performed in triplicate and the average values of were subjected to calculate for their bactericidal activity using the formula as "relative percent survival = 1-(enumerated streptococcus cell after incubation/initial inoculum)"

2.4.2. Expression analysis of COX-2, IL-1β, TNF-α and TGF-β encoded gene

2.4.2.1. RNA extraction

Total RNA were extracted from spleen of Nile tilapia using Invitrap® spin universal RNA mini kit (Stratec, Germany) according to manufacturer's instruction. RNA purity and concentration had been determined by 260/280 nm spectrophotometry and isolated RNA were preserved at -80 ^{0}C until use.

2.4.2.2. Quantitative RT-PCR of COX-2, IL-1β, TNF-α and TGF-β

Complementary DNA (cDNA) was constructed from 1 μg of total RNA using Omniscript® RT kit (QIAGEN, USA) according to the manufacturer's instruction. As a control for cDNA synthesizing process, the reverse transcription without reverse transcriptase was conducted simultaneously with normal relations in order to confirm whether genomic DNA was contaminated in the RNA sample. All cDNA samples were preserved at -20 ^{0}C until quantitative PCR (qPCR) was conducted.

To determine the expression of immune-related genes, the COX-2, IL-1β, TNF-α, and TGF-β encoded genes were chosen to applied in qRT-PCR analysis and L32 was selected as an endogenous gene for normalization. Detailed of primers used in this assays are presented in Table 4.1 The 20 μl qPCR reaction mixture contained 10 ng of cDNA, 0.2 μM of each forward and reverse primer, 1X Evagreen® real-time-PCR master mix E4 (GeneON, Germany). Quantitative PCR was performed in Real-Time PCR ABI Prism 7500® platform (Applied Biosystems, USA). The PCR condition beginning with denaturation step at 95 ^{0}C for 3 min, followed by 45 cycles of 95 ^{0}C denature for 15 sec, 58 ^{0}C annealing for 15 sec and 72 ^{0}C extension for 45 sec. Comparison of target genes expression were calculated by ΔΔCt method and the sample from '28 ^{0}C control group at 0 hpi' were employed as calibrator.

Table 4. 1 Primer using in gene expression study

Primer	Nucleotide sequence (5'→3')	Target length	References
COX-2			
-Forward	AGCAGCCAGAAGGAAGGCGG	130 bp	(Chuang and Pan, 2011)
-Reverse	GACTGAGTTGCAGTTCTCTTAGTGTGC		
TNF-α			
-Forward	GCTGGAGGCCAATAAAATCA	339 bp	(Pirarat et al., 2011)
-Reverse	CCTTCGTCAGTCTCCAGCTC		
IL-1β			
-Forward	TGCTGAGCACAGAATTCCAG	371 bp	(Pirarat et al., 2011)
-Reverse	GCTGTGGAGAAGAACCAAGC		
TGF-β			
-Forward	GACCTGGGATGGAAGTGGAT	225 bp	(Harms et al., 2003)
-Reverse	CAGCTGCTCCACCTTGTGTTG		
L32			
-Forward	GACCAAGTTCATGCTGCCAAC	151 bp	(Pirarat et al., 2011)
-Reverse	TGCCCTCTCCACACTCAGC		

2.5. Statistical analysis

Leukocyte numbers, serum killing activity, and expression of immune related genes group were compared using one-way ANOVA followed by the LSD multiple comparison analysis. Notably, the result of serum killing activity which exhibited as proportion has been transformed to a rational number using arc-sine transformation prior taking statistical analysis.

3. Results

3.1. Number of *S. agalactiae* in the brain

Streptococcus agalactiae inhabiting brain tissue had been recovered from experimentally infected animal. The bacterium can be detected in high numbers (10^4-10^5 cfu/g tissue) since 6 hpi. The bacterial numbers were continually increased and keeping stable when entered 96 hpi (Figure 19). In comparison, the numbers of viable streptococci was significantly higher about 100 times in experimental fish reared in 35 ^{0}C than in 28 ^{0}C condition.

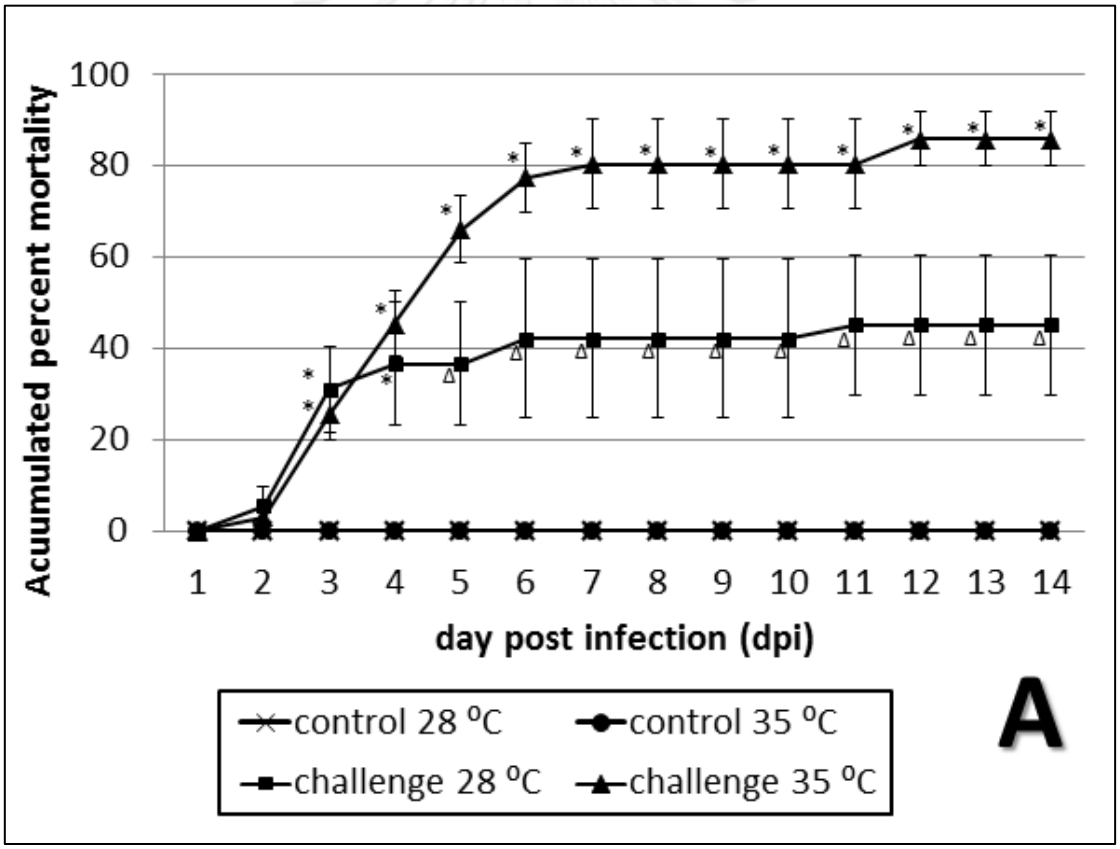


Figure 19 Quantities of *Streptococcus agalactiae* inhabited in brain tissue of experimentally infected tilapia. The symbols (* and Δ) represent the significant difference (P<0.05).

3.2. Leukocytes count

At 0-12 hpi, leukocyte numbers between challenged and control groups were insignificantly different. Total leukocytes had been decreased in the challenged groups since 24 hpi and continued declining until the end of experiment (96 hpi). At the 48-96 hpi, leukocyte numbers were dropped lower than 10000 cells/µl. The leukocytes count of Nile tilapia throughout the experiment is exhibited in Figure 20.

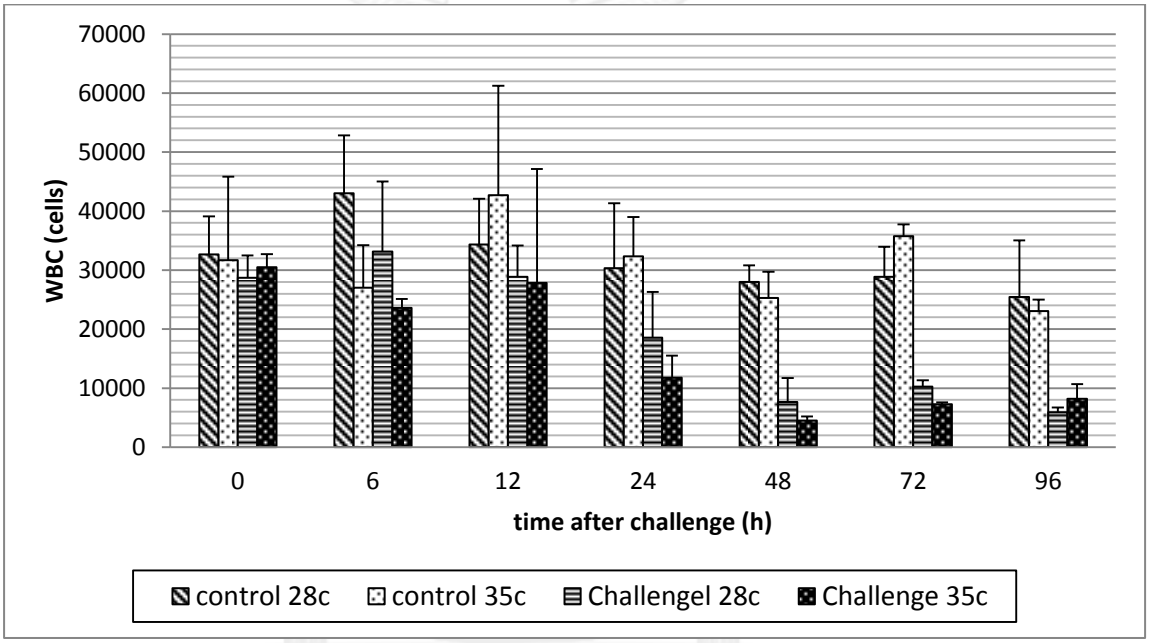


Figure 20 Total leukocyte of Nile tilapia infected with 10^6 CFU of pathogenic *S. agalactiae* and reared in 28 ^{0}C or 35 ^{0}C conditions. The animals were injected by sterile PBS in control groups.

3.3.Serum bactericidal activity

The effectiveness of serum bactericidal activity against *S. agalactiae* of Nile tilapia reared in different temperatures was determined at 0-96 hpi. No significantly different had been observed among control and experimental challenged fish at each specific time point due to the high standard deviation. However, most of tilapia serum exhibited low bactericidal activity with approximately 30% or lower had been measured throughout the experiment. The result for serum bactericidal assays is presented in Figure 21.

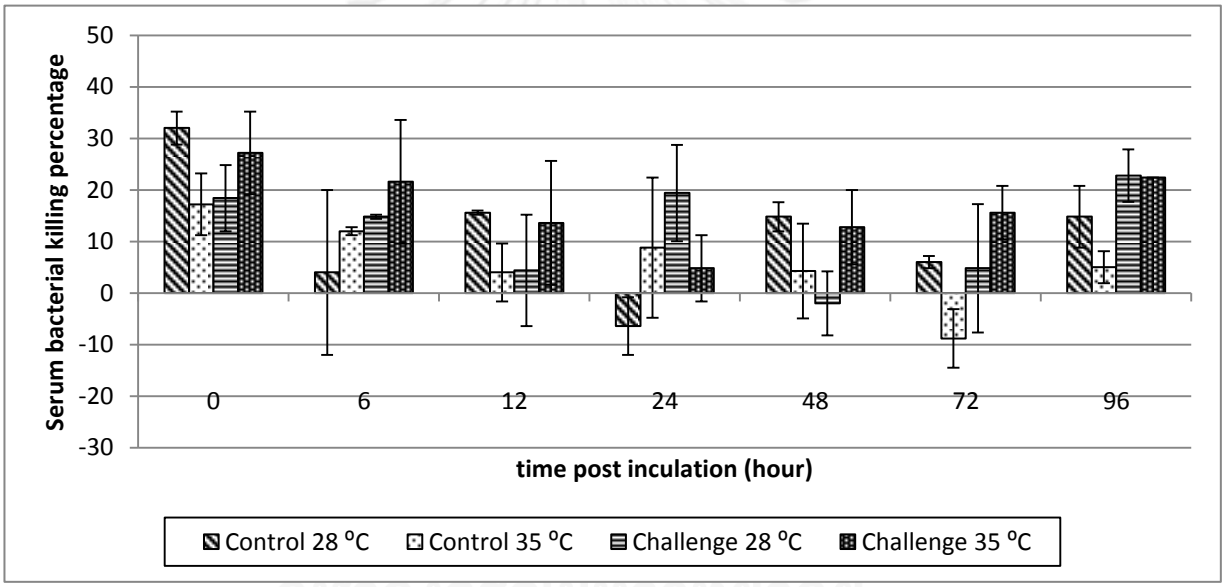
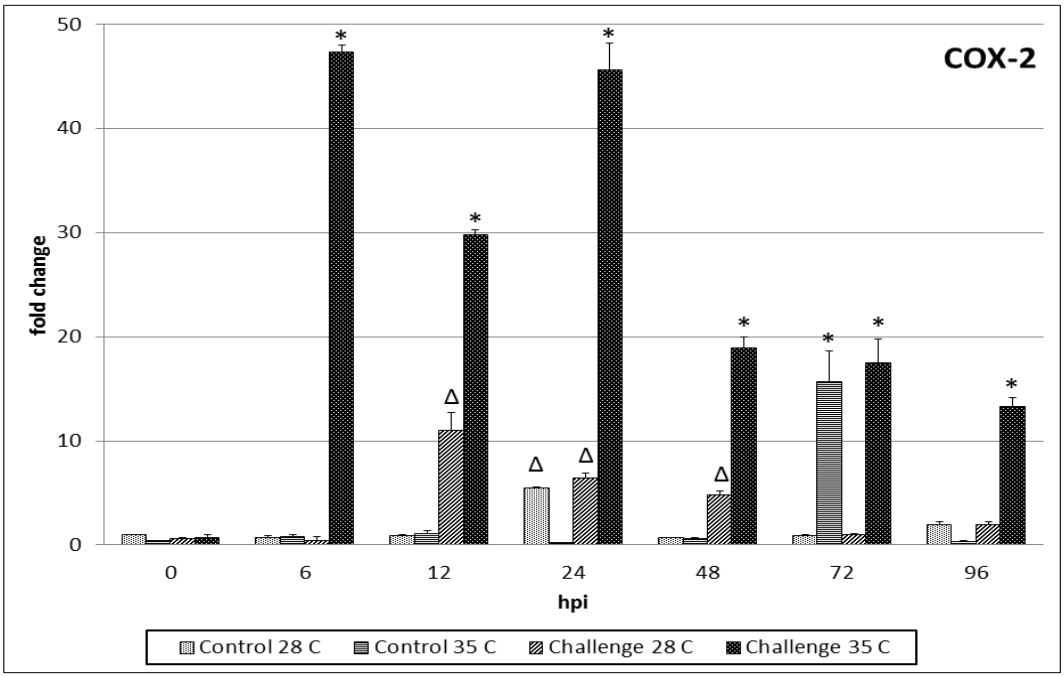


Figure 21 Serum bactericidal activity against *S. agalactiae* of streptococcosis Nile tilapia reared in different temperature conditions. The fish in control groups were intraperitoneally injected by sterilePBS instead of *S. agalactiae*.

3.4.Expression of COX-2, TNF-α, IL-1β and TGF-β

The trends of COX-2, TNF-α and IL-1β expression of streptococci infected Nile tilapia are almost identical. In control groups, the instant up-regulation of COX-2, TNF-α and IL1 had been detected only at 24 and 72 hpi of 28 ^{0}C and 35 ^{0}C reared fish, respectively. While the expression of these genes at other hpi remained constant at the normal expression level. On the other hand, COX-2, TNF-α and IL-1β expressions in 35 ^{0}C challenged group were extremely up-regulated since 6 hpi then started to decrease after 48 hpi until the end of experiments (96 hpi). The up-regulation of COX-2, TNF-α and IL-1β expressions in the in 28 ^{0}C challenged group were found but the expression level still significantly lowered than 35 ^{0}C challenged group. In 35 ^{0}C challenged group, the maximum COX-2, TNF-α, IL-1β expression were higher than 35-45 folds, respectively, while 10-15 folds up-regulation can be observed in 28 ^{0}C challenged group (Figure 22).

On the contrary, the expression of TGF-β among the experimental groups had no obvious difference. However, the up-regulation level of TGF-β among Nile tilapia was not quite high since only 5 folds-increasing had been measured as a maximum expression level.



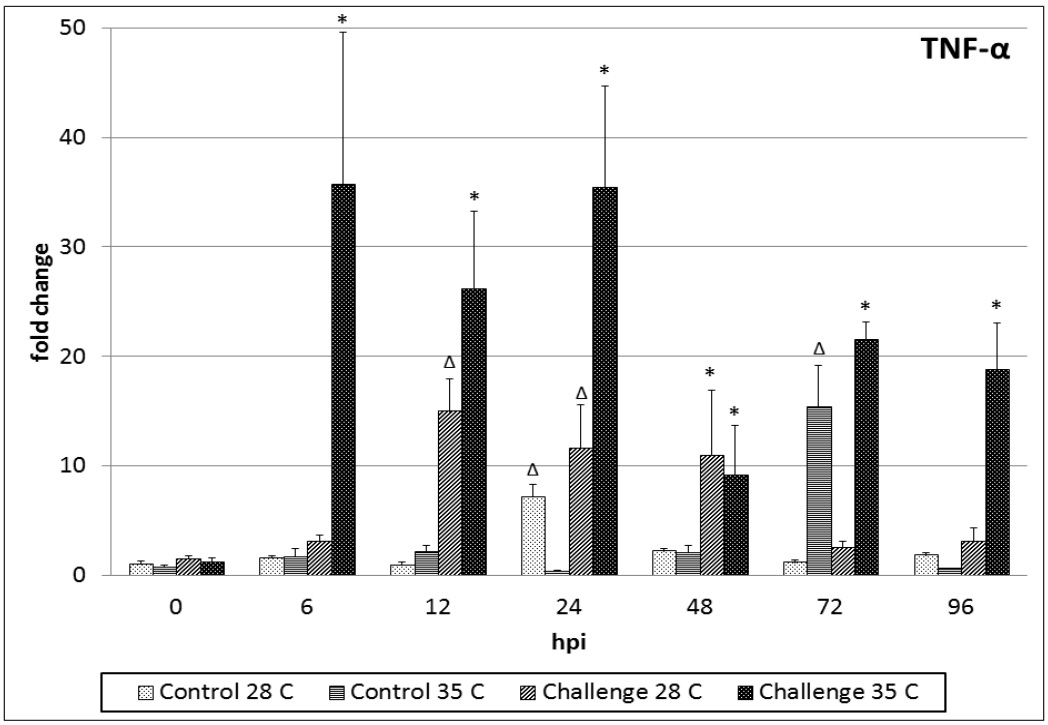


Figure 22 Expression analysis of COX-2 (top) and TNF-α (bottom) of Nile tilapia infected with *S. agalactiae* and reared in 28 ^{0}C or 35 ^{0}C conditions. The The symbols above S.D. bars represent the significant difference (P<0.05) of expression values.

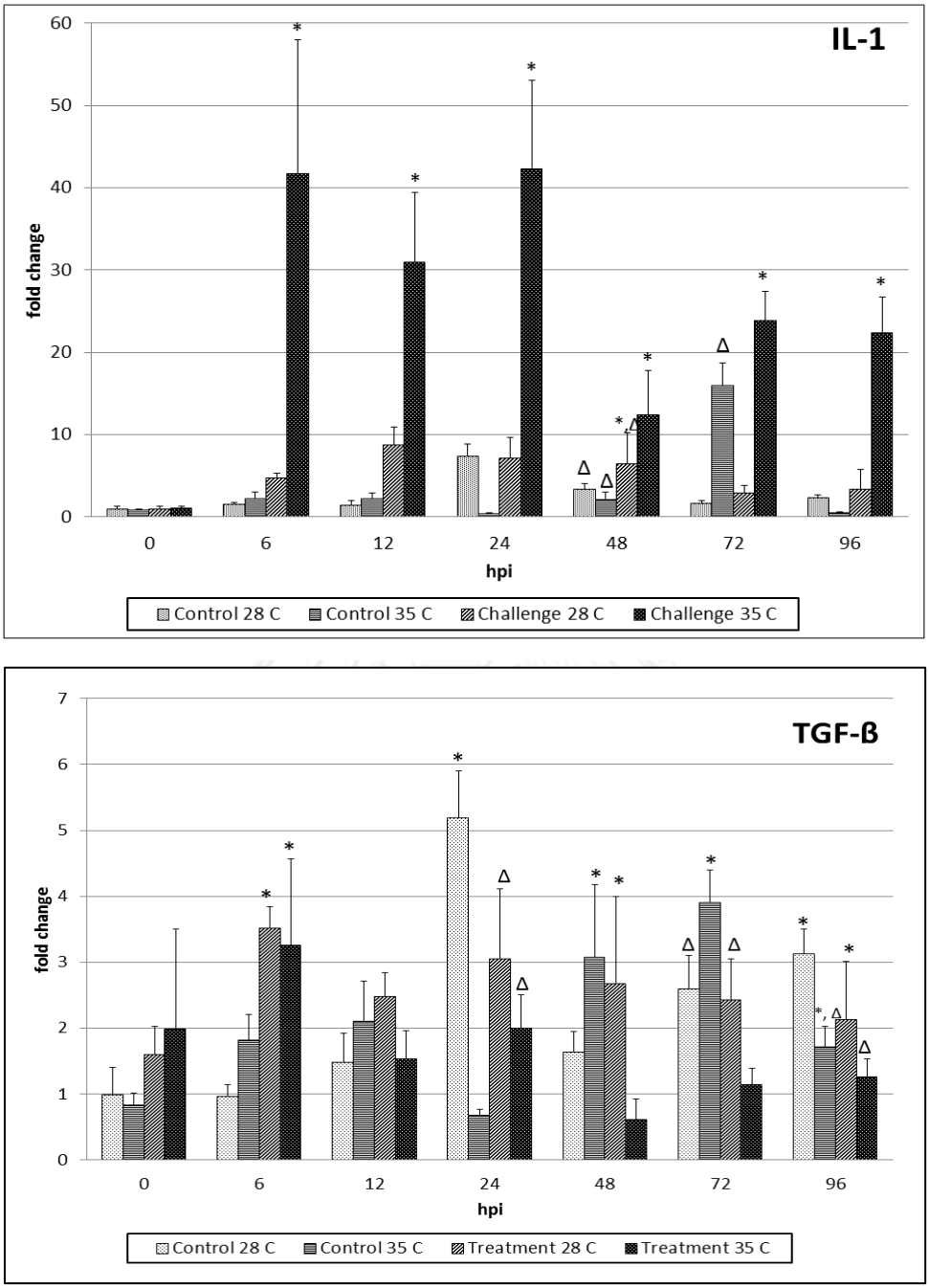


Figure 23 Expression analysis of IL-1β (C) and TGF-β (D) of Nile tilapia infected with *S. agalactiae* and reared in 28 ^{0}C or 35 ^{0}C conditions. The symbols above S.D. bars represent the significant difference (P<0.05) of expression values.

4. Discussion

Environmental managements are generally recognized as one of the key elements for the successful aquaculture farming. Several physical and chemical parameters such as pH, dissolved oxygen, ammonium and nitrite concentration have been approved to be associated with the production efficiency (Wendelaar Bonga, 1997; Barton, 2002). Among those environmental parameters, water temperature is the most serious factor influencing various physiological properties of the animal including metabolism, growing capability, reproductive system and immunological integrity (Bly and Clem, 1992; Le Morvan et al., 1998). The temperature promoting the beneficial effect to animal health has been designated as 'normal physiological temperatures' which may different among several host species (Karvonen et al., 2010). Therefore, changing of environmental condition into the particular temperatures could produce either beneficial or disadvantageous effects depends on the normal physiological threshold of the fish. In case of tilapia, the optimal temperature for maximum growth is between 29-31 ^{0}C and the lethal temperature is lower than 11 ^{0}C and higher than 38 ^{0}C (Allanson and Noble, 1964; Popma and Masser, 1999; Sifa et al., 2002). In our experiment, the maximum reared temperature at 35 ^{0}C may not reaching the lethal point since no mortality had occurred in control groups (no bacterial challenge).

In this study, the challenging experiment clearly indicated that the Nile tilapia reared at high temperature condition (35 ^{0}C) was more vulnerable to *S. agalactiae* than the fish kept in normal temperature (28 ^{0}C). The severity of disease at 35 ^{0}C was dreadfully high since the mortality had been found since 12 hpi and continuing until the end of experiment while, in 25 ^{0}C, mortality can be observed only at 12-72 hpi (data not shown). The number of streptococcus deposited in brain tissue of the fish kept in 35 ^{0}C was highest at 12 hpi and it was approximately about 10-100 folds higher than 28 ^{0}C group, which indicated that the streptococcal infection had been eradicated more effectively in normal temperature condition. Tremendous increase of host susceptibility to streptococcus infection in high-ambient temperatures can be affected by a rapid multiplication of bacterial pathogen. The optimal temperature

supporting *S. agalactiae* growth was reported to be 35-40 ^{0}C which is consistent to the results obtained in this study (Mereghetti et al., 2008b; Suanyuk, 2009). Meanwhile, high temperature may trigger the stressful conditions of tilapia which leads to the improper innate immune responses and failure of elimination of streptococci (Alcorn et al., 2002; Langston et al., 2002; Suanyuk, 2009).

In current study, there was no significant difference of *in vitro* immunogenic responses between the non-infected fish reared in 28 and 35 ^{0}C. However, leukocyte concentration of the streptococcus-challenged fish was significantly lower than non-infected group suggesting that the effect of streptococcal infection alone was enough to reduce the leukocyte numbers (figure 20). Our findings are inconsistent with various literatures because an infection of Gram positive cocci bacteria should induce leukocytosis due to the inflammatory response (Harvie et al., 2013). According to several publications, the normal leukocyte numbers of tilapia was varied due to genetic background, age, health and physiological status. Generally, the total leukocyte concentrations of 2 g and 300 g weight Nile tilapia were 3-4 X 10^{5} and 13-34 X 10^{3}, respectively (Abdel-Tawwab et al., 2008; Martins et al., 2009; Silva et al., 2009). In our experiment, the decreasing of leukocytes concentration in streptococcus-infected fish after 24 hpi may associated with 1) the distribution of leukocytes from circulation toward host tissue upon the acute inflammatory reaction which may responsible for the reduction of peripheral blood leukocyte in streptococcosis fish at 0-24 hpi (Dotta et al., 2011), 2) the continual infection induced the stressful condition which may responsible for leukopenia after 48 hpi (Esch and Hazen, 1980). The serum bactericidal activity of Nile tilapia detected in the current study exhibited the high variation without any particular trend. The approximately -10% to 30% of bactericidal activity with high standard deviations were examined throughout the experiment indicating that the large diversity had established among experimental animals (figure 21). Ordinarily, the bactericidal effects of fish serum are obtained from the activity of serum lysozyme, complements system and antigen-specific antibody of the host (Ellis, 2012). Since only a short duration of infection (96 hours) had been followed up in the current study, thus the serum bactericidal effects of tilapia largely relied on the innate immune responses, i.e. lysozyme and

complements activity, because tilapia needs at least 14 days to develop the antigen-specific antibody (Huang et al., 2013). The efficiency of humoral innate immunity of the fish was closely related to the water quality parameters, such as pH, DO, salinity and temperature (Uribe et al., 2011). Due to several publications, the gradual increasing of water temperature up to the upper-most of normal physiological point could enhanced the lysozyme and complement activities in various fish species such as Chinook salmon, Atlantic halibut and Sockeye salmon (Alcorn et al., 2002; Langston et al., 2002; Perez-Casanova et al., 2008). However, the changes of humoral immune response upon rearing temperature and streptococcal infection seem to have no significant effect to the serum bactericidal activity against *S. agalactiae* since the trendless data were detected in the current experiment.

It is commonly known that the COX-2, IL-1β and TNF-α cytokines have important roles in the orchestration of acute inflammatory responses (Secombes et al., 2011). These pro-inflammatory cytokines are responsible for the induction of several innate immune responses, such as leukocytic proliferation, phagocytosis and respiratory burst activity (García-Castillo et al., 2004; Buonocore et al., 2005). The COX-2, IL-1β and TNF-α were sharply up-regulated after bacterial inoculation, especially at 6-24 hpi which 40 folds up-regulation was found in 35 ^{0}C challenged group (figure 22-23). The constant expression levels of COX-2, IL-1β and TNF-α in 28 and 35 ^{0}C control groups throughout the experiment suggested that the increasing of water temperature alone has no- or very limited-effect on pro-inflammatory cytokines expression level of non-infected fish. On the other hand, the persistent up-regulation in the 28 and 35 ^{0}C challenged group indicated that the elevation of water temperature was contributed to the enhancing of pro-inflammatory expression in the *S. agarlactiae* infected tilapia. According to the results obtained in chapter 3 and 4, high temperature (35 ^{0}C) was the optimal condition allowing rapid multiplication of *S. agalactiae*, and inducing the immense production of bacterial hemolysin and numerous virulence factors which may activate massive inflammatory responses and tissue necrosis in the fish (Henderson et al., 1997; Mereghetti et al., 2008b). The enhancing of pro-inflammatory cytokines production due to the accumulation of bacterial pathogens within the host tissue was found to be the basic immunological

response in many disease models, such as *Yersinia ruckeri, Edwardsiella tarda* and *Aeromonas salmonisida* infections (Pressley et al., 2005; Raida and Buchmann, 2009; Secombes et al., 2011). However, the up-regulations of pro-inflammatory cytokines have the double-edged sword effects since the over-abundant of TNF-α secretion during inflammation may leads to the lethal tissue damage and multi-organ failures (Ishibe et al., 2009). According to recent publications, the level of pro-inflammatory cytokines expression was positively correlated with the severity of pathogenic *E. tarda* and *Y. ruckeri* infection (Pressley et al., 2005; Raida and Buchmann, 2009). On the contrary, the neutralization of inflammation by anti-TNF antibody has been firmly proved to prevent tissue injury and lethality (Schumann et al., 1998). Moreover, the limited inflammatory cytokines response coincide with the minimal pathological lesions have been reported in Edwardsiellosis-resistant fish and the fish challenged with the low-virulent strains of *E. tarda* (Ishibe et al., 2009). Therefore, it can be concluded that the vast severity and pathological changes found in streptococci infected tilapia rearing in 35 ^{0}C condition was mainly associated with the over-expression of pro-inflammatory cytokines (COX-2, IL-1ß and TNF-α). Additionally, the enhancing of IL-1ß expression can induce corticosteroid secretion which may leads to the inhibition of the fish immune response (Goshen and Yirmiya, 2009).

The biological functions of TGF-ß are known to be related with the control of proliferation and differentiation of several kinds of the cells, such as epithelium, endothelium, and lymphocytes, which, in turn, playing the important roles in the tissue development, bone remodeling, inflammation and carcinogenesis processes (Funkenstein et al., 2010). In aquatic animal, the TGF-ß secretion was induced by the pathogen-associated molecular pattern (PAMP) and pro-inflammatory cytokines (Maehr et al., 2013). The TGF-ß expression level can increase overtime after tissue damage, which was found to be related with the active-repairing process of host tissue (Li et al., 2006; Yang et al., 2012). However, in current study, the TGF-ß expression among infected and non-infected tilapia were extremely variate with no specific trend. In addition, the small level of TGF-ß up-regulation (5 folds at maximum) found in every experimental group (figure 23) suggesting the insignificant effects of *S. agalactiae* infection and high-temperature condition on the expression

of TGF-ß. According to recent publication, the expression level of the second TGF-ß1 paralogue (designated as 'TGF-ß1a') in head kidney macrophages remained unchanged even after the stimulation by strong-immunogenicity reagents (Maehr et al., 2013). Although the TGF-ß1a has not been reported in tilapia, but it is reasonable to believe that multiple TGF-ß1 paralogues could be existed in tilapia since the genetic process like fish-specific genome duplication events (FSGD) had driven the large genetic diversity among teleostean and may also give rise for more subtype of the TGF-ß family (Huminiecki et al., 2009; Santini et al., 2009). Therefore, further investigation of TGF-ß isoforms and paralogues in tilapia is necessary to elucidate the roles of TGF-ß in immune defense mechanisms.

In conclusion, this study suggested that Nile tilapia was highly susceptible to *S. agalactiae* infection in high temperature condition (35 °C). We also demonstrated that the rapid and significant up-regulation of pro-inflammatory cytokines upon *S. agalactiae* infection in high temperature condition were associated with mortality.

Chapter 5: General discussion

1. Summary

The aim of this study is to investigate about the effect of temperature to the occurrence of *S. agalactiae* in tilapia farms and its pathogenicity. To accomplish the objective, the study was started by the characterization of *S. agalactiae* isolated from tilapia farms since 2009. All *S. agalactiae* isolates belong to serotype Ia with ß-hemolysis while the γ-hemolytic serotype Ib, which was reported as the dominant strain in Vietnam, Brazil and Israel, has never been found (Vandamme et al., 1997; Godoy et al., 2013). In addition, the variety of genotypic characterization assays had been conducted and, despite the limited geographical diversity and number of the samples, a genotypic variation was confirmed among the *S. agalactiae* population. This genotypic variation might be the major obstacle of vaccine development since the very limited cross-protective immunity had established among different genotypes of *S. agalactiae* (Chen et al., 2012). Our surveillance study of streptococci occurrence in tilapia farms also indicated that the pathogenic strains of *S. agalactiae* can be viable opportunistically in the farming environments. Furthermore, the streptococci can be deposited in the environments for a long period of time which might be the complicated burden for the farming managements in order to eradicate the pathogenic *S. agalactiae* from their facilities.

Generally, the severity of infectious diseases in aquatic animals is relied on three factors, i.e. pathogen, host and environments. It is regarded as the rule of thumb that diseases will occur when the susceptible host exposes to the virulent pathogen in the appropriate environments (Snieszko, 1974). Therefore, to understand thoroughly about mechanisms lie behind the disease outbreak, the widely prudent consideration of the interaction among the multiple predisposing/causative factors is highly necessary.

The elevation of temperature from 28 ^{0}C (normal condition) to 35 ^{0}C (hot condition) firmly enhanced the virulence of *S. agalactiae* and the susceptibility of

Nile tilapia to the infection. The 35 ^{0}C condition was selected to imitate the high-temperature situation in Thailand during dry/hot season and it also based on the information obtained from our previous study indicating that Nile tilapia was more vulnerable to *S. agalactiae* infection when temperature was above 30 ^{0}C (Rodkhum et al., 2011). From our observation, the growing ability, hemolysis activity, viability in tilapia whole blood, production of surface capsular polysaccharide, virulence genes expression of *S. agalactiae* and the accumulated mortality in the infected Nile tilapia had increased sharply upon the high temperature condition (chapter 3). It is believed that the enhancing of the bacterial virulence due to the high temperature, rather than the reduction of host immune responses, takes the major responsibility of the disease progression since the significant changes of tilapia immune parameters (such as blood leukocyte concentration and serum bactericidal activity) had not been observed (chapter 4), while the tremendous up-regulation of pro-inflammatory cytokines in the infected fish reared in hot condition supports the hypothesis that the heightened-virulent *S. agalactiae* can produce massive inflammation in the host. The alteration of bacterial pathogenicity and the host-immune efficiency due to the temperature have been mentioned in several publications but our research is the first that examined both host and pathogen in parallel. The diagram of the putative effects of high temperature to the outcome of streptococcosis in tilapia is demonstrated in figure 24.

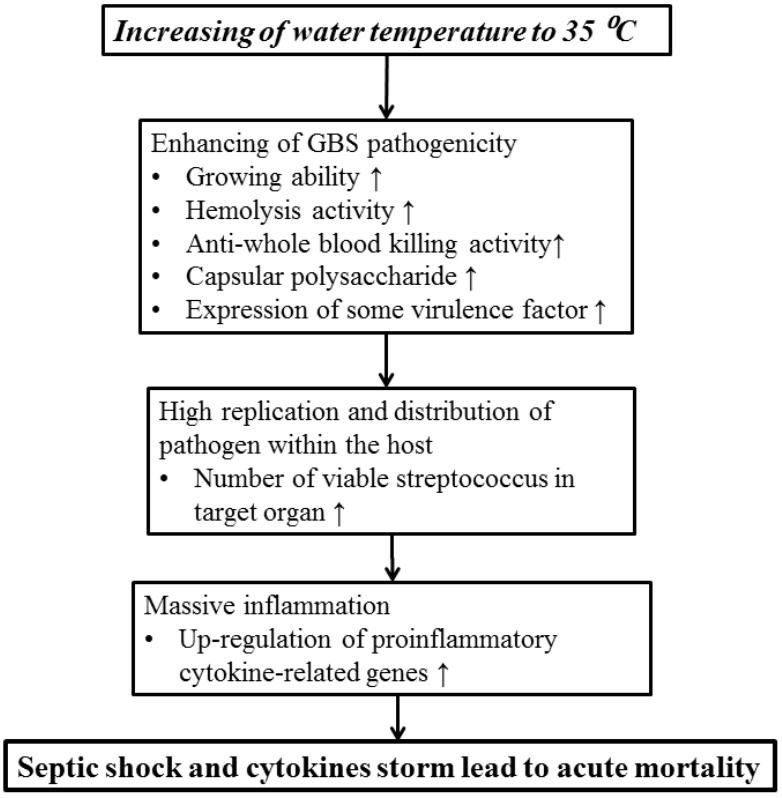


Figure 24 Effect of high water temperature to virulence of streptococcosis associated with *S. agalactiae* and the putative outcome of infection.

According to previous publication, the optimum temperature for tilapia culture is 29-31 ^{0}C (Popma and Masser, 1999). Nevertheless, the threshold of normal physiological temperatures may varied depends on many factors such as strain/breed of the fish, average temperature in the culturing site and the rapidity of temperature changes (Perez-Casanova et al., 2008). In this study, the local breed Jitrladar 3 was chosen to conduct the experiment and the highest temperature at 35 ^{0}C seems to have no directly detrimental effect to the fish. But, it should be noted that only some crude-immunological parameters were evaluated in current study while other important parameters, such as lysozyme and phagocytic activity, were not included in the experiment and some of those parameters have been proved to be associated with the resistant against *S. agalactiae* infection (Huang et al., 2013).

In conventional tilapia culturing system, an increasing of diseases susceptibility due to the high temperature condition is completely inevitable because the control of water temperature in the extremely large ponds in the open atmosphere is nearly impossible. However, as mentioned earlier, the vulnerability of the fish depends on many stressing factors such as high stocking density, high alkalinity, high salinity and low dissolved oxygen level (Chang and Plumb, 1996; Shoemaker et al., 2000; Yanong and Francis-Floyd, 2010). The intensive husbandry managements, such as the careful control of water qualities and the prudent use of antibiotics might be helpful to minimize the loss caused by bacterial infection. Moreover, the routine examination of pathogenic streptococcal contamination in the farming facilities along with the appropriate decontamination procedures and good biosecurity practices will provide the sustainable of streptococcosis-free status in the farms.

2. Further work

Since the experimental design in this study mainly focused only about the severe-acute streptococcosis, while the chronic infection model is far beyond our scope and did not mention in this research. Generally, the sub-acute/chronic streptococcosis can produce few mortality over several weeks, causing poor quality carcass due to the dark-nodule lesion in the muscle and also responsible for the emaciation of the fish with the heightened feed conversion ratio (Yanong and Francis-Floyd, 2010; Li et al., 2013). Despite the unfrightful-minimal mortality, the economic loss due to chronic infection is as large as (or may be higher than) the severe acute disease. Unfortunately, only a very few information about chronic streptococcosis in fish have been available to date. Therefore, the further work about the streptococcosis in aquaculture should aim for the topic about the factors influencing chronic infection and their impact on growing efficiency of the fish. Additionally, the development of animal model to reproduce the chronic infection of streptococcosis is also necessary.

For genotypic characterization of the pathogen, the further research using more standardized-powerful genotyping tools such as multilocus sequences typing (MLST) or Multiple Locus Variable-number Tandem Repeat Analysis (MLVA) will

provide the beneficial information to track down the geographical distribution of the pathogen not only in Thailand but in global scale. This epidemiological information is utterly useful to design the protection strategies against streptococcosis in the commercial farms. Furthermore, owning to the rapid development of the novel next-generation sequencing platforms lately, the cost of genome sequencing is becoming more reasonable for the general laboratories. The construction and comparison of whole genome of various *S. agalactiae* strains (for the example pathogenic- and nonpathogenic-strain) will provide the opportunity to discover the novel virulent-related antigen, which might be useful to design the universal-vaccine. At last, the comparative study of the immune responses between streptococcosis-resistant and susceptible tilapia will be helpful to verify the important immune-components responsible for disease protection.

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APPENDICES

Sample collection form

ใบบันทึกการเก็บตัวอย่าง

วันที่.....

ฟาร์ม.....จำนวนบ่อ.....

ที่อยู่.....เบอร์โทรศัพท์.....

บ่อที่.....ประเภทบ่อ.....ขนาดบ่อ.....ชนิดสัตว์.....

ประวัติ.....

.....เวลาเก็บตัวอย่าง.....

คุณภาพน้ำ		*คุณภาพน้ำ*	
DO		Nitrite	
pH		Ammonia	
Alkalinity		Hardness	
Salinity		Temperature	

รอยโรค.....

หมายเหตุ.....

บ่อที่.....ประเภทบ่อ.....ขนาดบ่อ.....ชนิดสัตว์.....

ประวัติ.....

.....เวลาเก็บตัวอย่าง.....

คุณภาพน้ำ		*คุณภาพน้ำ*	
DO		Nitrite	
pH		Ammonia	
Alkalinity		Hardness	
Salinity		Temperature	

รอยโรค.....

หมายเหตุ.....

Bacteriological media preparation

Trypticase soy agar with 5% sheep blood (TSA blood agar)

Composition per litre:

Difco™ TSA powder ..... 40 g

Sheep whole blood ..... 50 mL

Source: This medium is available as a pre-mixed powder form BD Difco™.

Medium preparation: Add components to distilled water and bring volume to 1.0L, as described by manufacturer instruction. Mix thoroughly. Gently heat and bring to boiling. Distribute into the 200 mL bottles. Autoclave for 15 min at 15 psi pressure, 121°C. Cool to 55-60^{0}C in the water bath. Before pouring medium into the petri dishes, one bottle of TSA is carefully mixed with 10-15 mL of sheep whole blood.

Use: For the isolation and cultivation of fastidious *Streptococcus sp*. The medium also used to observation of hemolytic reaction and performing CAMP test.

Trypticase soy agar with sheep blood plus colistin sulfate and oxolinic acid

Composition per litre:

Difco™ TSA powder ..... 40 g

Sheep whole blood ..... 50 mL

100 mg/mL of Sigma™ colistin sulfate ..... 500 µL

50 mg/mL of Sigma™ oxolinic acid in 0.5 N NaOH ..... 500 µL

Source: This medium is available as a pre-mixed powder form BD Difco™. Colistin sulfate and oxolinic acid are available as lyophilized powder.

Medium preparation: TSA blood agar is prepared as described above. Before pouring medium into the petri dishes, one bottle (200 mL) of TSA blood is throghly mixed with 100 µL of Sigma™ colistin sulfate and oxolinic acid.

Use: For the specific isolation and cultivation of fastidious *Streptococcus sp*. and *Enterococcus* sp. directly from the environmental samples. This medium has capable to eliminate or minimize the competitive growth of contaminated Gram negative and other Gram positive bacteria from the sample.

Trypticase soy broth (TSB)

Composition per litre:

Bacto™ TSB powder ..... 30 g

Source: This medium is available as a pre-mixed powder form BD Bacto™.

Medium preparation: Add components to distilled water and bring volume to 1.0L, as described by manufacturer instruction. Mix thoroughly. Gently heat and bring to boiling. Distribute into the tubes. Autoclave for 15 min at 15 psi pressure, 121°C.

Use: For the isolation and cultivation of fastidious *Streptococcus sp*.

Trypticase soy broth with 6.5% NaCl

Composition per litre:

Bacto™ TSB powder ..... 30 g

NaCl ..... 65 g

Medium preparation: Add components to distilled water and bring volume to 1.0L. Mix thoroughly. Gently heat and bring to boiling. Distribute into the tubes. Autoclave for 15 min at 15 psi pressure, 121°C.

Use: Used in biochemical assay. For determination of bacterial tolerance to the medium containing high concentration of salt.

Motility medium

Composition per liter:

Pancreatic digest of gelatin.....10.0g

NaCl.....5.0g

Agar.....4.0g

Beef extract.....3.0g

Triphenyltetrazolium chloride solution.....5.0mL

Preparation of Triphenyltetrazolium Chloride Solution: Add 2,3,5-triphenyltetrazolium chloride to distilled/deionized water and bring volume to 10.0mL. Mix thoroughly. Filter sterilize.

Medium preparation: Add components to distilled water and bring volume to 1.0L. Mix thoroughly. Gently heat and bring to boiling. Distribute into tubes in 2.0mL volumes. Autoclave for 15 min at 15 psi pressure, 121°C.

Use: Used in biochemical assay. For detection of the motility of bacteria.

OF medium (oxidative fermentative medium)

Composition per liter:

Glucose 10.0g

NaCl 5.0g

Agar 2.0g

Pancreatic digest of casein 2.0g

K_2HPO_4 ..... 0.3g

Bromthymol Blue 0.08g

pH 6.8 ± 0.2 at 25°C

Medium preparation: Add components (except glucose) to distilled water and bring volume to 1.0L. Mix thoroughly. Mix thoroughly. Gently heat and bring to boiling. Distribute into the tubes. Autoclave for 15 min at 15 psi pressure, 121°C. Cool to 55-60 ^{0}C before adding glucose. Aliquot medium into 10 mL test tubes.

Use: Used in biochemical assay. For determine the oxidative and fermentative metabolism of carbohydrates. Bacteria that ferment the carbohydrate turn the medium yellow.

VP Medium (Voges-Proskauer Medium)

Composition per liter:

Peptone 7.0g

K_2HPO_4 ..... 5.0g

Glucose 5.0g

pH 6.9 ± 0.2 at 25°C

Medium preparation: Add components to distilled water and bring volume to 1.0L. Mix thoroughly. Adjust pH to 6.9. Distribute into tubes in 3.0mL volumes. Autoclave for 15 min at 15 psi pressure, 121°C.

Use: Used in biochemical assay. For the cultivation and differentiation of bacteria based on their ability to produce acetoin.

Starch medium

Composition per liter:

Agar 12.0g

Soluble starch 10.0g

Beef extract 3.0g

pH 7.5 ± 0.2 at 25°C

Medium preparation: Add components to distilled water and bring volume to 1.0L. Mix thoroughly. Gently heat and bring to boiling. Distribute into tubes or flasks. Autoclave for 15 min at 15 psi pressure, 121°C. Pour into sterile Petri dishes.

Use: Used in biochemical assay. For the cultivation and differentiation of a bacterium based on amylase production. After incubation, starch hydrolysis is determined by the addition Lugol's iodine solution. Organisms that produce amylase appear as colonies surrounded by a clear zone.

Sodium Hippurate Broth (Hippurate Broth)

Composition per liter:

Beef heart, solids from infusion 500.0g

Tryptose 10.0g

Sodium hippurate 10.0g

NaCl 5.0g

pH 7.4 ± 0.2 at 25°C

Medium preparation: Add components to distilled water and bring volume to 1.0L. Mix thoroughly. Gently heat and bring to boiling. Distribute into 1.5 mL microcentrifuge tube. Autoclave for15 min at 15 psi pressure, 121°C.

Use: Used in biochemical assay. For the identification and differentiation of β-hemolytic streptococci based on hippurate hydrolysis. The hippurate broth are mixed with 100 µL of Streptococcus dissolved in TSB followed by adding of 5-6 droplets of 2% Ninhydrin solution (2,2-Dihydroxyindane-1,3-dione). The mixture is then incubating at 37 ^{0}C in water bath for 2 hours. The dark-blue color will appear if the bacterium can hydrolyze sodium hippurate contained in the solution.

Esculin Agar

Composition per liter:

Agar 15.0g

Pancreatic digest of casein 13.0g

NaCl 5.0g

Yeast extract 5.0g

Heart muscle, solids from infusion 2.0g

Esculin 1.0g

Ferric citrate 0.5g

pH 7.3 ± 0.2 at 25°C

Medium preparation: Add components to distilled water and bring volume to 1.0L. Mix thoroughly. Gently heat and bring to boiling. Distribute into screw-capped tubes in 3.0mL volumes. Autoclave for 15 min at 15 psi pressure, 121°C. Allow tubes to cool in a slanted position.

Use: Used in biochemical assay. For the cultivation and differentiation of bacteria based on their ability to hydrolyze esculin. Bacteria that hydrolyze esculin appear as colonies surrounded by a reddish-brown to dark brown zone.

Cystine Trypticase™ Agar (CTA) plus 1% sugar

Composition per liter:

BBL™ CTA medium powder.....28.5g

20% Sugar (Trehalose, Raffinose, Mannitol, Lactose, Galactose, Salicin or Sucrose) .....50 mL

pH 7.3 ± 0.2 at 25°C

Source: The medium is available as a premixed powder from BD BBL™.

Preparation of Medium: Add CTA medium powder to distilled water and bring volume to 1.0L. Mix thoroughly. Adjust pH to 7.3. Gently heat until boiling. Distribute into 5mL tubes. Autoclave for 15 min at 15 psi pressure, 121°C. Cool tubes to 55-60 ^{0}C in water bath then add 50 mL of 20% sugar contained solution. Store at 4 ^{0}C refrigerator.

Use: Used in biochemical assay. For the determination of sugar utilization ability of fastidious microorganisms such as *Streptococcus* sp. Culture the bacterium in CTA

medium and incubate in 37 ^{0}C for 24 hours. If the bacterium can utilize sugar, the color of CTA medium will changes from orange to yellow.

Water qualities data of Tilapia during January-September 2011

Date 30 January 2011

Farm: A

Location: Nakhon Pathom *Sampling time*: 10.40 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	25.5	8.5	7.3	170	0.4	0	2	700	+

Date 30 January 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 11.20 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	27.7	2.7	7.3	120	0.2	0	0	600	-
2	27.7	8	7.3	120	0.2	0	0	600	+
3	29.0	6.5	7.3	250	0.1	0	0	400	-

Date 30 January 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 12.15

History: Death occurred in some tilapia ponds with <10% accumulated mortality

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	28.4	6	7.5	170	0	0	0	200	-
2	28.4	6.3	7.5	170	0	0	0	200	+
3	28.8	10.3	8	272	0	0	0	300	+
4	28.5	7.4	8	194	0	0	0	200	-

Date 27 February 2011

Farm: A

Location: Nakhon Pathom *Sampling time*: 11.00 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	29.3	8.6	8.0	80	0.4	0	0.25	300	+

Date 27 February 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 11.30 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	30.3	3.6	7.3	120	0.2	0	0	500	-
2	30.6	4.2	7.3	150	0	0	0	400	-
3	30.9	2.7	7.3	230	0.1	0	0	400	-

Date 27 February 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 12300

History: Death occurred in some tilapia ponds with <10% accumulated mortality

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	31.8	3.2	7.3	180	0	0	0	200	-
2	32.5	14.8	8.6	170	0	0	0	200	+
3	32.5	15.5	8.6	170	0	0	0	200	-
4	32.0	NA	NA	NA	NA	NA	NA	NA	-

Date 03 April 2011

Farm: A

Location: Nakhon Pathom *Sampling time*: 10.30 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	31.5	15.7	8.6	110	0	0.05	0.5	900	-

Date 03 April 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 11.30 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	30.6	12.5	8	230	0	0	0	500	-
2	30.0	7.3	7.6	220	0	0	0	400	-
3	30.4	5.6	7.3	230	0	0	0	400	-

Date 03 April 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 12.20

History: Gradually dead for 1 week with 10% accumulated mortality

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	31.6	13.4	8	180	0	0	0	300	-
2	33.3	13.5	8.3	130	0	0.25	0	300	+

Date 24 April 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 10.30

History: Death occurred in some tilapia pond with <10% accumulated mortality

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	32.7	7.7	7.3	140	0	0	0	200	+
2	31.6	1.2	7.6	200	0	0	0	200	-
3	33.2	13.4	8.5	140	0	0	0	200	-
4	32.8	NA	NA	NA	NA	NA	NA	NA	-

Date 24 April 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 11.30

History: Death occurred in some tilapia ponds with <10% accumulated mortality

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	33.5	5.6	7.5	140	0	0	0	200	-
2	32.8	13.2	7.3	140	0	0	0	200	-
3	32.0	8.9	7.5	200	0	0	0	200	-
4	32.3	NA	NA	NA	NA	NA	NA	NA	-

Date 29 May 2011

Farm: A

Location: Nakhon Pathom *Sampling time*: 10.40 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	31.8	13.2	7.3	140	0	0.05	0.5	500	-

Date 29 May 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 11.10 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	32.3	5.5	7.6	140	0	0	0	200	-
2	31.7	6.1	8	160	0	0	1	500	-
3	31.5	4.8	8.2	160	0	0	0.5	500	+

Date 29 May 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 10.40

History: Gradually dead for 1 week with 10% accumulated mortality

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	32.1	6.7	7.3	110	0	0	0	200	-
2	31.8	13.2	8.0	140	0	0	0	200	+
3	32.3	7.8	7.3	110	0	0	0	200	+
4	33.0	NA	NA	NA	NA	NA	NA	NA	+

Date 26 June 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 11.50 *History*: Healthy

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	30.4	4.2	7.3	160	0	0	0	200	-
2	30.8	4.0	7.6	150	0	0	0	200	-
3	31.0	NA	NA	NA	NA	NA	NA	NA	-

Date 26 June 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 11.10

History: Gradually dead for 1 week with 10% accumulated mortality

Pond number	Temperature (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Streptococcus
1	30.9	3.0	7.0	170	1	0	0.25	100	-
2	30.5	4.5	7.6	230	0	0	0.25	300	-
3	31.0	NA	NA	NA	NA	NA	NA	NA	-
4	30.8	NA	NA	NA	NA	NA	NA	NA	-

Date 24 July 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 11.15 *History*: Healthy

Pond number	Tempera-ture (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Strepto-coccus
1	31.8	12.3	8.3	160	0	0	0	500	-
2	32.3	10.5	7.8	160	0	0	0	200	-
3	31.4	NA	NA	NA	NA	NA	NA	NA	-

Date 24 July 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 11.15

History: Death occurred in some tilapia ponds with <10% accumulated mortality

Pond number	Tempera-ture (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Strepto-coccus
1	32.8	12.3	8.3	190	0	0	0.25	300	-
2	33.3	9.8	8.0	120	0	0	0.25	100	+
3	33.0	NA	NA	NA	NA	NA	NA	NA	-
4	32.6	NA	NA	NA	NA	NA	NA	NA	-

Date 28 August 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 11.15 *History*: Healthy

Pond number	Tempera-ture (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Strepto-coccus
1	32.5	5.4	7.3	160	0	0	0	500	-
2	32.3	4.8	7.3	130	0	0	0	400	+

Date 28 August 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 11.15

History: Death occurred in some tilapia ponds with <10% accumulated mortality

Pond number	Tempera-ture (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Strepto-coccus
1	32.7	5.6	7.3	190	0	0	0	400	+
2	32.0	5.2	7.3	130	0	0	0	600	+
3	33.0	NA	NA	NA	NA	NA	NA	NA	-
4	32.6	NA	NA	NA	NA	NA	NA	NA	+

Date 25 September 2011

Farm: B

Location: Nakhon Pathom *Sampling time*: 11.15 *History*: Healthy

Pond number	Tempera-ture (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Strepto-coccus
1	32.1	5.8	6.5	160	0	0	0.25	300	-
2	31.2	12.1	8.6	140	0.1	0	0	500	-
3	31.5	NA	NA	NA	NA	NA	NA	NA	-

Date 28 August 2011

Farm: C

Location: Nakhon Pathom *Sampling time*: 11.15

History: Death occurred in some tilapia ponds with <10% accumulated mortality

Pond number	Tempera-ture (^{0}C)	DO	pH	Alkalinity (ppm)	Salinity (%)	Nitrite (ppm)	Ammonia (ppm)	Hardness (ppm)	Strepto-coccus
1	30.1	4	7.3	153	0.5	0	0	200	+
2	30.4	6.3	7.6	85	0	0.1	0	200	-
3	30.9	5.2	7.3	120	0	0	0	200	-
4	30.9	NA	NA	NA	NA	NA	NA	NA	-
5	30.2	NA	NA	NA	NA	NA	NA	NA	+

Protocol for Anthony's capsule stain

(http://www.microbelibrary.org/component/resource/laboratory-test/3041-capsule-stain-protocols)

A. General materials

1. Staining trayStaining rack
2. Slide holder
3. Disposable gloves

B. Staining reagents

1. Crystal violet 1% solution (primary stain)
2. Copper sulfate 20% (decolorizer agent)

C. Procedure

1. Prepare a smear from a 12- to 18-hour culture grown in milk broth or litmus milk. (Serum protein may be used to prepare the smear if the organism was not grown in milk broth or litmus milk.) This is to provide a proteinaceous background for contrast.

2. Allow the smear to air dry. DO NOT HEAT FIX (to avoid destroying or distorting the capsule or causing shrinkage).

3. Cover the slide with 1% crystal violet for 2 minutes.

4. Rinse gently with a 20% solution of copper sulfate.

5. Air dry the slide. DO NOT BLOT. (Blotting will remove the un-heat-fixed bacteria from the slide and/or cause disruption of the capsule.)

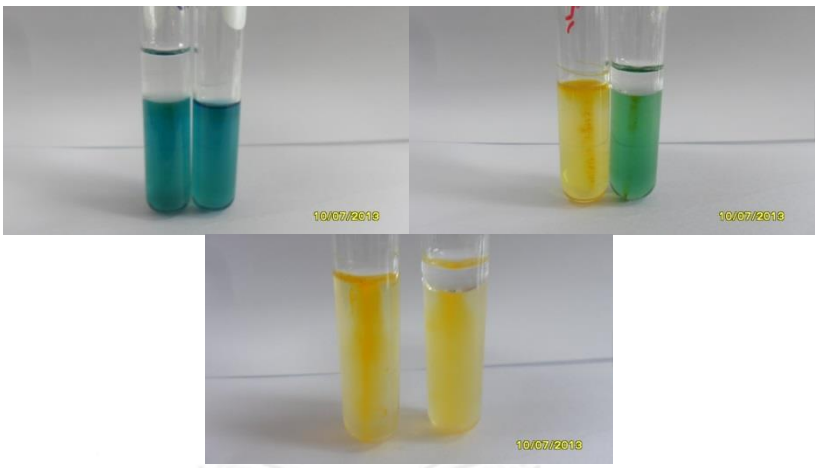
6. Examine the slide under an oil immersion lens. Bacterial cells and the proteinaceous background will appear purplish while the capsules will appear transparent.

Biochemical test pictures

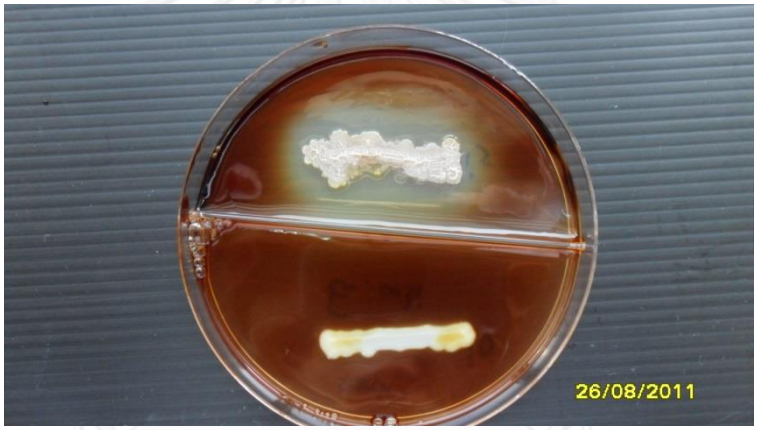
CAMP test positive result

Motility test positive (right) and negative (left) results

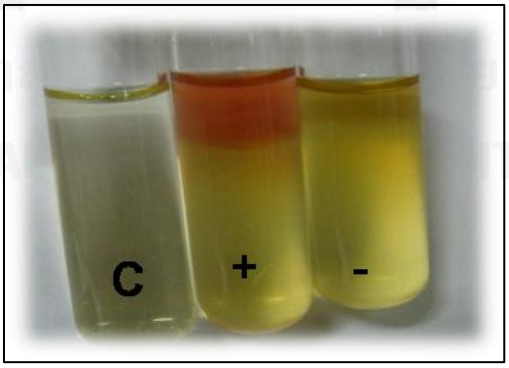
Catalase test positive result



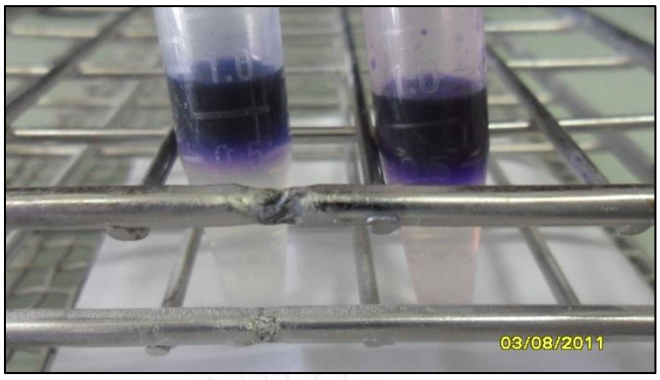
O/F test negative (left), Oxidation (middle) and Fermentation (right) results



Starch hydrolysis positive (upper) and negative (lower) results



VP test negative (right), positive (middle) results and uncultured control (left)



Hippurate hydrolysis test positive result

*inf*B allele sequence (position 1138-1641)

```
InfB_A_allele GAACCAGCGGAAATTGTTAAAAAACTCTTTATGATGGGAGTTATGGCA
InfB_D_allele ........A.....................................
InfB_I_allele ........A.....................................
InfB_T_allele ........A.....................................

InfB_A_allele ACGCAGAATCAATCATTAGATGGAGACACTATTGAGCTATTGATGGTT
InfB_D_allele ...........................................
InfB_I_allele ...........................................
InfB_T_allele ........................T..................

InfB_A_allele GACTACGGGATTGAAGCACATGCTAAGGTTGAAGTTGATGAAGCTGAT
InfB_D_allele ...........................................
InfB_I_allele ...........................................
InfB_T_allele ...........................................

InfB_A_allele ATTGAACGCTTCTTTGCAGATGAGGATTACCTTAATCCTGATAACTTA
InfB_D_allele ...........................................
InfB_I_allele ...........................................
InfB_T_allele ...........................................

InfB_A_allele ACGGAACGTCCGCCTGTTGTTACTATCATGGGACACGTTGATCATGGT
InfB_D_allele ......................................C......
InfB_I_allele ......................................C......
InfB_T_allele ...........................................

InfB_A_allele AAAACAACTCTTCTTGATACTCTACGTAATTCACGTGTCGCTACTGGC
InfB_D_allele ..........C................................
InfB_I_allele ......................................A....
InfB_T_allele ...........................................

InfB_A_allele GAAGCTGGTGGAATTACTCAACATATTGGTGCTTATCAGATTGAAGAA
InfB_D_allele ........................A..................
InfB_I_allele ...........................................
InfB_T_allele ...........................................

InfB_A_allele GCTGGCAAGAAAATTACTTTCTTGGATACACCAGGGCATGCGGCCTTT
InfB_D_allele ...........................................
InfB_I_allele ...........................................
InfB_T_allele ...........................................

InfB_A_allele ACTTCTATGCGTGCGCGTGGTGCATCAGTAACTGATATTACTATCCTT
InfB_D_allele ...........................................
InfB_I_allele ...........................................
InfB_T_allele ...........................................

InfB_A_allele ATCGTTGCCGCTGATGATGGTGTTATGCCGCAGACAGTTGAGGCAATT
InfB_D_allele ......................C........A.............
InfB_I_allele ...........................................
InfB_T_allele ...........................................

InfB_A_allele AATCACTCTAAAGCTGCGGGTGTC
InfB_D_allele ........................
InfB_I_allele ........................
InfB_T_allele ........................
```

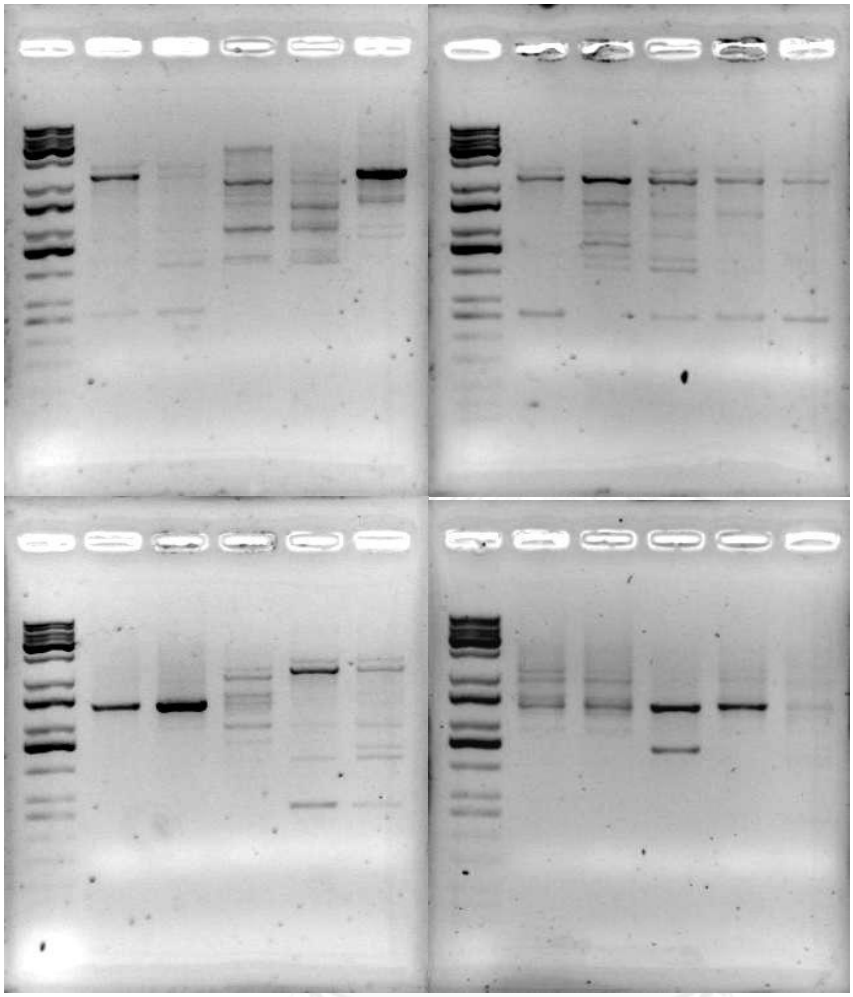
Translation initiation factor IF2 (*inf*B) nucleotide sequences at central variable region (position 1138-1641) of *S. agalactiae* isolated in this study. There were 4 allele types (A, D, I and T) presented among our *S. agalactiae* population. Consensus nucleotide among different allele types is annotated as dot symbol.

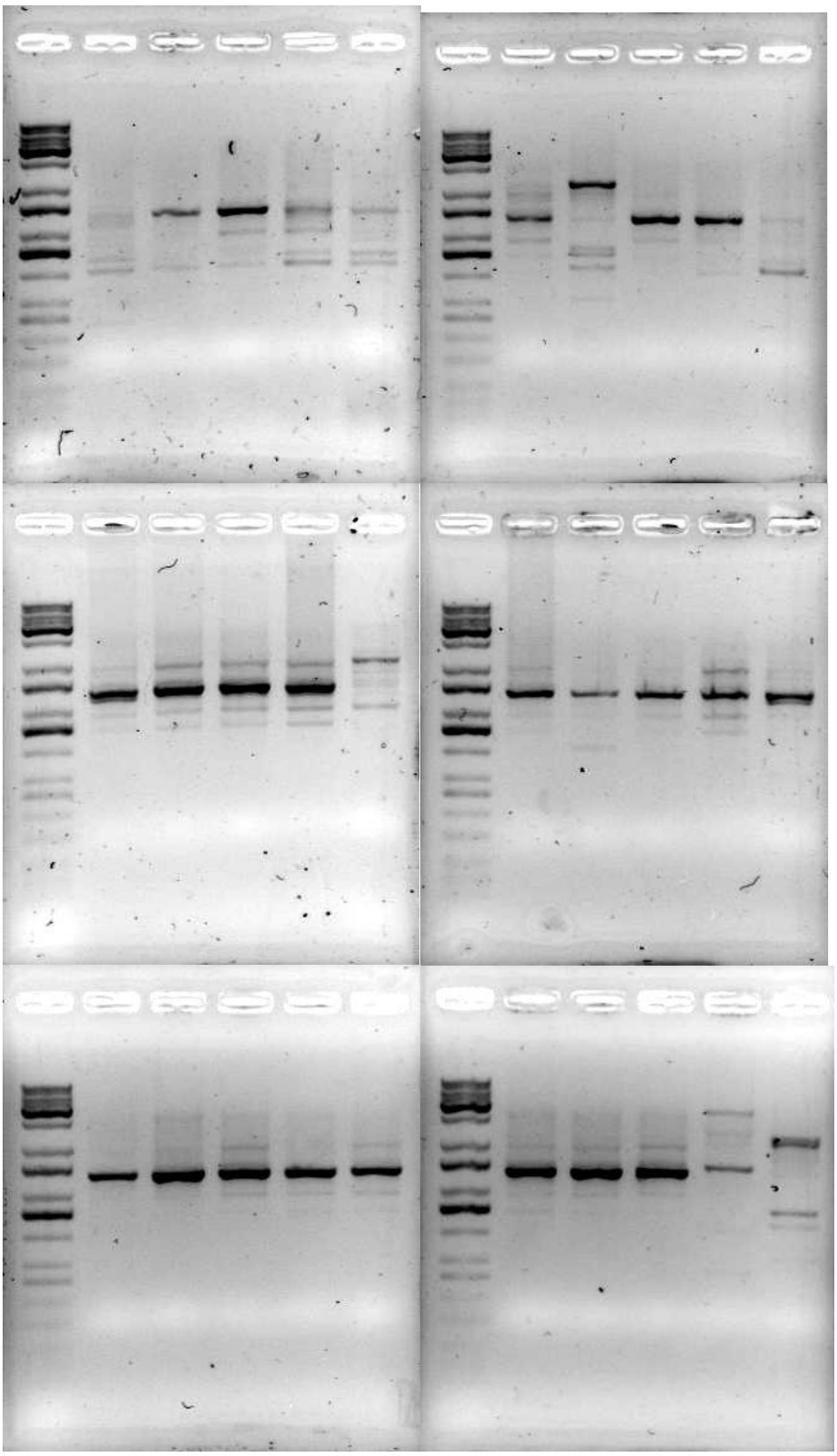
inf B translated amino acid sequences

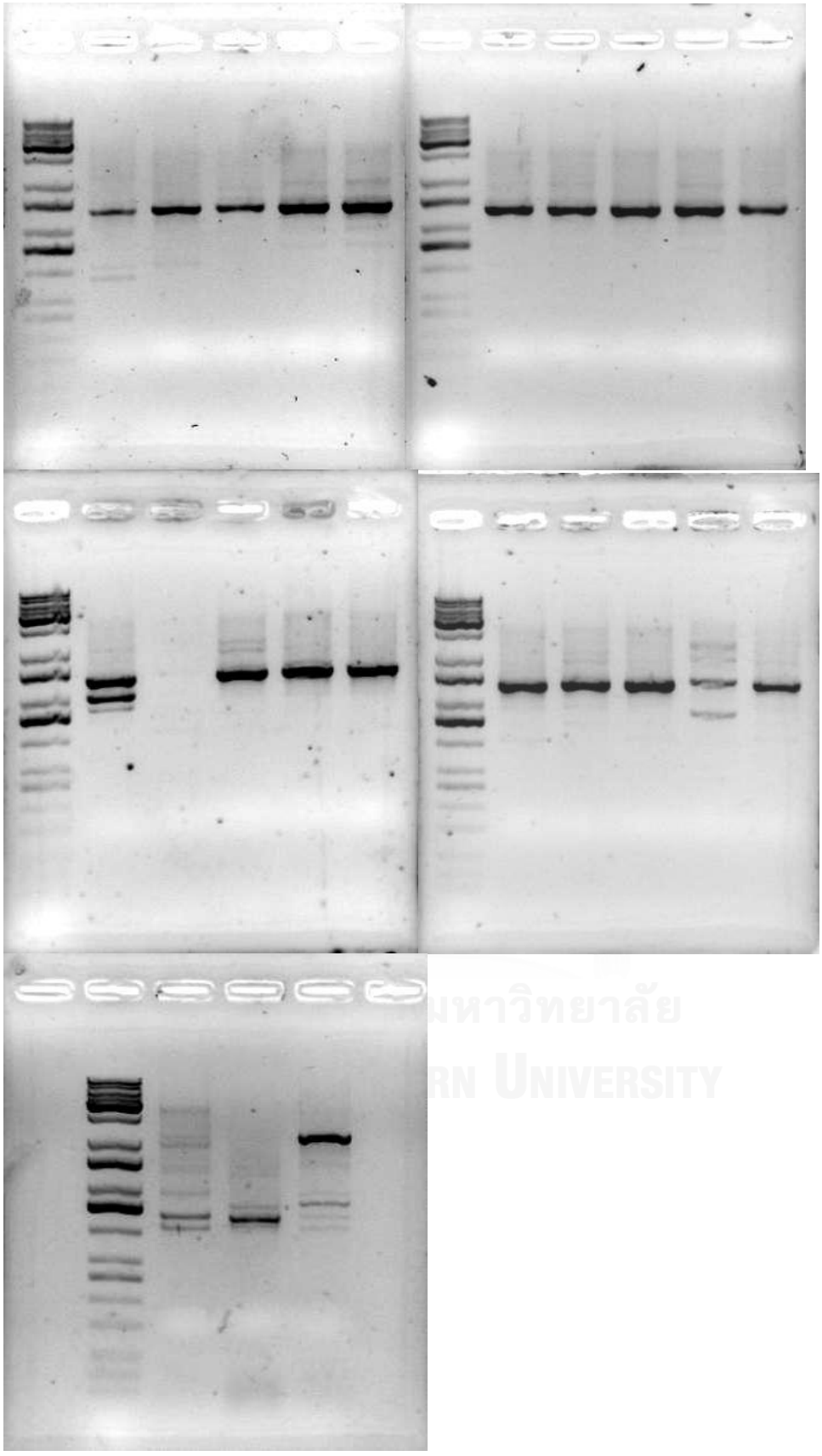
```
InfB_A_allele EPAEIVKKLFMMGVMATQNQSLDGDTIELLMVDYGIEAHAKVEVDEADIE
InfB_D_allele .............................................
InfB_I_allele .............................................
InfB_T_allele .............................................

InfB_A_allele RFFADEDYLNPDNLTERPPVVTIMGHVDHGKTTLLDTLRNSRVATGEAGG
InfB_D_allele .............................................
InfB_I_allele .............................................
InfB_T_allele .............................................

InfB_A_allele ITQHIGAYQIEEAGKKITFLDTPGHAAFTSMRARGASVTDITILIVAADD
InfB_D_allele .............................................
InfB_I_allele .............................................
InfB_T_allele .............................................

InfB_A_allele GVMPQTVEAINHSKAAGV
InfB_D_allele ..................
InfB_I_allele ..................
InfB_T_allele ..................
```

Translation initiation factor IF2 (*inf*B) translated amino acid sequences at central variable region of *S. agalactiae* isolated in this study. All four allele types (A, D, I and T) presented identical amino acid sequences.

RAPD fingerprinting patterns on 1.5% agarose gel







VITA

Pattanapon Kayansamruaj

Born: 18 March 1984

High Scool: Taweetapisek School, Bangkok

Bachelor: DVM, Faculty of Veterinary Science, Mahidol University

Doctoral: Veterinary Pathobiology, Faculty of Veterinary Science, Chulalongkorn University

Pattanapon Kayansamruaj studied in Taweetapisek high school and graduated in 2002 and continued his study in Faculty of Veterinary Science, Mahidol University in 2003. During six years in Veterinary school, Pattanapon had developed his passion in scientific and academic. Attending many student charity events, he learned the essence of benevolence and being brotherhood. In the last year of study, he had been chosen to be the president of Veterinary student club which given him the opportunity to work collaboratively. At 2009, he graduated from Mahidol University with the second class honours degree. Regarding an infinite hunger for knowledge, he decided to continue his study at Faculty of Veterinary Science, Chulalongkorn University. After five years of diligence and perspiration, he could fulfill the requirements of doctoral degree, alas.