ลักษณะพยาธิชีววิทยาของโรคสเตรปโตคอคโคซิสและการพัฒนาวัคซีนสำหรับปลานิลเพาะเลี้ยงในประเทศไทย

นางสาวหทัยรัตน์ ไม้สัก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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PATHOBIOLOGICAL CHARACTERISTICS OF STREPTOCOCCOSIS AND VACCINE DEVELOPMENT FOR FARMED TILAPIA *OREOCHROMIS NILOTICA* IN THAILAND

Miss Hathairat Maisak

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Medicine
Department of Veterinary Medicine
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Thesis Title	PATHOBIOLOGICAL CHARACTERISTICS OF STREPTOCOCCOSIS AND VACCINE DEVELOPMENT FOR FARMED TILAPIA *OREOCHROMIS NILOTICUS* IN THAILAND
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หทัยรัตน์ ไม้สัก : ลักษณะพยาธิชีววิทยาของโรคสเตรปโตคอคโคซิสและการพัฒนาวัคซีนสำหรับปลานิลเพาะเลี้ยงในประเทศไทย. (PATHOBIOLOGICAL CHARACTERISTICS OF STREPTOCOCCOSIS AND VACCINE DEVELOPMENT FOR FARMED TILAPIA OREOCHROMIS NILOTICUS IN THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.เจนนุช ว่องธวัชชัย, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.สพ.ญ.ดร.รุ่งทิพย์ ชวนชื่น, 115 หน้า.

ปลานิล *(Oreochromis niloticus)* เป็นสินค้าเกษตรอย่างหนึ่งที่มีบทบาทสำคัญในประเทศไทย ณ ปัจจุบัน ผลจากการเลี้ยงปลานิลอย่างหนาแน่นทำให้เกิดโรคระบาดและเป็นอุปสรรคสำคัญต่อการผลิตปลานิล โรคสเตรปโตคอคโคซิส เป็นสาเหตุของการสูญเสียทางเศรษฐกิจในหลายๆ ประเทศที่มีการผลิตปลานิล จากการตายจำนวนมากในทุกระบบของการเพาะเลี้ยง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาพยาธิชีววิทยาของโรคสเตรปโตคอคโคซิสและการตรวจวิเคราะห์ชนิดของเชื้อที่เป็นสาเหตุของการเกิดโรคระบาดโดยวิธีทางจุลชีววิทยาและจุลชีวโมเลกุล และการพัฒนาวัคซีนป้องกันโรคสเตรปโตคอคโคซิส ปลานิลป่วยจากอาการสันนิษฐานว่าเกิดโรคสเตรปโตคอคโคซิสช่วงปี พ.ศ. 2546 ถึง 2553 นำมาศึกษาลักษณะการเกิดโรคด้วยการชันสูตรซากร่วมกับวิธีทางจุลพยาธิวิทยา วิธีทางจุลชีววิทยา และวิธีปฏิกิริยาห่วงโซ่พอลิเมอเรส (PCR) พบว่าลักษณะอาการและวิการเป็นผลจากการติดเชื้อสเตรปโตคอคคัสในระบบเลือด โดยแสดงความผิดปกติ ได้แก่ ว่ายน้ำหมุนควง จุดเลือดออกทั่วร่างกายและอวัยวะภายใน ตาโปนและกระจกตาขุ่น มีของเหลวในช่องท้องทำให้ท้องขยายใหญ่ และอาจพบตุ่มหนองบริเวณลำตัว สามารถพบเชื้อแบคทีเรียสเตรปโตคอคคัสโดยการแยกเชื้อจากเนื้อเยื่อส่วนไตและสมอง การตรวจวินิจฉัยด้วยวิธีทางจุลชีววิทยา ด้วยชุดทดสอบ API และวิธี PCR จากจำนวน 139 ตัวอย่าง พบเชื้อ *S. agalactiae* จำนวน 131 ตัวอย่าง (94.24%) และ *S. iniae* จำนวน 8 ตัวอย่าง (5.76%) การศึกษาลักษณะของเชื้อ *S. agalactiae* และ *S. iniae* ทางพันธุกรรมโดยการวิเคราะห์ลำดับเบสของยีน 16S rRNA และยีน *sodA* พบว่ามีความเหมือนทางพันธุกรรม $\geq$97% จากการเปรียบเทียบลำดับเบสของยีน 16S rRNA (*S. agalactiae*; GenBank accession no. GQ169772-74, GQ338316-18 และ *S. iniae*; GenBank accession no. GQ169769-71, GQ338313-15) และยีน *sodA* (*S. agalactiae*; GenBank accession no. HM004089-94 และ *S. iniae*; GenBank accession no. HM004083-88) ของเชื้อชนิดนั้นๆ การทดสอบวัคซีนเชื้อตาย Formalin Killed Cell (FKC) และ Extracellular product (ECP) ผลิตจากเชื้อ *S. agalactiae* ที่แยกจากปลานิลป่วยในประเทศไทย พบว่าวัคซีนที่พัฒนาขึ้นสามารถให้ความคุ้มโรคในปลานิลทดลอง (ขนาดน้ำหนักตัว 200 กรัม) เป็นเวลาอย่างน้อย 10 สัปดาห์ หลังจากได้รับวัคซีน ทดสอบโดยการตรวจเซรั่มแอนติบอดี้ด้วยวิธีแอคกลูติเนชั่น และการฉีดเชื้อทับเข้าช่องท้องขนาด 1.5 X 10^8 *S. agalactiae* เซล/ตัว พบว่าปลาที่ได้รับวัคซีน มีอัตราการตาย 21.5% (4/19) ในขณะที่ปลากลุ่มที่ไม่ได้รับวัคซีนมีอัตราการตาย 95% (19/20) ไม่พบความแตกต่างของวัคซีนชนิด FKC และ FKC ผสม ECP ในการป้องกันโรค นอกจากนี้การทดสอบคุณภาพวัคซีนพบว่า วัคซีนมีปลอดภัยต่อปลาและปลาที่ได้รับวัคซีนมีอัตราการเจริญเติบโตตามปกติ

ภาควิชา อายุรศาสตร์..... ลายมือชื่อนิสิต.....

สาขาวิชา อายุรศาสตร์สัตวแพทย์..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2553..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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HATHAIRAT MAISAK: PATHOBIOLOGICAL CHARACTERISTICS OF STREPTOCOCCOSIS AND VACCINE DEVELOPMENT FOR FARMED TILAPIA *OREOCHROMIS NILOTICUS* IN THAILAND.

THESIS ADVISOR: ASSOCIATE PROFESSOR JANENUJ WONGTAVATCHAI, D.V.M., M.S., Ph.D. THESIS CO-ADVISOR: ASSISTANT PROFESSOR RUNGTIP CHUANCHEN, D.V.M., Ph.D. 115 pp.

Tilapia (*Oreochromis niloticus*) is presently a major aquaculture commodity in Thailand. Resulting from farming intensification, tilapia aquaculture has encountered with disease outbreaks. Streptococcosis, a disease caused by streptococci bacteria, has been reported in many countries and has economic consequences on mass mortality in all stages of tilapia farming. The purpose of this study is to identify streptococcal pathogens in farmed tilapia by using the conventional microbiological and molecular techniques. Streptococcosis vaccine development and its application were studied in tilapia farms. Diseased fish from overall culture areas reporting outbreaks between 2003 to 2010 were examined for pathological and microbiological characterization. Clinically infected fish showed septicemic condition, including generalized hemorrhage, exophthalmia with ocular opacity, abdominal distension, skin abscesses and erratically swimming. Bacterial isolates recovered from the kidney and brain tissue of the diseased tilapia were identified using API system and PCR assay. PCR amplification of the 16S rRNA gene with species-specific primers employed to 139 clinical isolates revealed that 131 isolates (94.24%) were *S. agalactiae* and 8 clinical isolates (5.75%) were *S. iniae*. The sequencing analysis of 16S rRNA gene and *sodA* gene suggested high sequence similarity ($\geq$ 97%) with the corresponding portion of fish pathogen genome, *S. agalactiae* (16S rRNA gene: GenBank accession no. GQ169772-74, GQ338316-18; *sodA* gene: GenBank accession no. HM004089-94) or *S. iniae* (16S rRNA gene: GenBank accession no. GQ169769-71, GQ338313-15; *sodA* gene: GenBank accession no. HM004083-88). Formalin killed cell (FKC) and extracellular product (ECP) were used as a vaccine against *S. agalactiae* infections. The vaccination of farmed fish (200 gm body weight) resulted in specific agglutinating antibodies for at least 10 weeks post-vaccination. The antibodies conferred protection against a single intraperitoneal challenge of 1.5 x 10^8 cell *S. agalactiae* / fish. Mortality of non-vaccinated fish reached 95% (19/20), whereas mortality of the vaccinated fish was 21.5% (4/19). The efficacy of FKC vaccine and FKC added with ECP vaccine was relatively not different. In addition, no evidence of negative impacts on health performance was observed in the vaccinated tilapia.

Department : Veterinary Medicine..... Student's Signature Hathairat Maisak

Field of Study : Veterinary Medicine..... Advisor's Signature

Academic Year : 2010..... Co-Advisor's Signature Rungtip Chuanchen

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CHAPTER I

IMPORTANCE AND RATIONALE

The nile tilapia is a freshwater fish native to Africa and the Middle East. The tilapia is well adjusted to different aquaculture systems, reproduced easily and able to take a variety of foods (Gupta and Acosta, 2004). Therefore, tilapia are one of the most produced and traded food fish worldwide (Gupta and Acosta, 2004; Josupeit, 2009). The total world tilapia aquaculture production in 2006 was 2,381,237 metric tons. While the major tilapia producers are China, Egypt, Indonesia, the Philippines, Thailand, Mexico, Taiwan and Brazil. Thailand is among the top five producers and production has been increasing over time, from 100,000 tons in 2006 to 120,000 tons in 2008 (Josupeit, 2009). The main export countries are the USA, Japan and the European community. Thai tilapia production supports both domestic and export demands. The income from their export value was approximately 800 million Baht in 2008 (Department of Foreign Trade, 2008).

Tilapia are usually reared at high density to serve the increasing demand from consumers worldwide, however, the intensify tilapia farming does not always implement the biosecurity system (Americulture, 1999; Evans *et al*., 2000). Bacterial infections are most significant diseases of cultured tilapia, causing the highest levels of both morbidity and mortality rate (Wildgoose, 2001; Yanong and Floyd, 2006). *Streptococcus* is one of the most significant bacterial diseases in tilapia culture worldwide and has caused economic loss to the world aquaculture industry (Yanong and Floyd, 2006). Streptococcosis is a septicemic disease caused by both the alpha-hemolytic and beta-hemolytic strains of streptococcal bacteria. Many species of *Streptococcus* are pathogenic to humans and animals. Some species have been proven or suspected to be zoonotic. The common isolates in fish infection are *S. agalactiae* and *S. iniae* (The Australian Ministry of Agriculture and Forestry, 1999; Yanong and Floyd, 2006). Streptococcal infections occur in many economically important species of marine and freshwater fish. Many studies have reported that the tilapia is more susceptible to streptococcosis than other fish (Yanong and Floyd, 2006). Chang and Plumb (1996) reported that tilapia had a greater mortality rate than channel catfish after bacterial inoculation and had a mortality rate of higher than 70% within 7 days post-inoculation (Evans *et al*, 2002).

Streptococcal infection in tilapia farming, particularly in intensive culture, causes a mortality rate of above 75% at 3 to 7 days post-infection (Yanong and Floyd, 2006). Chronic or subclinical infections dramatically reduce the appetite of the fish and, consequently, decrease growth rate significantly. In market or retail sites, the fish fillets from diseased fish have a short shelf life and poor meat quality yield. The physical appearance, e.g. missing one or both eyes or having hemorrhages all over the bodies of the diseased fish is not acceptable to the consumers (Americulture, 1999; Yanong and Floyd, 2006).

Streptococcosis is usually identified by its clinical signs, together with conventional microbiological tests; colony morphology, biochemical and Lancefield group characteristics. However, some species of *Streptococcus* cannot be identified by conventional tests. Molecular techniques have been developed for the detection and identification of many bacterial pathogens including the significant fish pathogens. All *Streptococcus* species can be confirmed correctly and rapidly by immunofluorescence, enzyme-linked immunosorbent assays (ELISA) and molecular techniques (Americulture, 1999; OIE, 2005; Yanong and Floyd, 2006).

Several strategies are employed to prevent streptococcosis in farmed fish, e.g. good maintenance of water quality and the environment, quarantining of new fish, rapid diagnostic tools with proper therapy and vaccination (Evans *et al*., 2004; Klesius *et al*., 2000). Fish vaccination has been successfully applied in the control of several bacterial diseases (Marsden *et al*., 1998; Nakanishi *et al*., 2002). In addition, vaccination has replaced the use of antibiotics for treatment and growth promotion that is sometimes ineffective or not safe to the consumer (Klesius *et al*., 2000). However, there are many factors that need to be considered for the vaccination, e.g. the types of antigen, the strain variation among the regions of disease outbreak, the route of administration and the size of fish (Evans *et al*., 2004; Klesius *et al*., 2000). Vaccination against streptococcosis has been used successfully in tilapia farming worldwide (Americulture, 1999; Gudding *et al*., 1999); hence, in addition to the pathobiological characteristics of the disease, this study will focus on the vaccine development and its application in farmed tilapia.

Objectives of study

The purposes of this study are;

(1) To identify streptococcal pathogens in farmed tilapia by using the conventional microbiological methods and molecular genetic-based techniques.

(2) To study the methodology for streptococcosis vaccine development and the application of vaccine in farmed tilapia.

Keywords (Thai): โรคสเตรปโตคอคโคซิส ปลานิล วัคซีน

Keywords (English): Streptococcosis, Tilapia, Vaccine

CHAPTER II

LITERATURE REVIEW

The tilapia is one of the most produced and internationally traded food fish in the world. The characteristics of tilapia aquaculture are that they are disease-resistant, reproduce easily and are widely acceptable in a variety of foods (Gupta and Acosta, 2004). Most tilapia can grow in both freshwater and brackish water (salinity $\leq$ 7 ppt) and, as a result, tilapia are suitable for culture in many countries (Bocek, 2008; Department of Fisheries, 2008; Gupta and Acosta, 2004). A greater part of tilapia production is consumed within Africa and Asia, but recently, tilapia consumption has become worldwide and includes the USA, Canada, Europe, Central and South America. The increasing demand of tilapia production is because it provides on important source of animal protein, foreign exchange and employment opportunities in several countries (Gupta and Acosta, 2004).

The world's total tilapia aquaculture production in 2006 was 2,381,237 metric tons. Tilapia are cultured in $\geq$ 100 countries and Asia has remained the number one producer. The major tilapia producers are China, Egypt, Indonesia, the Philippines, Thailand, Mexico, Taiwan and Brazil. Thailand is listed among the top five producers and its production has slightly increased overtime (100,000 tons in 2006 and 120,000 tons in 2008) (Josupeit, 2009). Thai tilapia production supports both domestic and export demands. Main export countries are the USA, Japan and the European community. The income from exports was approximately 800 million Baht in 2008 and is increasing overtime. The export products are whole frozen, fresh fillets and frozen fillets. Further value-added tilapia products are smoked products, sashimi, fried skins and frozen meals following consumer demand. In addition, the by-products, skin and scales of tilapia are used for leather goods and in the pharmaceutical industry. Other by-products are used for fertilizer in agriculture and for fish meal in animal food industry (Department of Fisheries, 2008; Department of Foreign Trade, 2008).

Tilapia is a freshwater fish native to Africa and the Middle East. There are about 70 species of tilapia and 9 species are used in aquaculture worldwide though tilapia production focuses mainly on 3 species; a warm water strain, the Nile tilapia (*Oreochromis nilotica*), a cold resistant strain, the Blue tilapia (*Oreochromis aureue*) and a hybrid reddish-colored strain, the Mozambique tilapia (*Oreochromis massambica*) (Gupta and Acosta, 2004). Nile tilapia comprise the majority of global tilapia production (the total Nile tilapia produced worldwide was about 83% in 2002, reported by FAO).

Tilapia can be developed to produce better performing species or strains and many techniques have been employed to manage reproduction. Nile tilapia are called mouth-brooders because the laid eggs are fertilized, and the female incubates and hatches its eggs in its mouth. The average spawning is about 3 times per year with approximately 750 to 6000 eggs produced per year. Tilapia have numerous large eggs and they are easy to culture until of marketable size. The rearing of tilapia is divided into 3 phases; the hatchery phase, the nursery phase and the grow-out phase. In the hatchery phase, the fertilized eggs are hatched over 3 to 5 days and are reared to fingerlings in about 7 days. Small fingerlings are cultured to a large size (ranging from 20 to 50 grams) in the nursery phase for 21 to 23 days. During the nursery phase, the fish are selected in monosex populations as the male tilapias grow faster and are more uniform in size than females. Monosex populations are achieved either by manual sexing, direct hormonal sex reversal, hybridization or genetic manipulation. The final phase is the grow-out phase. The fish are reared over 7 to 8 months to reach marketable size (800

to 1000 grams). The grow-out phase can be managed at different kinds of farms, such as; large cage farms, tilapia-shrimp polyculture farms, ponds, intensive tank culture and raceways systems (Bocek, 2008; Department of Fisheries, 2008; Gupta and Acosta, 2004). Although, tilapia are being reared at higher densities to support the increasing consumption demand worldwide, the intensified tilapia farming does not generally implement biosecurity systems. Consequently several significant diseases in tilapia culture are apparent (Americulture, 1999; Evans *et al*., 2002).

The significant tilapia pathogens fall into the general categories of protozoa, bacteria and ricketsial-like diseases. Bacterial diseases are the most significant pathogens of cultured fish, causing the highest levels of both morbidity and mortality. Bacterial diseases in fish are the result of many inducing factors such as environmental condition, stress, the susceptibility of the host and the virulence of the pathogen (Americulture, 1999; Wildgoose, 2001; Yanong and Floyd, 2006). *Streptococcus* is one of the most significant bacterial diseases in tilapia culture worldwide, and the disease causes economic loss to the world tilapia industry. Streptococcosis is associated with poor management, including high water temperature (water temperature of above 30^{O}C during the summer), high stocking density, harvesting or handling and poor water quality with high ammonia or nitrite concentrations (Romalde and Toranzo, 1999; Shoemaker *et al*., 2000; Wildgoose, 2001).

Streptococcosis is a septicemic disease caused by both alpha-hemolytic and beta-hemolytic strains of *Streptococcus* spp. *Streptococcus* is a gram positive cocci bacterium of the family Stretococcaceae. Many species of *Streptococcus* are pathogenic to humans and animals. Some species have been proven or suspected to be zoonotic. Species of *Streptococcus* that cause disease in fish are *Streptococcus agalactiae, S. dysgalactiae, S. equi, S.equisimilis, S. faecium, S.pyogenes, S. zooepidemicus* and *S. iniae*. The main common isolates from diseased fish are *S. agalactiae* and *S. iniae* (The Australian Ministry of Agriculture and Forestry, 1999; Yanong and Floyd, 2006). *S. agalactiae* can be found in both humans and animals including cattle, horses, dogs, rabbits, guinea pigs, mice and fish. *S. agalactiae* (beta-hemolytic and Lancefield group B) is an important cause of human infection and animal disease. *S. agalactiae* is hardly a zoonotic infection because the main infection of *S. agalactiae* in humans is limited from person to person transmission. *S. iniae*, a zoonotic infection, occurred in Asian people in Toronto, Ontario and Canada in 1997 (OIE, 2005). The cause of this human infection was associated with skin injury from the spines of fish or knives. The clinical sign of the infection was local inflammation like cellulitis around open wounds. Some cases that were susceptible to the disease had serious infection followed by septicemic disease. However, healthy people were at minimal risk of acquiring this disease from sick fish (OIE, 2005; Weinstein *et al*., 1997).

Streptococcal infections occur in many economically important species of marine and freshwater fish, e.g. striped mullet, sea trout, pinfish, spot, Atlantic croaker, gulf menhaden, yellowtail, striped bass, sea bream, salmon, rainbow trout, sea catfish, stingray, tilapia, eel, bluefish, golden shiner, silver sea trout, Japanese flounder and sturgeon (Edward, 2000; Romalde and Toranzo, 1999; Yanong and Floyd, 2006). Many studies support the theory that streptococcosis seriously affects tilapia. Chang and Plumb in 1996 reported that tilapias had a greater mortality rate than channel catfish within 24 hours of inoculation and had a mortality rate of upto 70% within 7 days of inoculation (Evans *et al*., 2002).

Streptococcosis outbreaks occur in many countries that conduct tilapia farming, including the USA, Japan, Israel and Italy (Edward, 2000; Romalde and Toranzo, 1999). In Thailand, the literature indicates that streptococcosis causes production loss in all regions of tilapia farming (Wongtavatchai *et al*., 2006). *Streptococcus* is found in a wide variety of sources e.g. environment (water, bottom mud of culture area), contaminated fish food and carrier fish. In addition, live or unprocessed (fresh or frozen) food can be a possible source of exposure and disease outbreak (Romalde and Toranzo, 1999). *Streptococcus* spp. can be transferred into fish by ingestion of the infected or dead fish in cannibalistic or predacious fish. Removing dead fish is suggested to be a possible mean of minimizing oral transmission (Evans *et al*., 2000; Yanong and Floyd, 2006).

The consequence of streptococcosis in tilapia farming, is that streptococcosis affects fish of any size or age. Streptococcal infections cause high mortality rates, particularly in intensive culture. A mortality rate (of upto 75%) was found over 3 to 7 days post-infection (Americulture, 1999; Romalde and Toranzo, 1999; Yanong and Floyd, 2006). Chronic or subclinical infections reduce fish appetite and consequently decrease growth rate significantly. The grow-out phase, which normally takes 7-8 months, retards to 10-12 months when the juvenile fish are subclinically infected. The diseased fish are injured easily during harvesting or transportation to market and have short shelf life. The physical appearance of infected fish, e.g. missing one or both eyes and hemorrhagic skin, are not acceptable to the consumer due to the poor meat quality yield. (Americulture, 1999; Yanong and Floyd, 2006).

The clinical signs of streptococcosis are those of bacteremia with widespread hemorrhaging and inflammation that occurs from its pathogen and exotoxin from bacteria as hemolysin. Streptococcosis shows clinical signs such as abnormal swimming behavior (spiraling or spinning), weakness, loss of appetite, imbalanced movement, lethargy, darkening, uni or bilateral exophthalmia (extruding eyes), corneal opacity (whitish eyes), hemorrhage in or around the eye, the operculum, the base of the fins, vent or anus and on the body, abdominal distension and ulceration. *Streptococci* can infect the brain and nervous system of fish resulting in abnormal swimming behavior during the last stage of infection. Internal lesions have revealed bloody fluid in the abdomen, an enlarged reddened spleen, a pale liver, kidney enlargement and hemorrhage over the heart, intestine, gut and brain (Edward, 2000; Yanong and Floyd,2006).

The kidney and brain are good sites for the detection of the causative bacteria. The bacteria infiltrates via the blood circulation of diseased fish and, as a result, it can be found in the kidney and other organs (Romalde and Toranzo, 1999; The Australian Ministry of Agriculture and Forestry, 1999; Yanong and Floyd, 2006). A presumptive diagnosis can be made by microscopic examination of the tissue imprint showing the gram positive cocci chain. *Streptococcus* may be found in wounds, blood or pustules. The definitive diagnosis depends on the bacterial identification of the pure culture. *Streptococcus* spp. can be identified by hemolytic patterns on blood agar, colony morphology, biochemical reaction, serology to detect antigens and molecular techniques (OIE, 2005). In hemolytic reaction, there are 3 types of hemolysis *Streptococcus* including beta, alpha and non-hemolysis. Beta-hemolysis *Streptococcus* can damage the red blood cells completely and found clear zone surrounding the colony on blood agar. Alpha-hemolysis *Streptococcus* causes a partial or green hemolysis around the colony since the activity is associated with the reduction of red blood cell hemoglobin (McKane and Kandel, 1996; OIE, 2005; Talaro and Talaro, 1996). As a serological method to detect antigens of *Streptococcus*, Lancefield grouping is used for the

grouping of *Streptococcus* and identified with the letters A to H and K to V. However, some streptococcal species do not have Lancefield group antigens (OIE, 2005). Fish streptococcosis are usually identified by clinical signs and confirmed by conventional tests such as culturing, colony morphology, biochemical test or Lancefield group (OIE, 2005; Songer and Post, 2005). However, the conventional tests cannot identify some species of *Streptococcus* and the methods do not provide rapid identification. All species of *Streptococcus* can be confirmed correctly and rapidly by immunofluorescence, enzyme-linked immunosorbent assays (ELISA) or molecular techniques (Americulture, 1999; OIE, 2005; Yanong and Floyd, 2006).

Molecular characterization as a phylogenetic system is based on the information carried in the genes. The methods are used to study the evolution of the organism, characterize the members of the bacteria and find out the taxonomic position of the bacterial strains (Alber *et al*., 2004; Sulultana *et al*., 1998). Genetic differences can be a database for the development of diagnostic tools and effective vaccines (Eldar *et al*., 1997). Homologous genes that among to different species are used in the construction of phylogenetic trees. The genes; encoding the 16S rRNA, the 23S rRNA and the 16S-23S rRNA intergenic spacer region; have been used for the genetic identification of bacteria including *Streptococci* (Alber *et al*., 2004) because these genes are highly conserved and pose variable sequence segments (Chatellier *et al*., 1998; Martinez *et al*., 2001; Sulultana *et al*., 1998). In addition, alternative target genes e.g. gene *sodA* encoding the superoxide dismutase A and gene *cpn60* encoding chaperonin 60 (60 KDa heat shock protein, HSP60) have been used for the molecular identification of *Streptococci* (Alber *et al*., 2004).

Although streptococcal infection responds to antibiotic therapy, the disease is not effectively controlled with antibiotics all the way to market. In addition, *Streptococcus* strains that break out in different areas may develop resistance to some antibiotics. Antibiotics used for treatment in food fish are regulated by Food and Drug Administration (FDA) to specify the species of fish and the disease to be treated, the method of administration, dosage and the withdrawal time in food fish (Bowser, 1999). Antibacterials are used in fish such as amoxicillin, oxytetracycline, sulphadiazine/trimethoprim and sulfadimethoxine/ormetoprim (Romet-30) (WHO, 1999). Feeding medication is usually the route of medical administration and the amount of antibacterial is calculated from drug/fish body weight per day for a specified number of days. Administration with an immersion bath of antibiotics is not suitable in aquaculture due to the large volume of water to be treated. Antibacterial susceptibility of bacterial isolates should be determined before each treatment to avoid any antimicrobial resistance problem (Bowser, 1999).

Prevention of streptococcosis in farmed fish can be achieved through several procedures; the maintaining of good water quality, the keeping of the environment clean, the quarantining of new fish before introducing farm, rapid diagnostic tools with proper therapy and vaccination (Evans *et al*., 2004; Klesius *et al*., 2000). Vaccination is mainly conducted and is successful against several bacterial diseases in the aquaculture system. Vaccination significantly reduces serious economic loss from diseases (Marsden *et al*., 1998; Nakanishi *et al*., 2002). In addition, vaccination may replace the use of antibiotic treatment that is ineffective or unsafe to consumer (Klesius *et al*., 2000).

Vaccination is an effective strategy to control diseases in large scale aquaculture and valuable brood stocks. Many types of bacterial vaccine are available commercially and routinely used in farmed fish, for instance; furunculosis vaccine, vibriosis vaccine, pasteurellosis vaccine and streptococcosis vaccine (Gudding *et al*., 1999). Fish vaccination is needed to be considered other factors; including types of antigens, strain variation in the region of disease outbreak, size of fish (Evans *et al*., 2004; Klesius *et al*., 2000).

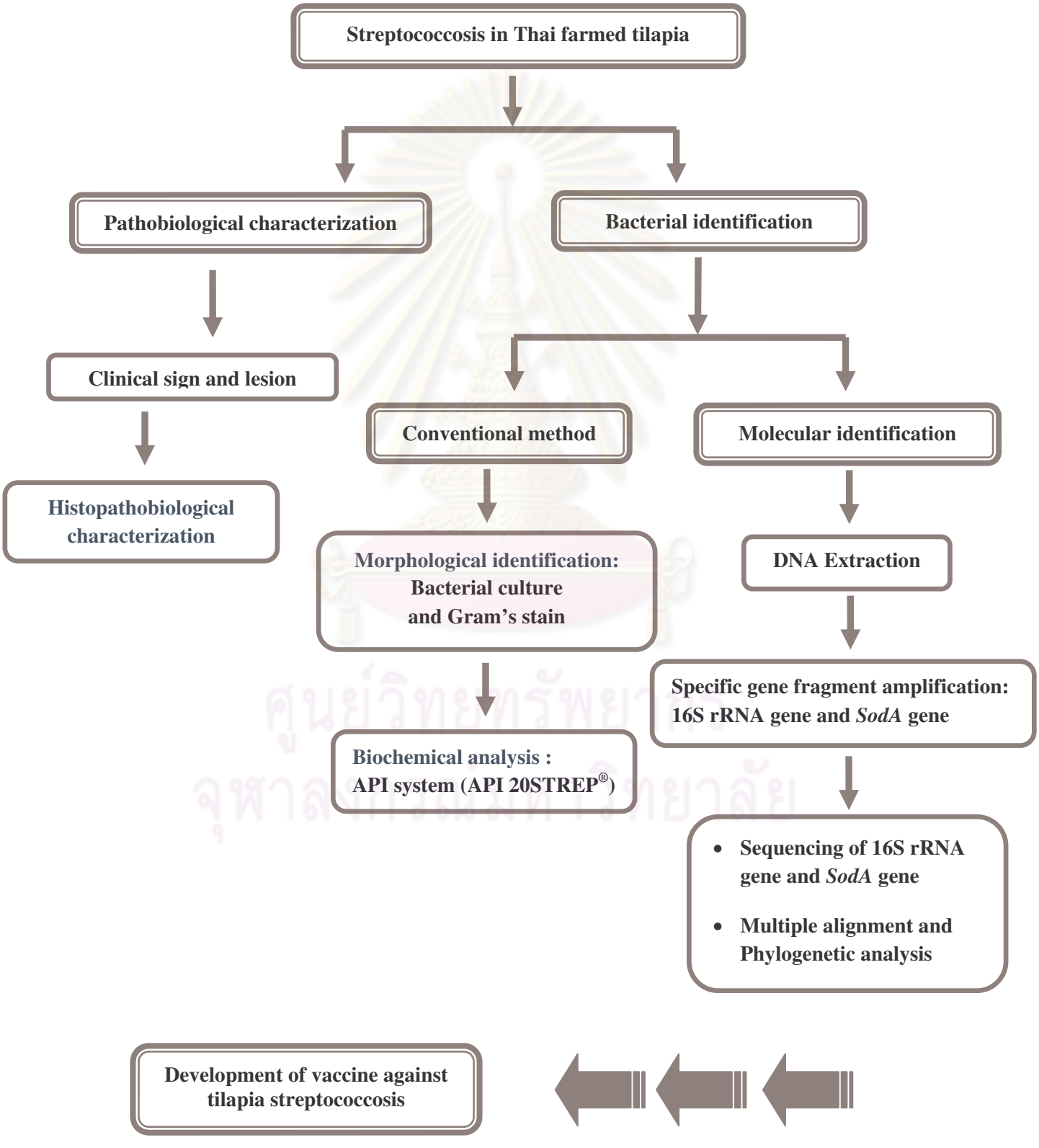
Fish can be immunized by injection, immersion or oral administration of vaccine. Different vaccine administration methods have their advantages and disadvantages depending on the level of protection, the side-effects, practicality and cost-efficiency. Intraperitoneal injection is the most commonly used method for administration of fish vaccine due to its high efficacy (Gudding *et al*., 1999; Nakanishi *et al*., 2002). The maturation of immunity is necessary for fish vaccination. The specific immune response in fish develops after the fish fry completely finish absorption of their yolk and started the meal feeding. The specific immune system depends more on fish weight rather on age (Ellis, 1989; Press and Lillehaug, 1995; Tort *et al*., 2003), thus the size of fish at vaccination day is an important factor for fish to be immunized (Evans *et al*., 2004; Gudding *et al*., 1999). For instance, salmon are able to possess immune response to justify vaccination at more than 5 grams of body weight (Press and Lillehaug,1995). More mature tilapia at 15-20 grams of body weight is required to provide effective response to streptococcosis vaccine (Americulture, 1999; Evans *et al*., 2004; Gudding *et al*., 1999).

Although, several streptococcosis vaccines have been developed for the protection of streptococcosis, many of these vaccines differ in their formulation; formalin-killed *S.iniae* vaccine, modified-killed *S.iniae* vaccine composed of whole cells and concentrated extracellular products, toxoid-enriched bacterin vaccine and vaccine in Freund's incomplete adjuvant. A formalin-killed bacterin was successful in controlling the disease for 2 years and the relative percentage survival (RPS) was higher than 80% (Romalde and Toranzo, 1999). Unvaccinated fingerlings have a greater cost of production than vaccinated fish due to the increasing cultural duration and use of chemicals and medicines for streptococcosis treatment. Unvaccinated fish show high mortality $\geq$ 75%, while streptococcal vaccinated fish have increased survival (Americulture, 1999). In contrast, the development of modified live vaccine against group B *S. agalactiae* or *S. iniae* vaccine is an unsuitable application due to risk of spreading to fish and environment (Toranzo *et al.*, 2009) and its lack of safety to human health (Evans *et al*., 2004).

CHAPTER III

MATERIALS AND METHODS

The study was conducted in 3 phases; Phase I, pathobiological characterization of streptococcosis in farmed tilapia, Phase II, phylogenetic analysis of streptococcal bacteria isolates with specific gene sequence comparison and Phase III, development of a streptococcosis vaccine.



Phase I : Pathobiological characterization of streptococcosis in farmed tilapia

Sample collection and bacterial isolation

Diseased tilapia from the culture areas reporting outbreaks between 2003 and 2010 were examined for clinical sign and gross lesion (external appearances and internal lesions). The bacterial isolates were cultured from target organ, including brain and kidney, on Tryptic Soy Agar (TSA, Oxoid®, USA) added with 5% (v/v) sheep blood (Romalde and Toranzo, 1999; Talaro and Talaro, 1996). The isolates were primarily examined by morphological characterization, e.g. colony characteristic (size, translucency, color and growth on media), bacterial cell morphology following the gram's stain, and biochemical tests. The catalase test were used to differentiate gram positive cocci bacteria, the staphylococcus and micrococcus presented positive for catalase production but *Streptococcus* was negative for catalase production (Stokes and Ridgway, 1980). The pure isolates of *Streptococcus* were stored in maintenance broth with 10% fetal bovine serum and 20% glycerol, at -80^{O}C for further study (Wongtavatchai *et al*., 2006).

Histopathological characterization

Organs from diseased fish e.g. brain, heart, spleen, kidney, liver, intestines and reproductive tissues were preserved in 10% formalin solution for histopathological study as shown in Figure 3.1. The tissue was prepared using histological procedures, chemical fixation and staining for microscopic examination. The tissues were immersed in multiple baths progressively with concentrate ethanol for dehydration of tissue, chloroform for cleaning of tissue and hot liquid paraffin for infiltering of tissue (Appendix B). The processed tissues were embedded and sectioned at 4 micrometer using a microtome. The sectioned tissues were stained with hematoxylin and eosin to reveal cellular components (Chang and Plumb, 1996; Gridley, 1949). Hematoxylin was used to stain nuclei blue and eosin stained cytoplasm and the extracellular connective tissue matrix pink (Gridley, 1949).

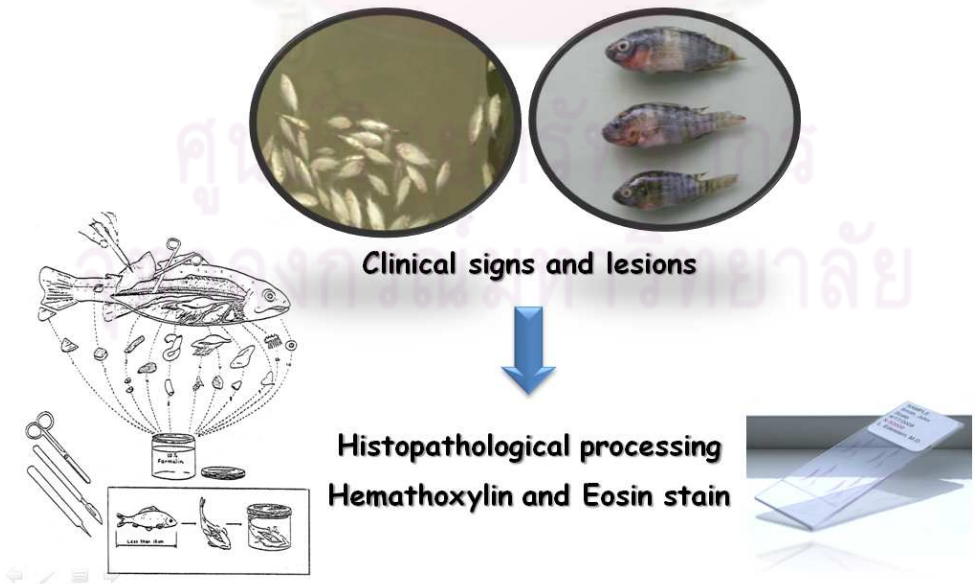


Figure 3.1 Processing of preservative tissues obtained from diseased fish for histopathology.

Biochemical analysis

The bacteria isolates were tested for biochemical characteristic using the enzymatic tests (i.e. acidification of carbohydrates) with the API system (BioMeieux, France). The system tested for activities of acetoin production, hippurate hydrolase, β-glucosidase, pyrrolidonyl arylaminidase, α-galactosidase, β-glucuronidase, β-galactosidase, alkaline phosphatase, leucine arylamidase, arginine dihydrolase, fermentation of carbohydrates, e.g. ribose, L-arabinose, manitol, sorbital, lactose, trehalose, inulin, raffinose, starch and glycogen. The commercial API 20STREP was applied following the manufacturer's instructions with a modification of the incubating temperature to 30$\pm$ 2^{O}C for the suitable bacterial growth (Wongtavatchai *et al*., 2006).

Molecular techniques

Bacterial isolates, those representing morphology and biochemical profiles of *Streptococcus* spp., were further confirmed by polymerase chain reaction (PCR). The chromosomal DNA from bacterial cells was separated with a NucleoSpin® Extract I kit (MACHEREY-NAGEL, Germany) following the manufacturer's instructions (Appendix C), and stored at -20^{O}C until use. PCR identification of the streptococcal DNA was amplified by genus specific oligonucleotide primers C1 and C2 (Meiri-Bendek *et al*., 2002); *S. iniae* 16S rRNA specific primers Sin-1 and Sin-2 (Zlotkin *et al*., 1998); *S. agalactiae* 16S rRNA specific primers F1 and IMOD (Martinez *et al*., 2001) as shown in Table 3.1. PCR was carried out in PCR Thermal Cycler (Whatman Biometra®, UK), and the PCR reaction mixture (20 µl) contained 2 µl of 10X PCR buffer (100 mM Tris HCl (pH 8.3), 500 mM KCl, 20 mM $MgCl_2$), 2 µl of dNTP 2.5 mM, 0.2 µl of Tag polymerase 5 U (iNtRON Biotechnology, USA), 1 µl of forward primer 10 µM, 1 µl of reverse primer 10 µM, 5 µl of DNA 50 ng/µl (Meiri-Bendek *et al*., 2002). *S. agalactiae* ATCC13813 and *S. iniae* ATCC29178 were used as positive control and distilled water was used as negative control of the reaction. PCR was conducted with the following program: 94℃ for 2 mins (initial denaturation step), 30 cycles at 94℃ for 20 secs (denaturation step), at 56℃ for 10 secs (annealing step), and at 72℃ for 30 secs (extension step), followed by a final extension at 72℃ for 2 mins. The PCR products were determined by the electrophoresis in 2% TBE agarose gel with Tris-Borate-EDTA buffer (TBE; 89 mM Tris-borate and 2 mM EDTA, pH 8.3) at 100 volt for 40 mins and 100 bp DNA ladder as a molecular marker (SibEnzyme, Russia). Gels were soaked in 0.5 µg/ml Ethidium bromide (Sigma Algrich Inc., USA) for 30 mins and visualized under UV illumination (Vilber Lourmat, Germany) and photographed.

Table 3.1 Species specific oligonucleotide primer for identification of 16S rRNA gene of *S. agalactiae* and *S. iniae*.

primer	sequence	PCR product (bp)	Reference
Streptococcus			
C1	5'-GCG TGC CTA ATA CAT GCA A-3'	202	Meiri-Bendek *et al*., 2002
C2	5'-TAC AAC GCA GGT CCA TCT-3'		
S. agalactiae			
F1	5'-GAG TTT GAT CAT GGC TCA G-3'	220	Martinez *et al*., 2001
IMOD	5'-ACC AAC ATG TGT TAA TTA CTC-3'		
S. iniae			
Sin-1	5'-CTA GAG TAC ACA TGT ACT AAG-3'	300	Zlotkin *et al*., 1998
Sin-2	5'-GGA TTT TCC ACT CCC ATT AC-3'		

Phase II: Phylogenetic analysis of streptococcal bacterial isolates with specific gene sequence comparison

Bacterial strains and growth conditions

The bacterial isolates used in the presented study were *Streptococcus agalactiae* and *S. iniae* as shown in Table 3.2. Six isolates of *S. agalactiae* and six isolates of *S. iniae* obtained from phase I study were chosen to represent different geographical distribution of tilapia farm in Thailand. These isolates were from the outbreaks occurring in tilapia farm located in middle, north-eastern and western part of Thailand. *S. agalactiae* ATCC13813 and *S. iniae* ATCC29178 were used as reference strains in this study.

Table 3.2 Source of the *Streptococcus agalactiae* and *S. iniae* applied to phylogenetic analysis.

ID	Source	Year of isolation	API identification	Molecular identification (PCR assay)
S. agalactiae				
JW10-SA2	Mukdahan	2004	*S. agalactiae*	*S. agalactiae*
JW13-SA71	Nakornpanum	2007	*S. agalactiae*	*S. agalactiae*
JW16-SA8	Nakornpathum	2004	*S. agalactiae*	*S. agalactiae*
JW19-SA32	Kanchanaburi	2004	*S. agalactiae*	*S. agalactiae*
JW22-SA35	Petchaburi	2005	*S. agalactiae*	*S. agalactiae*
JW25-SA65	Chachoengsao	2006	*S. agalactiae*	*S. agalactiae*
S. iniae				
JW1-SI1	Mukdahan	2003	*S. dys.equisimilis*	*S. iniae*
JW3-SI4	Mukdahan	2004	*S. agalactiae*	*S. iniae*
JW4-SI50	Nongkai	2006	*S. agalactiae*	*S. iniae*
JW6-SI52	Nongkai	2006	*S. agalactiae*	*S. iniae*
JW7-SI69	Nakornpanum	2007	*S. agalactiae*	*S. iniae*
JW9-SI76	Nakornpanum	2007	*S. agalactiae*	*S. iniae*

Preparation of DNA for PCR

Each strain was subcultured on Tryptic Soy Agar (TSA, Oxoid®, USA) added with 5% (v/v) sheep blood for 18-24 hours at 30± 2^{O}C and then grew in Tryptic Soy Broth (TSB, Oxoid®, USA) overnight at 30± 2^{O}C with shaking at 100 RPM. Isolation of the chromosomal DNA from pure cultures was performed using genomic DNA kit (NucleoSpin® Extract I, MACHEREY-NAGEL, Germany), according to the manufacturer's protocol as described previously.

PCR primer

Molecular characterization of the tilapia pathogenic streptococcal bacteria was performed by sequencing of the 16S rRNA and *sodA* gene. The *sodA* gene was examined to differentiate gram positive bacteria, particularly, between streptococci and enterococci (Alber *et al*., 2004). Both genes were amplified by PCR with specific primers designed by Primer3 program (available at http://frodo.wi.mit.edu/primer3). Oligonucleotide primers used to amplify fragments of 16S rRNA (Table 3.3) and *sodA* gene (Table 3.4) of *Streptococcus* were designed on the conserved sequences of the particular genes reported in GenBank nucleotide database. The designed primers were synthesized by Sigma-Genosys, Singapore. Appendix D shows the oligonucleotide primer designs for *Streptococcus* 16S rRNA and *sodA* gene amplification.

Oligonucleotide primers design for the amplification of 16S rRNA gene

The full length of *S. agalactiae* and *S. iniae* 16S rRNA gene (approximately 1450 to 1530 bp) were determined from multiple sequence alignments of this gene from many sources (GenBank accession no. AB002479, AF015927, EF092913, DQ985468 for *S. agalactiae* and AF335572, AY762259, EU075069 for *S. iniae*). The consensus sequence was used to generate primers with Primer3 program. The designed primers for DNA sequencing of 16S rRNA gene were JW_16S_F (5'-AAC GGG TGA GTA ACG CGT AG-3') as a forward primer, and JW_16S_R (5'-TTC ATG TAG GCG AGT TGC AG-3') as a reverse primer, and had the 1234 bp expected PCR product.

Table 3.3 16S rRNA gene sequences of *Streptococcus* species in GenBank nucleotide sequence database used for PCR primer design.

GenBank no.	Information	region	source	sequence	Size (bp)
Streptococcus agalactiae					
AB002479	ATCC13813 Non-hemolytic strain	U.S.A	ND	Partial	1450
AF015927	ATCC27956 Hemolytic strain	U.S.A	Bovine	Partial	1472
EF092913	CMS002	China	Tilapia	Partial	1457
EU075069	Strain 14 (streptococcal carriage)	Australia	Human	Partial	1449
Streptococcus iniae					
AF335572	ATCC29178	U.S.A	Marine fish	Partial	1536
AY762259	SCCF5L	Taiwan	Frog	Partial	1486
DQ985468	CGX	China	ND	Partial	1447

ND, Not data

Oligonucleotide primers design for the amplification of internal part of sodA gene

The internal part of *sodA* gene associated with Mn-dependent superoxide dismutase was amplified using forward primer, JW_*sodA*F1 (5'-TGA TGC TTT AGA GCC ACA ATT TGA T-3') and reverse primer, JW_*sodA*R1 (5'-CAT TGA TGT AGT TTG GAC GAA CA-3'), and yielded a 512 bp PCR product.

Table 3.4 Internal part of *sodA* gene sequences of *Streptococcus* species in GenBank nucleotide sequence database used for PCR primer design.

GenBank no.	Information	region	source	sequence	Size (bp)
Streptococcus agalactiae					
Z95893	Mn-dependent superoxide dismutase	France	ND	Partial	435
Streptococcus iniae					
EU661272	sodA gene	Australia	ND	Partial	609
Z99176	Mn-dependent superoxide dismutase	France	ND	Partial	435
AM490314	Mn-dependent superoxide dismutase	France	Fish	Partial	429

ND, Not data

DNA amplification and sequencing

DNA amplification was performed in a final volume of 40 µl containing 50 ng of genomic DNA, 0.5 µM of each primer (Sigma-Genosys, Singapore), 250 µM of each dNTP, and 1 units of Taq DNA polymerase in a 1X amplification buffer (10 mM Tris HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$) (iNtRON Biotechnology, USA). The PCR mixture was then amplified 30 cycles on the PCR Thermalcycler (Whatman Biometra®, UK) with programmes. PCR condition for 16S rRNA gene was conducted with the following program: 94℃ for 3 mins (initial denaturation step), 30 cycles at 94℃ for 30 secs (denaturation step), at 58℃ for 30 secs (annealing step), and at 72℃ for 60 secs (extension step), followed by a final extension at 72℃ for 5 mins. PCR condition for internal part of *sodA* gene associated with Mn-dependent superoxide dismutase was conducted with the following program: 94℃ for 2 mins (initial denaturation step), 30 cycles at 94℃ for 20 secs (denaturation step), at 54℃ for 10 secs (annealing step), and at 72℃ for 30 secs (extension step), followed by a final extension at 72℃ for 2 mins.

The presence of PCR products was observed by electrophoresis of 5 µl product in 2% TBE agarose gel, a 100 bp DNA-ladder was a molecular marker. The gel was stained with 5 mg/ml of ethidium bromide for visualization of the PCR products. The PCR products were purified using NucleoSpin® Extract II (MACHEREY-NAGEL, Germany) and submitted for DNA sequencing. The sequencing reactions were performed by the 1st BASE DNA Sequencing (Singapore). The sequencing data were analyzed using the biosystems DNA sequencing analysis Software v5.2 with KB basecaller.

Sequence data analysis

Forward and reverse sequences were aligned with the consensus sequences allocated in GenBank. Comparative sequence analysis was carried out using the Bioedit Sequence Alignment Editor V.7.0.5.3 and phylogenetic analysis was performed using the CLUSTAL V program (DNASTAR, Madison, WI). Sequence data was submitted to GenBank/EMBL for the appointment of their accession numbers.

Phase III : The development of streptococcosis vaccine

Results obtained from previous works in phenotypic characterization (phase I) and genotypic characterization of streptococcal isolates (phase II) were employed for selection of the vaccine strain(s).

Bacterial isolates

The selected streptococcal isolates were used as seeds for vaccine production (Table 3.5). Seeds were tested for their biochemical characteristics by conventional microbiological procedures as described in phase I (Evans *et al*., 2004).

Table 3.5 Selected isolates of the *Streptococcus agalactiae* obtained from diseased tilapia were used as seeds for vaccine production.

ID	Year of isolation	Source
71	2007	Nakornpanum
72	2007	Nakornpanum
119	2008	Petchaburi
120	2008	Petchaburi
121	2008	Petchaburi
122	2008	Petchaburi

Streptococcal vaccine

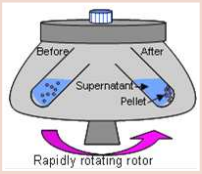
Two types of streptococcal vaccine were developed in this study. The formalin killed cells (FKC) and the extracellular products (ECP) were tested for their efficacy. Figure 3.2 shows steps in vaccine preparation, 0.5 McFarland (1-2 x 10^8 bacteria/ml) of the pure streptococcal isolate were added 1 ml in 100 ml Tryptic Soy Broth (TSB) (Oxoid®, USA). The cultures were incubated at 30$\pm$ 2^{O}C for 10 hours in a 100 RPM shaking incubator (Shel Lab, USA). The bacteria cells were harvested by centrifugation 1000g (Sartorius 3-16K, Sigma, USA) at 4^{O}C for 30 mins and inactivated in 3% formalin at 4^{O}C, overnight. Formalin was removed from the cells by washing 3 times with normal saline solution (NSS) at the 1000g centrifugation 4^{O}C 30 mins. FKC was resuspended in NSS to make 3 x 10^9 cell/ml FKC vaccine (Evans *et al.,* 2004; Klesius *et al.,* 2000). ECP was prepared from heat inactivated supernatant (separating 1 x 10^9 bacterial per 1 ml supernatant) at 56^{O}C for 30 mins and filtered sterile with microfilter at 0.45 and 0.22 µm (modified from Cryz *et al*., 1982 and Evans *et al*., 2004). The mixed vaccine was prepared by resuspending FKC in the ECP (3 x 10^9 streptococcal formalin-killed cell in 1 ml of ECP). The sterility of the produced vaccine was tested by plate culture on blood agar at 30$\pm$ 2^{O}C for 24-48 hours (Evans *et al*., 2004).

Figure 3.2 Preparation of Streptococcal vaccine

Step 1 *S. agalactiae* culture on blood agar 30 ^{O}C 24 hours
Seed test : hemolytic zone, bacterial morphology
and biochemical property

Step 2 *Streptococcus* cell suspension 1 ml (0.5 McFarland, ~1-2 x 10^8 CFU/ml) in 100 ml TSB shaking incubator 30 ^{O}C 100 rpm 10 hours.

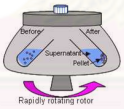
Step 3 Streptococcal culture 100 ml (~ 1-2 x 10^9 CFU/ml) was centrifuged to separate the bacterial cell and supernatant
(1000 g 4 ^{O}C 30 mins)



Step 4 Vaccine production

Streptococcal cells
Wash cell and treat with
3% formalin 4 ^{O}C 24 hours

Wash cell with
normal saline 3 times



Streptococcal cells

Resuspend in normal saline

FKC vaccine

Sterility test : Culture on blood agar

Supernatant

Heat 56 ^{O}C 30 mins

Sterile culture fluid with
microfilter 0.45 and 0.22 µm

Sterile supernatant (ECP)

Streptococcal cells mix
sterile ECP

Mixed vaccine

Sterility test : Culture on blood agar

Step 5 *In vivo* safety test
Intraperitoneal injection 1X dose in 20 gram tilapia; monitor
the vaccinated fish for 2 weeks

Vaccine Quality

The developed vaccine was assessed for its safety and efficacy in experimental animals (nile tilapia) and in field trials.

Safety test

Fish and management

Tilapia (*Oreochromis niloticus*) fingerlings with a mean body weight of 20 grams obtained from local farm were used for the safety test of the produced vaccine. Fish were stocked in 40 l square plastic tanks and fed daily to apparent satiation. Rearing water was aerated continuously and the water quality was measured as indicated in Table 3.7. Fish were acclimated to the experimental condition for 7 days prior to the trial started. The experiment was conducted in the Wet Lab facility of department of Veterinary Medicine, faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

Vaccination

Fish from the storage tank were randomly assigned to 8 groups, each of 10 fish as shown in Table 3.6. These groups were duplicates of 2 controls and 2 treatments. During the acclimatization, fish were examined for health status e.g. parasitic infestation and bacterial infections particularly the streptococcal infection. After 7 days of acclimatization, the healthy fish were vaccinated by a single intraperitoneal (i.p.) injection (Figure 3.6). Formulated clove oil solutions were used to anesthetize the fish for vaccination. Vaccine was administered 0.1 ml per fish (approximately 3 X 10^8 CFU per fish). The control groups were non-injected fish and TSB injected fish. Clinical signs and dead fish were monitored daily for 2 weeks post vaccination. Anterior kidney and brain tissues from the dead fish were collected for microbiological procedures to confirm the streptococcal infection as described in phase I.

Table 3.6 Experimental groups for the safety test of the produced vaccine.

Group		Replicate tanks
1	Formalin Killed Cell (FKC)	2 (n = 10 x 2)
2	Formalin Killed Cell (FKC) mixed Extracellular product (ECP)	2 (n = 10 x 2)
3	Placebo vaccinated control (TSB)	2 (n = 10 x 2)
4	Untreated control	2 (n = 10 x 2)

Efficacy test

Efficacy of the streptococcal vaccine against challenge test

Fish and management

Juvenile tilapia (*Oreochromis niloticus*) of approximately 200 grams body weight were hold, in a commercial fish farm, 80 fish per net pen (length 2 m x width 2 m x depth 2 m, water level 1.5 m) in 6.5 CLAS (unit of area equal to 1,600 square meters) supplied with well aerated brackish water (salinity 7 ppt) (Figure 3.5). Fish were fed commercial dry pellet feed twice daily at 2% body weight per day. Recirculation aquaculture systems (RAS) was applied in the farm with the water quality showed in Table 3.7.

Table 3.7 Water quality parameters

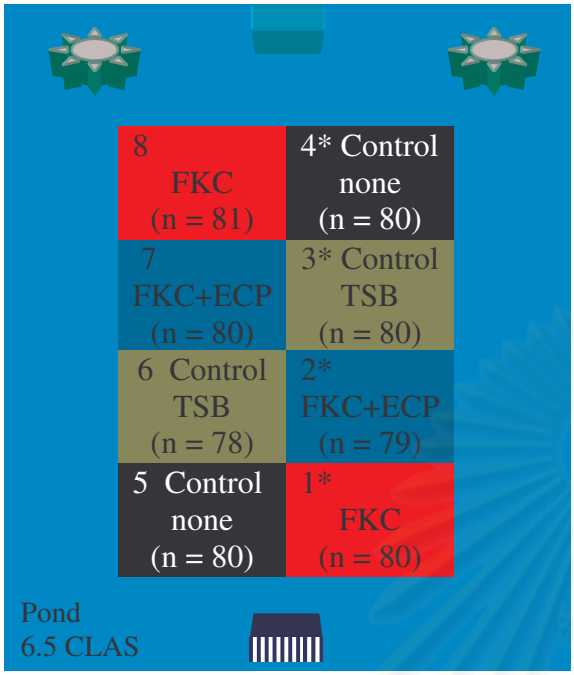
Water quality parameters	Reference value*
pH	8-8.5
Nitrite (ppm)	≤ 0.3
Ammonia (ppm)	≤ 1.5
Dissolved oxygen (ppm)	≥ 5
Salinity (ppt)	≤ 7
Water temperature (^{O}C)	28-32
Total bacterial count (CFU/ml)	$< 10^4$

* Water quality suggested for tilapia culture.

Vaccination

Juvenile tilapia were randomly assigned to 8 groups, each of 80 fish (Figure 3.3). These groups represented duplicates of 2 controls and 2 treatments. Treated groups received intraperitoneally 0.2 ml of FKC vaccine (treatment group 1) or mixed vaccine (treatment group 2) with a dose of approximately 6 x 10^8 CFU per fish. Two control groups were an untreated control and a placebo vaccinated control. Fish were deprived from feeding 24 hours before the vaccination. The placebo vaccinated control received intraperitoneally 0.2 ml of TSB. All fish were anaesthetized with formulated clove oil solution prior to the vaccine or TSB injection. Following the vaccination, the fish were weighed every 2 weeks to evaluate growth performance (Average Daily Gain, ADG and Feed Conversion Rate, FCR). Serum for checking the antibody titer was taken from 10% of the experimental fish, at 0, 3rd, 5th, 8th, 10th and 12th week post vaccination. Clinical signs and mortality were monitored daily for 12 weeks post vaccination. Anterior kidney (Figure 3.7) and brain tissues (Figure 3.8) from dead fish were microbiological examined to confirm death associated with the streptococcal infection as described above.

Figure 3.3 Diagram of the experimental net pens used for efficacy test of the streptococcal vaccine



8 Net pen (length 2 m X width 2 m X depth 2 m, water level 1.5 m) in 6.5 CLAS pond

Net pen 1* Formalin Killed Cell (FKC)
Net pen 2* FKC mixed Extracellular product (ECP)
Net pen 3* Placebo vaccinated control
Net pen 4* Untreated control
Net pen 5 Untreated control
Net pen 6 Placebo vaccinated control
Net pen 7 FKC mix Extracellular product (ECP)
Net pen 8 Formalin Killed Cell (FKC)

Aerator

Water input

Water output

Collection of serum sample

Peripheral blood was collected by caudal venous puncture (Figure 3.4). The needle was inserted at a point on or just below the lateral line and blood samples were aspirated into a microsyringe. The serum was separated from blood clotting for 1 hour at 25^{O}C and centrifuged at 1000g for 5 mins. The serum was stored at -20^{O}C until application.

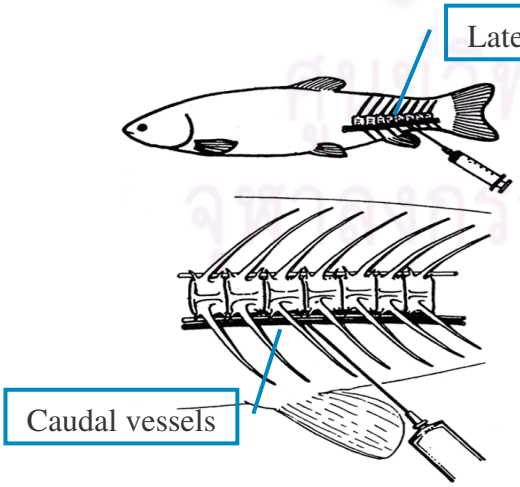


Figure 3.4 Blood sampling in fish of more than 200 g weight by the method of punction of caudal blood vessels. (Svobodová and Vykusová, 1991, online available from http://www.fao.org/docrep/field/003/ac160e/AC160E00.htm#TOC)

3.5	3.6
3.7	3.8

Figure 3.5 Net pens in the pond used for the efficacy test of streptococcal vaccine
Figure 3.6 Fish are vaccinated by intraperitoneal (i.p.) injection.
Figure 3.7-8 Anterior kidney (Figure 3.7) and brain (Figure 3.8) tissues of the fish were processed for microbiological identification of the *Streptococcus* pathogens.

Challenge test

At 10^{th} week post vaccination, vaccinated and control tilapia (average 500 grams body weight) were transported to the Wet Lab Facility, faculty of Veterinary Science, Chulalongkorn University, Nakornpathom, Thailand. Fish were placed in 1200 l cement tanks (length 200 cm x width 100 cm x depth 60 cm) and fed daily to apparent satiation. The running freshwater aerated with air stones was used in the facility, therefore, the fish were acclimatized with gradually decreasing salinity to 0 ppt within 8 days (Table 3.8). Groups of 10 fish each were anaesthetized and challenged at dosage of 1.5 x 10^8 CFU per fish with homologous live streptococcal strains to the vaccine seeds by intraperitoneal injection. The mortality of the challenged fish was monitored for 21 days. Dead fish was examined for streptococcal infection by clinical signs, external and internal pathological lesions. Streptococcal bacteria isolated from the target organs (kidney and brain) were identified with biochemical profile and PCR assay. The mean percent mortality and mean percent cumulative mortality of vaccinated and non-vaccinated fish were determined over a 21-day period. The efficacy of vaccine to prevent infection was evaluated based on the relative percent survival (RPS).

$$\text{Relative Percent Survival} = \left(1 - \frac{\text{Mortality in vaccinated group}}{\text{Mortality in non-vaccinated group}}\right) \times 100$$

Table 3.8 Fish acclimatization with gradually decreasing salinity to 0 ppt rearing water.

Day	Salinity (ppt)	Total Salt (kg/tank)
1	4.00	5.00
2	2.00	2.50
3	1.50	1.87
4	1.25	1.56
5	0.75	0.94
6	0.50	0.62
7	0.25	0.31
8	0.00	0.00

Detection of serum antibody titer by direct agglutination

The specific antibody titer against *Streptococcus agalactiae* was quantified using a direct agglutination test modified from Eldar *et al*. (1997), Klesius *et al*. (2000) and Whittington *et al*. (2005). The particle agglutination assay employed in this study was based on the theory that antigen coated particles are agglutinated in the presence of the complementary soluble antibody. The results are read by macroscopic visual for agglutination in the bottom of the microwell (Figure 3.9).

S. agalactiae seed strains were grown on Tryptic Soy Agar (TSA, Oxoid®, USA) added with 5% (v/v) sheep blood. Following an overnight culture, pure isolates were added in Tryptic Soy Broth (TSB) (Oxoid®, USA) and incubated in the incubator shaker (100 RPM, Shel Lab, USA) at 30± 2^{O}C for 24 hours. The bacterial pellets were separated by a centrifugation at 1000g (Satorius 3-16K, Sigma, USA) for 15 mins at 4^{O}C and washed 2 times with Phosphate Buffer Saline (PBS) solution. To prepare the antigen particles, the turbidity of streptococcal cells was adjusted to 0.5 McFarland (approximately 1-2 x 10^8 CFU of *S. agalactiae*/ml) with PBS solution. The agglutination test was performed in U-shaped microtiter plates (Corning®, Sigma-Aldrich, USA). Tilapia serum was diluted in PBS solution to provide 2 fold serial dilutions of the tested serum (1:2 to 1:4096). One hundred µl of the antigen particle suspension (approximately 1-2 x 10^7 *S. agalactiae* cell/ml) was added to each well and thoroughly mixed. The plate was then covered with a lid and incubated for 6-8 hours at 30± 2^{O}C. The highest dilution of sera showing bacteria agglutination was used to evaluate the antibody titer, a $\log_{10}$ of the reciprocal of such dilution.

The appearance of a fuzzy edge at the bottom of the well was considered as a positive reaction, whereas the formation of a round precipitate with sharp contours was evaluated as a negative reaction. Serum obtained from tilapia infected with *S. agalactiae* was used as a positive control, while the negative controls were the serum obtained from healthy, non-infected fish and PBS.

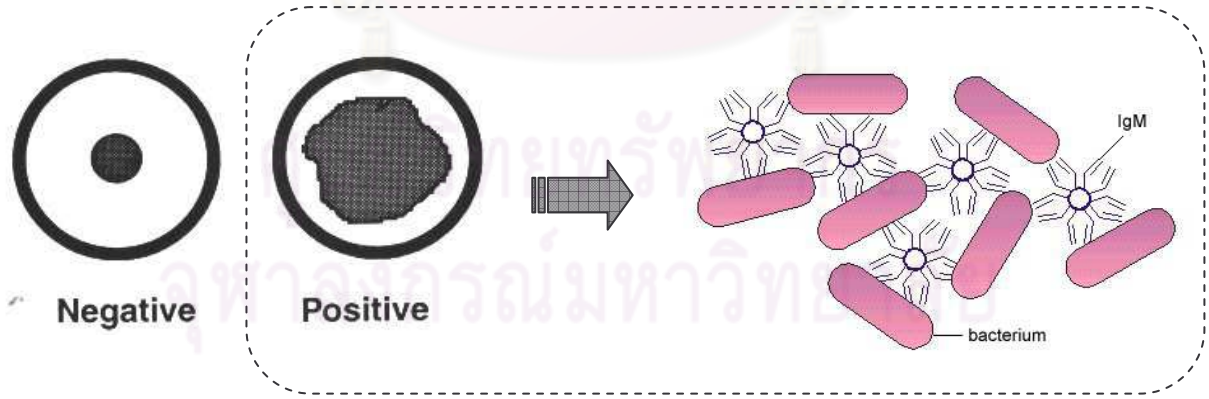


Figure 3.9 Agglutination result: The appearance of a fuzzy edge at the bottom of the well as a positive reaction, whereas the formation of a round precipitate with sharp contours as a negative reaction (online available from http://www.emro.who.int/publications)

Statistical analysis

The results of this study were analyzed by descriptive statistics in phase I and phase II. Phase III was analyzed mortality rate using the relative percentage survival (RPS) rate and nonparametric test for comparison between treatment groups and control groups. Growth performances; body weight, ADG and FCR were analyzed by comparison with mean value. Antibody titers were analyzed by ANOVAR.

CHAPTER IV

RESULTS

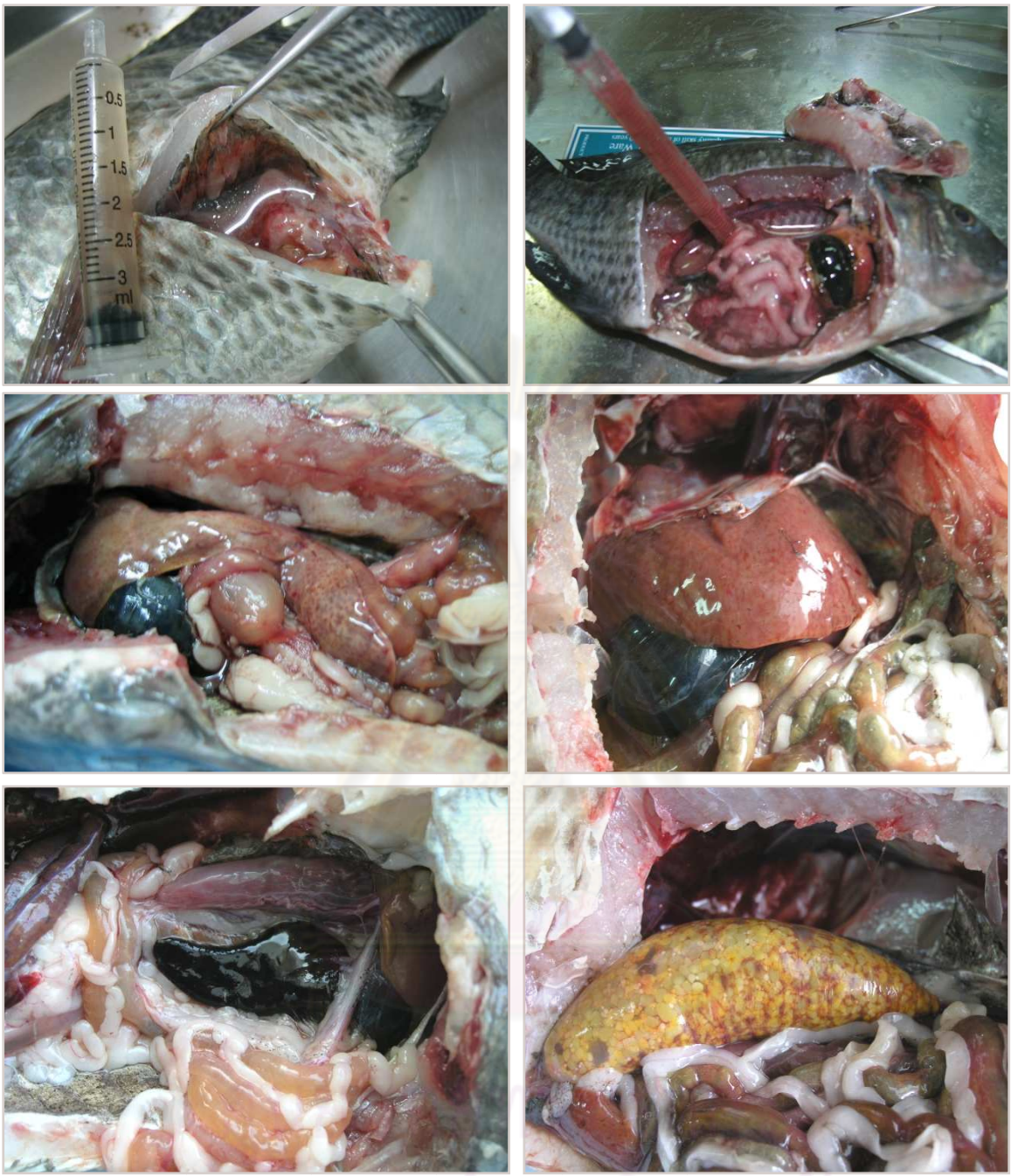
Phase I : Pathobiological characterization of streptococcosis in farmed tilapia

Clinical disease

A continuing mortality in Thai cultured tilapia has been observed in country-wide tilapia farm since 2003 to date. Disease outbreaks are evident repeatedly in summer months or when the water temperature is higher than 30 ^{O}C. The disease is found in all stages of rearing tilapia, both in pond and floating cage culture. Some cases are associated with the carrier fish that was transported from another region. Streptococcosis showed a rapid appearance of clinical sign (1 or 2 days after the infection) and high mortality. Streptococcosis in tilapia shows clinical symptoms include abnormal swimming near the water surface with an increased rate of respiration or stationary at bottom, erratic swimming behavior, low acceptance or refusal of food, dark skin coloration. Diseased fish showed generalized hemorrhagic septicemia, e.g. numerous hemorrhages at the base of fin, anus and operculum, hemorrhages on the body surface, exophthalmia with ocular opacity (cloudy eye) and hemorrhagic fluid around eyes, abdominal distension, ulceration and skin abscesses as shown in Figure 4.1. At necropsy, moribund tilapia had hemorrhage in the internal organ (i.e. brain, heart, liver, stomach, intestine and genital tract) and hepatomegaly with showing pale necrotic on tissue, development of ascites (serosanguneous and blood tinged abdominal fluid). Enlargement of spleen and kidney are common pathological changes in the diseased fish (Figure 4.2).

a | b
c | d

Figure 4.1 Clinical signs and external lesions observed in the streptococcal infected tilapia. (a) Moribund tilapia associated with the streptococcal infection; (b) Dark skin coloration; (c) Exophthalmia with ocular opacity; (d) Generalized hemorrhages on the body surface and at the base of fin and the operculum.



a	b
c	d
e	f

Figure 4.2 Internal lesions commonly observed in the streptococcal infected tilapia. (a) Serosanguneous (yellow) abdominal fluid; (b) Blood tinged (reddish) abdominal fluid; (c) Enlarged liver with pale necrotic on tissue; (d) Enlarged liver with petechial hemorrhage; (e) Enlarged spleen and intestinal edema, (f) Septicemia conditions cause generalized hemorrhages of the visceral organs, ovary and intestine.

Histopathology

Histopathologic study revealed a septicemia with numbers of cocci bacteria and inflammatory cells (predominantly macrophage and lymphocyte) infiltrated in multiple organs of diseased tilapia. Numerous cocci were seen in blood vessels of the liver, gut, intestine, ovary and brain. Bacteria were surrounded by macrophages in multiple organs including spleen, liver, digestive tract, kidney, brain and ovary (Figure 4.3).

Liver

The liver had granular degeneration (swelling of hepatocyte caused by accumulation of intracellular water in response to cell injury) with focal necrosis and infiltration of macrophage and lymphocyte. Bacteria were found in the hepatic vein and a branch of the hepatic vein (called the central vein) (Figure 4.3a).

Digestive tract

A variable amount of lymphocytic and macrophage infiltration occurred in serosal intestine and gut. The mild lymphocyte infiltration was observed in submucosal digestive tract, from the stomach to rectal intestine. Gastrointestinal lesions were found necrosis and erosion of mucosa with numerous inflammatory cells in lumen. Mucosal edema with increased thickness of the submucosal layer was observed in gastric lumen. Bacterial clumps and bacterial emboli were observed in capillary congestion (Figure 4.3b and 4.3c).

Spleen and kidney

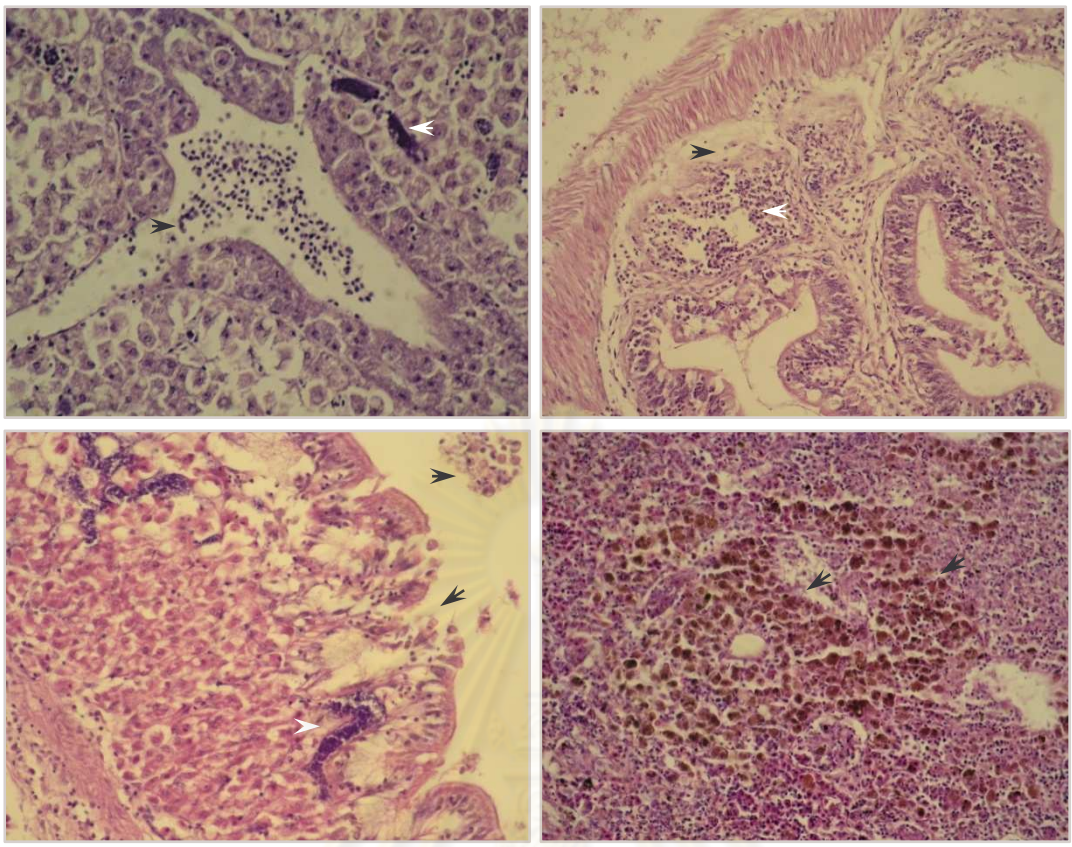
Splenitis and nephritis were characterized by infiltration of macrophages, lymphocytes and erythrocytes. Focal macrophage infiltration was noted in the spleen. Erythrocytes and yellow-brown pigment were prominent in the splenic red pulp (Figure 4.3d). Invasion of large number of bacteria and inflammatory cells was also found in the pronephros. Hyaline droplet or eosinophilic droplet degeneration associated with tubular epithelial degeneration was accumulated in the lumen of kidney (Figure 4.3e and 4.3f).

Brain

Hypertrophic thickening of the meninges were associated with the accumulation of large numbers of erythrocytes, bacteria, inflammatory cells and fibroblasts. Diffused vascular congestion with bacterial emboli was observed on brain tissue (Figure 4.3g).

Ovary

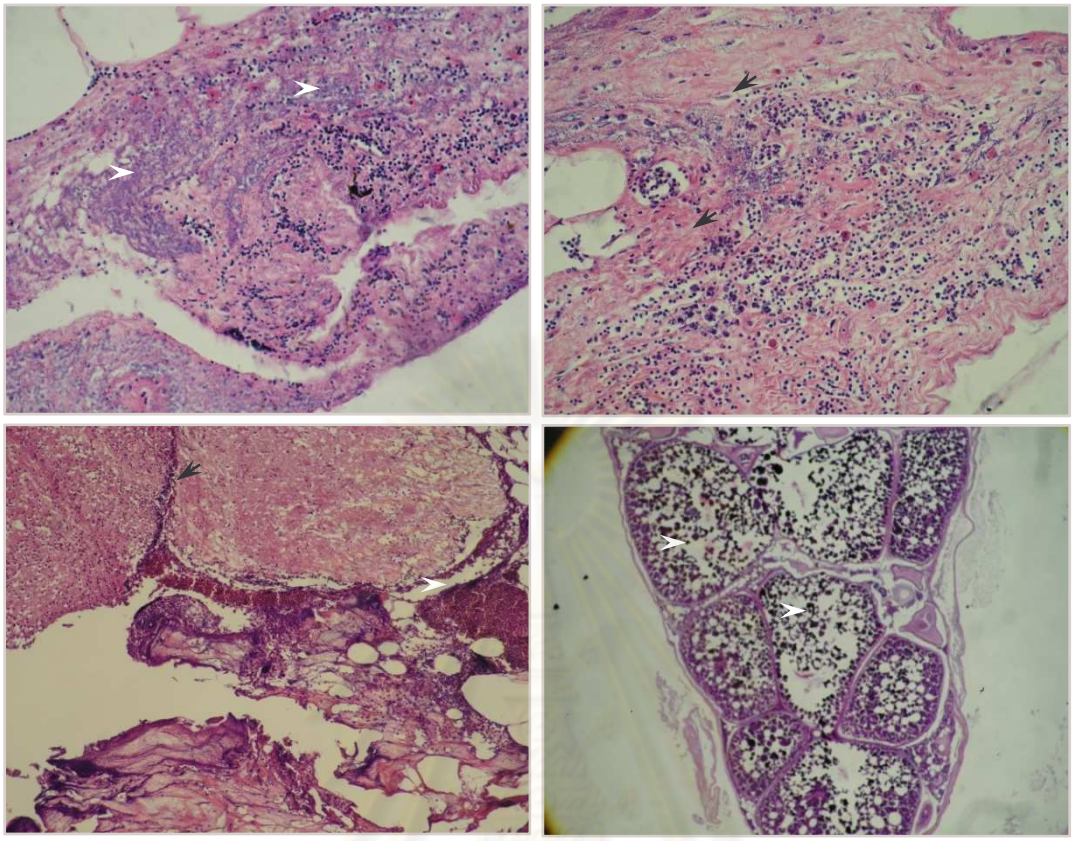
Macrophage and fibroblast infiltrations were seen in ovaries. Ovary was found necrosis and erosion of mucosa with numerous inflammatory cells with the ovarian follicles (Figure 4.3h).



a	b
c	d

Figure 4.3 Histopathologic lesions of the *Streptococcus agalactiae* infected tilapia.

Figure 4.3a, hepatocytes were vacuolated and swollen by the accumulation of intracellular fluid. White arrows point bacterial cocci in a branch of hepatic portal vein. Inflammatory cells, including macrophages and lymphocytes, and erythrocytes infiltrated in the lumen of hepatic portal vein (black arrow) (H&E x100). Figure 4.3b, acute enteritis characterized by the infiltration of inflammatory cells and edematous fluid in the intestinal submocosa (black arrow). The white arrows point clusters of darkly stained coccus-shaped bacteria in the capillary (H&E x100). Figure 4.3c, acute gastritis; black arrows indicate regions of necrosis and erosion of the gastric mucosa. Inflammatory cells and tissue debris were found in gastric lumen. Mucosa and submucosa were infiltrated by inflammatory cells and edematous fluid. The white arrow points bacterial emboli within capillary vessels (H&E x100). Figure 4.3d, focal macrophage infiltration was noted in the spleen. Erythrocytes and yellow-brown pigment were prominent in the splenic red pulp (black arrow) (H&E x100).



e	f
g	h

Figure 4.3 Histopathologic lesions of the *Streptococcus agalactiae* infected tilapia.

Figure 4.3e and 4.3f, nephritis; white arrows show invasion of bacterial and inflammatory cells in the pronephros (Figure 4.3e) (H&E x100). The renal tubular epithelium revealed cell degeneration with accumulation of hyaline droplet (Figure 4.3f) (H&E x100). Figure 4.3g, exudative meningitis; macrophage, erythrocyte and fibroblast infiltrations were seen in meninges (black arrow). The white arrow point shows active hyperemia and edema (H&E x40). Figure 4.3h, ovary was found necrosis and erosion of mucosa with numerous inflammatory cells within the ovarian follicles (white arrow) (H&E x40).

Microbiological analysis

Presumptive identification

The pathogen from 139 clinical cases was isolated initially from the anterior kidney and brain of diseased tilapia by streaking on a blood agar plate (TSA added with 5% sheep blood). Colonies of streptococcus appeared on blood agar were pinpointed, translucent to slightly opaque, whitish, round, convex, 1-2 mm in diameter with hemolytic zone (Figure 4.4a). Gram-stain of the pure culture smear revealed the characteristic chain of Gram-positive cocci (0.6-0.9 µm diameter) as shown in Figure 4.4b. The catalase test was negative.

Biochemical identification

Presumptive streptococci from diseased tilapia were further biochemically characterized using the API 20STREP kit (Figure 4.4c) and the results were compared with the analytical profile index of the system. The biochemical characteristics of streptococcal isolates were shown in Table 4.1. Fifty streptococcal isolates from 139 isolates were identified to be *S.agalactiae* 46 isolates and *S. dys.equisimilis* 4 isolates (Table 4.2). All isolates showed positive reaction to the arginine dihydrolase and fermentation of ribose and trehalose. Isolates identified as *S. dys.equisimilis* posing biochemical profiles similar to those of the *S.iniae* ATCC29178. None of the tested isolates could not be identified for *S.iniae* by the API 20STREP because the test does not provide data on biochemical characteristics of *S.iniae*. Therefore, further identification with PCR assay was attempted to provide the confirmative diagnosis. In this study, stock isolate no. 63 to 144 was only confirmed identification with PCR assay because this method confirmed correctly and provided rapid identification.

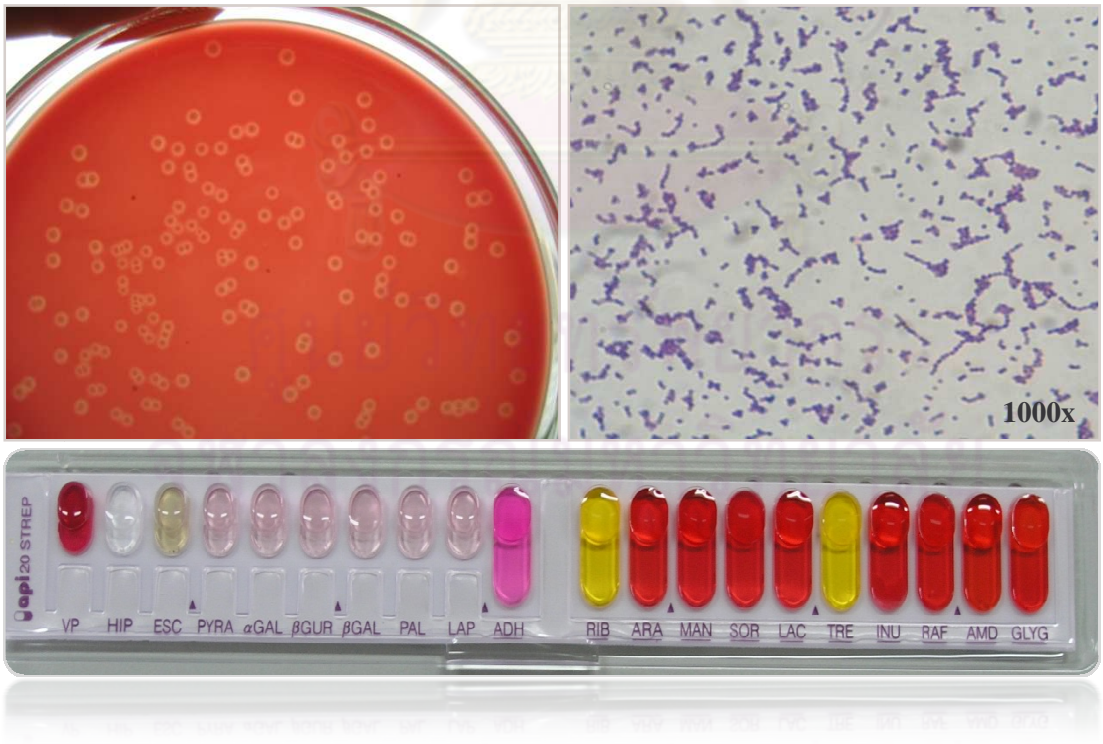


Figure 4.4 Microbiological analysis of streptococcus infection from diseased tilapia.
(a) Streptococcus colonies on blood agar; (b) Gram-stained smear of *Streptococcus* sp. from the pure culture; (c) Biochemical reaction of *Streptococcus* sp. tested with the API STREP20 (BioMeieux).

Table 4.1 Biochemical characteristics of *Streptococcus* sp. isolated from diseased tilapia tested with the API STREP20 (BioMeieux) (+ : positive reaction; - : negative reaction)

Species of streptococcus	Biochemical properties																				
	VP	HIP	ESC	PYRA	αGAL	βGUR	βGAL	PAL	LAP	ADH	RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG	βHEM
S. agalactiae ATCC13813	+	-	-	-	-	+	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
S. iniae ATCC29178	+	-	-	-	-	+	-	-	-	+	+	-	+	-	-	+	-	-	+	+	+
S. dys.equisimilis[1]	-	-	+	+	-	+	-	+	+	+	+	-	-	-	-	+	-	-	+	+	+
S. dys.equisimilis[2]	-	-	+	+	+	+	+	+	-	+	+	-	-	-	-	+	-	-	+	+	+
S. agalactiae[1]	+	-	-	-	+	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	+
S. agalactiae[2]	+	+	+	-	+	+	-	+	+	+	+	-	-	-	-	+	-	-	-	-	+
S. agalactiae[3]	+	-	-	-	+	-	-	+	+	+	+	-	-	-	+	+	-	+	-	-	+
S. agalactiae[4]	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	+	-	-	+
S. agalactiae[5]	+	+	-	-	+	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	+
S. agalactiae[6]	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	+
S. agalactiae[7]	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	+	-	-	-	-	+
S. agalactiae[8]	+	-	-	-	+	+	-	+	+	+	+	-	-	-	-	+	-	-	-	-	+
S. agalactiae[9]	+	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-	-	-	+

VP: acetoin production (Voges Proskauer), HIP: hydrolysis (hippuric acid), ESC: β-glucosidase hydrolysis (esculin), PYRA: pyrolidonyl arylamidase, αGAL: α-galactosidase, βGUR: β-giucuronidase, βGAL: β-galactosidase, PAL: alkaline phosphatase, RIB: acidification (ribose), ARA: acidification (arabinose), MAN: acidification (mannitol), SOR: acidification (sorbitol), LAC: acidification (lactose), TRE: acidification (trehalose), INU: acidification (inulin), RAF: acidification (raffinose), LAP: leucine aminopeptidase, AMN: acidification (amidon), ADH: arginine dihydrolase, GLYG: acidification (glycogen), βHEM: β-hemolysis

Note: *S. dys.equisimilis*[1], stock isolate no. 1; *S. dys.equisimilis*[2], stock isolate no. 50-52; *S. agalactiae*[1], stock isolate no. 2-7, 12-17, 21, 22, 27-30, 35, 38-41, 47, 49; *S. agalactiae*[2], stock isolate no. 18-20, 54-57; *S. agalactiae*[3], stock isolate no. 24; *S. agalactiae*[4], stock isolate no. 26; *S. agalactiae*[5], stock isolate no. 31; *S. agalactiae*[6], stock isolate no. 32-34; *S. agalactiae*[7], stock isolate no. 45; *S. agalactiae*[8], stock isolate no. 58, 59; *S. agalactiae*[9], stock isolate no. 42-44, 60-62.

Table 4.2 Biochemical identification of *Streptococcus* sp. isolated from diseased tilapia using the API STREP20 (BioMeieux)

No.	Stock isolates	Date	Region	Identification	
				Streptococcus sp.	Identification profile (%)
1	1	25/12/2003	Mukdahan	*S. dys.equisimilis*	99.8
2	2	6/2/2004	Mukdahan	*S. agalactiae*	98.9
3	3	6/2/2004	Mukdahan	*S. agalactiae*	98.9
4	4	6/2/2004	Mukdahan	*S. agalactiae*	98.9
5	5	6/2/2004	Mukdahan	*S. agalactiae*	98.9
6	6	3/5/2004	Prachinburi	*S. agalactiae*	98.9
7	7	3/5/2004	Prachinburi	*S. agalactiae*	98.9
8	12	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
9	13	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
10	14	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
11	15	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
12	16	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
13	17	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
14	18	12-31/5/2004	Mukdahan	*S. agalactiae*	83.3
15	19	12-31/5/2004	Mukdahan	*S. agalactiae*	83.3
16	20	12-31/5/2004	Mukdahan	*S. agalactiae*	83.3
17	21	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
18	22	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
19	24	31/5/2004	Singburi	*S. agalactiae*	99.1
20	26	31/5/2004	Singburi	*S. agalactiae*	97.1
21	27	28/5/2004	Khonkaen	*S. agalactiae*	98.9
22	28	28/5/2004	Khonkaen	*S. agalactiae*	98.9
23	29	28/5/2004	Khonkaen	*S. agalactiae*	98.9
24	30	28/5/2004	Khonkaen	*S. agalactiae*	98.9
25	31	7/6/2004	Kanchanaburi	*S. agalactiae*	99.9
26	32	14/6/2004	Kanchanaburi	*S. agalactiae*	97.8
27	33	14/6/2004	Kanchanaburi	*S. agalactiae*	97.8
28	34	9/7/2004	Kanchanaburi	*S. agalactiae*	97.8
29	35	07/7/2005	Petchaburi	*S. agalactiae*	98.9
30	36	07/7/2005	Petchaburi	*S. agalactiae*	98.9
31	38	07/10/2005	Nakornpathom	*S. agalactiae*	98.9
32	39	16/11/2005	Petchaburi	*S. agalactiae*	98.9
33	40	16/11/2005	Petchaburi	*S. agalactiae*	98.9
34	41	16/11/2005	Petchaburi	*S. agalactiae*	98.9
35	42	30/11/2005	Petchaburi	*S. agalactiae*	96.7
36	43	30/1/2006	Petchaburi	*S. agalactiae*	96.7
37	44	30/1/2006	Petchaburi	*S. agalactiae*	96.7
38	45	28/2/2006	Petchaburi	*S. agalactiae*	99.9
39	47	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
40	49	12-31/5/2004	Mukdahan	*S. agalactiae*	98.9
41	50	20/3/2006	Petchaburi	*S. dys.equisimilis*	unacceptable profile
42	51	20/3/2006	Nongkai	*S. dys.equisimilis*	unacceptable profile
43	52	20/3/2006	Nongkai	*S. dys.equisimilis*	unacceptable profile
44	54	29/3/2006	Petchaburi	*S. agalactiae*	83.3
45	55	29/3/2006	Petchaburi	*S. agalactiae*	83.3
46	56	29/3/2006	Petchaburi	*S. agalactiae*	83.3
47	57	29/3/2006	Petchaburi	*S. agalactiae*	83.3
48	58	26/4/2006	Petchaburi	*S. agalactiae*	95.9
49	59	26/4/2006	Petchaburi	*S. agalactiae*	95.9
50	60	5/7/2006	Samutprakan	*S. agalactiae*	96.7
51	61	5/7/2006	Samutprakan	*S. agalactiae*	96.7
52	62	5/7/2006	Samutprakan	*S. agalactiae*	96.7

Molecular analysis with PCR assay

The PCR assay using genus specific oligonucleotide primers, *S.agalactiae* and *S.iniae* 16S rRNA specific primers were employed to 139 clinical isolates. PCR identification using primers C1/C2 presented 207 bp amplicon for *Streptococcus* spp., primers F1/IMOD yielded 220 bp amplicon for *S. agalactiae* and 300 bp amplicon was obtained using primers Sin-1/Sin-2 for *S. iniae*, as shown in Figure 4.5. The DNA amplication showed all 139 clinical isolates were positive for streptococcus bacteria as preliminarily indicated by the API system, whilst the amplifications observed in 131 isolates were specific for *S. agalactiae* and 8 isolates were specific for *S. iniae* (Table 4.3). Sequences of randomly selected isolates (18 *S. agalactiae* isolates and 9 *S. iniae* isolates) were compared to the sequences cited in GenBank. *S. agalactiae* isolates observed in the present study acquired 98% sequence similarity with the corresponding portion of *S. agalactiae* isolated from tilapia (GenBank accession no. EF092913). High sequence similarity (99%) was also observed in the typing of the observed *S. iniae* sequences against the ATCC29178 *S. iniae* type strain (GenBank accession no. DQ303187).

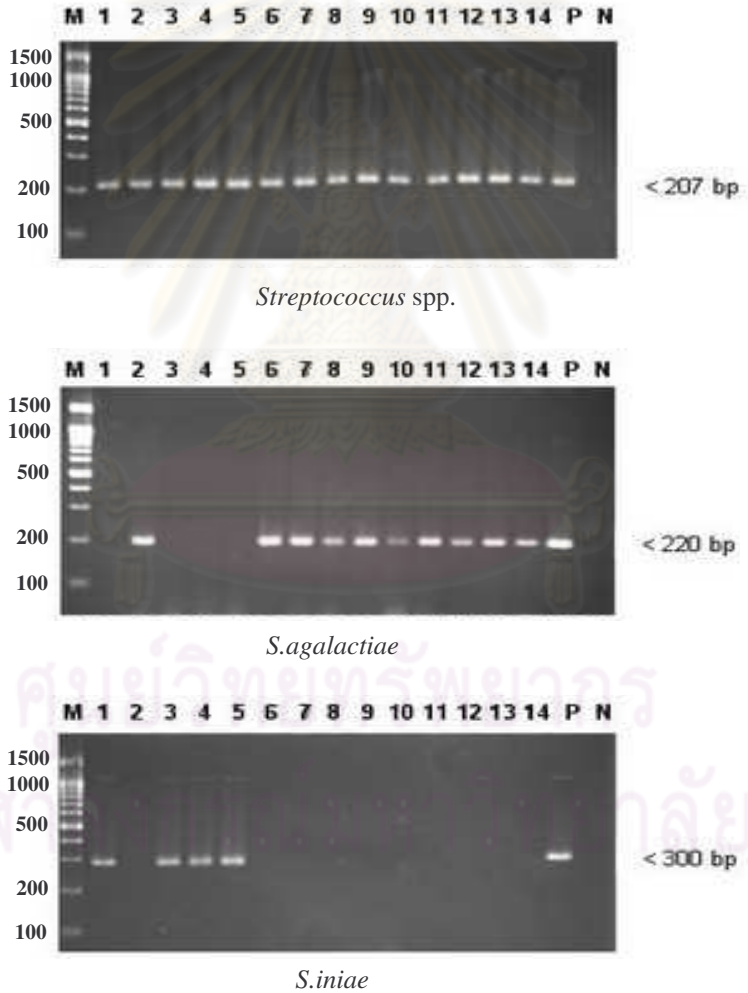


Figure 4.5 Direct PCR assay using 16S rRNA specific primers to identify tilapia streptococcal pathogen genome. Left side, 100-bp DNA ladder; Lane P, positive control (*S.agalactiae* ATCC 13813, 220 bp and *S.iniae* ATCC 29178, 300 bp); Lane N, negative control (distilled water).

Table 4.3 PCR identification for streptococcal bacteria isolated from diseased tilapia.

No.	Stock isolates	Date	Rearing Stage	Region	Polymerase Chain Reaction (PCR)		
					Streptococcus	*S. agalactiae*	*S. iniae*
1	1	25/12/2003	ND	Mukdahan	/	-	/
2	2	6/2/2004	ND	Mukdahan	/	/	-
3	3	6/2/2004	ND	Mukdahan	/	-	/
4	4	6/2/2004	Grow-out	Mukdahan	/	-	/
5	5	6/2/2004	Grow-out	Mukdahan	/	-	/
6	6	3/5/2004	ND	Prachinburi	/	/	-
7	7	3/5/2004	ND	Prachinburi	/	/	-
8	8	12/5/2004	Grow-out	Nakornpathom	/	/	-
9	9	12/5/2004	Grow-out	Nakornpathom	/	/	-
10	10	12/5/2004	Grow-out	Nakornpathom	/	/	-
11	11	12/5/2004	Grow-out	Nakornpathom	/	/	-
12	12	12-31/5/2004	Grow-out	Mukdahan	/	/	-
13	13	12-31/5/2004	Grow-out	Mukdahan	/	/	-
14	14	12-31/5/2004	Grow-out	Mukdahan	/	/	-
15	15	12-31/5/2004	Grow-out	Mukdahan	/	/	-
16	16	12-31/5/2004	Nursery	Mukdahan	/	/	-
17	17	12-31/5/2004	Nursery	Mukdahan	/	/	-
18	18	12-31/5/2004	Grow-out	Mukdahan	/	/	-
19	19	12-31/5/2004	Grow-out	Mukdahan	/	/	-
20	20	12-31/5/2004	Grow-out	Mukdahan	/	/	-
21	21	12-31/5/2004	Grow-out	Mukdahan	/	/	-
22	22	12-31/5/2004	Grow-out	Mukdahan	/	/	-
23	23	31/5/2004	Grow-out	Singburi	/	/	-
24	24	31/5/2004	Grow-out	Singburi	/	/	-
25	25	31/5/2004	Grow-out	Singburi	/	/	-
26	26	31/5/2004	Grow-out	Singburi	/	/	-
27	27	28/5/2004	ND	Khonkaen	/	/	-
28	28	28/5/2004	ND	Khonkaen	/	/	-
29	29	28/5/2004	ND	Khonkaen	/	/	-
30	30	28/5/2004	ND	Khonkaen	/	/	-
31	31	7/6/2004	ND	Kanchanaburi	/	/	-
32	32	14/6/2004	ND	Kanchanaburi	/	/	-
33	33	14/6/2004	ND	Kanchanaburi	/	/	-
34	34	9/7/2004	ND	Kanchanaburi	/	/	-
35	35	07/7/2005	Nursery	Petchaburi	/	/	-
36	36	07/7/2005	Nursery	Petchaburi	/	/	-
37	37	07/10/2005	Grow-out	Nakornpathom	/	/	-
38	38	07/10/2005	Grow-out	Nakornpathom	/	/	-
39	39	16/11/2005	Nursery	Petchaburi	/	/	-
40	40	16/11/2005	Nursery	Petchaburi	/	/	-

ND, Not data

Table 4.3 PCR identification for streptococcal bacteria isolated from diseased tilapia (continued).

No.	Stock isolates	Date	Rearing Stage	Region	Polymerase Chain Reaction (PCR)		
					Streptococcus	*S. agalactiae*	*S. iniae*
41	41	16/11/2005	Nursery	Petchaburi	/	/	-
42	42	30/11/2005	Nursery	Petchaburi	/	/	-
43	43	30/1/2006	Grow-out	Petchaburi	/	/	-
44	44	30/1/2006	Grow-out	Petchaburi	/	/	-
45	45	28/2/2006	Nursery	Petchaburi	/	/	-
46	46	28/2/2006	Nursery	Petchaburi	/	/	-
47	47	12-31/5/2004	Grow-out	Mukdahan	/	/	-
48	49	12-31/5/2004	Grow-out	Mukdahan	/	/	-
49	50	20/3/2006	Grow-out	Nongkai	/	/	/
50	51	20/3/2006	Grow-out	Nongkai	/	/	/
51	52	20/3/2006	Grow-out	Nongkai	/	/	/
52	54	29/3/2006	Nursery	Petchaburi	/	/	-
53	55	29/3/2006	Nursery	Petchaburi	/	/	-
54	56	29/3/2006	Nursery	Petchaburi	/	/	-
55	57	29/3/2006	Nursery	Petchaburi	/	/	-
56	58	26/4/2006	Nursery	Petchaburi	/	/	-
57	59	26/4/2006	Nursery	Petchaburi	/	/	-
58	60	5/7/2006	Grow-out	Samutprakan	/	/	-
59	61	5/7/2006	Grow-out	Samutprakan	/	/	-
60	62	5/7/2006	Grow-out	Samutprakan	/	/	-
61	63	5/7/2006	Grow-out	Samutprakan	/	/	-
62	64	5/7/2006	Grow-out	Samutprakan	/	/	-
63	65	8/11/2006	Broodstock	Chachoengsao	/	/	-
64	66	9/1/2007	Nursery	Chachoengsao	/	/	-
65	67	9/1/2007	Nursery	Chachoengsao	/	/	-
66	68	9/1/2007	Nursery	Chachoengsao	/	/	-
67	69	9/4/2007	Grow-out	Nakornpanum	/	/	/
68	70	9/4/2007	Grow-out	Nakornpanum	/	/	/
69	71	9/4/2007	Nursery	Nakornpanum	/	/	-
70	72	9/4/2007	Nursery	Nakornpanum	/	/	-
71	73	9/4/2007	Grow-out	Nakornpanum	/	/	/
72	74	9/4/2007	Grow-out	Nakornpanum	/	/	/
73	75	9/4/2007	Grow-out	Nakornpanum	/	/	/
74	76	9/4/2007	Grow-out	Nakornpanum	/	-	/
75	77	9/4/2007	Nursery	Petchaburi	/	/	-
76	78	9/4/2007	Nursery	Petchaburi	/	/	-
77	79	25/4/2007	Nursery	Petchaburi	/	/	-
78	80	25/4/2007	Nursery	Petchaburi	/	/	-
79	81	5/5/2007	Nursery	Chachoengsao	/	/	-
80	82	5/5/2007	Nursery	Chachoengsao	/	/	-

Table 4.3 PCR identification for streptococcal bacteria isolated from diseased tilapia (continued).

No.	Stock isolates	Date	Rearing Stage	Region	Polymerase Chain Reaction (PCR)		
					Streptococcus	*S. agalactiae*	*S. iniae*
81	83	17/5/2007	Nursery	Petchaburi	/	/	-
82	84	17/5/2007	Nursery	Petchaburi	/	/	-
83	85	9/1/2007	Nursery	Chachoengsao	/	/	-
84	86	9/1/2007	Nursery	Chachoengsao	/	/	-
85	87	9/1/2007	Nursery	Chachoengsao	/	/	-
86	88	26/2/2007	Nursery	Chachoengsao	/	/	-
87	89	26/2/2007	Nursery	Chachoengsao	/	/	-
88	90	26/2/2007	Nursery	Chachoengsao	/	/	-
89	91	12/6/2007	Nursery	Prachinburi	/	/	-
90	92	12/6/2007	Nursery	Prachinburi	/	/	-
91	93	12/6/2007	Nursery	Prachinburi	/	/	-
92	94	12/6/2007	Nursery	Prachinburi	/	/	-
93	95	12/6/2007	Grow-out	Ubonratchathani	/	/	-
94	96	12/6/2007	Grow-out	Ubonratchathani	/	/	-
95	97	26/5/2007	Grow-out	Ubonratchathani	/	/	-
96	98	25/6/2007	Grow-out	Ratchaburi	/	/	-
97	99	25/6/2007	Grow-out	Ratchaburi	/	/	-
98	100	25/6/2007	Grow-out	Ratchaburi	/	/	-
99	101	25/6/2007	Grow-out	Ratchaburi	/	/	-
100	102	26/6/2007	Nursery	Petchaburi	/	/	-
101	103	30/6/2007	Nursery	Chachoengsao	/	/	-
102	104	30/6/2007	Nursery	Chachoengsao	/	/	-
103	105	30/6/2007	Nursery	Chachoengsao	/	/	-
104	106	30/6/2007	Nursery	Chachoengsao	/	/	-
105	107	18/7/2007	Grow-out	Nakornpanum	/	/	-
106	108	18/7/2007	Grow-out	Nakornpanum	/	/	-
107	109	18/7/2007	Grow-out	Nakornpanum	/	/	-
108	110	18/7/2007	Grow-out	Nakornpanum	/	/	-
109	111	18/7/2007	Grow-out	Nakornpanum	/	/	-
110	112	18/7/2007	Grow-out	Nakornpanum	/	/	-
111	113	18/7/2007	Grow-out	Nakornpanum	/	/	-
112	114	18/7/2007	Grow-out	Nakornpanum	/	/	-
113	115	19/11/2007	Grow-out	Petchaburi	/	/	-
114	116	26/1/2008	ND	Petchaburi	/	/	-
115	117	26/2/2008	ND	Petchaburi	/	/	-
116	118	26/2/2008	ND	Petchaburi	/	/	-
117	119	26/3/2008	Nursery	Petchaburi	/	/	-
118	120	26/3/2008	Nursery	Petchaburi	/	/	-
119	121	26/3/2008	Nursery	Petchaburi	/	/	-
120	122	26/3/2008	Nursery	Petchaburi	/	/	-

ND, Not data

Table 4.3 PCR identification for streptococcal bacteria isolated from diseased tilapia (continued).

No.	Stock isolates	Date	Rearing Stage	Region	Polymerase Chain Reaction (PCR)		
					Streptococcus	*S. agalactiae*	*S. iniae*
121	123	6/5/2008	Grow-out	Chachoengsao	/	/	-
122	124	6/5/2008	Broodstock	Chachoengsao	/	/	-
123	125	6/5/2008	Broodstock	Chachoengsao	/	/	-
124	126	6/5/2008	Broodstock	Chachoengsao	/	/	-
125	127	6/5/2008	Broodstock	Chachoengsao	/	/	-
126	128	26/3/2008	ND	Petchaburi	/	/	-
127	129	26/3/2008	ND	Petchaburi	/	/	-
128	133	2/11/2009	Nursery	Petchaburi	/	/	-
129	134	2/11/2009	Nursery	Petchaburi	/	/	-
130	135	18/11/2009	Nursery	Petchaburi	/	/	-
131	136	18/11/2009	Nursery	Petchaburi	/	/	-
132	137	18/11/2009	Nursery	Petchaburi	/	/	-
133	138	16/3/2010	Nursery	Petchaburi	/	/	-
134	139	16/3/2010	Nursery	Petchaburi	/	/	-
135	140	16/3/2010	Nursery	Petchaburi	/	/	-
136	141	6/4/2010	Nursery	Chachoengsao	/	/	-
137	142	9/4/2010	Grow-out	Chachoengsao	/	/	-
138	143	9/4/2010	Nursery	Chachoengsao	/	/	-
139	144	9/4/2010	Nursery	Chachoengsao	/	/	-

ND, Not data

Streptococcosis in Thai cultured tilapia

The study of 139 clinical cases during the period from 2003 to 2010 has shown that streptococcosis occurred in tilapia farming located in the western, north-eastern and middle of Thailand. The areas of tilapia aquaculture, both freshwater and brackish water, all have experiences in streptococcosis outbreaks. Streptococcosis outbreaks were reported in Nakornpathom, Prachinburi, Singburi, Petchaburi, Kanchanaburi, Ratchaburi, Khonkean, Mukdahan, Nongkai, Nakornpanum, Samutprakan, Ubonratchathani and Chachoengsao (Figure 4.6). The PCR technique demonstrated that at least 2 species of streptococcus bacteria were involved in tilapia streptococcal infection. *S. agalactiae* were found in 131 cases (94.24%) and 8 cases were associated with *S. iniae* (5.76%). The infections were evident in the nursery stage, the grow-out stage (juvenile tilapia rearing up to market-sized tilapia in pond or floating case for 6 to 8 months) and bloodstock.

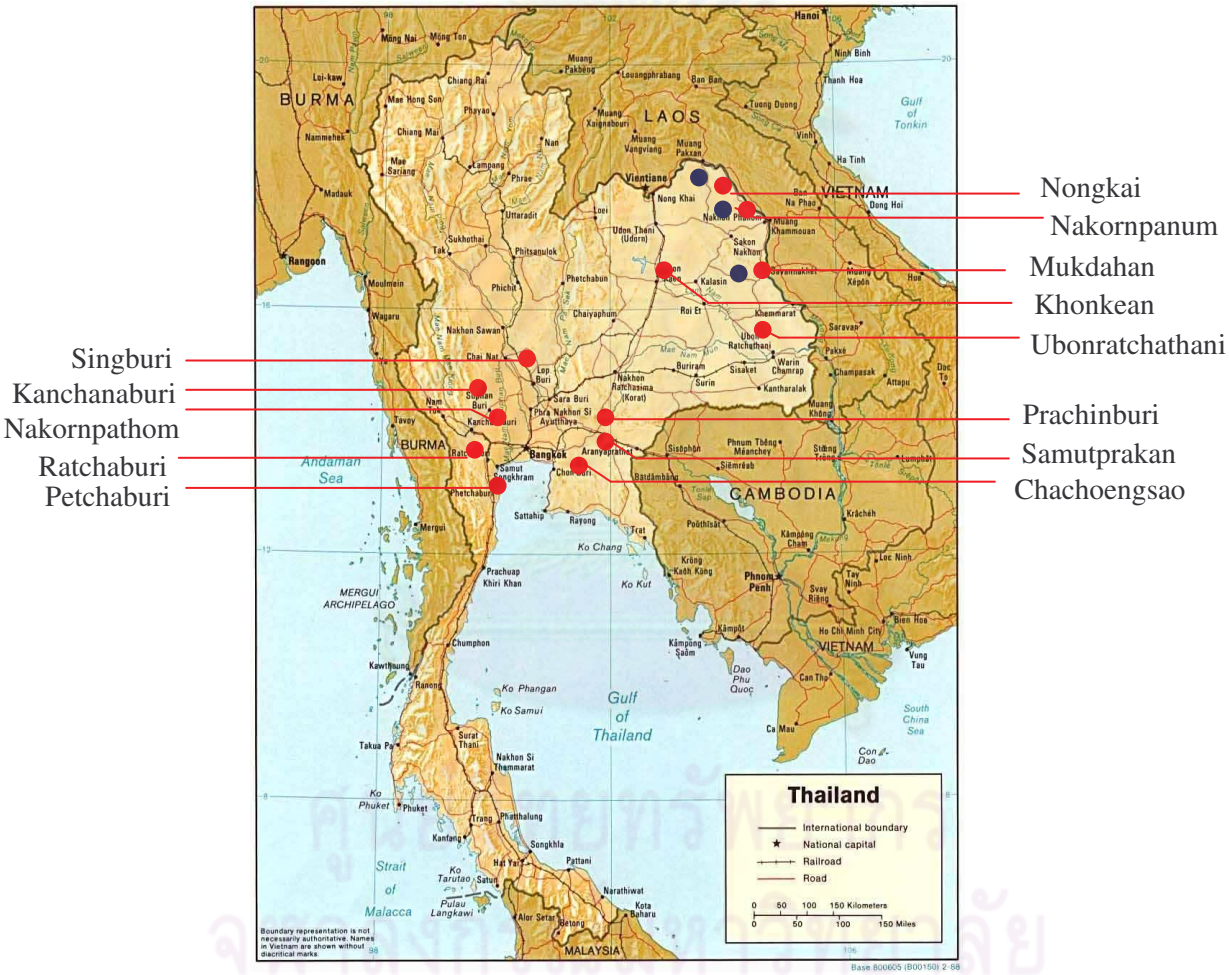


Figure 4.6 Distribution of the streptococcosis in farmed tilapia *Oreochromis nilotica* of Thailand. The PCR identification showed that the clinical isolates were mainly *S.agalacitae* (•) whereas *S.iniae* infections (•) were confined within the north-eastern part of Thailand.

Phase II : Phylogenetic analysis of streptococcus isolates by specific gene sequence comparison

***Streptococcus agalactiae* obtained from diseased tilapia**

PCR determination of 16S rRNA gene and *sodA$_{int}$ fragment*

Results from the molecular microbiological study suggested that *S.agalactiae* were isolated from diseased tilapia farm in the middle, the north-eastern and the western part of Thailand. All six strains of *S.agalactiae* were used for the amplification of 16S rRNA gene (for comfirmative of genus streptococcus) and *sodA$_{int}$* fragment encoding superoxide dismutase A (for strain variation). The amplified PCR products of the 16S rRNA gene and *sodA$_{int}$* fragment were 1234 and 512 bps, respectively (Figure 4.7).

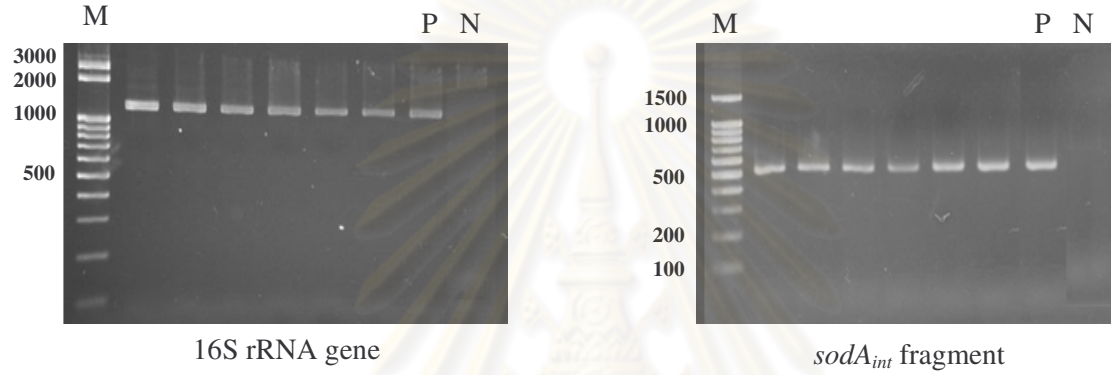


Figure 4.7 Amplification of 16S rRNA gene (1234 bp) and *sodA$_{int}$* fragment (512 bp) of *S.agalactiae* isolated from diseased tilapia. The molecular size marker was a 100 bps DNA ladder (left sides); Lane P, positive control (*S.agalactiae* ATCC 13813); Lane N, negative control (distilled water).

Sequence determination and similarity analysis of 16S rRNA gene and *sodA$_{int}$ fragment*

Sequence determination and similarity analysis of 16S rRNA gene

Six strains of *S.agalactiae* from various region of tilapia farming (Mukdahan, Nakornpanum, Nakornpathom, Kanchanaburi, Petchaburi and Chachoengsao) showed 99.1-99.6% the 16S rRNA gene sequences similarity with *S.agalactiae* ATCC13813 (GenBank accession no. AB002479). The identical sequences (100% sequence identity) were demonstrated within all six strains of *S.agalactiae* isolated from Thai cultured tilapia and some other fish species (GenBank accession no. EU622516, EF092913 and AB297817, Table 4.4). Comparison of 16S rRNA gene sequences of *S.agalactiae* obtained from other susceptible hosts, including fish, bovine, human and canine, showed 100, 95, 100 and 83% of similarity, respectively.

Table 4.4 Similarities and dissimilarities among 16S rRNA sequences of *S.agalactiae* obtained from Thai tilapia, type strain, other fish species and other susceptible host species cited from GenBank databases.

Percent Identity

Divergence	1	2	3	4	5	6	7	8	9	10	11	12	13		
1		100.0	100.0	100.0	100.0	100.0	99.5	99.9	99.9	99.9	95.4	99.9	83.0	**1**	SA JW10.seq
2	0.0		100.0	100.0	100.0	100.0	99.6	99.9	99.9	99.9	95.4	99.9	82.8	**2**	SA JW13.seq
3	0.0	0.0		99.9	100.0	99.7	99.1	99.5	99.5	99.5	95.0	99.5	82.2	**3**	SA JW16.seq
4	0.0	0.0	0.1		100.0	99.9	99.2	99.6	99.6	99.6	95.1	99.6	82.3	**4**	SA JW19.seq
5	0.0	0.0	0.0	0.0		100.0	99.5	99.9	99.9	99.9	95.3	99.9	83.2	**5**	SA JW22.seq
6	0.0	0.0	0.1	0.0	0.0		99.5	99.8	99.8	99.8	95.4	99.8	82.5	**6**	SA JW25.seq
7	0.5	0.5	0.7	0.6	0.5	0.4		97.7	98.1	98.2	94.7	94.3	79.2	**7**	AB002479.seq
8	0.1	0.1	0.4	0.3	0.1	0.1	0.8		99.9	99.5	95.6	96.4	79.5	**8**	EF092913.seq
9	0.1	0.1	0.4	0.3	0.1	0.1	0.8	0.0		99.5	95.6	96.4	79.3	**9**	EU622516.seq
10	0.1	0.1	0.4	0.3	0.1	0.1	1.2	0.0	0.0		95.6	98.8	80.9	**10**	AB297817.seq
11	4.2	4.2	4.4	4.3	4.3	4.1	4.1	4.0	4.0	4.0		95.5	81.0	**11**	AB002480.seq
12	0.1	0.1	0.4	0.3	0.1	0.1	1.4	0.1	0.1	0.1	4.1		82.5	**12**	EU075069.seq
13	13.1	13.2	13.7	13.6	12.9	13.5	14.2	14.2	14.2	14.0	15.5	14.2		**13**	EU075070.seq
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**		

Note : SA JW10 isolated from Mukdahan; SA JW13 isolated from Nakornpanum; SA JW16 isolated from Nakornpathom; SA JW19 isolated from Kanchanaburi; SA JW22 isolated from Petchaburi; SA JW25 isolated from Chachoengsao; AB002479, isolated from human in USA (ATCC13813); EF092913, isolated from aquatic animal in China; EU622516, isolated from tilapia in China; AB297817, isolated from aquatic animal in Japan; AB002480, isolated from bovine in USA; EU075069, isolated from human in Australia; EU075070, isolated from canine in Australia.

Sequence determination and similarity analysis of *sodA$_{int}$* fragment

Sequences covering 512 bp of *sodA$_{int}$* fragment were used in the analyses. Six strains of *S.agalactiae* from tilapia farming presented 100% sequence homology (Table 4.5). However, nucleotide divergences were found when the fragments acquired from tilapia were typed against the fragment of the *S.agalactiae* ATCC13813 (GenBank accession no. Z95893) at 7 single nucleotide positions, position 111, 114, 126, 243, 259, 414 and 420, and no gaps were present (Figure 4.8). The ATCC13813 reference strain was non-hemolytic *S.agalactiae*, whereas the fish strains were beta-hemolytic *S.agalactiae*. The result showed that comparison of the nucleotide sequences from *sodA$_{int}$* fragment can differentiate *S.agalactiae* between non-hemolytic and hemolytic strains with 1.6% of sequence divergence, whereas the comparison of 16S rRNA gene showed 0.5% of sequence divergence.

Nucleotide sequences of the *sodA$_{int}$* DNA fragment from *S.agalactiae* were deduced to amino acid sequences, and then compared with Mn-SOD from ATCC13813 reference strain (GenBank accession no. CAB09346). The multiple amino acid alignment revealed that these amino acid sequences were clearly related to Mn-SOD protein, suggesting that the amplified PCR products were the internal fragment of gene encoding manganese-dependent superoxide dismutase.

Table 4.5 Similarities and dissimilarities among *sodA$_{int}$* sequences of *S.agalactiae* obtained from Thai tilapia and the ATCC13813 reference strain.

Percent Identity

	1	2	3	4	5	6	7		
1		99.8	100.0	99.8	100.0	100.0	98.4	1	SA JW10.seq
2	0.0		100.0	100.0	100.0	100.0	98.4	2	SA JW13.seq
3	0.0	0.0		100.0	100.0	100.0	98.4	3	SA JW16.seq
4	0.0	0.0	0.0		100.0	100.0	98.4	4	SA JW19.seq
5	0.0	0.0	0.0	0.0		100.0	98.4	5	SA JW22.seq
6	0.0	0.0	0.0	0.0	0.0		98.4	6	SA JW25.seq
7	1.6	1.6	1.6	1.6	1.6	1.6		7	Z95893.seq
	1	2	3	4	5	6	7		

Divergence

Note : SA JW10 isolated from Mukdahan; SA JW13 isolated from Nakornpanum; SA JW16 isolated from Nakornpathom; SA JW19 isolated from Kanchanaburi; SA JW22 isolated from Petchaburi; SA JW25 isolated from Chachoengsao; Z95893, isolated from human in USA (ATCC13813).

Figure 4.8 Multiple sequence alignment of *S.agalactiae sodA*$_{int}$ fragments. Nucleotide divergences were found between nucleic acid position 110-420 of the sequence.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
SA JW10          AAGACTTAGA GGCGCTCTTA GCTGATGTTT CTCAAATTCC AGAAGATATT
SA JW13          AAGACTTAGA GGCGCTCTTA GCTGATGTTT CTCAAATTCC AGAAGATATT
SA JW16          AAGACTTAGA GGCGCTCTTA GCTGATGTTT CTCAAATTCC AGAAGATATT
SA JW19          AAGACTTAGA GGCGCTCTTA GCTGATGTTT CTCAAATTCC AGAAGATATT
SA JW22          AAGACTTAGA GGCGCTCTTA GCTGATGTTT CTCAAATTCC AGAAGATATT
SA JW25          AAGACTTAGA GGCGCTCTTA GCTGATGTTT CTCAAATTCC AGAAGATATT
Z95893           AAGACTTAGA AGCACTCTTA GCTGATATTT CTCAAATTCC AGAAGATATT
Clustal Co       ********** ** ****** ****** *** ********** **********
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         210        220        230        240        250
SA JW10          CTGGGAATTG ATGTCACCAG AAGAAACTCA AATTTCACAA GAGTTATCTG
SA JW13          CTGGGAATTG ATGTCACCAG AAGAAACTCA AATTTCACAA GAGTTATCTG
SA JW16          CTGGGAATTG ATGTCACCAG AAGAAACTCA AATTTCACAA GAGTTATCTG
SA JW19          CTGGGAATTG ATGTCACCAG AAGAAACTCA AATTTCACAA GAGTTATCTG
SA JW22          CTGGGAATTG ATGTCACCAG AAGAAACTCA AATTTCACAA GAGTTATCTG
SA JW25          CTGGGAATTG ATGTCACCAG AAGAAACTCA AATTTCACAA GAGTTATCTG
Z95893           CTGGGAATTG ATGTCACCAG AAGAAACTCA AATTTCACAA GACTTATCTG
Clustal Co       ********** ********** ********** ********** ** *******
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         260        270        280        290        300
SA JW10          AAGACATTAA TGCAACTTTT GGTTCATTTG AAGACTTTAA AGCTGCTTTC
SA JW13          AAGACATTAA TGCAACTTTT GGTTCATTTG AAGACTTTAA AGCTGCTTTC
SA JW16          AAGACATTAA TGCAACTTTT GGTTCATTTG AAGACTTTAA AGCTGCTTTC
SA JW19          AAGACATTAA TGCAACTTTT GGTTCATTTG AAGACTTTAA AGCTGCTTTC
SA JW22          AAGACATTAA TGCAACTTTT GGTTCATTTG AAGACTTTAA AGCTGCTTTC
SA JW25          AAGACATTAA TGCAACTTTT GGTTCATTTG AAGACTTTAA AGCTGCTTTC
Z95893           AAGACATTGA TGCAACTTTT GGTTCATTTG AAGACTTTAA AGCTGCTTTC
Clustal Co       ******** * ********** ********** ********** **********
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450
SA JW10          CAATTATGGA AGGCAAGAAA CCTATTTTAG GGCTTGATGT ATGGGAGCAT
SA JW13          CAATTATGGA AGGCAAGAAA CCTATTTTAG GGCTTGATGT ATGGGAGCAT
SA JW16          CAATTATGGA AGGCAAGAAA CCTATTTTAG GGCTTGATGT ATGGGAGCAT
SA JW19          CAATTATGGA AGGCAAGAAA CCTATTTTAG GGCTTGATGT ATGGGAGCAT
SA JW22          CAATTATGGA AGGCAAGAAA CCTATTTTAG GGCTTGATGT ATGGGAGCAT
SA JW25          CAATTATGGA AGGCAAGAAA CCTATTTTAG GGCTTGATGT ATGGGAGCAT
Z95893           CAATTATGGA AGGTAAGAAG CCTATTTTAG GGCTT----- ----------
Clustal Co       ********** *** *****  ********** *****
```

SA JW10 isolated from Mukdahan; SA JW13 isolated from Nakornpanum; SA JW16 isolated from Nakornpathom; SA JW19 isolated from Kanchanaburi; SA JW22 isolated from Petchaburi; SA JW25 isolated from Chachoengsao; Z95893, isolated from human in USA (type strain of *S.agalactiae* ATCC13813).

Figure 4.9 Multiple alignments of manganese-dependent superoxide dismutase (Mn-SOD). Amino acid sequences were deduced from the sequencing data of *S.agalactiae* $sodA_{int}$ fragments.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
SA JW10          HIDAETMTLH HDKHHATYVA NANAALEKHP EIGEDLEALL ADVSQIPEDI
SA JW13          HIDAETMTLH HDKHHATYVA NANAALEKHP EIGEDLEALL ADVSQIPEDI
SA JW16          HIDAETMTLH HDKHHATYVA NANAALEKHP EIGEDLEALL ADVSQIPEDI
SA JW19          HIDAETMTLH HDKHHATYVA NANAALEKHP EIGEDLEALL ADVSQIPEDI
SA JW22          HIDAETMTLH HDKHHATYVA NANAALEKHP EIGEDLEALL ADVSQIPEDI
SA JW25          HIDAETMTLH HDKHHATYVA NANAALEKHP EIGEDLEALL ADVSQIPEDI
SA CAB0934       HIDAETMTLH HDKHHATYVA NANAALEKHP EIGEDLEALL ADISQIPEDI
Clustal Co       ********** ********** ********** ********** **:*******
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
SA JW10          RQAVINNGGG HLNHALFWEL MSPEETQISQ ELSEDINATF GSFEDFKAAF
SA JW13          RQAVINNGGG HLNHALFWEL MSPEETQISQ ELSEDINATF GSFEDFKAAF
SA JW16          RQAVINNGGG HLNHALFWEL MSPEETQISQ ELSEDINATF GSFEDFKAAF
SA JW19          RQAVINNGGG HLNHALFWEL MSPEETQISQ ELSEDINATF GSFEDFKAAF
SA JW22          RQAVINNGGG HLNHALFWEL MSPEETQISQ ELSEDINATF GSFEDFKAAF
SA JW25          RQAVINNGGG HLNHALFWEL MSPEETQISQ ELSEDINATF GSFEDFKAAF
SA CAB0934       RQAVINNGGG HLNHALFWEL MSPEETQISQ DLSEDIDATF GSFEDFKAAF
Clustal Co       ********** ********** ********** :*****:*** **********
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
SA JW10          TAAATGRFGS GWAWLVVNAE GKLEVLSTAN QDTPIMEGKK PILGLDVWEH
SA JW13          TAAATGRFGS GWAWLVVNAE GKLEVLSTAN QDTPIMEGKK PILGLDVWEH
SA JW16          TAAATGRFGS GWAWLVVNAE GKLEVLSTAN QDTPIMEGKK PILGLDVWEH
SA JW19          TAAATGRFGS GWAWLVVNAE GKLEVLSTAN QDTPIMEGKK PILGLDVWEH
SA JW22          TAAATGRFGS GWAWLVVNAE GKLEVLSTAN QDTPIMEGKK PILGLDVWEH
SA JW25          TAAATGRFGS GWAWLVVNAE GKLEVLSTAN QDTPIMEGKK PILGLDVWEH
SA CAB0934       TAAATGRFGS GWAWLVVNAE GKLEVLSTAN QDTPIMEGKK PILGL-----
Clustal Co       ********** ********** ********** ********** *****
                 ....|....| ..
                        160
SA JW10          AYYLNYRNVR P-
SA JW13          AYYLNYRNVR P-
SA JW16          AYYLNYRNVR P-
SA JW19          AYYLNYRNVR PN
SA JW22          AYYLNYRNVR P-
SA JW25          AYYLNYRNVR P-
SA CAB0934       ---------- --
Clustal Co
```

Note: Ala (A), Cys (C), Asp (D), Glu (E), Phe (F), Gly (G), His (H), Ile (I), Lys (K), Leu (L), Met (M), Asn (N), Pro (P), Gln (Q), Arg (R), Ser (S), Thr (T), Val (V), Trp (W), Tyr (Y)

SA JW10 isolated from Mukdahan; SA JW13 isolated from Nakornpanum; SA JW16 isolated from Nakornpathom; SA JW19 isolated from Kanchanaburi; SA JW22 isolated from Petchaburi; SA JW25 isolated from Chachoengsao; CAB09346, isolated from human in USA (type strain of *S.agalactiae* ATCC13813).

Phylogenetic analysis of Streptococcus agalactiae obtained from diseased tilapia

Partial nucleotide sequences of 16S rRNA gene (1,234 bp) and $sodA_{int}$ fragment (512 bp) were used in the phylogenetic analysis. Figure 4.10 and 4.11 illustrate phylogenetic tree generated from the selected isolates. The dendrogram revealed that all *S.agalactiae* strains of both diseased tilapia and reference strain were distinctively apart from other streptococcus species listed in Table 4.6. The phylogenetic trees showing relationships of the 16S rRNA gene and the $sodA_{int}$ fragment among streptococcal isolates demonstrate that *S.agalactiae* obtained from clinical isolates of this study and reference strains were indistinguishable (Table 4.7 and 4.8). Sequence similarities between *S.agalactiae* and other species of streptococcus were less than 97% for the 16S rRNA gene, and 75% for the $sodA_{int}$ fragment. According to phylogenetic analysis, partial sequences of $sodA_{int}$ fragment contain dissimilarity of the sequence, therefore typing of the sequence can distinguish *S.agalactiae* from other streptococcal species (Figure 4.11).

Nucleotide sequence accession numbers

The determined nucleotide sequences from amplified PCR product were deposited in GenBank database under GQ169772 to GQ169774 and GQ338316 to GQ338318 (16S rRNA) and HM004089 to HM004094 ($sodA_{int}$).

Table 4.6 The GenBank accession number for 16S rRNA gene and *sodA* partial gene of reference strains used for phylogenetic analysis.

Streptococcus sp.	16S rRNA		$sodA_{int}$	
	Reference no.	GenBank accession no.	Reference no.	GenBank accession no.
S.agalactiae	ATCC13813	AB002479	ATCC13813	Z95893
S.agalactiae	ATCC27956	AF015927	-	-
S.dysgalactiae supsp. dys.	ATCC43078	AB002485	dys. No. 110	AB334741
S.dysgalactiae supsp. equi.	NCFB1356	AB008926	equisimilis No. 125	AB334742
S.iniae	ATCC29178	AF335572	ATCC29178	Z99176
S.porcinus	ATCC43138	AB002523	ATCC43138	Z99177
S.canis	ATCC43498	AB002483	ATCC43496	Z99175
S.pyogenes	ATCC12344	AB002521	ATCC12344	Z95915
S.equi subsp. zooepidemicus	ATCC43079	AB002516	AZB-01	AB334743
S.suis	NCTC10234	AF009477	ATCC43765	Z95920
S.salivarius	NCDO 1779(T)	X58320	ATCC7073	Z95916
S.bovis	NCDO 597(T)	X58317	ATCC33317	Z95896
S.constellatus	ATCC27823	Z69041	ATCC27823	Z95897

ATCC; American Type Culture Collection
NCFB; The National Collection of Food Bacteria
NCTC; The National Collection of Type Cultures
NCDO; The National Collection of Dairy Organisms

Figure 4.10 The phylogenetic tree generated based on the sequences of the *S.agalactiae* **16S rRNA gene** and other species of streptococcus.

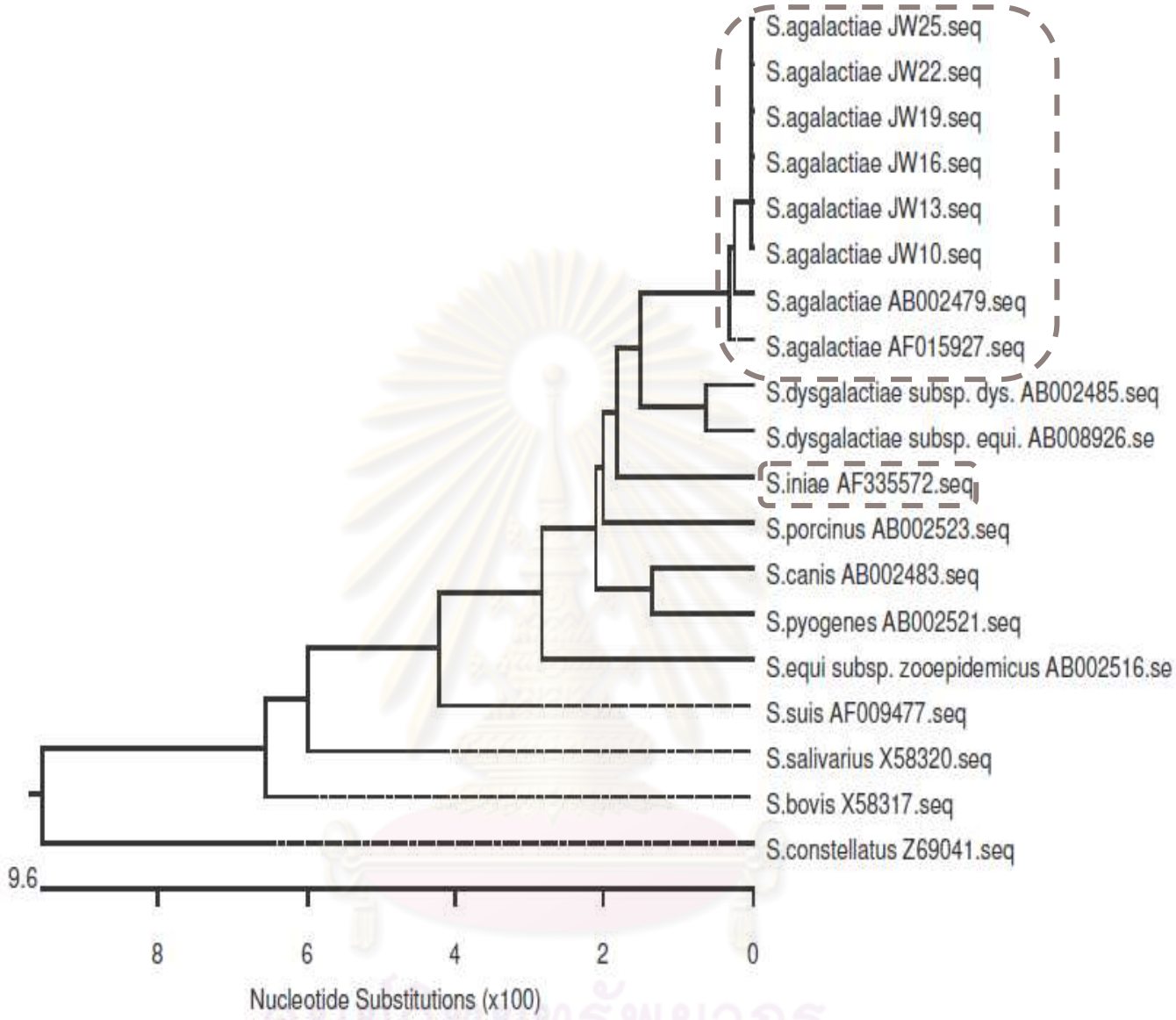


Figure 4.11 The phylogenetic tree generated based on the sequences of the *S.agalactiae* ***sodA$_{int}$*** **fragment** and other species of streptococcus.

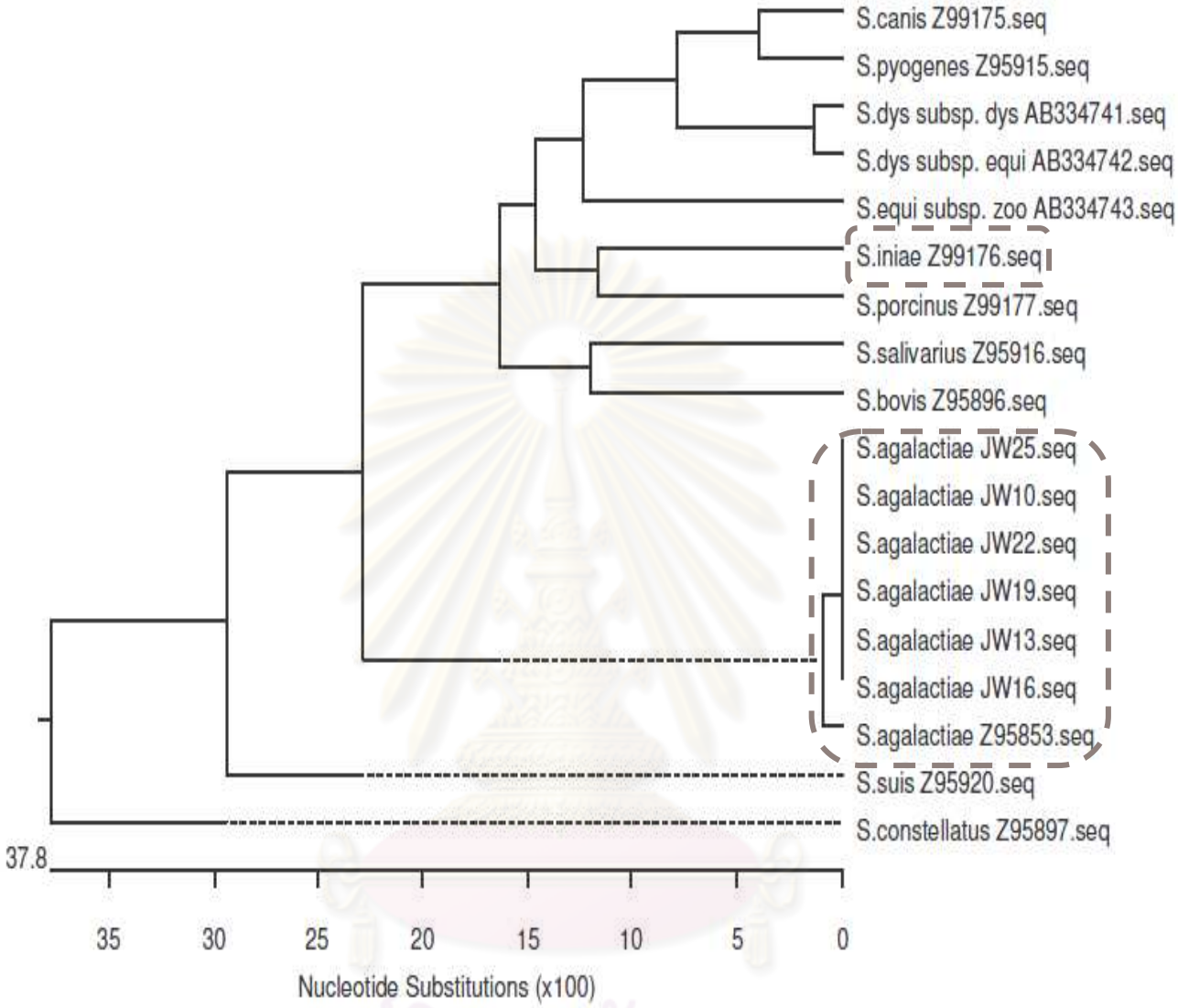


Table 4.7 Similarities and dissimilarities among **16S rRNA gene** sequences of *S.agalactiae* obtained from Thai tilapia and type strains.

The sequence similarities between *S.agalactiae* and other species of streptococcus were 95.7% (*S.iniae*), 91.4% (*S.bovis*), 94% (*S.canis*), 70% (*S.constellatus*), 96.4% (*S.dysgalatiae* subsp. *dys*galactiae), 97% (*S.dysgalatiae* subsp. *equisimitis*), 93.4% (*S.euisimitis* supsp. *zooepidemicus*), 95.6% (*S.porcinus*), 96.4% (*S.pyogenes*), 89% (*S.salivarius*) and 94.4% (*S.suis*)

The sequence divergence between *S.agalactiae* and other species of streptococcus were 3.6% (*S.iniae*), 5.1% (*S.bovis*), 5.4% (*S.canis*), 9.6% (*S.constellatus*), 3.3% (*S.dysgalatiae* subsp. *dys*galactiae), 2.2% (*S.dysgalatiae* subsp. *equisimitis*), 6.1% (*S.euisimitis* supsp. *zooepidemicus*), 3.9% (*S.porcinus*), 3.5% (*S.pyogenes*), 7.3% (*S.salivarius*) and 5.3% (*S.suis*)

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		100.0	100.0	100.0	100.0	100.0	99.5	99.8	95.7	91.4	94.1	69.8	96.4	97.2	93.4	95.6	96.4	89.1	94.4	**1**	S.agalactiae JW10.seq
2	0.0		100.0	100.0	100.0	100.0	99.5	99.8	95.7	91.4	94.1	69.8	96.4	97.2	93.4	95.6	96.4	89.1	94.4	**2**	S.agalactiae JW13.seq
3	0.0	0.0		100.0	100.0	100.0	99.5	99.8	95.7	91.4	94.1	69.8	96.4	97.2	93.4	95.6	96.4	89.1	94.4	**3**	S.agalactiae JW16.seq
4	0.0	0.0	0.0		100.0	100.0	99.5	99.8	95.7	91.4	94.1	69.8	96.4	97.2	93.4	95.6	96.4	89.1	94.4	**4**	S.agalactiae JW19.seq
5	0.0	0.0	0.0	0.0		100.0	99.5	99.8	95.7	91.4	94.0	69.8	96.4	97.1	93.4	95.6	96.4	89.1	94.3	**5**	S.agalactiae JW22.seq
6	0.0	0.0	0.0	0.0	0.0		99.5	99.8	95.7	91.4	94.1	69.8	96.4	97.2	93.4	95.6	96.4	89.1	94.4	**6**	S.agalactiae JW25.seq
7	0.5	0.5	0.5	0.5	0.5	0.5		97.2	95.6	91.7	93.0	84.1	94.9	94.2	91.7	94.7	93.1	90.0	93.8	**7**	S.agalatiae AB002479.seq
8	0.2	0.2	0.2	0.2	0.2	0.2	1.3		96.0	91.8	94.4	84.1	96.2	96.5	91.2	96.0	95.1	90.2	95.2	**8**	S.agalactiae AF015927.seq
9	3.6	3.6	3.6	3.6	3.6	3.6	3.8	3.4		90.9	94.6	67.7	96.1	95.2	93.7	95.9	95.0	89.8	93.8	**9**	S.iniae AF335572.seq
10	5.1	5.1	5.1	5.1	5.1	5.1	4.8	4.5	5.7		91.0	82.4	90.6	87.1	91.0	91.1	92.2	91.5	92.4	**10**	S.bovis X58317.seq
11	5.4	5.4	5.4	5.4	5.4	5.4	6.0	5.1	5.1	5.7		84.1	94.9	95.0	93.2	95.5	96.1	90.3	93.3	**11**	S.canis AB002483.seq
12	9.6	9.6	9.6	9.6	9.6	9.6	10.8	12.6	12.2	5.0	13.6		82.2	83.8	83.4	82.2	82.8	78.3	83.8	**12**	S.constellatus Z69041.seq
13	3.3	3.3	3.3	3.3	3.3	3.3	4.1	3.4	3.5	6.1	4.8	15.2		98.2	92.1	95.9	96.2	90.9	93.4	**13**	S.dysgalactiae subsp. dys. AB00248
14	2.2	2.2	2.2	2.2	2.2	2.2	3.5	2.9	3.7	5.5	4.4	12.2	1.2		91.3	95.5	93.2	86.1	93.3	**14**	S.dysgalactiae subsp. equi. AB00891
15	6.1	6.1	6.1	6.1	6.1	6.1	6.2	6.3	5.8	5.5	5.2	11.7	6.5	5.7		93.4	91.7	90.6	91.9	**15**	S.equi subsp. zooepidemicus AB002
16	3.9	3.9	3.9	3.9	4.0	3.9	4.4	3.8	3.9	5.2	4.5	14.8	3.7	3.9	4.6		94.8	91.1	94.8	**16**	S.porcinus AB002523.seq
17	3.5	3.5	3.5	3.5	3.5	3.5	3.9	3.1	4.5	4.4	2.7	9.7	3.2	2.9	5.0	3.9		91.4	94.8	**17**	S.pyogenes AB002521.seq
18	7.3	7.3	7.3	7.3	7.4	7.3	6.7	6.5	6.8	3.4	6.3	8.0	5.9	6.6	5.8	5.4	4.8		91.7	**18**	S.salivarius X58320.seq
19	5.3	5.3	5.3	5.3	5.3	5.3	5.4	4.4	6.0	4.2	6.3	12.6	6.2	5.9	6.4	4.8	4.7	4.8		**19**	S.suis AF009477.seq
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**		

Table 4.8 Similarities and dissimilarities among ***sodA*$_{int}$ fragment** sequences of *S.agalactiae* obtained from Thai tilapia and type strains.

The sequence similarities between *S.agalactiae* and other species of streptococcus were 72% (*S.bovis*), 71.7% (*S.canis*), 73.1% (*S.constellatus*), 72% (*S.dysgalatiae* subsp. *dys*galactiae), 69.4% (*S.dysgalatiae* subsp. *equisimitis*), 66.7% (*S.euisimitis* supsp. *zooepidemicus*), 71.7% (*S.iniae*), 71.5% (*S.porcinus*), 71.5% (*S.pyogenes*), 74.9% (*S.salivarius*) and 74.7% (*S.suis*)

The sequence divergence between *S.agalactiae* and other species of streptococcus were 30.6% (*S.bovis*), 29.5% (*S.canis*), 30% (*S.constellatus*), 29.2% (*S.dysgalatiae* subsp. *dys*galactiae), 30.1% (*S.dysgalatiae* subsp. *equisimitis*), 36% (*S.euisimitis* supsp. *zooepidemicus*), 29.9% (*S.iniae*), 30.6% (*S.porcinus*), 29.9% (*S.pyogenes*), 27% (*S.salivarius*) and 25.9% (*S.suis*)

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
1		99.8	100.0	99.8	100.0	100.0	98.4	72.0	71.7	73.1	72.0	69.4	66.7	71.7	71.5	71.5	74.9	74.7	**1**	S.agalactiae JW10.seq
2	0.0		100.0	100.0	100.0	100.0	98.4	72.0	71.7	73.1	72.0	69.4	66.7	71.7	71.5	71.5	74.9	74.7	**2**	S.agalactiae JW13.seq
3	0.0	0.0		100.0	100.0	100.0	98.4	72.0	71.7	73.1	72.0	69.4	66.7	71.7	71.5	71.5	74.9	74.7	**3**	S.agalactiae JW16.seq
4	0.0	0.0	0.0		100.0	100.0	98.4	72.0	71.7	73.1	72.0	69.4	66.7	71.7	71.5	71.5	74.9	74.7	**4**	S.agalactiae JW19.seq
5	0.0	0.0	0.0	0.0		100.0	98.4	72.0	71.7	73.1	72.0	69.4	66.7	71.7	71.5	71.5	74.9	74.7	**5**	S.agalactiae JW22.seq
6	0.0	0.0	0.0	0.0	0.0		98.4	72.0	71.7	73.1	72.0	69.4	66.7	71.7	71.5	71.5	74.9	74.7	**6**	S.agalactiae JW25.seq
7	1.6	1.6	1.6	1.6	1.6	1.6		72.6	71.7	73.1	71.5	69.1	66.2	71.7	71.3	70.8	74.5	74.7	**7**	S.agalactiae Z95853.seq
8	30.6	30.6	30.6	30.6	30.6	30.6	30.3		72.0	70.3	68.7	68.1	66.2	68.0	68.5	70.6	77.5	70.3	**8**	S.bovis Z95896.seq
9	29.5	29.5	29.5	29.5	29.5	29.5	29.6	30.7		64.1	84.8	82.4	80.2	78.9	74.0	92.2	74.0	67.8	**9**	S.canis Z99175.seq
10	30.0	30.0	30.0	30.0	30.0	30.0	30.6	31.5	40.0		66.0	66.3	62.1	66.9	69.2	66.7	70.3	71.5	**10**	S.constellatus Z95897.seq
11	29.2	29.2	29.2	29.2	29.2	29.2	28.8	33.4	16.2	38.1		96.2	75.6	77.2	72.4	86.7	72.0	65.7	**11**	S.dys subsp. dys AB334741.seq
12	30.1	30.1	30.1	30.1	30.1	30.1	29.7	32.8	17.9	36.4	2.6		74.0	77.0	71.4	84.7	71.9	64.8	**12**	S.dys subsp. equi AB334742.seq
13	36.0	36.0	36.0	36.0	36.0	36.0	36.0	36.7	20.8	43.6	26.4	27.7		71.7	69.4	77.5	69.9	67.1	**13**	S.equi subsp. zoo AB334743.seq
14	29.9	29.9	29.9	29.9	29.9	29.9	29.9	35.2	22.8	35.2	24.9	23.8	31.2		77.0	79.3	72.4	71.0	**14**	S.iniae Z99176.seq
15	30.6	30.6	30.6	30.6	30.6	30.6	31.0	33.7	27.6	33.0	30.5	30.1	33.2	23.3		74.9	71.0	71.3	**15**	S.porcinus Z99177.seq
16	29.9	29.9	29.9	29.9	29.9	29.9	30.2	31.3	7.8	38.2	14.1	14.9	23.6	22.7	27.2		74.5	66.7	**16**	S.pyogenes Z95915.seq
17	27.0	27.0	27.0	27.0	27.0	27.0	26.9	23.9	28.2	31.5	29.6	29.0	33.3	30.1	32.1	27.6		73.3	**17**	S.salivarius Z95916.seq
18	25.9	25.9	25.9	25.9	25.9	25.9	25.2	30.3	35.3	29.5	37.5	36.7	35.0	31.1	31.0	36.1	25.7		**18**	S.suis Z95920.seq
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**		

***Streptococcus iniae* obtained from diseased tilapia**

PCR determination and sequence determination of 16S rRNA gene and *sodA$_{int}$ fragment*

Results from the molecular microbiological study suggested that *S.iniae* were isolated from diseased tilapia farm in the north-eastern part of Thailand (Mukdahan, Nongkai and Nakornpanum), while the tilapia streptococcosis occurred in other culture areas was found associated with *S.agalactiae*. All six strains of *S.iniae* were used for the amplification of 16S rRNA gene (for confirmative of genus streptococcus) and *sodA$_{int}$* fragment encoding superoxide dismutase A (for strain variation). The amplified PCR products of the 16S rRNA gene and *sodA$_{int}$* fragment were 1234 and 512 bps, respectively (Figure 4.12).

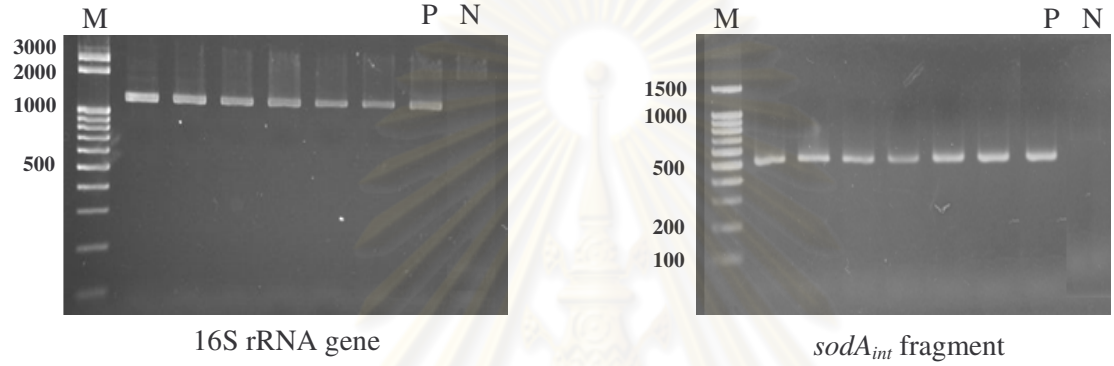


Figure 4.12 Amplification of 16S rRNA gene (1234 bp) and *sodA$_{int}$* fragment (512 bp) of *S.iniae* isolated from diseased tilapia. The molecular size marker was a 100 bps DNA ladder (left sides); Lane P, positive control (*S.iniae* ATCC 29178); Lane N, negative control (distilled water).

Sequence determination and similarity analysis of 16S rRNA gene and *sodA$_{int}$ fragment*

Sequence determination and similarity analysis of 16S rRNA gene

Six strains of *S.iniae* from the north-eastern region of tilapia farming i.e. Mukdahan, Nakornpanum, and Nongkai were determined sequence similarity of 16S rRNA gene. The results showed that these strains were > 98 to 100% similar to the ATCC29178 type strain and 98 to 100% similar to aquatic animal strains of the GenBank databases (GenBank accession no. EU622514, EU622515, AY762259, EU622508, AF335572 and AF335573) (Table 4.9). The similarity of the sequences ranged from 98 to 99% was also observed when sequences were compared with *S.iniae* from human origin (GenBank accession no. DQ193527).

Table 4.9 Similarities and dissimilarities among 16S rRNA sequences of *S.iniae* obtained from Thai tilapia, reference strain, other fish species and human strain cited from GenBank databases.

Percent Identity / Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		99.7	99.2	99.7	99.5	99.7	99.7	99.7	99.7	99.7	98.9	**1**	SI JW1.seq
2	0.3		98.9	100.0	99.7	100.0	99.5	99.5	99.5	99.5	99.2	**2**	SI JW3.seq
3	0.8	1.1		98.9	99.2	98.9	98.9	98.9	98.9	98.9	98.1	**3**	SI JW4.seq
4	0.3	0.0	1.1		99.7	100.0	99.5	99.5	99.5	99.5	99.2	**4**	SI JW6.seq
5	0.5	0.3	0.8	0.3		99.7	99.2	99.2	99.2	99.2	98.9	**5**	SI JW7.seq
6	0.3	0.0	1.1	0.0	0.3		99.5	99.5	99.5	99.5	99.2	**6**	SI JW9.seq
7	0.3	0.5	1.1	0.5	0.8	0.5		99.6	99.8	99.9	99.0	**7**	AF335572.seq
8	0.3	0.5	1.1	0.5	0.8	0.5	0.4		98.6	99.5	99.0	**8**	AF335573.seq
9	0.0	0.3	0.8	0.3	0.5	0.3	0.1	0.5		99.9	99.2	**9**	AY762259.seq
10	0.3	0.5	1.1	0.5	0.8	0.5	0.1	0.5	0.1		99.0	**10**	EU622508.seq
11	0.8	0.5	1.6	0.5	0.8	0.5	1.1	1.1	0.5	1.1		**11**	DQ193527.seq
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**		

Note : SI JW1 and SI JW3 isolated from Mukdahan; SI JW4 and SI JW6 isolated from Nakornpanum; SI JW7 and SI JW9 isolated from Nongkai; AF335572, ATCC29178 isolated from dolphin in USA; AF335573, isolated from Rainbow trout in Israel; AY762259, isolated from frog in Taiwan; EU622508, isolated from freshwater fish in China; DQ193527, isolated from human in Singapore.

Sequence determination and similarity analysis of *sodA*$_{int}$ fragment

Sequences covering 512 bp of *sodA*$_{int}$ fragment were used in the analyses. Six strains of *S.iniae* from tilapia farming presented 100% sequence homology. Comparing of *sodA*$_{int}$ sequences from other fish strains cited in GenBank databases, including USA (Z99176), France (AM490314) and Australia (EU661272) showed nearly 100% of similarity; and 99.5-99.7% similarity corresponded to the ATCC29178 reference strain (GenBank accession no. Z99176) (Table 4.10).

Their nucleotide sequences of the *sodA*$_{int}$ DNA fragment from *S.iniae* were deduced to amino acid sequences. The derived amino acid sequences were compared with Mn-SOD from ATCC29178 reference strain (CAB16320) and another fish strain (CAM32425) as shown in Figure 4.13. The multiple amino acid alignments suggested that these amino acid sequences were related to Mn-SOD protein, thus implying the amplified PCR products were *sodA*$_{int}$ fragments.

Table 4.10 Similarities and dissimilarities among *sodA*$_{int}$ sequences of *S.iniae* obtained from Thai tilapia, reference strain and aquatic animals cited from GenBank databases.

Percent Identity

Divergence	1	2	3	4	5	6	7	8	9		
1		100.0	100.0	99.7	99.7	100.0	99.7	100.0	100.0	1	SI JW1.seq
2	0.0		100.0	99.7	99.7	100.0	99.7	100.0	100.0	2	SI JW3.seq
3	0.0	0.0		99.7	99.7	100.0	99.7	100.0	100.0	3	SI JW4.seq
4	0.3	0.3	0.3		100.0	99.7	99.5	99.7	99.7	4	SI JW6.seq
5	0.3	0.3	0.3	0.0		99.7	99.5	99.7	99.7	5	SI JW7.seq
6	0.0	0.0	0.0	0.3	0.3		99.7	100.0	100.0	6	SI JW9.seq
7	0.3	0.3	0.3	0.5	0.5	0.3		99.7	99.7	7	Z99176.seq
8	0.0	0.0	0.0	0.3	0.3	0.0	0.3		100.0	8	AM490314.seq
9	0.0	0.0	0.0	0.3	0.3	0.0	0.3	0.0		9	EU661272.seq
	1	2	3	4	5	6	7	8	9		

Note : SI JW1 and SI JW3 isolated from Mukdahan; SI JW4 and SI JW6 isolated from Nakornpanum; SI JW7 and SI JW9 isolated from Nongkai; Z99176, ATCC29178 isolated from dolphin in USA; AM490314, isolated from fish in France; EU661272, isolated from barramundi fish in Australia.

Figure 4.13 Multiple amino acid alignments of the manganese-dependent superoxide dismutase (Mn-SOD) deduced from the *sodA*$_{int}$ fragment of *S.iniae* obtained from aquaculture.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
SI JW1           ---------- --KHHATYVA NANAALEKHP EIGENLEELL ANVESIPADI
SI JW3           ---------- ---HHATYVA NANAALEKHP EIGENLEELL ANVESIPADI
SI JW4           ---------- ---HHATYVA NANAALEKHP EIGENLEELL ANVESIPADI
SI JW6           ---------- --KHHATYVA NANAALEKHP EIGENLEVLL ANVESIPADI
SI JW7           ---------- --KHHATYVA NANAALEKHP EIGENLEVLL ANVESIPADI
SI JW9           ---------- ---HHATYVA NANAALEKHP EIGENLEELL ANVESIPADI
SI CAB1632       QFDQETMTLH HDKHHATYVA NANAALEKHP EIGENLEELL ANVESIPADI
SI CAM3242       --DPETMTLH HDKHHATYVA NANAALEKHP EIGENLEELL ANVESIPADI
Clustal Co                  ******* ********** ******* ** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
SI JW1           RQALINNGGG HLNHALFWEL LSPEKTEVTK EVASAIDQAF GSFDAFKEQF
SI JW3           RQALINNGGG HLNHALFWEL LSPEKTEVTK EVASAIDQAF GSFDAFKEQF
SI JW4           RQALINNGGG HLNHALFWEL LSPEKTEVTK EVASAIDQAF GSFDAFKEQF
SI JW6           RQALINNGGG HLNHALFWEL LSPEKTEVTK EVASAIDQAF GSFDAFKEQF
SI JW7           RQALINNGGG HLNHALFWEL LSPEKTEVTK EVASAIDQAF GSFDAFKEQF
SI JW9           RQALINNGGG HLNHALFWEL LSPEKTEVTK EVASAIDQAF GSFDAFKEQF
SI CAB1632       RQALINNGGG HLNHALFWEL LSPEKTEVTK EVASAIDQAF GSFDAFKEQF
SI CAM3242       RQALINNGGG HLNHALFWEL LSPEKTEVTK EVASAIDQAF GSFDAFKEQF
Clustal Co       ********** ********** ********** ********** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
SI JW1           AAAATGRFGS GWAWLVVTKE GSLEITSTAN QDTPISEGKK PILALDVWEH
SI JW3           AAAATGRFGS GWAWLVVTKE GSLEITSTAN QDTPISEGKK PILALDVWEH
SI JW4           AAAATGRFGS GWAWLVVTKE GSLEITSTAN QDTPISEGKK PILALDVWEH
SI JW6           AAAATGRFGS GWAWLVVTKE GSLEITSTAN QDTPISEGKK PILALDVWEH
SI JW7           AAAATGRFGS GWAWLVVTKE GSLEITSTAN QDTPISEGKK PILALDVWEH
SI JW9           AAAATGRFGS GWAWLVVTKE GSLEITSTAN QDTPISEGKK PILALDVWEH
SI CAB1632       AAAATGRFGS GWAWLVVTKE GSLEITSTAN QDTPISEGKK PILAL-----
SI CAM3242       AAAATGRFGS GWAWLVVTKE GSLEITSTAN QDTPISEGKK PIFST-----
Clustal Co       ********** ********** ********** ********** **::

                 ....|....| ..
                         160
SI JW1           AYYLNYRNVR PN
SI JW3           AYYLNYRNVR P-
SI JW4           AYYLNYRNVR P-
SI JW6           AYYLNYRNVR --
SI JW7           AYYLNYRNVR P-
SI JW9           AYYLNYRNVR P-
SI CAB1632       ---------- --
SI CAM3242       ---------- --
Clustal Co
```

Note: Ala (A), Cys (C), Asp (D), Glu (E), Phe (F), Gly (G), His (H), Ile (I), Lys (K), Leu (L), Met (M), Asn (N), Pro (P), Gln (Q), Arg (R), Ser (S), Thr (T), Val (V), Trp (W), Tyr (Y)

SI JW1 and SI JW3 isolated from Mukdahan; SI JW4 and SI JW6 isolated from Nakornpanum; SI JW7 and SI JW9 isolated from Nongkai; CAB16320, type strain of ATCC29178; CAM32425, fish strain from France

Phylogenetic analysis of Streptococcus iniae obtained from diseased tilapia

The phylogenetic relationships of *Streptococcus iniae* in Thai cultured tilapia based on 16S rRNA gene (1234 bp) and $sodA_{int}$ fragment (512 bp). Nucleotide sequences of all 6 isolates were addressed phylogenetic positions as shown in Figure 4.14 and 4.15. The phylogenetic tree showed that all *S.iniae* strains were clearly separated from other species of streptococcus. The dendrogram generated from the typing of 16S rRNA gene and $sodA_{int}$ fragment revealed that *S.iniae* obtained from clinical isolates and reference strains were indifferent (Table 4.11 and 4.12). The sequence divergences between *S.iniae* and other species of streptococcus evaluated in this study were less than 13% (4-12.5%) upon the 16S rRNA gene typing, but were more than 20% (23-34.6%) upon the $sodA_{int}$ fragment comparison. According to phylogenetic analysis, the partial sequences of $sodA_{int}$ could be applied to differentiate the genotype of *S.iniae* from other streptococcal species (Figure 4.15).

Nucleotide sequence accession numbers

The typed nucleotide sequences from the amplified PCR products were addressed in GenBank database under GQ169769 to GQ169771 and GQ338313 to GQ338315 (16S rRNA) and HM004083 to HM004088 ($sodA_{int}$).

Figure 4.14 The phylogenetic tree generated based on the sequences of the *S.iniae* **16S rRNA gene** and other species of streptococcus.

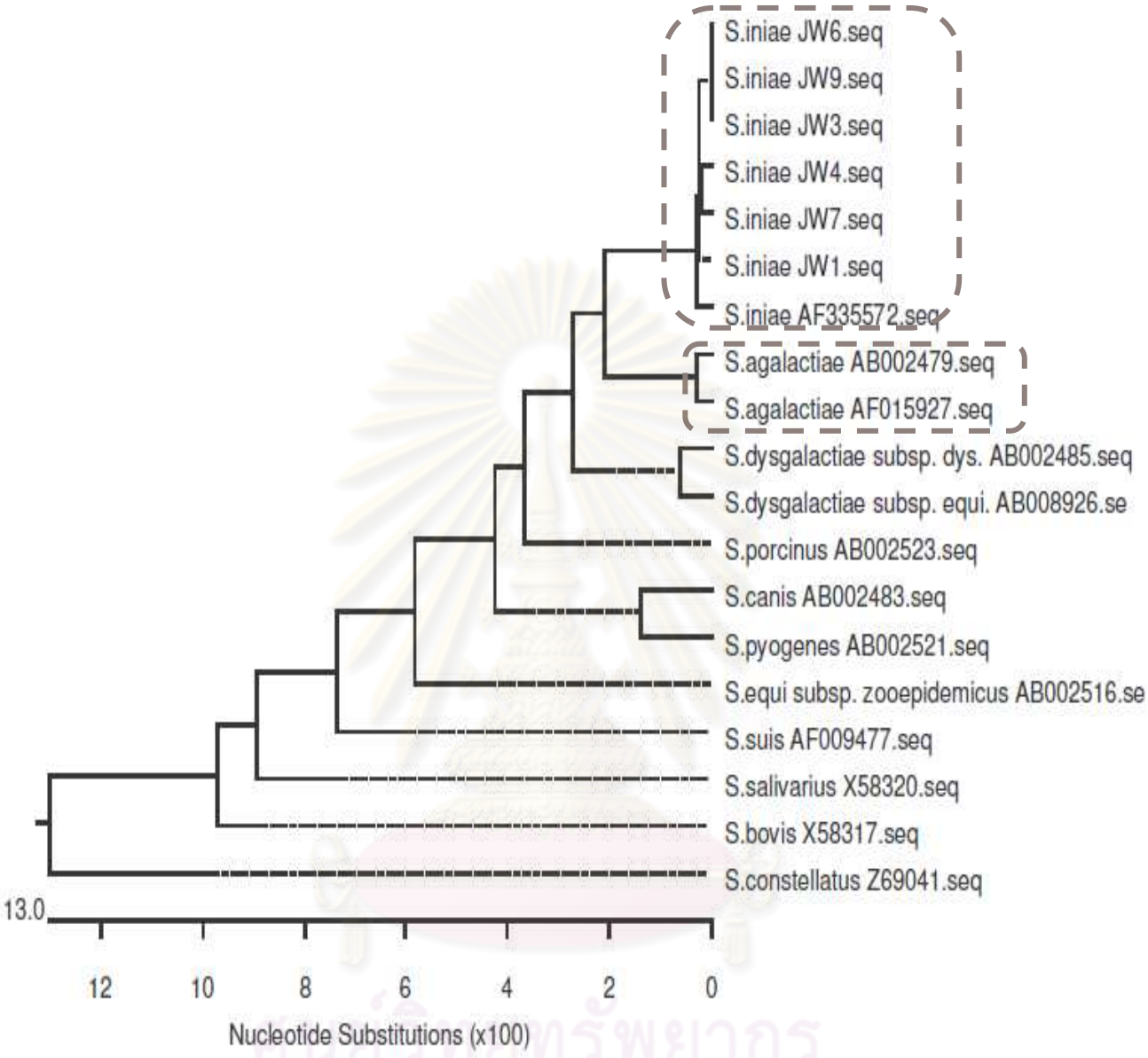


Figure 4.15 The phylogenetic tree generated based on the sequences of the *S.iniae* ***sodA***$_{int}$ **fragment** and other species of streptococcus.

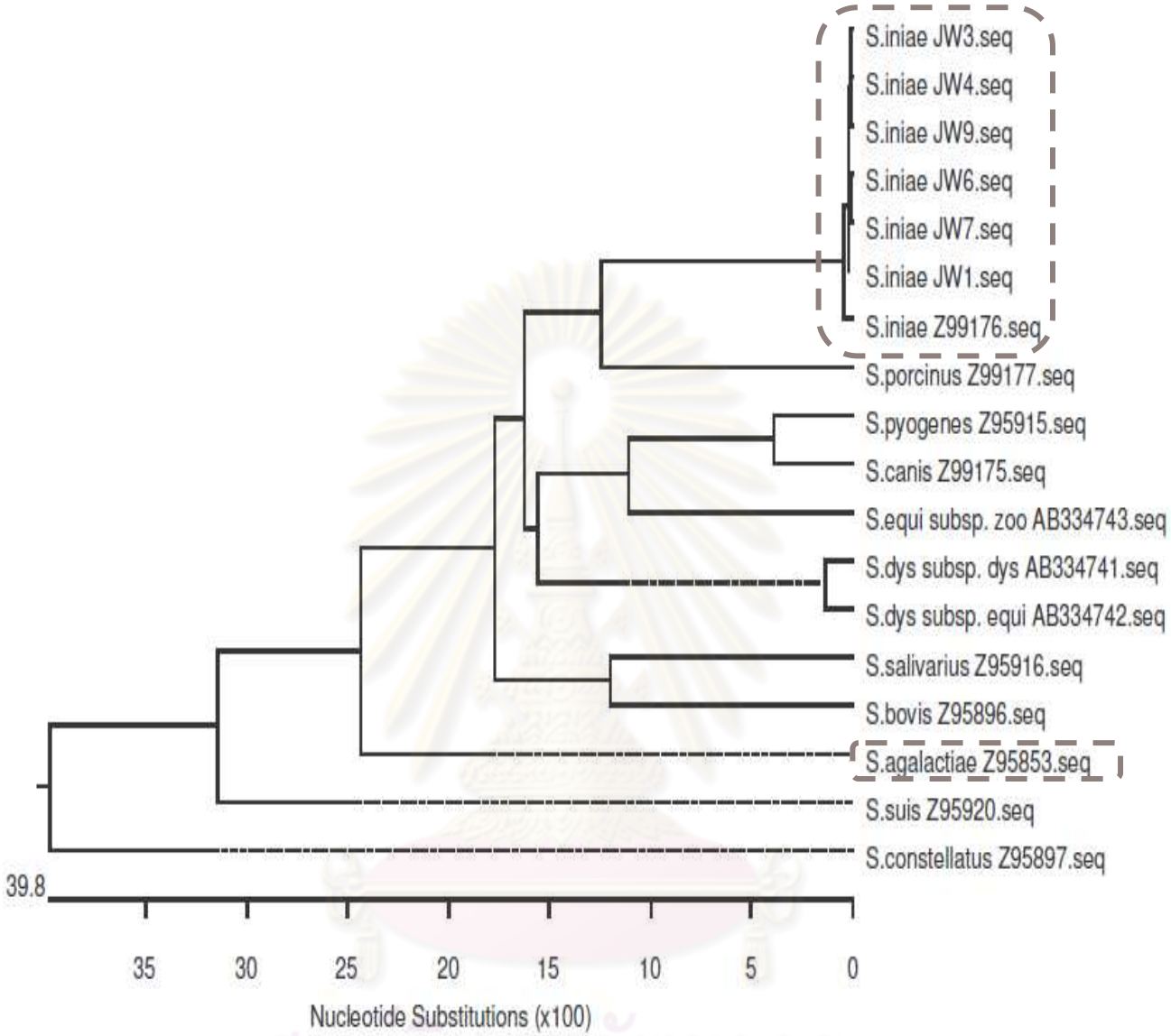


Table 4.11 Similarities and dissimilarities among **16S rRNA gene** sequences of *S.iniae* obtained from Thai tilapia and type strains.

The sequence similarities between *S.iniae* and other species of streptococcus were 95% (*S.agalactiae*), 90% (*S.bovis*), 94% (*S.canis*), 67.5% (*S.constellatus*), 95.5% (*S.dysgalatiae* subsp. *dys*galactiae), 94.5% (*S.dysgalatiae* subsp. *equisimitis*), 93% (*S.euisimitis* supsp. *zooepidemicus*), 95.4% (*S.porcinus*), 94% (*S.pyogenes*), 88.5% (*S.salivarius*) and 93% (*S.suis*)

The sequence divergence between *S.iniae* and other species of streptococcus were 4% (*S.agalactiae*), 6.3% (*S.bovis*), 5.6% (*S.canis*), 12.5% (*S.constellatus*), 4% (*S.dysgalatiae* subsp. *dys*galactiae), 4.2% (*S.dysgalatiae* subsp. *equisimitis*), 6.4% (*S.euisimitis* supsp. *zooepidemicus*), 4.3% (*S.porcinus*), 5.3% (*S.pyogenes*), 7.5% (*S.salivarius*) and 6.7% (*S.suis*)

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
1		99.9	99.7	99.9	99.8	99.9	99.9	95.1	95.3	90.4	94.1	67.5	95.5	94.5	93.0	95.4	94.3	88.8	93.1	**1**	S.iniae JW1.seq
2	0.1		99.6	100.0	99.9	100.0	99.8	95.0	95.2	90.3	94.1	67.5	95.5	94.5	93.0	95.4	94.2	88.7	93.0	**2**	S.iniae JW3.seq
3	0.3	0.4		99.6	99.7	99.6	99.6	94.9	95.1	90.2	93.9	67.1	95.1	94.3	92.8	95.4	94.0	88.4	92.9	**3**	S.iniae JW4.seq
4	0.1	0.0	0.4		99.9	100.0	99.8	95.0	95.2	90.3	94.1	67.5	95.5	94.5	93.0	95.4	94.2	88.7	93.0	**4**	S.iniae JW6.seq
5	0.2	0.1	0.3	0.1		99.9	99.7	95.0	95.2	90.3	94.1	67.5	95.3	94.5	93.0	95.6	94.1	88.5	93.0	**5**	S.iniae JW7.seq
6	0.1	0.0	0.4	0.0	0.1		99.8	95.0	95.2	90.3	94.1	67.5	95.5	94.5	93.0	95.4	94.2	88.7	93.0	**6**	S.iniae JW9.seq
7	0.1	0.2	0.4	0.2	0.3	0.2		95.6	96.0	90.9	94.6	67.7	96.1	95.2	93.7	95.9	95.0	89.8	93.8	**7**	S.iniae AF335572.seq
8	4.1	4.2	4.4	4.2	4.3	4.2	3.8		97.2	91.7	93.0	84.1	94.9	94.2	91.7	94.7	93.1	90.0	93.8	**8**	S.agalatiae AB002479.seq
9	3.9	4.0	4.2	4.0	4.1	4.0	3.4	1.3		91.8	94.4	84.1	96.2	96.5	91.2	96.0	95.1	90.2	95.2	**9**	S.agalactiae AF015927.seq
10	6.1	6.3	6.4	6.3	6.3	6.3	5.7	4.8	4.5		91.0	82.4	90.6	87.1	91.0	91.1	92.2	91.5	92.4	**10**	S.bovis X58317.seq
11	5.6	5.6	5.8	5.6	5.6	5.6	5.1	6.0	5.1	5.7		84.1	94.9	95.0	93.2	95.5	96.1	90.3	93.3	**11**	S.canis AB002483.seq
12	12.5	12.5	13.1	12.5	12.5	12.5	12.2	10.9	12.4	5.0	13.4		82.2	83.8	83.4	82.2	82.8	78.3	83.8	**12**	S.constellatus Z69041.seq
13	4.0	4.0	4.3	4.0	4.1	4.0	3.5	4.1	3.4	6.1	4.8	15.0		98.2	92.1	95.9	96.2	90.9	93.4	**13**	S.dysgalactiae subsp. dys. AB00248
14	4.2	4.2	4.5	4.2	4.2	4.2	3.7	3.5	2.9	5.5	4.4	12.3	1.2		91.3	95.5	93.2	86.1	93.3	**14**	S.dysgalactiae subsp. equi. AB00892
15	6.4	6.4	6.5	6.4	6.3	6.4	5.8	6.3	6.4	5.5	5.3	12.1	6.6	5.7		93.4	91.7	90.6	91.9	**15**	S.equi subsp. zooepidemicus AB002
16	4.3	4.3	4.4	4.3	4.2	4.3	3.9	4.4	3.8	5.2	4.5	14.5	3.7	3.9	4.7		94.8	91.1	94.8	**16**	S.porcinus AB002523.seq
17	5.2	5.3	5.5	5.3	5.4	5.3	4.5	3.9	3.1	4.4	2.7	9.7	3.2	2.9	5.1	3.9		91.4	94.8	**17**	S.pyogenes AB002521.seq
18	7.4	7.5	7.7	7.5	7.6	7.5	6.8	6.7	6.5	3.4	6.3	8.0	5.9	6.6	5.8	5.4	4.8		91.7	**18**	S.salivarius X58320.seq
19	6.6	6.7	6.8	6.7	6.7	6.7	6.0	5.4	4.4	4.2	6.3	12.4	6.2	5.9	6.5	4.8	4.7	4.8		**19**	S.suis AF009477.seq
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**		

Table 4.12 Similarities and dissimilarities among ***sodA$_{int}$*** **fragment** sequences of *S.iniae* obtained from Thai tilapia and type strains.

The sequence similarities between *S.iniae* and other species of streptococcus were 64.5% (*S.agalactiae*), 63% (*S.bovis*), 72% (*S.canis*), 62% (*S.constellatus*), 70.3% (*S.dysgalatiae* subsp. *dys*galactiae), 77% (*S.dysgalatiae* subsp. *equisimitis*), 64.4% (*S.euisimitis* supsp. *zooepidemicus*), 69.2% (*S.porcinus*), 72.4% (*S.pyogenes*), 66.4% (*S.salivarius*) and 64.5% (*S.suis*).

The sequence divergence between *S.iniae* and other species of streptococcus were 31.1% (*S.agalactiae*), 35% (*S.bovis*), 23% (*S.canis*), 34.6% (*S.constellatus*), 25% (*S.dysgalatiae* subsp. *dys*galactiae), 23.9% (*S.dysgalatiae* subsp. *equisimitis*), 33% (*S.euisimitis* supsp. *zooepidemicus*), 25% (*S.porcinus*), 23% (*S.pyogenes*), 30% (*S.salivarius*) and 32% (*S.suis*) **.**

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
1		100.0	99.8	99.8	99.8	100.0	99.8	65.1	63.0	72.4	61.8	70.8	77.0	64.8	69.9	72.9	66.9	64.8	**1**	S.iniae JW1.seq
2	0.0		100.0	99.6	99.8	100.0	100.0	64.4	62.5	72.0	61.6	70.3	77.0	64.1	69.2	72.4	66.4	64.1	**2**	S.iniae JW3.seq
3	0.0	0.0		99.6	99.6	100.0	99.8	64.4	62.5	72.0	61.6	70.3	77.0	64.1	69.2	72.4	66.4	64.1	**3**	S.iniae JW4.seq
4	0.2	0.2	0.2		100.0	99.6	99.8	64.8	63.2	71.7	61.8	70.3	77.0	64.4	69.2	72.4	66.7	64.6	**4**	S.iniae JW6.seq
5	0.2	0.2	0.2	0.0		99.8	99.8	64.8	63.2	71.7	61.8	70.3	77.0	64.4	69.2	72.4	66.7	64.6	**5**	S.iniae JW7.seq
6	0.0	0.0	0.0	0.2	0.2		100.0	64.4	62.5	72.0	61.6	70.3	77.0	64.1	69.2	72.4	66.4	64.1	**6**	S.iniae JW9.seq
7	0.2	0.0	0.0	0.2	0.2	0.0		71.5	68.3	79.1	67.1	77.2	77.0	71.3	77.5	79.5	72.6	71.3	**7**	S.iniae Z99176.seq
8	30.9	31.1	31.2	31.1	31.1	31.1	29.5		72.6	71.7	73.1	71.5	69.1	66.2	71.3	70.8	74.5	74.7	**8**	S.agalactiae Z95853.seq
9	35.1	35.4	35.5	35.0	35.0	35.4	34.5	30.3		72.0	70.3	68.7	68.1	66.2	68.5	70.6	77.5	70.3	**9**	S.bovis Z95896.seq
10	22.8	22.9	23.0	23.2	23.2	22.9	22.5	29.3	30.1		64.1	84.8	82.4	80.2	74.0	92.2	74.0	67.8	**10**	S.canis Z99175.seq
11	34.8	34.7	34.8	34.6	34.6	34.7	34.2	30.7	31.6	39.5		66.0	66.3	62.1	69.2	66.7	70.3	71.5	**11**	S.constellatus Z95897.seq
12	25.0	25.1	25.2	25.5	25.5	25.1	24.9	28.9	32.8	16.2	38.0		96.2	75.6	72.4	86.7	72.0	65.7	**12**	S.dys subsp. dys AB334741.seq
13	23.9	23.9	23.9	23.9	23.9	23.9	23.8	29.8	32.5	17.9	36.1	2.6		74.0	71.4	84.7	71.9	64.8	**13**	S.dys subsp. equi AB334742.seq
14	32.8	33.0	33.1	33.4	33.4	33.0	31.6	35.8	36.5	20.8	43.9	26.4	27.7		69.4	77.5	69.9	67.1	**14**	S.equi subsp. zoo AB334743.seq
15	24.7	24.9	24.9	25.2	25.2	24.9	23.0	30.7	33.1	27.6	32.4	30.5	30.1	33.2		74.9	71.0	71.3	**15**	S.porcinus Z99177.seq
16	22.7	22.8	22.9	23.1	23.1	22.8	22.4	30.0	30.7	7.8	37.7	14.1	14.9	23.6	27.2		74.5	66.7	**16**	S.pyogenes Z95915.seq
17	29.6	29.8	29.8	29.8	29.8	29.8	29.4	26.9	23.9	27.6	31.6	29.0	28.7	33.0	31.5	26.9		73.3	**17**	S.salivarius Z95916.seq
18	31.8	32.0	32.1	32.0	32.0	32.0	30.3	26.2	30.9	35.7	30.3	38.2	37.5	35.0	31.4	36.5	26.0		**18**	S.suis Z95920.seq
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**		

Phase III : The development of streptococcosis vaccine

Safety test

Safety of the injectable *Streptococcus agalactiae* FKC vaccine (whole cell *S.agalactiae* vaccine) was tested by an intraperitoneal administration of 3 X 10^8 cell/20 gm body weight tilapia. Water quality was monitored and carefully controlled during the period of the test (Table 4.13). All moribund fish were sacrificed and examined for macroscopic lesions. External gross lesions were ulcerative skin and fin erosion that may have caused by the stress response to status under experiment conditions. Isolation of bacterial pathogens from brain and kidney of the moribund fish was negative for streptococcus, however the infection of *Aeromonas hydrophila* was found in cases with external gross lesions. Groups of non-injected and TSB-injected fish were maintained as controls. The vaccinated fish, both with FKC vaccine and FKC+ECP vaccine, retained survival rates similar to those of control groups (Table 4.14). The result demonstrated that the injectable *S. agalactiae* FKC vaccine prepared from local strains under the laboratory conditions as described previously, was safe for tilapia.

Table 4.13 The mean ± standard deviation of water quality parameters (pH, ammonia, nitrite and water temperature) in each group.

	Groups			
Water qualities	FKC	FKC+ECP	TSB	None
pH	7.49±0.12	7.32±0.10	7.42±0.12	7.41±0.13
Ammonia (mg/l)	0.51±0.24	0.54±0.27	0.27±0.07	0.39±0.21
Nitrite (mg/l)	0.06±0.05	0.09±0.05	0.01±0.03	0.03±0.03
Water temperature (^{O}C)	29.1±0.47	29.1±0.62	29.5±0.50	29.3±0.47

Note: Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.

Table 4.14 Safety test of the vaccine was evaluated in laboratory conditions. Following an intraperitoneal vaccination of 3 X 10^8 CFU per fish (20 gm body weight), the survival rates of vaccinated group were compared to the placebo vaccinated control (TSB intraperitoneal injection) and untreated control. Dead fish were removed daily for pathogen identification.

Group		Number of dead fish							
		FKC		FKC+ECP		Control : TSB		Control : None	
		R1	R2	R1	R2	R1	R2	R1	R2
Day	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-
	4	2	1	1	2	-	-	-	-
	5	-	-	-	-	1	-	-	-
	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-
	12	-	-	-	-	1	1	-	-
	13	-	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-	-
Total number of fish		10	10	10	10	10	10	10	10
Number of dead fish		2	1	1	2	2	1	0	0
Survival rates (%)*		80	90	90	80	80	90	100	100

R = replication

*Survival rates of each group were not different statistically ($\alpha = 0.05$) (the Kruskal-Wallis one way analysis of variance by range).

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.

Efficacy test

Determination of streptococcal vaccine efficacy with challenge test

Vaccine efficacy in the field trial was preliminary evaluated on the survival rates of the vaccinated fish (two types of vaccines, FKC vaccine and FKC+ECP vaccine, at dosage of 6.0 X 10^8 CFU per fish) and the non-vaccinated fish (two control groups, placebo vaccinated control and untreated control). In addition, vaccinated and non-vaccinated juvenile hybrid tilapias (200 gm body weight) were compared the growth performance i.e. individual body weight, average daily gain (ADG) and feed conversion ratio (FCR) during 12 weeks of rearing. The cumulative mortality of the vaccinated and non-vaccinated fish reared in floating cages was shown in Table 4.15. Comparison of mortality between the vaccinated and non-vaccinated fish was not statistically significant (α = 0.05). Percentage of survival following 12 weeks post vaccination was 98.1% for vaccinated group and 96.8% for non-vaccinated group. Macroscopic examination in all moribund fish revealed mainly hemorrhage at fin, operculum and brain. Streptococci were found in the moribund fish, both the experimental (only mixed vaccine and control group) and the wild fish, suggesting that the streptococcal bacteria was present and pre-existing culture condition of the field trial. The water quality parameters, including salinity (ranged from 6 to 7) and pH (ranged from 8 to 8.3) were suitable for tilapia rearing.

The growth performance, including average body weight, ADG and FCR of tilapia at 2nd, 3rd, 5th, 8th, 10th and 12th week post-vaccination are given in Table 4.16 to 4.18 and Figure 4.16 to 4.18. The average body weight between vaccinated and non-vaccinated fish was 277 and 274 grams (at 2nd week), 302 and 298 grams (at 3rd week), 367 and 348 grams (at 5th week), 475 and 460 grams (at 8th week), 497 and 483 grams (at 10th week), 539 and 540 grams (at 12th week). The ADG between vaccinated and non-vaccinated fish was 7.04 and 6.70 grams (at 2nd week), 5.11 and 4.90 grams (at 3rd week), 4.93 and 4.40 grams (at 5th week), 5.09 and 4.80 grams (at 8th week), 4.31 and 4.13 grams (at 10th week), 4.08 and 4.10 grams (at 12th week). The FCR between vaccinated and non-vaccinated fish was 0.90 and 0.96 grams (at 2nd week), 1.16 and 1.17 grams (at 3rd week), 1.23 and 1.40 grams (at 5th week), 1.13 and 1.21 grams (at 8th week), 1.32 and 1.38 grams (at 10th week), 1.41 and 1.41 grams (at 12th week). The growth performance evaluated at different periods post vaccination, did not differ between the vaccinated and non-vaccinated groups. The results showed that vaccination did not cause adverse effect on the growth performance of rearing tilapia.

Table 4.15 Vaccine efficacy in cultured tilapia was evaluated in field trial following an intraperitoneal vaccination of 6 X 10^8 CFU per fish (200 gm body weight). Vaccinated groups were compared with non-vaccinated groups, including placebo vaccinated control and untreated control. Dead fish were removed daily for pathogen identification.

Group	Number of dead fish							
	FKC		FKC+ECP		TSB		None	
	R1	R2	R1	R2	R1	R2	R1	R2
Total of fish	78	79	77	78	78	76	78	77
Week 1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	1*	-	-	-	1	-	2	-
4	-	-	-	-	1	1	-	-
5	-	-	-	-	-	1	-	-
6	-	-	-	-	-	-	-	-
7	-	-	-	1	-	-	-	-
8	-	-	-	1	2	-	-	-
9	-	-	-	-	-	-	1	-
10	-	-	-	-	-	-	1	-
11	-	-	-	-	-	-	-	-
12	-	-	3	-	-	-	-	-
Total of dead fish	1	0	3	2	4	2	4	0
Total of survive fish	77	79	74	76	74	74	74	77
Survival rate (%)**	99.4		96.8		96.1		97.4	

R = replication
* Not found bacterial infection
** Survival rate of each group are not different statistically ($\alpha = 0.05$) (the Kruskal-Wallis one way analysis of variance by range).

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.

Table 4.16 and Figure 4.16 Body weight of vaccinated fish (at dose of 6 X 10^8 CFU per fish) compared with non-vaccinated groups, including placebo vaccinated control and untreated control, at week 0, 2, 3, 5, 8, 10 and 12 post-vaccination.

Group		Body weight of fish * (grams)							
		FKC		FKC+ECP		TSB		None	
		1	2	1	2	1	2	1	2
Week	2	290	270	280	270	285	255	280	275
	3	310	291	308	300	300	300	291	300
	5	381	360	390	340	330	330	350	380
	8	510	460	480	450	420	480	450	488
	10	510	460	520	500	520	460	460	490
	12	526	519	552	558	553	565	521	524

* Average body weight was determined on a pooled sample of eight fish per measurement. Four measurements were achieved for an average.

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.

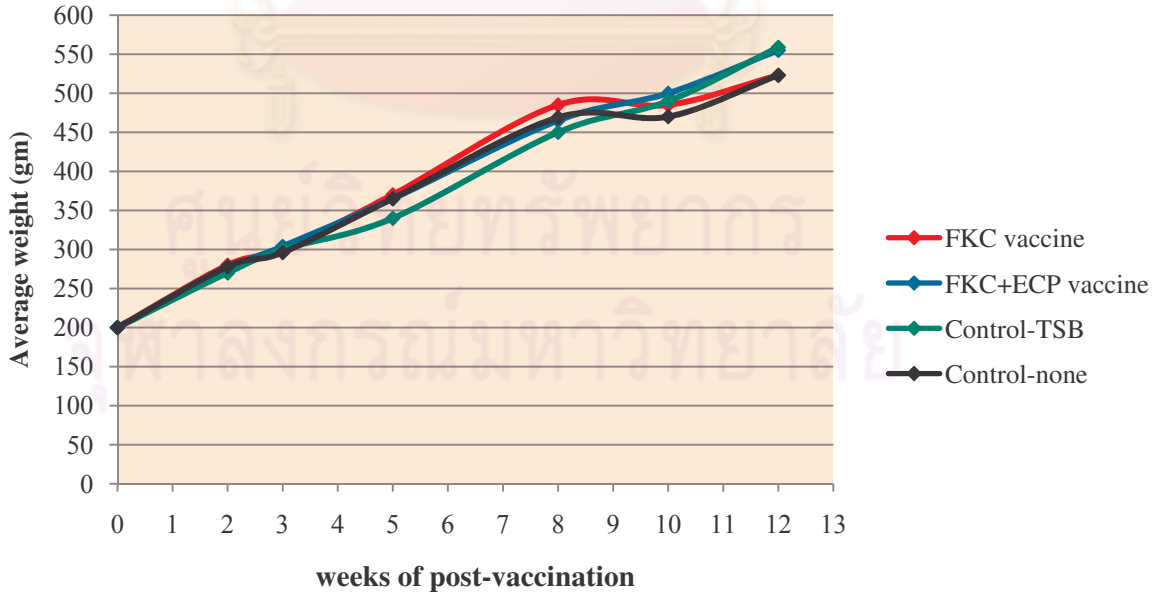


Table 4.17 and Figure 4.17 The Average daily gain (ADG) of vaccinated fish (at dose of 6 X 10^8 CFU per fish) compared with non-vaccinated groups, including placebo vaccinated control and untreated control, at week 0, 2, 3, 5, 8, 10 and 12 post-vaccination.

Group	Average daily gain (ADG) * (grams per day)							
	FKC		FKC+ECP		TSB		None	
	1	2	1	2	1	2	1	2
Week 2	8.18	6.36	7.27	6.36	7.73	5.00	7.27	6.82
3	5.50	4.55	5.40	5.00	5.00	5.00	4.55	5.00
5	5.32	4.71	5.59	4.12	3.82	3.82	4.41	5.29
8	5.74	4.81	5.19	4.63	4.07	5.19	4.63	5.33
10	4.49	3.77	4.64	4.35	4.64	3.77	3.90	4.20
12	3.93	3.84	4.24	4.31	4.25	4.40	3.87	3.90

*The average daily gain (ADG) is a significant factor in assessing growth rates in most food animal species. The ADG was calculated using:

$$\text{ADG (grams per day)} = \frac{\text{Final weight (grams)} - \text{Initial weight (grams)}}{\text{Rearing period (day)}}$$

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.

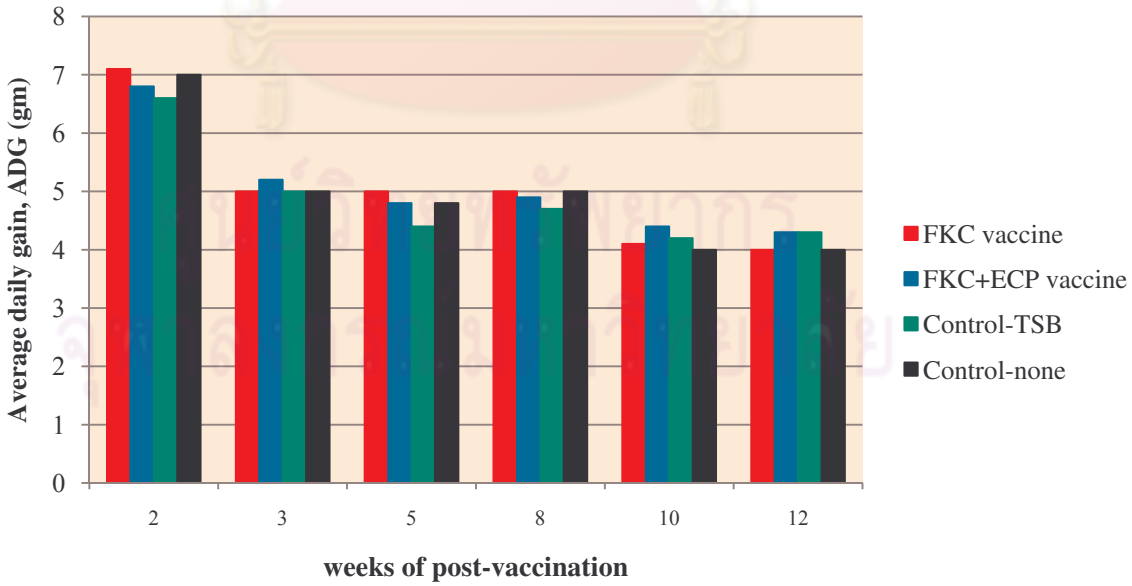
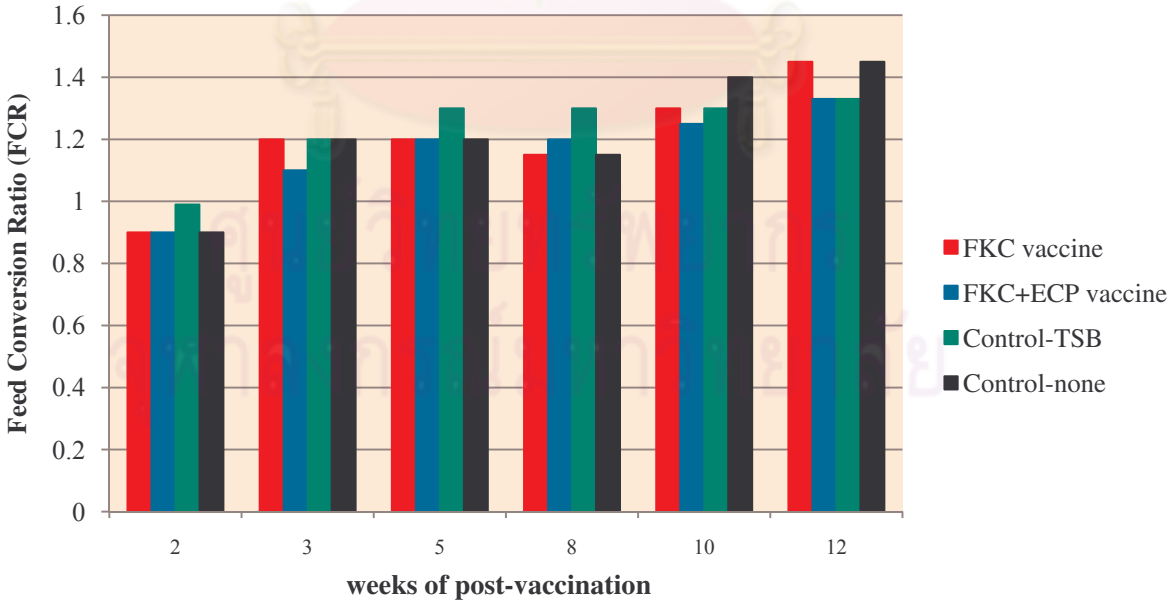


Table 4.18 and Figure 4.18 The Feed Conversion Ratio (FCR) of vaccinated fish (at dose of 6 X 10^8 CFU per fish) compared with non-vaccinated groups, including placebo vaccinated control and untreated control, at week 0, 2, 3, 5, 8, 10 and 12 post-vaccination.

Group		Feed Conversion Ratio (FCR)*							
		FKC		FKC+ECP		TSB		None	
		1	2	1	2	1	2	1	2
Week	2	0.76	0.98	0.86	0.98	0.81	1.25	0.86	0.92
	3	1.08	1.30	1.10	1.19	1.19	1.19	1.30	1.19
	5	1.12	1.27	1.07	1.45	1.56	1.56	1.35	1.13
	8	1.00	1.19	1.11	1.24	1.41	1.11	1.24	1.08
	10	1.26	1.50	1.22	1.30	1.22	1.50	1.45	1.34
	12	1.47	1.50	1.36	1.33	1.35	1.31	1.49	1.48

*The Feed Conversion Ratio (FCR) was calculated from the amount of kilos of feed that are used to produce one kilo of whole fish. The standard of FCR for intensive cultured tilapia is ranged from 1.6 to 1.8).

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.



Specific antibody response post-vaccination

Serum agglutination titers of tilapia immunized intraperitoneally with FKC vaccine and FKC+ECP vaccine (at dose of 6 X 10^8 CFU per fish) were tested with homologous *S. agalactiae* isolates. Humoral immune response (HIR) after vaccination against streptococcus presented by the agglutinating antibody were tittered at week 0, 3, 5, 8, 10 and 12 post-vaccination. The agglutination titers of the different groups were shown in Table 4.19 and Figure 4.19. The titers of serum antibody were reported as $\log_{10}$ of reciprocal of the highest serum dilution causing agglutination (Annex 7). Prior to the vaccination, agglutinating antibody against *S. agalactiae* ranged from 0.564 to 0.752. The mean agglutination titers continuously increased reaching higher level at 3 weeks post-vaccination. The agglutination titers remained at significantly high levels until 10 weeks post-vaccination. After a plateau phase, the titers decreased subsequently at 12 weeks post-vaccination (1.184$\pm$0.551 for FKC vaccine and 1.279$\pm$0.636 for FKC+ECP vaccine), however, were significantly higher than pre-vaccination titers. The agglutination titer of the placebo vaccinated control (TSB injection) was ranged 0.338 to 0.752 and comparable to the titer of untreated control (0.432-0.639). Fish vaccinated with FKC vaccine and FKC+ECP vaccine had significant higher agglutinating antibody titers against *S. agalactiae* than the non-vaccinated ($p<0.05$) (the Analysis of Variance, ANOVA). The results showed that an injectable *S. agalactiae* vaccine developed in the present study immunized tilapia to produce specific antibody against *S. agalactiae* at $\leq$ 3 weeks post-vaccination, and the immunized titers remained for $\geq$ 12 weeks post-vaccination.

The kinetic levels of antibody response against *S. agalactiae* in tilapia after vaccination with FKC vaccine and FKC+ECP vaccine was given in Figure 4.20. A primary antibody response after vaccination was detected early at $\leq$ 3 weeks and maintained a significantly high level until 10 weeks post-vaccination. Antibody titers decreased subsequently when evaluated at 12 weeks post-vaccination. The kinetic levels of agglutinating antibody response in vaccinated tilapia is a primary consideration to programme the vaccination in tilapia culture.

Table 4.19 and Figure 4.19 Agglutination titers against *S.agalactiae* assayed by a direct agglutination test. The titer reported as $\log_{10}$ of reciprocal of the highest serum dilution causing agglutination. The antibody titer of vaccinated group differ significantly from non-vaccinated group evaluated at week 3, 5, 8, 10 and 12 weeks post-vaccination ($p<0.05$).

	Antibody titer by direct agglutination test ($\log_{10}$)			
Group	FKC	FKC+ECP	TSB	None
Week 0	0.583±0.257	0.695±0.436	0.752±0.527	0.564±0.362
3	1.655±0.503	1.561±0.614	0.639±0.502	0.527±0.258
5	1.749±0.643	1.599±0.500	0.414±0.308	0.432±0.219
8	1.655±0.677	1.467±0.649	0.545±0.506	0.451±0.329
10	1.542±0.424	1.486±0.596	0.338±0.150	0.639±0.424
12	1.184±0.551	1.279±0.636	0.545±0.384	0.489±0.288

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.

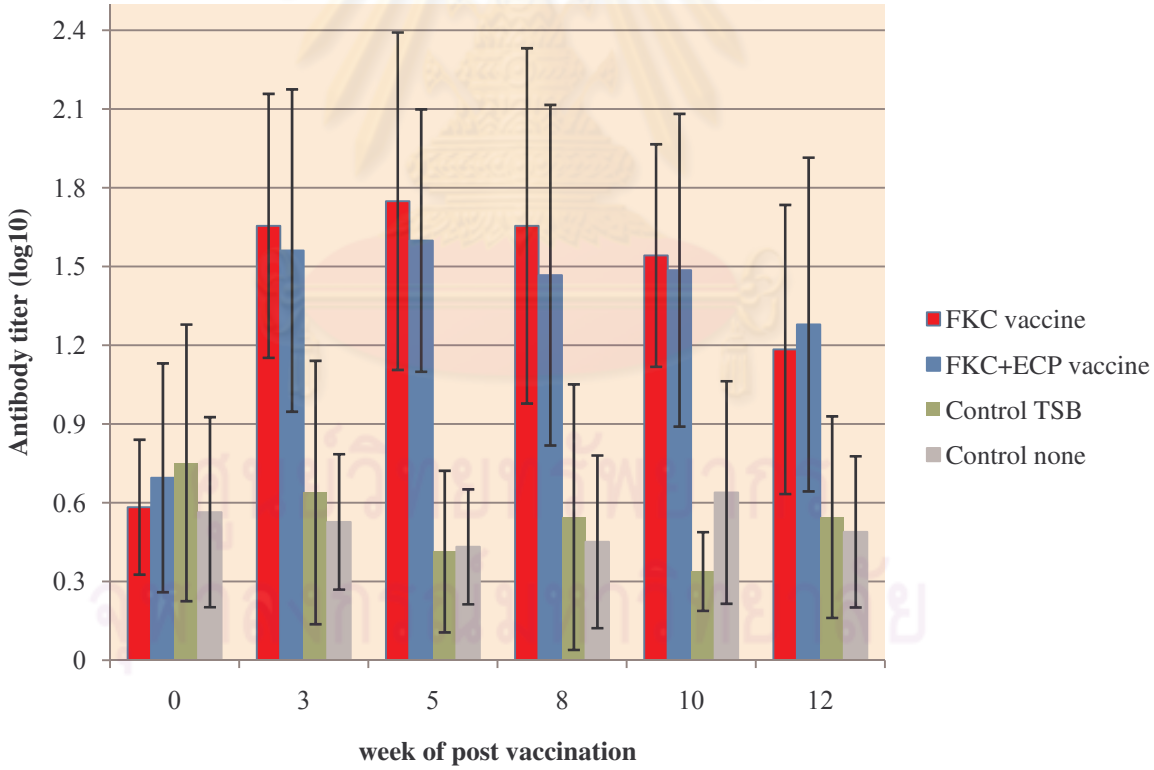
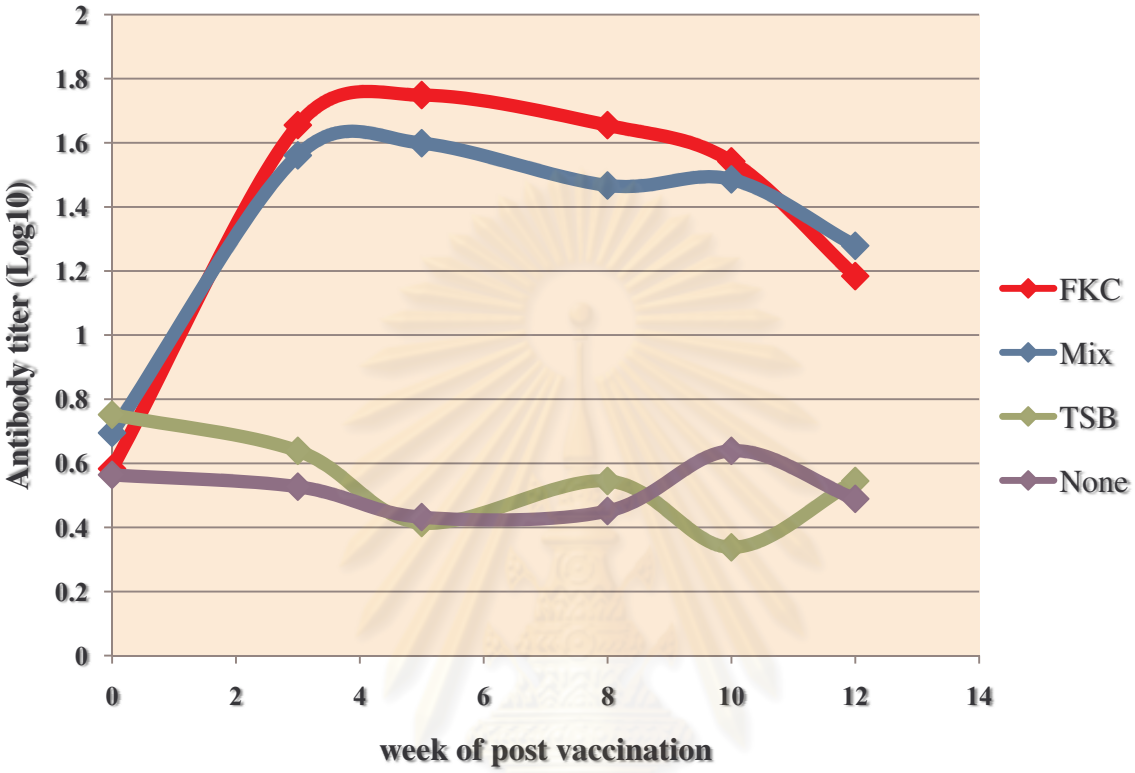


Figure 4.20 The kinetic levels of antibody response against *S.agalactiae* in tilapia after vaccination with FKC vaccine and FKC+ECP vaccine. The placebo vaccinated fish (TSB injection) and untreated fish were evaluated as control groups.



Protection of streptococcosis in vaccinated tilapia

Challenge test was performed 10 weeks post-vaccination. Ten fish of each group were intraperitoneal challenged with 0.5 ml of 1.5 X 10^8 *S. agalactiae* cells per fish. Mortality was observed in non-vaccinated fish at 24 hours after challenge. The intraperitoneal challenge causes 100% mortality in untreated control and 90% in the placebo vaccinated control. Fish immunized with FKC vaccine presented no mortality for 7 days after the challenge, whereas fish immunized with FKC+ECP vaccine show 22% mortality. At the end of the experiment period (at the 21st day post-challenge), cumulative mortality of FKC vaccinated group was 10% (FKC vaccine) and the FKC+ECP vaccinated group was 33%. Streptococcal was not found in blood sample taken from all vaccinated survivors. No significant difference in survival was noted between FKC vaccine and FKC+ECP vaccine ($\alpha = 0.01$) (the Mann-Whitney Test). However, gross examination in survivors revealed skin abscess containing streptococci (Table 4.22). Overall, the survival rates of vaccinated fish were a significantly greater than the non-vaccinated fish (Table 4.21 and Figure 4.21). External and internal macroscopic examination of all moribund fish revealed mainly hemorrhage of at the base of fin, operculum and brain. All dead fish were positive for streptococcus infection (Table 4.23).

The level of circulating specific antibody response after challenge was measured by direct agglutination method and reported at $\log_{10}$ of reciprocal of the highest serum dilution causing agglutination (Annex 7). The agglutination titers of vaccinated fish were found to be 3.461 ± 0.158 (FKC vaccine) and 3.431 ± 0.165 (FKC+ECP vaccine) at the end of trial (21 days post-challenge). This challenge resulted in a significant increase of the secondary response. In addition, the non-vaccinated survivor showed a significantly increase of primary antibody response to the challenge. The results showed that the challenge of virulent *S. agalactiae* strain stimulated immune response in both the vaccinated and non-vaccinated fish within 21 days post-challenge.

Table 4.21 and Figure 4.21 The percent cumulative mortalities in both vaccinated fish (at dose of 6.0 X 10^8 CFU per fish) and non-vaccinated fish were recorded after a challenge test at 10 weeks post-vaccination. Each group of tilapia was challenged virulent *S. agalactiae* strain at dose of 1.5 X 10^8 CFU per fish by intraperitoneal injection. Three weeks after challenge, the percent cumulative mortalities ranged from 10 to 33% of the vaccinated and 90 to 100% of non-vaccinated group. The relative percent survival (RPS) of the vaccinated fish was significantly greater than the non-vaccinated fish ($p<0.01$).

		Mortality post-challenge			
Group		FKC	FKC+ECP	TSB	None
Week	1	-	2	9	10
	2	-	-	-	-
	3	1	1	-	-
Total of fish		10	9	10	10
Total of dead fish		1	3	9	10
Total of survive fish**		9 (NF)	6 (NF)	1 (S)	0
Cumulative mortality (%)		10	33	90	100
Relative percent survival (% RPS)*		90	67	0	0

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC mixed Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.

* Survival rate in vaccinated group (FKC vaccine and FKC added with ECP vaccine) were different significantly from non-vaccinated fish (placebo vaccinated control and untreated control) ($p<0.01$) (the Kruskal-Wallis one way analysis of variance by range and the Mann-Whitney Test).

** All survivors were confirmed the streptococcosis infection by microbiological identification of the bacteria in blood sample at the end of trial (21 days post-challenge); NF, Not found; S, Streptococcus positive.

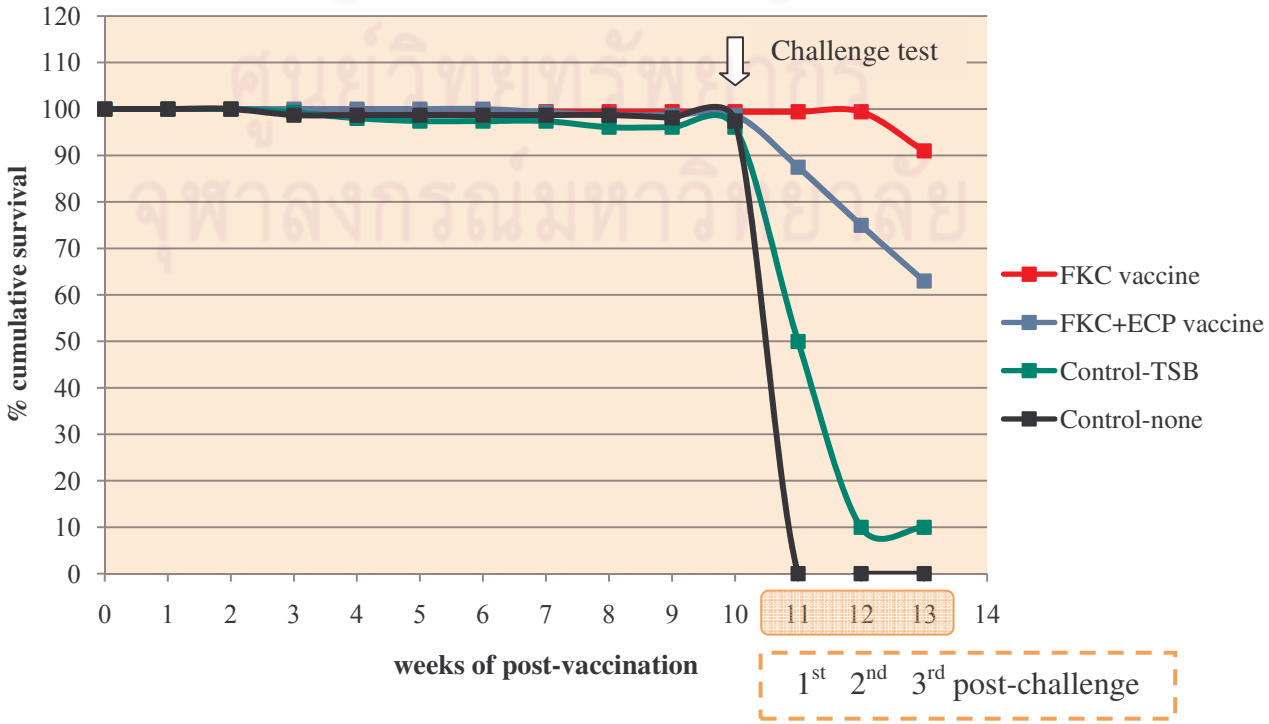


Table 4.22 All survivors from the challenge were confirmed the streptococcal infection by conventional microbiological identification of the blood sample. Bacterial pathogens, including streptococcus, was not found in all vaccinated survivors. External lesions were noted in some survivors. The vaccinated survivors those presented skin abscess neither retained systemic streptococcal infection identified using the bacterial culture nor histological alteration of the visceral organs, spleen and intestine. Culture of the abscess content failed to recover streptococcal bacteria whilst the histological examination of the skin abscess revealed fibrous connective tissue surrounding debris (Appendix H).

Group	Fish	External lesions	Streptococcus in blood sample
FKC	1	NF	NF
	2	Ulcerative skin; Hemorrhage at operculum and fin; Fin erosion	NF
	3	Abscess around the mouth; Fin erosion	NF
	4	Abscess around the mouth; Fin erosion	NF
	5	Abscess around the mouth and base of fin; Fin erosion	NF
	6	Abscess around the mouth and base of fin; Fin erosion	NF
	7	Abscess around the mouth; Fin erosion	NF
	8	Abscess around the mouth	NF
	9	NF	NF
FKC+ECP	1	Abscess around the mouth	NF
	2	Abscess around the mouth	NF
	3	NF	NF
	4	Abscess around the mouth	NF
	5	NF	NF
	6	NF	NF
TSB	1	Abscess around the mouth	(+)

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.
NF; Not found, (+); positive

Table 4.23 Bacterial pathogens were isolated from target organs (brain and kidney) of the moribund fish using conventional microbiological method. All dead fish of the challenge were cultured positive for streptococcus.

Groups	Fish	Date of death*	Body weight (gram)	Body length (cm.)	Diagnosis
FKC	1	25/8/2009	-	-	Streptococcus
FKC+ECP	1	6/8/2009	507.48	25.0	Streptococcus + Aeromonas
	2	6/8/2009	419.46	25.0	Streptococcus + Aeromonas
	3	25/8/2009	-	-	Streptococcus
TSB	1	6/8/2009	456.33	25.0	Streptococcus
	2	6/8/2009	422.77	27.0	Streptococcus
	3	6/8/2009	345.87	24.0	Streptococcus + Aeromonas
	4	6/8/2009	437.90	27.0	Streptococcus
	5	7/8/2009	538.14	30.0	Streptococcus + Aeromonas
	6	7/8/2009	467.78	28.0	Streptococcus
	7	7/8/2009	431.44	25.5	Streptococcus
	8	7/8/2009	500.30	27.5	Streptococcus
	9	7/8/2009	313.36	26.0	Streptococcus
None	1	6/8/2009	533.50	28.0	Streptococcus
	2	6/8/2009	344.15	24.0	Streptococcus
	3	6/8/2009	400.79	26.0	Streptococcus + Aeromonas
	4	6/8/2009	545.73	25.0	Streptococcus + Aeromonas
	5	6/8/2009	425.81	26.0	Streptococcus
	6	7/8/2009	394.70	26.0	Streptococcus + Aeromonas
	7	7/8/2009	387.47	25.0	Streptococcus + Aeromonas
	8	7/8/2009	457.85	28.0	Streptococcus + Aeromonas
	9	7/8/2009	517.97	28.0	Streptococcus + Aeromonas
	10	7/8/2009	498.66	25.0	Streptococcus

* Fish were challenged on 4/8/2009 with 0.5 ml innocula per fish (1.5×10^8 CFU per fish).

Group FKC, Formalin Killed Cell (FKC) vaccine; Group FKC+ECP, FKC added with Extracellular product (ECP) vaccine; Group TSB, Placebo vaccinated control; Group None, Untreated control.

CHAPTER V

DISCUSSION

Phase 1: Pathobiological characterization of streptococcosis in farmed tilapia

Tilapia farming is major aquaculture in Thailand because tilapia are more resistant to stressors than other commercial fish species. They are tolerant to physical or biological changes in the quality of water and diseases commonly attack the fish culture. Resulting from intensification of tilapia production in Thailand, the disease outbreaks have been evident in several culture areas. Outbreak of streptococcosis in farmed tilapia have been reported in all stages of tilapia farming and cause severe economic loss. During the period from 2003 to 2010, clinical cases of streptococcosis were examined in tilapia farmed in the middle, north-eastern and western parts of Thailand. The disease usually occurred in summer, resulting in acute mortality during the first few days after the initial infection. Clinically infected fish showed septicemic condition erratically swimming, exophthalmia with ocular opacity and abdominal distension. Skin pigmentation, ulceration and skin abscesses were also found in some cases. Generalized haemorrhage was found in many visceral organs particularly brain, liver, gastrointestinal tract and genital tract. Clinical features and pathologic findings of streptococcosis in farmed tilapia reported in this study were similar to many literatures (Bromage and Owens, 2002; Duremdez *et al*., 2004; Filho *et al*., 2009; Lahav *et al*., 2004). Clinical streptococcosis has been reported in some freshwater and marine fish, and symptoms of the disease appear in later stages of the illness (Bromage and Owens, 2002; Evans *et al*., 2000). Many studies have proposed that streptococcosis seriously affects tilapia more than other fish species. Chang and Plumb in 1996 reported that tilapia had a greater mortality rate than channel catfish within 24 hours of an inoculation and had a mortality rate of upto 70% within 7 days of an inoculation. At the later stage of infection, many streptococci infect the nervous system via blood circulation causing damage of the central nervous system; as a result, the infected fish elicits erratic swimming (Eldar *et al*., 1994).

Histopathologic findings observed in diseased tilapia were similar to previous reports of Perera *et al*. (1998), Stoffregen *et al*. (1996) and Bromage and Owens (2002). The lesions consisting of inflammatory cells (predominantly macrophage and lymphocyte) and cocci bacteria (presumably *Streptococcus*) appear in blood vessels of many organs e.g. liver, gut, intestine, ovary and brain. This histological lesion supports that *Streptococcus* infection in tilapia is severe generalized septicemic disease. Chang and Plumb (1996) explained that streptococci attached to erythrocytes of tilapia and septicemia was related to the circulation of erythrocytes. The bacterial cell and its extracellular product can damage many tissues thorough blood circulation, inducing edematous swelling of cell in the liver, spleen, kidney, gastrointestinal tract and brain. For instance, the lesion of exudative bacterial meningitis showed damaging of brain tissue and infiltration of inflammatory cell around tissue that related to clinical sign of erratic swimming (Bromage and Owens, 2002; Neely *et al*., 2002; Filho *et al*., 2009). Histological evidence from hyaline droplet degeneration appeared from the absorption of excessive amounts of protein that related to bacterial toxin causing tissue

damage on the progressive disease (Filho *et al*., 2009). Kusuda and Hamaguchi (1989) supported the hypothesis that *Streptococcus* can produce toxic substances, including intracellular and extracellular toxin. Toxin produced from streptococcal bacteria could kill yellowtail within 10 to 34 hours. Filho *et al*. (2009) explained the occurrences of melanopigment in the liver and spleen of diseased tilapia that melanomacrophages in the liver and spleen show phagocytic properties by proliferation and hypertrophy of macrophage for antigen trapping and presentation to lymphocytes. Consequences of phagocytic properties show cellular degeneration products as melanin pigment.

In this study, streptococcal bacteria were recovered from target tissue, including the haematopoietic tissue of the head kidney and the neural tissue of the brain from diseased tilapia. Streptococci grew on blood agar display pinpoint, whitish, circular colonies holding hemolytic activity. The bacteria was gram-positive aerobic cocci chain and was negative for catalase activity. Many published reports supported that the kidney and brain are good sites for the detection of the causative bacteria in fish (Romalde and Toranzo, 1999; Yanong and Floyd, 2006). The bacteria can infiltrate via blood circulation of diseased fish and, as a result, it can be found in the kidney and other organs. Nguyen and Kanai (1999) reported that *S. iniae* was obtained aseptically from brain and kidney. An enriched medium as blood agar, containing nutritionally rich whole blood supplemented with other basic nutrients (including tryptic soy, heart infusion or peptone) is suitable cultivation for streptococci because streptococci are fastidious with respect to their nutritional requirements (Facklam and Washington, 1991). Nguyen and Kanai (1999) demonstrated that the percentage recovery of number of streptococci colonies on blood agars was near 100%. Furthermore, *Streptococcus* spp. could be identified presumptively by hemolytic patterns on blood agar and a concentration of 3% blood was enough to enable observation of hemolysis types of colonies (Facklam and Washington, 1991; Nguyen and Kanai, 1999).

In this work, a collection of *Streptococcus* spp. isolated from diseased tilapia in different regions was biochemically and genetically characterized. Forty-six of 50 tested isolates were identified *S. agalactiae* and 4 were *S. dysgalactiae subsp. equisimilis* based on the API 20STREP analytical profile. The biochemical patterns of *S. dysgalactiae subsp. equisimilis* were tested similar to *S. iniae* ATCC29178. Mata *et al*. (2004) explained that laboratory identification of *S. iniae* from diseased fish can be difficult, especially when using commercial identification systems, because no currently available commercial system includes *S. iniae* in its database. At the molecular level, the PCR amplification with primers that are species-specific to 16S rRNA gene of *S. agalactiae* (Martinez *et al*., 2001) and *S. iniae* (Zlotkin *et al*., 1998) amplified a single band from all the tilapia isolates as well as *S. agalactiae* ATCC13813 and *S. iniae* ATCC29178. Sequencing of the PCR products revealed 98-99% homology of the sequence to the reference strains, thus indicating that the employed PCR assay was specific for the corresponding streptococcal pathogens. The PCR identification demonstrated that 2 different species of *Streptococcus* were involved in 139 clinical cases, *S. agalactiae* was found in 131 cases (94.24%) and *S. iniae* found in 8 cases (5.75%). It was interesting to note that streptococcosis in farmed tilapia commonly occurred in summer when the water temperature could be upto 33^{O}C. Filho *et al*. (2009) and

Shoemaker *et al*. (2000) suggested that high temperature water with higher than 28 ^{O}C, poor water quality and fish density were considered as stressing factors that contribute to streptococcosis. Unsuitable density of intensive cultured system led to the high levels of mortality. Shoemaker *et al*. (2001) reported significant increases in mortality due to *Streptococcus* infection occurred in tilapia held at density higher than 11.2 g/l because of direct contact transmission.

Pathobiological study on streptococcosis occurring in farmed tilapia of Thailand indicates clinical and histological findings as have been reported elsewhere. The disease was identified by the conventional microbiological and DNA based techniques. Based on the PCR assay, at least 2 species of streptococcal bacteria, *S. agalactiae* and *S. iniae*, were involved in tilapia streptococcal infection. The genotyping of the etiologic agents showed that *S. agalactiae* was the dominant species that caused streptococcosis in Thai tilapia culture.

Phase 2 : Phylogenetic analysis of *Streptococcus* isolates by specific gene sequence comparison

Phylogenetic system is used to study the evolution of the organism, characterize the members of the bacteria and find out the taxonomic position of the bacterial strains (Alber *et al*., 2004; Sulultana *et al*., 1998). The genes encoding the 16S rRNA have been used for the genetic identification of many bacteria, including streptococci. In addition, gene *sodA* encoding the superoxide dismutase A ($sodA_{int}$ fragment) has been used for the molecular identification of streptococci (Alber *et al*., 2004). Both genes, the 16S rRNA gene and *sodA* gene were analyzed in this study. PCR products obtained from amplification of the 16S rRNA gene and $sodA_{int}$ fragment by specific primers were 1234 and 512 bps. DNA sequence of the PCR products were used for phylogenetic analysis. Yoshiaki *et al*. (1999) supported that a size less than 400 bps was enough to determine the phylogenetic position of a strain.

The taxonomic analysis based on 16S rRNA gene sequences was used to determine phylogenetic relationships among members of the genus *Streptococcus*. For instance, classification of *Streptococcus* with 16S rRNA sequencing was analyzed in *S.phocae* from Atlantic salmond (Romalde *et al*., 2008; Gibello *et al.,* 2005), *S. iniae* from fish (Kvitt and Colorni, 2004), *S. agalactiae* from human and bovine (Sukhnannand *et al*., 2005), the mitis group within the genus *Streptococcus* from human (Kawamura *et al*., 1999). In the present study, the partial 16S rRNA gene sequences were determined in 6 isolates of *S. agalactiae* and 6 isolates of *S. iniae* from diseased tilapia. These sequences were compared with reference strains reported in other animal species, including fish. All 6 strains of *S. agalactiae* exhibited more than 99% DNA similarity with reference strains (other fish strains and human strains) but showed more than 5% divergence to bovine strain and feline strain. Level of 16S rRNA sequence homology of *S. iniae* in all 6 strains presented more than 98% DNA homology with reference strains (other fish strains and human strain). A 100% homology was found in every isolates compared to the corresponding species of the reference strains, *S. agalactiae* strain (ATCC13813) and *S. iniae* strain (ATCC29178). The results clearly demonstrated that 16S rRNA sequencing for classification of both *S. agalactiae* and

S. iniae clinical isolates based on accurate type strain sequences showed nearly 100% DNA similarity. Stackbrandt and Goebel (1994) started that 16S rRNA sequence analysis can be used to determine the phylogenetic divergence of prokaryotic species when the levels of sequence homology are less than 97%. Song *et al*. (2003) explained that the 16S rRNA gene sequence analysis is normally use for bacterial classification and identification owing to its high accuracy. The features of this molecular target (particularly 16S rRNA gene) and variable regions at the presence of species specific were useful and essential task for bacterial phylogenetic analysis and detection of clinical isolates (Weisburg *et al*., 1991).

Alternative target genes, partial *sodA* gene encoding the superoxide dismutase A ($sodA_{int}$ fragment) which has been proven to be useful for molecular identification and phylogenetic analysis of various gram-positive bacteria (Goh *et al*., 1998; Poyart *et al*., 2000). For instance, classification of *Streptococcus* with $sodA_{int}$ fragment was analyzed in *S. dysgalactiae subsp. dysgalactiae* from farmed fish (Nomoto *et al*., 2008), *Streptococcus* isolates from human (Hoshino *et al*., 2005) and *S. agalactiae* (Poyart *et al*., 2001). In addition, the use of species-specific oligonucleotide primers for *sodA* genes to improve future diagnosis of *Streptococcus* species was suggested by Alber *et al*. (2004). In the present study, $sodA_{int}$ fragment amplitication was applied to assign the phylogenetic relationships of *S. agalactiae* and *S. iniae* obtained from Thai diseased tilapia. All strains of *S. agalactiae* presented more than 98.4% DNA similarity with reference strain (ATCC13813, non-hemolytic strain), the DNA similarity may suggest similar charateritics of the testd strains and the reference strain, such as haemolytic. Sequence homology of all tilapia *S. iniae* also showed nearly 100% DNA homology with reference strain (ATCC29178, fish strain). The amplified PCR products of this result were confirmed an internal fragment of the gene encoding manganese-dependent superoxide dismutase (Mn-SOD). Sequences of $sodA_{int}$ fragment from both *S. agalactiae* and *S. iniae* displayed a close relationship within the species. This result could also be confirmed by dendrogram analysis of $sodA_{int}$ fragment sequences of both species and various other streptococci as shown in Figure 4.11 and 4.15. Smith and Doolittle (1992) explained that superoxide dismutases (SODs) were metalloenzymes, of which MnSOD and FeSOD were present in prokaryotes that probably diverged from a common ancestor. MnSOD was useful to characterize the gram-positive bacteria (Nakayama, 1992). Kawamura *et al*. (1999) suggested that the *sodA* partial gene sequencing, particularly, $sodA_{int}$ fragment, was useful for the identification of members of *Streptococcus*.

Comparative analysis of internal parts of the *sodA* gene and 16S rRNA gene of *S. agalactiae* and *S. iniae* from diseased tilapia with corresponding sequences of other *Streptococcus* species appeared that representative strains of *S. agalactiae* or *S. iniae* formed a single cluster that was distinguished clearly from other species of genus *Streptococcus* in phylogenetic tree. Clinical *S. agalactiae* or *S. iniae* strains from different areas did not indicate geographic variation. Kawamura *et al*. (1999) concluded that the evolution rate of the *sodA* gene was much faster than the 16S rRNA gene, and would be useful in differentiating genetically closely related organisms.

In conclusion, Typing of *Streptococcus*-specific PCR products by DNA sequencing revealed that 16S rRNA gene and the *sodA* partial sequence analysis are applicable for genetic classification of the streptococcal bacteria. Phylogenetic tree generated based on 16S rRNA gene and $sodA_{int}$ fragment discriminated *S. agalactiae* or *S. iniae* from other species of genus *Streptococcus*. Sequencing the chromosomal regions of 16S rRNA gene and $sodA_{int}$ fragment suggested that the strains of *S. agalactiae* and *S. iniae* from different areas of tilapia rearing in Thailand did not exhibit geographic variation. Phenotypic and genotypic differences of streptococcal pathogens from tilapia reared in Thailand could be considered for the development of suitable streptococcosis vaccine for tilapia aquaculture.

Phase 3: The development of streptococcosis vaccine

Streptococcus vaccine has been developed and commonly applied to control streptococcosis in aquaculture. The development of vaccines is complicated by the variety of streptococcal species and their antigenicity. Pathobiological study of streptococcosis in Thai tilapia farming indicates that *S. agalactiae* are dominant species causing streptococcosis in cultured tilapia. Sequence analysis of 16S rRNA gene and the $sodA_{int}$ fragment of *S. agalactiae* from different geographic regions of tilapia rearing in Thailand are identical. In the present study, the formalin-killed vaccine developed from whole cell and extracellular product of the local strain *S. agalactiae* was evaluated for its safety and efficacy.

Safety test of vaccine was assessed in healthy tilapia fingerings, survival rate post-vaccination in each group; FKC vaccinated, FKC+ECP vaccinated, placebo vaccinated control and non-vaccinated control was not different ($\alpha = 0.05$). Overall of the experiment, no serious adverse effects were identified thorough 14 days post-vaccination. The prepared vaccines (FKC vaccine and combined FKC+ECP vaccine) were also found no evidence of negative impacts on tilapia performance. The results are in consistency with other studies in fish vaccination, for instance, survival rate after vaccination with streptococcal vaccine showed 91.7% in trout (Akhlaghi *et al*., 1996) and 100% in tilapia (Shelby *et al*., 2002). In the present study, vaccine efficacy was determined on titer of specific agglutinating antibody. Vaccinated fish (FKC vaccinated and FKC+ECP vaccinated) showed increasing agglutinating antibody titers against homologous strain of *S. agalactiae* for 12 weeks post-vaccination and continue high titer levels for 3 months, whereas non-vaccinated maintained a low level titer. The producing time of antibody in the immunized fish could be used to determine the time for vaccination (Pasnik *et al*., 2005). This result is in agreement with those recorded by Akhlaghi *et al*. (1996); Klesius *et al*. (2000) and Shelby *et al*. (2002). They found that antibody titers stimulated active immunity by a single intraperitoneal (IP) injection of *Streptococcus* vaccine were significantly higher than non-vaccinated group in tilapia (Akhlaghi *et al*.,1996; Klesius *et al*., 2000) and trout (Shelby *et al*., 2002). Agglutination reactions between homologous and heterologous strains of *S. agalactiae* were indifferent. The results of immunological analysis were correlated with molecular characterization of *S. agalactiae* as described previously.

The efficacy of vaccine was also proven by an intraperitoneal challenge and detection of antibody level following a challenge on vaccinated group. Nordmo and Ramstad (1997) indicated that the determination of vaccine efficacy with challenge by an intraperitoneal injection was a reproducible and reliable means because each individual fish received certainly a uniform challenges. Tilapia vaccinated with FKC vaccine and FKC+ECP vaccine were challenged by intraperitoneal route at 10 weeks post-vaccination. The fish were protected against lethal challenge with the virulence of *S. agalactiae* strains ($3X10^8$ CFU per fish). Survival rates of non-vaccinated fish (0-10%) were significantly lower than FKC vaccinated group (90%) and FKC+ECP vaccinated group (67%) ($\alpha = 0.01$), and survival rates of both vaccinated groups were not different. Many published reports indicated that injectable *Streptococcus* formalin-killed vaccine by intraperitoneal route provided a high survival rate in tilapia and other species of fish. For instance, FKC and combined FKC+ECP vaccines of *S. agalactiae* and *S. iniae* increased the survival of tilapia and stimulated systemically immunity (Evans *et al*., 2004). Combined FKC+ECP vaccine from of *S. iniae* showed 90% survival rates after challenge (Klesius *et al*., 1999). Vaccine produced from formalin-killed *S.difficile* and its protein extract efficiently protected tilapia against a lethal challenge (Eldar *et al*., 1995). Formalin killed cells with and without adjuvant vaccine against β-hemolytic streptococcal disease showed 89% protection in rainbow trout after challenge with live bacteria (Akhlaghi *et al*., 1996). The present study indicated that anti - *S. agalactiae* antibody titers stimulated with a single intraperitoneal injection of FKC vaccine or combined FKC+ECP vaccine were higher than non-vaccinated serum significantly ($p<0.05$) at 21st day post-challenge. It was also found that the vaccine had a therapeutic effect to protect tilapia against subsequent pathogen challenge for at least 10 weeks post-vaccination. For the kill whole vaccine, it may be necessary for time period of a therapeutic vaccination effect to stimulate a response and can clear the infection (Evans *et al*., 2006; Rhodes *et al*., 2004). Agglutinating antibody titer of survivors from vaccinated and non-vaccinated tilapia evaluated post-challenge were higher than prior to challenge. This phenomenon was explained by Shelby *et al*. (2002) that tilapia could response with a primary and secondary anti-streptococcal antibody response after active immunization and challenge with virulent *Streptococcus*. The secondary response was significantly higher than the primary antibody response and coincided with immunity to *Streptococcus*.

The fact that fish have adaptive and innate cellular immune defenses that is not unlike those of the mammalian species. Fish have immunoglobins, antigen-processing cells, T cells and B cells as well as complement and phagocytic cells and leucocytes. The predominant immunoglobulin in blood is IgM produced from immunological organs, including thymus, the anterior part of kidney and spleen. A secondary antibody response in fish shows enhanced antibody titers and accelerated antibody responses, its immunological memory to control infectious disease (Press and Lillehaug, 1995). Thus, immunization with *S. agalactiae* vaccine alone resulted in the enhancement of specific antibodies (as protective antibody) and protection of Nile tilapia against *S. agalactiae* infection. Vaccine efficacy can antagonize bacterial replication or eliminate bacteria thereby reducing mortality (Evans *et al*.,2006; Pasnik *et al*., 2005; Sako, 1998) and decreasing of disease outbreak (Pasnik *et al*., 2005). By an injectable vaccination, significant protection is achieved by actively

immunizing fish, which could induce humoral mediated responses, cell mediated responses and possibly non-specific responses of fish to the immunogen components of bacterial cells (Kakuta *et al*., 2004). The protective antibody in the serum acts as opsonins and antibacterial factors may act in conjunction with complement in a direct bactericidal response or aid phagocytes in their ability to engulf and kill *Streptococcus* (Shelby *et al*., 2002; Shutou *et al*., 2007).

Although the comparative efficacy between FKC and combined FKC+ECP vaccine showed that the FKC+ECP vaccinated fish had lower survival rates than the FKC vaccinated fish, the ECP generally recognized as soluble antigenic factors produced from pathogens. Klesius *et al*. (2000) suggest that protective antibody response is dependent on the antigenic composition of the *Streptococcus* used to prepare the vaccine. Extracellular products (ECP) from *Streptococcus* were important virulence factors of fish pathogens (Pasnik *et al*., 2005). For example, ECP produced from *Streptococcus* was selected at 30 KDa of *S. iniae* and 20 KDa of *S. agalactiae*. Many reports supported that components of ECP from *Streptococcus* were therapeutic or prophylactic vaccines against *Streptococcus* by the stimulation of both innate and acquired immune response because ECP was able to stimulate phagocytic activity following macrophage receptors recognition of ECP (Evans *et al*., 2006) and to provide antibody against challenge with virulent pathogen after inoculation (Eldar *et al*., 1995; Evans *et al*., 2006; Pasnik *et al*., 2005).

Formalin killed cell vaccine against *S. agalactiae* administered by intraperitoneal injection provided sustained protection for at least 10 weeks post-vaccination. The immunization protected the vaccinated tilapia from a lethal dose challenge of streptococcal pathogens and a concurrent secondary response was apparently over the primary response to the vaccine, thus, indicating the role of memory and recognition of the vertebrate immune system in tilapia. The injectable vaccine appeared to be a potential measurement for disease control in Thai tilapia aquaculture.

CHAPTER VI

CONCLUSION

Streptococcosis has been well recognized with the intensification of aquaculture and has economic consequences on fisheries in many areas of the world. Different species of streptococcal bacteria are pathogenic to fish, however *S. iniae* has been reported as a significant contributor to the damage of aquacultured fish. Due to the commercial growth of tilapia culture in Thailand, streptococcal infections are becoming a major threat to Thai tilapia industry. Study has indicated that streptococcosis causes production loss in all regions of tilapia farming in Thailand. The subclinical infections result in a retarded growth rate and poor meat quality yield, while mortality up to 75% is evident in acute cases.

Pathobiological study on streptococcosis occurring in farmed tilapia of Thailand indicates clinical and histological findings as have been reported elsewhere. The disease was identified by the conventional microbiological and DNA based techniques. Based on the PCR assay, at least 2 species of streptococcal bacteria, *S. agalactiae* and *S. iniae*, were involved in tilapia streptococcal infection. The genotyping of the etiologic agents showed that *S. agalactiae* was the dominant species that caused streptococcosis in Thai tilapia culture.

Typing of streptococcus-specific PCR products by DNA sequencing revealed that 16S rRNA gene and the *sodA* partial sequence analysis are applicable for genetic classification of the streptococcal bacteria. Phylogenetic tree generated based on 16S rRNA gene and $sodA_{int}$ fragment discriminated *S. agalactiae* or *S. iniae* from other species of genus streptococcus. Sequencing the chromosomal regions of 16S rRNA gene and $sodA_{int}$ fragment suggested that the strains of *S. agalactiae* and *S. iniae* from different areas of tilapia rearing in Thailand did not exhibit geographic variation.

Formalin killed cell vaccine against *S. agalactiae* administered by intraperitoneal injection provided sustained protection for at least 10 weeks post-vaccination. The immunization protected the vaccinated tilapia from a lethal dose challenge of streptococcal pathogens; and a concurrent secondary response was apparently over the primary response to the vaccine, thus, indicating the role of memory and recognition of the vertebrate immune system in tilapia. In addition to the vaccine efficacy, the developed vaccine was shown safe in clinically healthy juvenile tilapia. The intraperitoneal injectable vaccine did not induce any observed histopathological effect to the vaccinated fish, and appeared to be a potential measurement for streptococcosis control in Thai tilapia aquaculture. However, further studies may resolve the time kinetics of protective antibody response in tilapia fingering following an intraperitoneal vaccination to allow an appropriate vaccination programme for tilapia rearing.

This investigation documented the first prevalence of streptococcosis in tilapia culture in Thailand whereby the genotyping of the etiologic agents showed that *S. agalactiae* was the dominant species that caused streptococcosis in Thai tilapia culture. Phylogenetic analysis to characterize the member of bacteria and their taxonomic portion revealed high sequence similarity ($\geq$ 97%) with the corresponding portion of fish pathogens, *S. agalactiae* and *S. iniae* genome. Streptococcosis vaccine developed from formalin killed whole cell was demonstrated for its efficacy in protection against streptococcosis. Active stimulation of specific antibody titers were evidently sustainable for at least 10 weeks following a single intraperitoneal vaccination. The immunized tilapia were shown to possess protective antibody level with a lethal dose challenge of streptococcal pathogens. Whilst the current control of streptococcosis in farmed tilapia is limited to antimicrobial treatments, the success in active immunization against streptococcosis in the present study elucidates an alternative mean to control this disease in Thai tilapia culture.

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APPENDICES

APPENDIX A

Equipments and Chemicals

Equipments

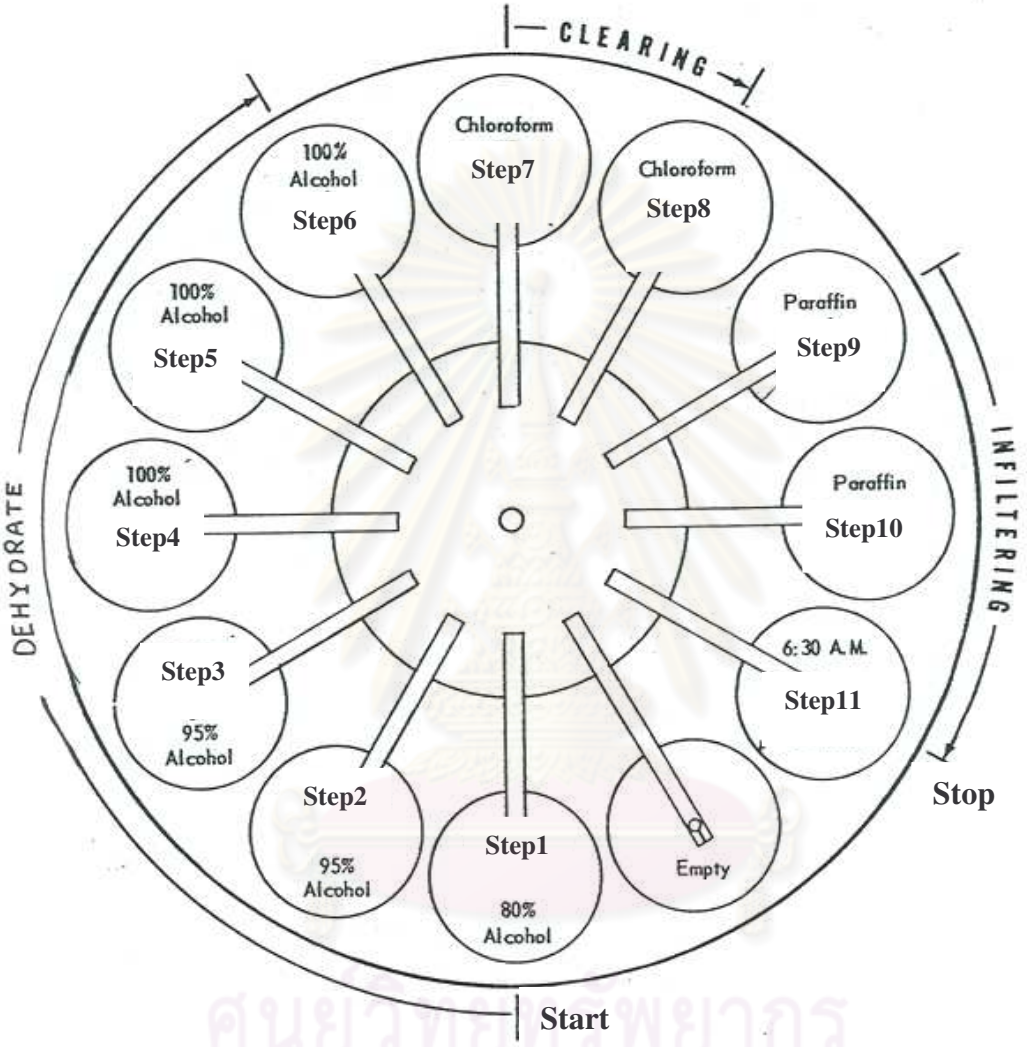
- Incubator (SANYO ELECTRIC, Japan), Shaking incubator (BIOSAN, Latvia), Centrifuge (IEC, USA)
- Vortex mixer Geinie 2 (Scientific Industries, USA)
- PCR thermalcycler (Perkin Elmer), Electrophoresis (Bio-Rad, USA), UV transilluminator
- Analytical Profile Identification (API) 20 STREP (Biomerieux, France)
- McFarland standard : 0.5, 4 McFarland
- Qiagen DNeasy Tissue kit (Qiagen Valencia, UK)
- Volumetric flask, Beaker, Microtube, PCR tube with domed cap, Micropipette
- Petridish, 96-well microtiter plates

Chemicals

- Tryptic Soy Agar (TSA) (Oxoid, England)
- Sodium chloride (NaCl) (Merck, Germany)
- Gram's stain : Crystal-violet, Lugol solution (Gram's iodine), Decolorizer (Ethyl alcohol 70%) and Safranin solution
- Hydrogen peroxide (H_2O_2) 3%
- Tag polymerase, dNTP and PCR buffer (iNtRON Biotechnology, USA)
- Primer (Sigma-Genosys, Singapore)
- Loading dye and DNA ladder (SibEnzyme, Russia)
- Agarose gel (Molecular Biology Grade) (Research organics, USA)
- Ethidium bromide (Sigma Aldrich Inc., USA)
- Absolute ethanol (Merck, Germany)
- Tris base (Merck, Germany)
- 0.5 M EDTA, pH 8.0 (GIBCO®, USA)
- Lysozyme (Bio Basic Inc., Canada)
- Proteinase K (Roche, USA)
- 10X TBE buffer (Bio Basic Inc., Canada)
- Anesthesia reagent (AquaNes®)

APPENDIX B

Processing of tissues preparation for histology, including of dehydration, cleaning and infiltering (Modified from Gridley, 1949)



APPENDIX C

Procedures of DNA extraction with DNA-binding spin column
(NucleoSpin® Extract I, Germany)

Day 1

Subculture streptococcus in Tryptic Soy broth (TSB)

↓ Incubate for 18-24 hours at 33°C in shaking incubator at 100 RPM

↓ Centrifuge for 15 mins at 15,000 g and Discard supernatant (TSB)

Wash streptococcal cell with normal saline solution (NSS) 3 times

Day 2

↓

Adjust density of streptococcal cell at 0.5 McFarland (approximately 10^8 cell/ml)

↓ Centrifuge for 15 mins at 15,000 g and Discard supernatant

Add 180 µl of *Buffer T1* and 25 µl of *Proteinase K*

↓ Mix and Incubate for 3 hours at 56°C in shaking incubator at 100 RPM

Add 200 µl of *Buffer B3*

↓ Mix and Incubate for 10 mins at 70°C

Add 210 µl of *Absolute Ethanol*

↓ Mix and Transfer to column Centrifuge for 1 minute at 11,000 g and Discard solution

Add 500 µl of *Buffer BW*

↓ Centrifuge for 1 minute at 11,000 g and Discard solution

Add 600 µl of *Buffer B5*

↓ Centrifuge for 1 minute at 11,000 g and Discard solution

Transfer DNA-binding column to new microtube

↓

Add 100 µl of *Buffer BE* (warm buffer at 70°C before use)

↓ Incubate for 1 minute at room temperature and Centrifuge for 1 minute at 11,000 g

Store bacterial DNA at -20°C for PCR identification

APPENDIX D

PCR primer design

PCR primer design for 16S rRNA gene of *S.agalactiae* and *S.iniae*

1. The full length of *S.agalactiae* and *S.iniae* 16S rRNA gene (approximately 1450 to 1530 bp) obtained from GenBank nucleotide database (Accession no. of *S.agalactiae* e.g. AB002479, AF015927, EF092913, DQ985468 and *S.iniae* e.g. AF335572, AY762259, EU075069, respectively) was aligned all of the sequences and reported consensus sequence. The red label showed the variable region of 16S rRNA gene between *S.agalactiae* and *S.iniae.*

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                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                     5          15         25         35         45         55
EU075069        AGAGTTTGAA TCNTGGCTCA GGACGAACGC TGGCGGCGTG CCTAATACAT GCAAGTAGAA
AB002479        ---------- ---------- ---------- ---------- ---------- ------AGAA
AF015927        ---------- ---------- -GACGAACGC TGGCGGCGTG CCTAATACAT GCAAGTAGAA
EF092913        ---------- ---------- ---------- ---------- -GCTATACAT GCA-GTAGAA
DQ985468        ---------- ---------- ---------- ---------- ---------- -------GAA
AF335572        -AGAGTTTGA TCCTGGCTCA GGACGAACGC TGGCGGCGTG CCTAATACAT GCAAGTAGAA
AY762259        ---------- ---------- ---------- ---------- -CTAATACAT GCAAGTAGAA
Clustal Co                                                                    ***
Consensus       ARRRKTTKRA TCCTGGCTCA GGACGAACGC TGGCGGCGTG CSYWATACAT GCAAGTAGAA

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                     65         75         85         95         105        115
EU075069        CGCTGATGTT TGGTGTTTAC ACTAGA--CT GATGAGTTGC GAACGGGTGA GTAACGCGTA
AB002479        CGC--GAGGT TGGTGTACCA CCTAGATCCT GATGAGTTGC GAACGGGTGA GTAACGCGTA
AF015927        CGCTGAGGTT TGGTGTTTAC ACTAGA--CT GATGAGTTGC GAACGGGTGA GTAACGCGTA
EF092913        CGCTGAGGTT TGGTGTTTAC ACTAGA--CT GATGAGTTGC GAACGGGTGA GTAACGCGTA
DQ985468        CGCTGAGGAT TGGTGCTTGC ACTAAT--CC AAAGAGTTGC GAACGGGTGA GTAACGCGTA
AF335572        CGCTGAGGAT TGGTGCTTGC ACTAAT--CC AAAGAGTTGC GAACGGGTGA GTAACGCGTA
AY762259        CGCTGAGGAT TGGTGCTTGC ACTAAT--CC AAAGAGTTGC GAACGGGTGA GTAACGCGTA
Clustal Co      ***    * * *****       ***    *   * ******* ********** **********
Consensus       CGCTGRDGDT TGGTGYWYVM MCTARWTCCY RAWGAGTTGC GAACGGGTGA GTAACGCGTA

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   125        135        145        155        165        175
EU075069        GGTAACCTGC CTCATAGCGG GGGATAACTA TTGGAAACGA TAGCTAATAC CGCATAAGAG
AB002479        GGTAGCCTGC CTCATAGCGG GGGATAACTA TTGGAAACGA TAGCTAATAC CGCATAAGAG
AF015927        GGTAACCTGC CTCATAGCGG GGGATAACTA TTGGAAACGA TAGCTAATAC CGCATAAGAG
EF092913        GGTAACCTGC CTCATAGCGG GGGATAACTA TTGGAAACGA TAGCTAATAC CGCATAAGAG
DQ985468        GGTAACCTAC CTCATAGCGG GGGATAACTA TTGGAAACGA TAGCTAATAC CGCATGANAC
AF335572        GGTAACCTAC CTCATAGCGG GGGATAACTA TTGGAAACGA TAGCTAATAC CGCATGACAC
AY762259        GGTAACCTAC CTCATAGCGG GGGATAACTA TTGGAAACGA TAGCTAATAC CGCATGAYAC
Clustal Co      **** *** * ********** ********** ********** ********** ***** * *
Consensus       GGTARCCTRC CTCATAGCGG GGGATAACTA TTGGAAACGA TAGCTAATAC CGCATRASAS

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   185        195        205        215        225        235
EU075069        TAATTAACAC ATGTTAGTTA TTTAAAAGGA GCAATTGCTT CACTGTGAGA TGGACCTGCG
AB002479        TAATTAACAC ATGTTAGTTA TTTAAAAGGA GCAATTGCTT CACTGTGAGA TGGACCTGCG
AF015927        TAATTAACAC ATGTTGGTTA TTTAAAAGGA GCAATTGCTT CACTGTGAGA TGGACCTGCG
EF092913        TAATTAACAC ATGTTAGTTA TTTAAAAGGA GCAATTGCTT CACTGTGAGA TGGACCTGCG
DQ985468        TAGAGTACAC ATGTACTTAA GTTAAAAGGA GCAANNGCTT CACTATGAGA TGGACCTGCG
AF335572        TAGAGTACAC ATGTACTTAA TTTAAAAGGA GCAATTGCTT CACTATGAGA TGGACCTGCG
AY762259        TAGAGTACAC ATGTACTTAA GTTAAAAGGA GCAATTGCTT CACTATGAGA TGGACCTGCG
Clustal Co      **    **** ****   * *  ********* ****  **** **** ***** **********
Consensus       TARWKWACAC ATGTWVKTWA KTTAAAAGGA GCAATTGCTT CACTRTGAGA TGGACCTGCG
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                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   245         255         265         275         285         295
EU075069        TTGTATTAGC TAGTTGGTGA GGTAAAGGCT CACCAAGGCG ACGATACATA GCCGACCTGA
AB002479        TTGTATTAGC TAGTTGGTGA GGTAAAGGCT CACCAAGGCG ACGATACATA GCCGACCTGA
AF015927        TTGTATTAGC TAGTTGGTGA GGTAAAGGCT CACCAAGGCG ACGATACATA GCCGACCTGA
EF092913        TTGTATTAGC TAGTTGGTGA GGTAAAGGCT CACCAAGGCG ACGATACATA GCCGACCTGA
DQ985468        TTGTATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCG ACGATACATA GCCGACCTGA
AF335572        TTGTATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCG ACGATACATA GCCGACCTGA
AY762259        TTGTATTAGC TAGTTGGTGA GGTAACGGCT CACCAAGGCG ACGATACATA GCCGACCTGA
Clustal Co      ********** ********** ***** **** ********** ********** **********
Consensus       TTGTATTAGC TAGTTGGTGA GGTAAMGGCT CACCAAGGCG ACGATACATA GCCGACCTGA

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
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EU075069        GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
AB002479        GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
AF015927        GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
EF092913        GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
DQ985468        GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
AF335572        GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
AY762259        GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       GAGGGTGATC GGCCACACTG GGACTGAGAC ACGGCCCAGA CTCCTACGGG AGGCAGCAGT

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
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EU075069        AGGGAATCTT CGGCAATGGA CGGAAGTCTG ACCGAGCAAC GCCGCGTGAG TGAAGAAGGT
AB002479        AGGGAATCTT CGGCAATGGA CGGAAGTCTG ACCGAGCAAC GCCGCGTGAG TGAAGAAGGT
AF015927        AGGGAATCTT CGGCAATGGA CGGAAGTCTG ACCGAGCAAC GCCGCGTGAG TGAAGAAGGT
EF092913        AGGGAATCTT CGGCAATGGA CGGAAGTCTG ACCGAGCAAC GCCGCGTGAG TGAAGAAGGT
DQ985468        AGGGAATCTT CGGCAATGGA CGGAAGTCTG ACCGAGCAAC GCCGCGTGAG TGAAGAAGGT
AF335572        AGGGAATCTT CGGCAATGGA CGGAAGTCTG ACCGAGCAAC GCCGCGTGAG TGAAGAAGGT
AY762259        AGGGAATCTT CGGCAATGGA CGGAAGTCTG ACCGAGCAAC GCCGCGTGAG TGAAGAAGGT
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       AGGGAATCTT CGGCAATGGA CGGAAGTCTG ACCGAGCAAC GCCGCGTGAG TGAAGAAGGT

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
                   425         435         445         455         465         475
EU075069        TTTCGGATCG TAAAGCTCTG TTGTTAGAGA AGAACGTTGC TAGGAGTGGA AAATCTACCA
AB002479        TTTCGGATCG TAAAGCTCTG TTGTTAGAGA AGAACGTTGG TAGGAGTGGA AAATCTACCA
AF015927        TTTCGGATCG TAAAGCTCTG TTGTTAGAGA AGAACGTTGG TAGGAGTGGA AAATCTACCA
EF092913        TTTCGGATCG TAAAGCTCTG TTGTTAGAGA AGAACGTTGG TAGGAGTGGA AAATCTACCA
DQ985468        TTTCGGATCG TAAAGCTCTG TTGTTAGAGA AGAACGGTAA TGGGAGTGGA AAATCCATTA
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AY762259        TTTCGGATCG TAAAGCTCTG TTGTTAGAGA AGAACGGTAA TGGGAGTGGA AAATCCATTA
Clustal Co      ********** ********** ********** ****** *   * ******** ***** *  *
Consensus       TTTCGGATCG TAAAGCTCTG TTGTTAGAGA AGAACGKTRR TRGGAGTGGA AAATCYAYYA

                ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|
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DQ985468        CGTGACGGTA ACTAACCAGA AAGGGACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG
AF335572        CGTGACGGTA ACTAACCAGA AAGGGACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG
AY762259        CGTGACGGTA ACTAACCAGA AAGGGACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG
Clustal Co       ********* ********** ********** ********** ********** **********
Consensus       MGTGACGGTA ACTAACCAGA AAGGGACGGC TAACTACGTG CCAGCAGCCG CGGTAATACG
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   545        555        565        575        585        595
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AB002479        TAGGTCCCGA GCGTTGTCCG GATTTATTGG GCGTAAAGCG AGCGCAGGCG GTTCTTTAAG
AF015927        TAGGTCCCGA GCGTTGTCCG GATTTATTGG GCGTAAAGCG AGCGCAGGCG GTTCTTTAAG
EF092913        TAGGTCCCGA GCGTTGTCCG GATTTATTGG GCGTAAAGCG AGCGCAGGCG GTTCTTTAAG
DQ985468        TAGGTCTCGA GCGTTGTCCG GATTTATTGG GCGTAAAGCG AGCGCAGGCG GTTCTATAAG
AF335572        TAGGTCTCGA GCGTTGTCCG GATTTATTGG GCGTAAAGCG AGCGCAGGCG GTTCTATAAG
AY762259        TAGGTCTCGA GCGTTGTCCG GATTTATTGG GCGTAAAGCG AGCGCAGGCG GTTCTATAAG
Clustal Co      ****** *** ********** ********** ********** ********** ***** ****
Consensus       TAGGTCYCGA GCGTTGTCCG GATTTATTGG GCGTAAAGCG AGCGCAGGCG GTTCTWTAAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
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AB002479        TCTGAAGTTA AAGGCAGTGG CTTAACCATT GTACGCTTTG GAAACTGGAG GACTTGAGTG
AF015927        TCTGAAGTTA AAGGCAGTGG CTTAACCATT GTACGCTTTG GAAACTGGAG GACTTGAGTG
EF092913        TCTGAAGTTA AAGGCAGTGG CTTAACCATT GTACGCTTTG GAAACTGGAG GACTTGAGTG
DQ985468        TCTGAAGTAA AAGGCAGTGG CTCAACCATT GTATGCTTTG GAAACTGTAG AACTTGAGTG
AF335572        TCTGAAGTAA AAGGCAGTGG CTCAACCATT GTATGCTTTG GAAACTGTAG AACTTGAGTG
AY762259        TCTGAAGTAA AAGGCAGTGG CTCAACCATT GTATGCTTTG GAAACTGTAG AACTTGAGTG
Clustal Co      ******** * ********** ** ******* *** ****** ******* **  *********
Consensus       TCTGAAGTWA AAGGCAGTGG CTYAACCATT GTAYGCTTTG GAAACTGKAG RACTTGAGTG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   665        675        685        695        705        715
EU075069        CAGAAGGGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGATATA TGGAGGAACA
AB002479        CAGAAGGGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGATATA TGGAGGAACA
AF015927        CAGAAGGGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGATATA TGGAGGAACA
EF092913        CAGAAGGGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGATATA TGGAGGAACA
DQ985468        CAGAAGGGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGATATA TGGAGGAACA
AF335572        CAGAAGGGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGATATA TGGAGGAACA
AY762259        CAGAAGGGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGATATA TGGAGGAACA
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       CAGAAGGGGA GAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGATATA TGGAGGAACA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   725        735        745        755        765        775
EU075069        CCGGTGGCGA AAGCGGCTCT CTGGTCTGTA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG
AB002479        CCGGTGGCGA AAGCGGCTCT CTGGTCTGTA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG
AF015927        CCGGTGGCGA AAGCGGCTCT CTGGTCTGTA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG
EF092913        CCGGTGGCGA AAGCGGCTCT CTGGTCTGTA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG
DQ985468        CCGGTGGCGA AAGCGGCTCT CTGGTCTGTA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG
AF335572        CCGGTGGCGA AAGCGGCTCT CTGGTCTGTA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG
AY762259        CCGGTGGCGA AAGCGGCTCT CTGGTCTGTA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       CCGGTGGCGA AAGCGGCTCT CTGGTCTGTA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   785        795        805        815        825        835
EU075069        CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA GGTGTTAGGC
AB002479        CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA GGTGTTAGGC
AF015927        CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA GGTGTTAGGC
EF092913        CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA GGTGTTAGGC
DQ985468        CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA GGTGTTAGGC
AF335572        CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA GGTGTTAGGC
AY762259        CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA GGTGTTAGGC
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA GGTGTTAGGC
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   845        855        865        875        885        895
EU075069        CCTTTCCGGG GCTTAGTGCC GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGACC
AB002479        CCTTTCCGGG CCTTAGTGCC GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGACC
AF015927        CCTTTCCGGG GCTTAGTGCC GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGACC
EF092913        CCTTTCCGGG GCTTAGTGCC GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGACC
DQ985468        CCTTTCCGGG GCTTAGTGCC GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGACC
AF335572        CCTTTCCGGG GCTTAGTGCC GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGACC
AY762259        CCTTTCCGGG GCTTAGTGCC GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGACC
Clustal Co      **********   ******** ********** ********** ********** **********
Consensus       CCTTTCCGGG SSTTAGTGCC GCAGCTAACG CATTAAGCAC TCCGCCTGGG GAGTACGACC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   905        915        925        935        945        955
EU075069        GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT
AB002479        GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT
AF015927        GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT
EF092913        GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT
DQ985468        GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT
AF335572        GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT
AY762259        GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       GCAAGGTTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   965        975        985        995       1005       1015
EU075069        AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCTTCTGACC GGCCTAGAGA
AB002479        AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCTTCTGACC GGCCTAGAGA
AF015927        AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCTTCTGACC GGCCTAGAGA
EF092913        AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCTTCTGACC GGCCTAGAGA
DQ985468        AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCCTCTGACC GTCCTAGAGA
AF335572        AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCCTCTGACC GTCCTAGAGA
AY762259        AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCCTCTGACC GTCCTAGAGA
Clustal Co      ********** ********** ********** ********** ** ******* * ********
Consensus       AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCYTCTGACC GKCCTAGAGA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  1025       1035       1045       1055       1065       1075
EU075069        TAGGCTTTCT CTTCGGAGCA GAAGTGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG
AB002479        TAGGCTTTCT CTTCGGAGCA GAAGTGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG
AF015927        TAGGCTTTCT CTTCGGAGCA GAAGTGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG
EF092913        TAGGCTTTCT CTTCGGAGCA GAAGTGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG
DQ985468        TAGGATTTTC CTTCGGGACA GAGGAGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG
AF335572        TAGGATTTTC CTTCGGGACA GAGGAGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG
AY762259        TAGGATTTTC CTTCGGGACA GAGGAGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG
Clustal Co      **** ***   ******  ** ** * ***** ********** ********** **********
Consensus       TAGGMTTTYY CTTCGGRRCA GARGWGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  1085       1095       1105       1115       1125       1135
EU075069        TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTAA
AB002479        TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTAA
AF015927        TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTAA
EF092913        TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTAA
DQ985468        TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTAA
AF335572        TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTAA
AY762259        TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTAA
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCCTA TTGTTAGTTG CCATCATTAA
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   1145       1155       1165       1175       1185       1195
EU075069        GTTGGGCACT CTAGCGAGAC TGCCGGTAAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA
AB002479        GTTGGGCACT CTAGCGAGAC TACCGGTAAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA
AF015927        GTTGGGCACT CTAGCGAGAC TGCCGGTAAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA
EF092913        GTTGGGCACT CTAGCGAGAC TGCCGGTAAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA
DQ985468        GTTGGGCACT CTAGCGAGAC TGCCGGTAAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA
AF335572        GTTGGGCACT CTAGCGAGAC TGCCGGTAAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA
AY762259        GTTGGGCACT CTAGCGAGAC TGCCGGTAAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA
Clustal Co      ********** ********** * ******** ********** ********** **********
Consensus       GTTGGGCACT CTAGCGAGAC TRCCGGTAAT AAACCGGAGG AAGGTGGGGA TGACGTCAAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   1205       1215       1225       1235       1245       1255
EU075069        TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA ATGGTTGGTA CAACGAGTCG
AB002479        TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTAGA ATGGTTGGTA CAACGAGTCG
AF015927        TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA ATGGTTGGTA CAACGAGTCG
EF092913        TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA ATGGTTGGTA CAACGAGTCG
DQ985468        TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA ATGGTTGGTA CAACGAGTCG
AF335572        TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA ATGGTTGGTA CAACGAGTCG
AY762259        TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTACA ATGGTTGGTA CAACGAGTCG
Clustal Co      ********** ********** ********** ******** * ********** **********
Consensus       TCATCATGCC CCTTATGACC TGGGCTACAC ACGTGCTASA ATGGTTGGTA CAACGAGTCG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   1265       1275       1285       1295       1305       1315
EU075069        CAAGCCGGTG ACGGCAAGCT AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA
AB002479        CAAGCCGGTG ACGGCAAGCT AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA
AF015927        CAAGCCGGTG ACGGCAAGCT AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA
EF092913        CAAGCCGGTG ACGGCAAGCT AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA
DQ985468        CAAGCCGGTG ACGGCAAGCT AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA
AF335572        CAAGCCGGTG ACGGCAAGCT AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA
AY762259        CAAGCCGGTG ACGGCAAGCT AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       CAAGCCGGTG ACGGCAAGCT AATCTCTTAA AGCCAATCTC AGTTCGGATT GTAGGCTGCA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   1325       1335       1345       1355       1365       1375
EU075069        ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGGATAC
AB002479        ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGAATAC
AF015927        ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGAATAC
EF092913        ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGAATAC
DQ985468        ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGAATAC
AF335572        ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGAATAC
AY762259        ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGAATAC
Clustal Co      ********** ********** ********** ********** ********** ***** ****
Consensus       ACTCGCCTAC ATGAAGTCGG AATCGCTAGT AATCGCGGAT CAGCACGCCG CGGTGRATAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   1385       1395       1405       1415       1425       1435
EU075069        GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
AB002479        GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
AF015927        GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
EF092913        GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
DQ985468        GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
AF335572        GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
AY762259        GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
Clustal Co      ********** ********** ********** ********** ********** **********
Consensus       GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   1445       1455       1465       1475       1485       1495
EU075069        GTGAGGTAAC C--------- ---------- ---------- ---------- ----------
AB002479        GTGAGGTAAC CTTTTAGGAG CCAGCCGCCT AAGGTGGGAT AGATGATTGG GGTGAAGTCG
AF015927        GTGAGGTAAC CTTTTAGGAG CCAGCCGCCT AAGGTGGGAT AGATGATTGG GGTGA-----
EF092913        GTGAGGTAAC CTTTTAGGAG CCAGCCGCCT AAGGTGGGAT AGATGATTGG GGTGAAGTCG
DQ985468        GTGAGGTAAC CTTTTAGGAG CCAGCCGCCT AAGGTGGGAT AGATGATTGG GGTGAAGTCG
AF335572        GTGAGGTAAC CTTTTAGGAG CCAGCCGCCT AAGGTGGGAT AGATGATTGG GGTGAAGTCG
AY762259        GTGAGGTAAC CTTTTAGGAG CCAGCCGCCT AAGGTGGGAT AGATGATTGG GGTGAAGTCG
Clustal Co      ********** *
Consensus       GTGAGGTAAC CTTTTAGGAG CCAGCCGCCT AAGGTGGGAT AGATGATTGG GGTGAAGTCG
                ....|....| ....|....| ....|....| ....|....
                   1505       1515       1525       1535
EU075069        ---------- ---------- ---------- ---------
AB002479        TAACAAGG-- ---------- ---------- ---------
AF015927        ---------- ---------- ---------- ---------
EF092913        T--------- ---------- ---------- ---------
DQ985468        -AACAAG--- ---------- ---------- ---------
AF335572        TAACAAGGTA GCCGTATCGG AAGCTGCGGC TGGATCACC
AY762259        TAACAAGGTA GCCGTATCGG AAGGTGCGG- ---------
Clustal Co
Consensus       TAACAAGGTA GCCGTATCGG AAGSTGCGGC TGGATCACC
```

2. The consensus sequence used to generate the primers with Primer3 program (available at http://frodo.wi.mit.edu/primer3). Many designed pairs of primer were selected specifically for amplification of 16S rRNA gene both *S.agalactiae* and *S.iniae* to use in the present study that considered in product size (cover mainly 16S rRNA gene), primer length (18-30 nucleotide in length), GC content (within 30-80%), annealing and melting temperature (Tm) (within 58°C to 60°C), numerous bases at the 3 prime end (no more than two G's or C's). The selected primer for this study had 1234 bps of product size, 20 bps of length both forward and reverse primer, 50-55% of GC content, 60°C of Tm and 3 bases and 2 bases at the 3 prime end of forward and reverse primer.

3. The selected primer was double checked specificity to 16S rRNA of *S.agalactiae* and *S.iniae* by NCBI BLAST that showed 99-100% of specificity.

```
                start  len    tm    gc%   any   3' seq

1 LEFT PRIMER     45   20   60.19  55.00 7.00  3.00 AACGGGTGAGTAACGCGTAG
  RIGHT PRIMER  1278   20   60.01  50.00 4.00  2.00 TTCATGTAGGCGAGTTGCAG
  PRODUCT SIZE: 1234, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00
```

PCR primer design for *sodA* gene of *S.agalactiae* and *S.iniae*

The *sodA gene* of *S.agalactiae* and *S.iniae* was designed two subregions, including whole *sodA* gene and internal part of *sodA* gene associated with Manganese (Mn)-dependent superoxide dismutase.

Whole *sodA* gene

1. The full length of *S.agalactiae* and *S.iniae sodA* gene (approximately 600 to 3103 bps) obtained from GenBank nucleotide database (Accession no. of *S.agalactiae* Y12224 and *S.iniae* EU661272) was aligned all of the sequences and reported consensus sequence. The red label showed the variable region of 16S rRNA gene between *S.agalactiae* and *S.iniae.*

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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1860       1870       1880       1890       1900
Y12224           GATTTCGACA TGGCAATTAT TTTACCAGAT TTACCCTATG CATATGATGC
EU661272         ---------A TGGCTATTAT TTTACCAGAA CTTCCATATG CATATGATGC
Clustal Co                * **** ***** *********   * ** **** **********
Consensus        GATTTCGACA TGGCWATTAT TTTACCAGAW YTWCCMTATG CATATGATGC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1910       1920       1930       1940       1950
Y12224           GCTTGAGCCA CATATTGATG CTGAGACAAT GACACTACAT CATGATAAGC
EU661272         TTTAGAGCCA CAATTTGATC AAGAAACAAT GACACTTCAT CATGATAAAC
Clustal Co         * ****** **  *****    ** ***** ****** *** ******** *
Consensus        KYTWGAGCCA CAWWTTGATS MWGARACAAT GACACTWCAT CATGATAARC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         1960       1970       1980       1990       2000
Y12224           ACCATGCAAC TTATGTTGCT AATGCAAATG CTGCTCTTGA CAAACATCCT
EU661272         ACCATGCGAC TTATGTTGCT AATGCTAATG CTGCCTTAGA AAAACAGCCA
Clustal Co       ******* ** ********** ***** **** ****  * **  ***** **
Consensus        ACCATGCRAC TTATGTTGCT AATGCWAATG CTGCYYTWGA RAAACAYCCW

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2010       2020       2030       2040       2050
Y12224           GAAATTGGAG AAGACTTAGA GGCGCTCTTA GCTGATGTTT CTCAAATTCC
EU661272         GAAATTGGTG AAAACTTAGA GGAGCTCTTG GCAAATGTTG AGTCTATTCC
Clustal Co       ******** * ** ******* ** ******  **  *****       *****
Consensus        GAAATTGGWG AARACTTAGA GGMGCTCTTR GCWRATGTTK MKYMWATTCC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2060       2070       2080       2090       2100
Y12224           AGAAGATATT GCTCAGGCAG TCATCAATAA CGGTGGTGGA CATCTTAACC
EU661272         AGCCGATATT CGTCAAGCTT TAATCAATAA TGGTGGCGGA CACTTGAATC
Clustal Co       **  ******   *** **   * ********  ***** *** **  * ** *
Consensus        AGMMGATATT SSTCARGCWK TMATCAATAA YGGTGGYGGA CAYYTKAAYC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2110       2120       2130       2140       2150
Y12224           ACGCTCTTTT CTGGGAATTG ATGTCACCAG AAGAAACTCA AATTTCACAA
EU661272         ATGCTTTGTT CTGGGAATTA TTATCACCTG AGAAAACTGA AGTAACAAAA
Clustal Co       * *** * ** *********   * ***** * *  ***** * * *  ** **
Consensus        AYGCTYTKTT CTGGGAATTR WTRTCACCWG ARRAAACTSA ARTWWCAMAA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2160       2170       2180       2190       2200
Y12224           GAGTTATCTG AAGACATTAA TGCAACTTTT GGTTCATTTG AAGACTTTAA
EU661272         GAAGTTGCAA GTGCAATTGA CCAAGCTTTT GGATCTTTTG AAGCTTTTAA
Clustal Co       **  *  *     *  *** *    * ***** ** ** **** * *  *****
Consensus        GARKTWKCWR RWGMMATTRA YSMARCTTTT GGWTCWTTTG AWGMYTTTAA
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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2210       2220       2230       2240       2250
Y12224           AGCTGCTTTC ACAGCAGCAG CAACAGGACG TTTTGGTTCA GGTTGGGCTT
EU661272         AGAACAATTT GCAGCAGCAG CAACTGGCCG TTTTGGTTCT GGTTGGGCTT
Clustal Co       **     **   ********* **** ** ** *********  **********
Consensus        AGMWSMWTTY RCAGCAGCAG CAACWGGMCG TTTTGGTTCW GGTTGGGCTT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2260       2270       2280       2290       2300
Y12224           GGCTTGTTGT TAATGCTGAA GGCAAACTTG AAGTGCTTTC AACTGCCAAT
EU661272         GGTTAGTTGT TACTAAAGAA GGAAGTCTTG AAATTACTTC AACTGCAAAT
Clustal Co       ** * ***** ** *   *** ** *  **** ** *   *** ****** ***
Consensus        GGYTWGTTGT TAMTRMWGAA GGMARWCTTG AARTKMYTTC AACTGCMAAT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2310       2320       2330       2340       2350
Y12224           CAAGATACTC CAATTATGGA AGGTAAGAAA CCTATTTTAG CACTTGATGT
EU661272         CAAGATACGC CTATTTCAGA AGGTAAGAAA CCTATTTTAG CACTTGATGT
Clustal Co       ******** * * ***   ** ********** **********  *********
Consensus        CAAGATACYC CWATTWYRGA AGGTAAGAAA CCTATTTTAG SACTTGATGT
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2360       2370       2380       2390       2400
Y12224           ATGGGAGCAT GCTTATTACT TAAACTATCG TAATGTTCGT CCTAACTACA
EU661272         TTGGGAGCAT GCTTATTACC TTAACTACCG TAATGTTCGT CCAAACTACA
Clustal Co        ********* *********  * ***** ** ********** ** *******
Consensus        WTGGGAGCAT GCTTATTACY TWAACTAYCG TAATGTTCGT CCWAACTACA

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2410       2420       2430       2440       2450
Y12224           TCAAAGCTTT CTTTGAAATT ATTAACTGGA ATAAAGTAAA TGAACTTTAC
EU661272         TCAATGCTTT CTTTGAAATC ATTAACTGGA ATAAAGTAGA TGAATTATTT
Clustal Co       **** ***** *********  ********** ******** * **** * *
Consensus        TCAAWGCTTT CTTTGAAATY ATTAACTGGA ATAAAGTARA TGAAYTWTWY

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         2460       2470       2480       2490       2500
Y12224           CAAGCTGCTA AAGCTTAATA AAAAAACGAG TCGATAGGCT CGTTTTTTTA
EU661272         AAAGCTGCTA AAGCATAA-- ---------- ---------- ----------
Clustal Co        ********* **** ***
Consensus        MAAGCTGCTA AAGCWTAATA AAAAAACGAG TCGATAGGCT CGTTTTTTTA
```

2. The consensus sequence used to generate the primers with Primer3 program (available at http://frodo.wi.mit.edu/primer3). Many designed pairs of primer were selected specifically for amplification of *sodA* gene both *S.agalactiae* and *S.iniae* to use in the present study that considered in product size (cover mainly *sodA* gene), primer length (18-30 nucleotide in length), GC content (within 30-80%), annealing and melting temperature (Tm) (within 58°C to 60°C), numerous bases at the 3 prime end (no more than two G's or C's). The selected primer for this study had 1199 bps of product size, 20 bps of forward and reverse primer, 50-55% of GC content, 60°C of Tm and 2 bases at the 3 prime end both forward and reverse primer.

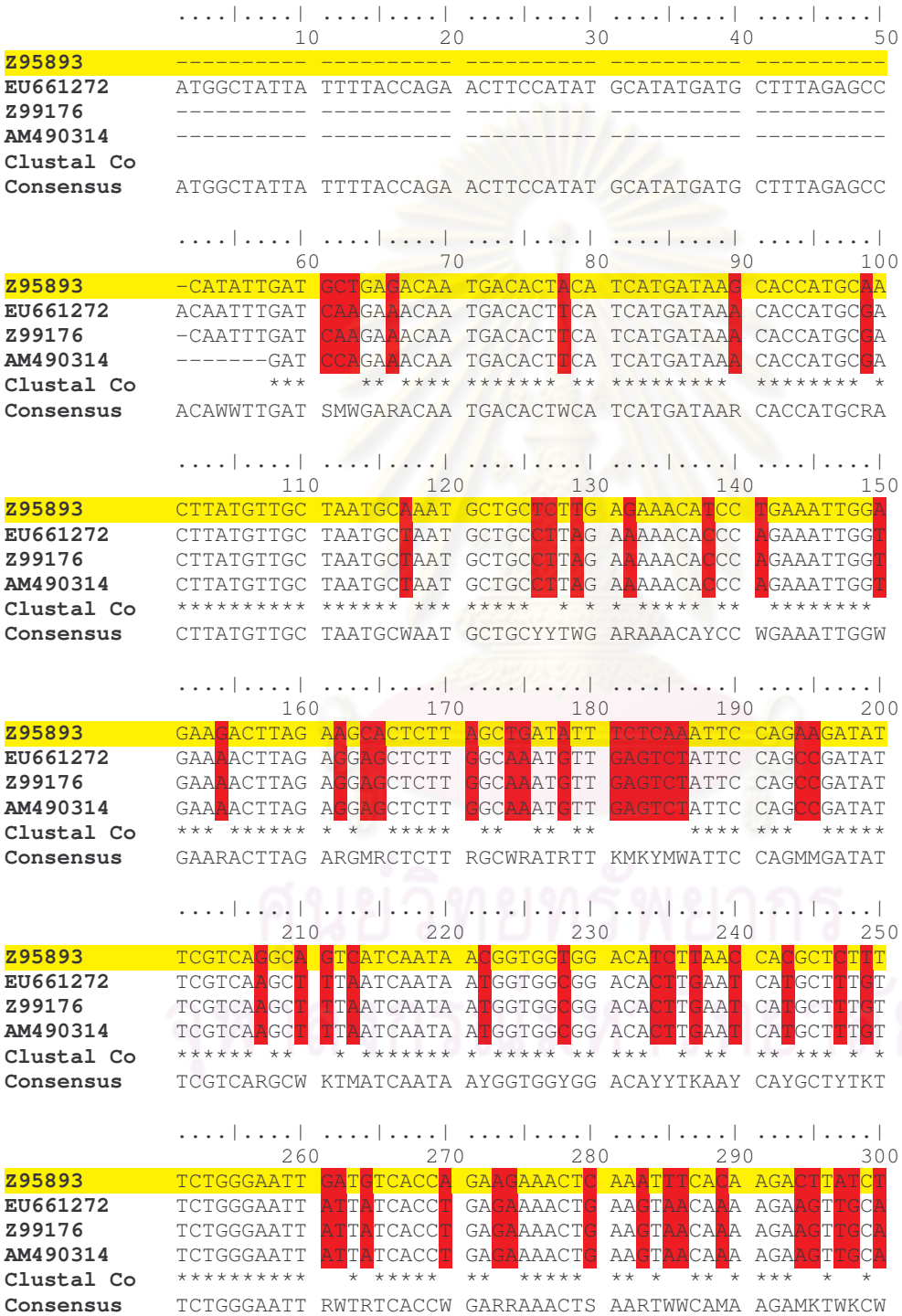
3. The selected primer was double checked specificity to *sodA* of *S.agalactiae* and *S.iniae* by NCBI BLAST that showed 90-100% of specificity.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO            start  len      tm      gc%    any    3'  seq
LEFT PRIMER       1044   20   60.15    55.00   6.00   2.00 gcttggaagcctacttgcag
RIGHT PRIMER      2242   20   60.01    50.00   4.00   2.00 cctgaaccaaaacgtcctgt
SEQUENCE SIZE: 3103
INCLUDED REGION SIZE: 3103

PRODUCT SIZE: 1199, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00
```

Internal part of *sodA* gene associated with Mn-dependent superoxide dismutase

1. The full length of *S.agalactiae* and *S.iniae* Internal part of *sodA* gene associated with Mn-dependent superoxide dismutase gene (approximately 429 to 600 bp) obtained from GenBank nucleotide database (Accession no. of *S.agalactiae* Z95893 and *S.iniae* EU661272, Z99176 and AM490314) was aligned all of the sequences and reported consensus sequence. The red label showed the variable region of 16S rRNA gene between *S.agalactiae* and *S.iniae.*



```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         310        320        330        340        350
Z95893           GAAGACATTG ATGCAACTTT TGGTTCATTT GAAGACTTTA AAGCTGCTTT
EU661272         AGTGCAATTG ACCAAGCTTT TGGATCTTTT GATGCTTTTA AAGAACAATT
Z99176           AGTGCAATTG ACCAAGCTTT TGGATCTTTT GATGCTTTTA AAGAACAATT
AM490314         AGTGCAATTG ACCAAGCTTT TGGATCTTTT GATGCTTTTA AAGAACAATT
Clustal Co           *  **** *   * **** *** ** *** ** *  **** ***     **
Consensus        RRWGMMATTG AYSMARCTTT TGGWTCWTTT GAWGMYTTTA AAGMWSMWTT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         360        370        380        390        400
Z95893           CACAGCAGCA GCAACAGGAC GTTTTGGTTC AGGTTGGGCT TGGCTTGTTG
EU661272         TGCAGCAGCA GCAACTGGCC GTTTTGGTTC TGGTTGGGCT TGGTTAGTTG
Z99176           TGCAGCAGCA GCAACTGGCC GTTTTGGTTC TGGTTGGGCT TGGTTAGTTG
AM490314         TGCAGCAGCA GCAACTGGCC GTTTTGGTTC TGGTTGGGCT TGGTTAGTTG
Clustal Co        ******** ***** ** * **********  ********* *** * ****
Consensus        YRCAGCAGCA GCAACWGGMC GTTTTGGTTC WGGTTGGGCT TGGYTWGTTG

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450
Z95893           TTAATGCTGA AGGCAAACTT GAAGTGCTTT CAACTGCCAA TCAAGATACT
EU661272         TTACTAAAGA AGGAAGTCTT GAAATTACTT CAACTGCAAA TCAAGATACC
Z99176           TTACTAAAGA AGGAAGTCTT GAAATTACTT CAACTGCAAA TCAAGATACC
AM490314         TTACTAAAGA AGGAAGTCTT GAAATTACTT CAACTGCAAA TCAAGATACC
Clustal Co       *** *   ** *** *  *** *** *   ** ******* ** *********
Consensus        TTAMTRMWGA AGGMARWCTT GAARTKMYTT CAACTGCMAA TCAAGATACY

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         460        470        480        490        500
Z95893           CCAATTATGG AAGGTAAGAA GCCTATTTT- AGGGCTT--- ----------
EU661272         CCTATTTCAG AAGGTAAGAA ACCTATTTT- AGCACTTGAT GTTTGGGAGC
Z99176           CCTATTTCAG AAGGTAAGAA GCCTATTTT- AGCACTT--- ----------
AM490314         CCTATTTCAG AAGGTAAGAA ACCTATTTTT AGCACT---- ----------
Clustal Co       ** ***   * **********  ********  **  **
Consensus        CCWATTWYRG AAGGTAAGAA RCCTATTTTT AGSRCTTGAT GTTTGGGAGC

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         510        520        530        540        550
Z95893           ---------- ---------- ---------- ---------- ----------
EU661272         ATGCTTATTA CCTTAACTAC CGTAATGTTC GTCCAAACTA CATCAATGCT
Z99176           ---------- ---------- ---------- ---------- ----------
AM490314         ---------- ---------- ---------- ---------- ----------
Clustal Co
Consensus        ATGCTTATTA CCTTAACTAC CGTAATGTTC GTCCAAACTA CATCAATGCT

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         560        570        580        590        600
Z95893           ---------- ---------- ---------- ---------- ----------
EU661272         TTCTTTGAAA TCATTAACTG GAATAAAGTA GATGAATTAT TTAAAGCTGC
Z99176           ---------- ---------- ---------- ---------- ----------
AM490314         ---------- ---------- ---------- ---------- ----------
Clustal Co
Consensus        TTCTTTGAAA TCATTAACTG GAATAAAGTA GATGAATTAT TTAAAGCTGC

                 ....|....|
                         610
Z95893           ----------
EU661272         TAAAGCATAA
Z99176           ----------
AM490314         ----------
Clustal Co
Consensus        TAAAGCATAA
```

2. The consensus sequence used to generate the primers with Primer3 program (available at http://frodo.wi.mit.edu/primer3). Many designed pairs of primer were selected specifically for amplification of internal part of *sodA* gene associated with Mn-dependent superoxide dismutase gene both *S.agalactiae* and *S.iniae* to use in the present study that considered in product size (cover mainly *Mn-sodA* gene), primer length (18-30 nucleotide in length), GC content (within 30-80%), annealing and melting temperature (Tm) (within 58°C to 60°C), numerous bases at the 3 prime end (no more than two G's or C's). The selected primer for this study had 512 bps of product size, 25 and 23 bps of forward and reverse primer, 36-38% of GC content, 56°C of Tm and 3 bases and 5 bases at the 3 prime end of forward and reverse primer.

3. The selected primer was double checked specificity to internal part of *sodA* gene associated with Mn-dependent superoxide dismutase of *S.agalactiae* and *S.iniae* by NCBI BLAST that showed 90-100% of specificity.

```
No mispriming library specified
Using 1-based sequence positions
OLIGO               start  len     tm     gc%    any    3'  seq
LEFT PRIMER            36   25  56.90   38.00   6.00 3.00  tgatgctttagagccacaatttgat
RIGHT PRIMER          547   23  56.00   36.00   4.00 5.00  cattgatgtagtttggacgaaca
SEQUENCE SIZE: 610
INCLUDED REGION SIZE: 610

PRODUCT SIZE: 512, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00
```

APPENDIX E

Purification of nucleic acid with DNA-binding spin column
(NucleoSpin® Extract II, Germany)

Mix 40 µl of sample (PCR product) with 80µl of *Buffer NT*

↓

Place a column into a collection tube

↓ Centrifuge for 1 minute at 11,000 g and
Discard solution in collection tube

Add 600 µl of *Buffer NT3*

↓ Centrifuge for 1 minute at 11,000 g and
Discard solution in collection tube

Centrifuge for 2 mins at 11,000 g to remove *Buffer NT3* and dry column

↓

Transfer DNA-binding column to new microtube

↓

Add 20 µl of *Elution Buffer NE*

↓ Incubate for 1 minute at room temperature and
Centrifuge for 1 minute at 11,000 g

Purified nucleic acid for sequencing

APPENDIX F

Safety test of vaccine

Water quality (water temperature, pH, amount of ammonia and nitrite) was monitored daily during the experiment.

	Group			
Water quality	FKC	FKC+ECP	TSB	None
Day 1st of testing				
pH	7.5	7.6	7.5	7.6
Ammonia (mg/l)	1.00	1.00	0.25	0.25
Nitrite (mg/l)	0.10	0.05	0.10	0.05
Water temperature (^{O}C)	28.5	28.3	29.0	29.0
Day 2nd of testing				
pH	7.3	7.3	7.6	7.3
Ammonia (mg/l)	0.50	0.50	0.25	0.50
Nitrite (mg/l)	0.10	0.05	0.05	0.05
Water temperature (^{O}C)	29.0	29.0	29.5	29.0
Day 3th of testing				
pH	7.5	7.3-7.6	7.3	7.5
Ammonia (mg/l)	1.00	1.00	0.50	1.00
Nitrite (mg/l)	0.05	0.05	0.05	0.00
Water temperature (^{O}C)	30.0	30.0	30.0	30.0
Day 4th of testing				
pH	7.3	7.3	7.3	7.3
Ammonia (mg/l)	0.50	0.50	0.25	0.25
Nitrite (mg/l)	0.05	0.05	0.00	0.05
Water temperature (^{O}C)	30.0	30.0	30.0	30.0
Day 5th of testing				
pH	7.3-7.6	7.3	7.3-7.6	7.3-7.6
Ammonia (mg/l)	0.25	0.50	0.25	0.25
Nitrite (mg/l)	0.10	0.10	0.00	0.05
Water temperature (^{O}C)	29.5	29.0	29.0	29.0
Day 6th of testing				
pH	7.3	7.3	7.3	7.3
Ammonia (mg/l)	0.50	0.25	0.25	0.25
Nitrite (mg/l)	0.10	0.05	0.00	0.00
Water temperature (^{O}C)	29.0	29.0	29.5	29.0
Day 7th of testing				
pH	7.3-7.6	7.0-7.3	7.3-7.6	7.3-7.6
Ammonia (mg/l)	0.5-1.0	0.5-1.0	0.25	0.25
Nitrite (mg/l)	0.1-0.25	0.1-0.25	0.00	0.10
Water temperature (^{O}C)	28.5	29.0	28.5	28.5

Water quality (water temperature, pH, amount of ammonia and nitrite) was monitored daily during the experiment.

	Group			
Water quality	FKC	FKC+ECP	TSB	None
Day 8th of testing				
pH	7.6	7.3	7.6	7.3
Ammonia (mg/l)	0.25	0.50	0.25	0.50
Nitrite (mg/l)	0.05	0.05	0.00	0.05
Water temperature (^{O}C)	29.0	28.5	29.0	29.0
Day 9th of testing				
pH	7.6	7.3	7.3	7.6
Ammonia (mg/l)	0.25	0.50	0.25	0.50
Nitrite (mg/l)	0.00	0.10	0.00	0.00
Water temperature (^{O}C)	29.0	29.0	29.0	29.0
Day 10th of testing				
pH	7.3-7.6	7.3	7.3	7.3
Ammonia (mg/l)	0.5-1.0	1.00	0.25	0.25
Nitrite (mg/l)	0.05	0.1-0.25	0.00	0.05
Water temperature (^{O}C)	28.5	30.0	30.0	29.5
Day 11th of testing				
pH	7.6	7.3	7.3	7.3
Ammonia (mg/l)	0.25	0.25	0.25	0.25
Nitrite (mg/l)	0.00	0.10	0.00	0.00
Water temperature (^{O}C)	29.0	29.0	29.5	29.5
Day 12th of testing				
pH	7.6	7.3	7.3-7.6	7.3-7.6
Ammonia (mg/l)	0.5-1.0	0.25	0.25	0.50
Nitrite (mg/l)	0.05	0.10	0.00	0.00
Water temperature (^{O}C)	29.0	28.0	30.0	29.0
Day 13th of testing				
pH	7.6	7.3	7.6	7.3
Ammonia (mg/l)	0.50	0.50	0.25	0.50
Nitrite (mg/l)	0.00	0.10	0.00	0.00
Water temperature (^{O}C)	29.0	29.5	30.0	30.0
Day 14th of testing				
pH	7.6	7.3	7.3-7.6	7.6
Ammonia (mg/l)	0.50	0.25	0.25	0.25
Nitrite (mg/l)	0.05	0.10	0.00	0.05
Water temperature (^{O}C)	29.0	29.5	29.5	29.0

The survival and dead tilapia of vaccinated tilapia with Formalin Killed Cell (FKC) vaccine and FKC mixed Extracellular product (ECP) (at a dose of 3 X 10^8 CFU per fish, by intraperitoneal route) was analyzed statistically with **the Kruskal-Wallis one way analysis of variance by range (non-parametric method)** to evaluate the vaccine safety. The vaccinated group was compared with non-vaccinated group (control group) i.e. Placebo vaccinated control and untreated control at 14 days post-vaccination. The result showed that the vaccinated and non-vaccinated group are not difference statistically ($\alpha = 0.05$).

NPar Tests

Kruskal-Wallis Test

Ranks

	GROUP	N	Mean Rank
survival rate	FKC	20	42.00
	FKC+ECP	20	42.00
	TSB	20	42.00
	None	20	36.00
	Total	80	

Test Statistics[a,b]

	survival rate
Chi-Square	3.338
df	3
Asymp. Sig.	.342

a. Kruskal Wallis Test

b. Grouping Variable: GROUP

APPENDIX G

Efficacy test of vaccine:

Determine streptococcal vaccine efficacy with challenge test

Antibody titer

The antibody titer against streptococcosis by direct agglutination test (log_{10}) of 200 gram-tilapia fish with Formalin Killed Cell (FKC) vaccine (at dose of 6 X 10^8 CFU per fish) at pre-vaccination, 3rd, 5th, 8th, 10th and 12th week post-vaccination.

serum	Antibody titer by direct agglutination test													
	0 wk		3 wk		5 wk		8 wk		10 wk		12 wk		Post challenge	
	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}
1	1	0.301	3	0.903	8	2.408	8	2.408	4	1.204	8	2.408	12	3.612
2	3	0.903	4	1.204	8	2.408	7	2.107	8	2.408	4	1.204	12	3.612
3	3	0.903	4	1.204	4	1.204	5	1.505	3	0.903	3	0.903	11	3.311
4	2	0.602	6	1.806	5	1.505	6	1.806	7	2.107	4	1.204	12	3.612
5	1	0.301	3	0.903	4	1.204	2	0.602	3	0.903	3	0.903	11	3.311
6	3	0.903	5	1.505	4	1.204	5	1.505	4	1.204	4	1.204	11	3.311
7	3	0.903	5	1.505	6	1.806	2	0.602	6	1.806	2	0.602	12	3.612
8	2	0.602	5	1.505	3	0.903	2	0.602	4	1.204	3	0.903	11	3.311
9	2	0.602	6	1.806	8	2.408	3	0.903	4	1.204	4	1.204	12	3.612
10	1	0.301	6	1.806	3	0.903	5	1.505	5	1.505	2	0.602	11	3.311
11	2	0.602	7	2.107	8	2.408	9	2.709	6	1.806	8	2.408		
12	1	0.301	8	2.408	8	2.408	6	1.806	6	1.806	5	1.505		
13	1	0.301	8	2.408	5	1.505	6	1.806	5	1.505	3	0.903		
14	2	0.602	4	1.204	8	2.408	8	2.408	5	1.505	3	0.903		
15	1	0.301	6	1.806	3	0.903	7	2.107	6	1.806	3	0.903		
16	3	0.903	8	2.408	8	2.408	7	2.107	6	1.806	NA	NA		
Mean	1.94	0.583	5.5	1.655	5.81	1.749	5.50	1.655	5.12	1.542	3.93	1.184	11.50	3.461
SD	0.85	0.257	1.67	0.503	2.13	0.643	2.25	0.677	1.41	0.424	1.83	0.551	0.53	0.158

NA, not available

The antibody titer against streptococcosis by direct agglutination test (log_{10}) of 200 gram-tilapia fish with FKC mixed Extracellular product (ECP) vaccine (at dose of 6 X 10^8 CFU per fish) at pre-vaccination, 3rd, 5th, 8th, 10th and 12th week post-vaccination.

serum	Antibody titer by direct agglutination test													
	0 wk		3 wk		5 wk		8 wk		10 wk		12 wk		Post challenge	
	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}
1	1	0.301	8	2.408	2	0.602	3	0.903	6	1.806	5	1.505	12	3.612
2	1	0.301	3	0.903	5	1.505	2	0.602	6	1.806	2	0.602	11	3.311
3	4	1.204	3	0.903	6	1.806	5	1.505	4	1.204	1	0.301	11	3.311
4	2	0.602	5	1.505	7	2.107	5	1.505	4	1.204	3	0.903	11	3.311
5	2	0.602	1	0.301	6	1.806	2	0.602	8	2.408	2	0.602	12	3.612
6	4	1.204	3	0.903	8	2.408	8	2.408	6	1.806	4	1.204		
7	5	1.505	5	1.505	6	1.806	7	2.107	4	1.204	9	2.709		
8	3	0.903	4	1.204	8	2.408	8	2.408	4	1.204	3	0.903		
9	4	1.204	6	1.806	5	1.505	7	2.107	8	2.408	5	1.505		
10	4	1.204	8	2.408	6	1.806	5	1.505	7	2.107	5	1.505		
11	1	0.301	5	1.505	4	1.204	6	1.806	6	1.806	7	2.107		
12	1	0.301	5	1.505	4	1.204	4	1.204	1	0.301	5	1.505		
13	1	0.301	8	2.408	6	1.806	2	0.602	2	0.602	7	2.107		
14	1	0.301	7	2.107	5	1.505	6	1.806	5	1.505	3	0.903		
15	1	0.301	6	1.806	4	1.204	6	1.806	3	0.903	4	1.204		
16	2	0.602	6	1.806	3	0.903	2	0.602	5	1.505	3	0.903		
Mean	2.31	0.695	5.2	1.561	5.31	1.599	4.88	1.467	4.94	1.486	4.25	1.279	11.4	3.431
SD	1.45	0.436	2.04	0.614	1.66	0.500	2.15	0.649	1.98	0.596	2.11	0.636	0.55	0.165

The antibody titer against streptococcosis by direct agglutination test (log_{10}) of 200 gram-tilapia fish with Placebo vaccinated control (TSB injection) at pre-vaccination, 3rd, 5th, 8th, 10th and 12th week post-vaccination.

serum	Antibody titer by direct agglutination test													
	0 wk		3 wk		5 wk		8 wk		10 wk		12 wk		Post challenge	
	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}
1	4	1.204	1	0.301	5	1.505	6	1.806	1	0.301	2	0.602	11	3.311
2	4	1.204	5	1.505	1	0.301	1	0.301	1	0.301	2	0.602		
3	3	0.903	3	0.903	1	0.301	1	0.301	1	0.301	1	0.301		
4	4	1.204	5	1.505	1	0.301	1	0.301	1	0.301	1	0.301		
5	2	0.602	4	1.204	1	0.301	6	1.806	3	0.903	2	0.602		
6	7	2.107	1	0.301	2	0.602	2	0.602	1	0.301	2	0.602		
7	4	1.204	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301		
8	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301	6	1.806		
9	3	0.903	5	1.505	1	0.301	2	0.602	1	0.301	1	0.301		
10	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301		
11	1	0.301	2	0.602	1	0.301	1	0.301	1	0.301	3	0.903		
12	1	0.301	1	0.301	1	0.301	2	0.602	1	0.301	2	0.602		
13	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301		
14	1	0.301	1	0.301	2	0.602	1	0.301	1	0.301	1	0.301		
15	2	0.602	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301		
16	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301	2	0.602		
Mean	2.50	0.752	2.12	0.639	1.37	0.414	1.81	0.545	1.12	0.338	1.81	0.545	11	3.311
SD	1.75	0.527	1.67	0.502	1.02	0.308	1.68	0.506	0.50	0.150	1.27	0.384	-	-

The antibody titer against streptococcosis by direct agglutination test (log_{10}) of 200 gram-tilapia fish with Untreated control at pre-vaccination, 3rd, 5th, 8th, 10th and 12th week post-vaccination.

serum	Antibody titer by direct agglutination test													
	0 wk		3 wk		5 wk		8 wk		10 wk		12 wk		Post challenge	
	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}	Log_2	Log_{10}
1	2	0.602	3	0.903	1	0.301	1	0.301	1	0.301	2	0.602		
2	1	0.301	2	0.602	1	0.301	4	1.204	2	0.602	1	0.301		
3	1	0.301	2	0.602	3	0.903	1	0.301	2	0.602	4	1.204		
4	3	0.903	1	0.301	3	0.903	1	0.301	1	0.301	3	0.903		
5	2	0.602	1	0.301	1	0.301	1	0.301	3	0.903	2	0.602		
6	5	1.505	2	0.602	1	0.301	4	1.204	3	0.903	3	0.903		
7	1	0.301	1	0.301	1	0.301	1	0.301	3	0.903	1	0.301		
8	1	0.301	1	0.301	2	0.602	1	0.301	6	1.806	1	0.301		
9	3	0.903	2	0.602	2	0.602	1	0.301	1	0.301	1	0.301		
10	3	0.903	3	0.903	1	0.301	1	0.301	4	1.204	1	0.301		
11	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301		
12	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301		
13	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301	1	0.301		
14	3	0.903	1	0.301	2	0.602	1	0.301	2	0.602	1	0.301		
15	1	0.301	3	0.903	1	0.301	1	0.301	2	0.602	2	0.602		
16	1	0.301	3	0.903	1	0.301	3	0.903	1	0.301	1	0.301		
Mean	1.87	0.564	1.75	0.527	1.44	0.432	1.50	0.451	2.12	0.639	1.62	0.489		
SD	1.20	0.362	0.85	0.258	0.73	0.219	1.09	0.329	1.41	0.424	0.96	0.288		

Water quality (water temperature, pH, hardness, alkalinity, amount of ammonia and nitrite) was monitored monthly during the experiment before challenge test.

Month (date of collection)	Groups	Water quality parameter						
		pH	Ammonia (mg/l)	Nitrite (mg/l)	Salinity (g/l)	DO (mg/l)	Water temperature (^{O}C)	Total bacterial count (CFU/ml)
Month 1 (28/6/2551)	FKC	8.0	0	0	7.0	7	29-31	-
	FKC+ECP	8.0	0	0	7.0	7	29-31	2.25×10^3
	TSB	8.0	0	0	7.0	7	29-31	1.00×10^3
	None	8.0	0	0	7.0	7	29-31	2.20×10^3
Month 2 (18/7/2551)	FKC	8-8.3	0	0	7	7.1	29-32	0.80×10^3
	FKC+ECP	8-8.3	0	0	6	7.1	29-32	1.39×10^3
	TSB	8.3	0	0	7	7.1	29-32	10.3×10^3
	None	8-8.3	0	0	6	7.1	29-32	1.21×10^3
Month 3 (2/8/2551)	FKC	8.3	0	0	7	7.0	30-32	1.00×10^3
	FKC+ECP	8.3	0	0	7	7.0	30-32	1.00×10^3
	TSB	8.0	0	0	7	7.0	30-32	2.00×10^3
	None	8.3	0	0	7	7.0	30-32	5.00×10^3

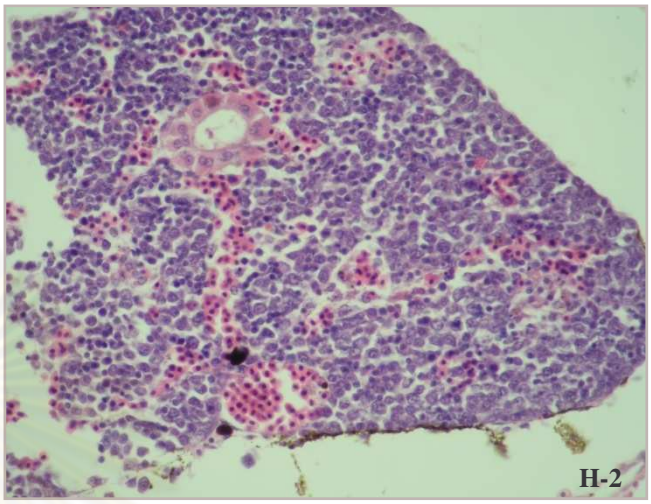
Water quality (water temperature, pH, hardness, alkalinity, amount of ammonia and nitrite) was monitored weekly during the challenge test.

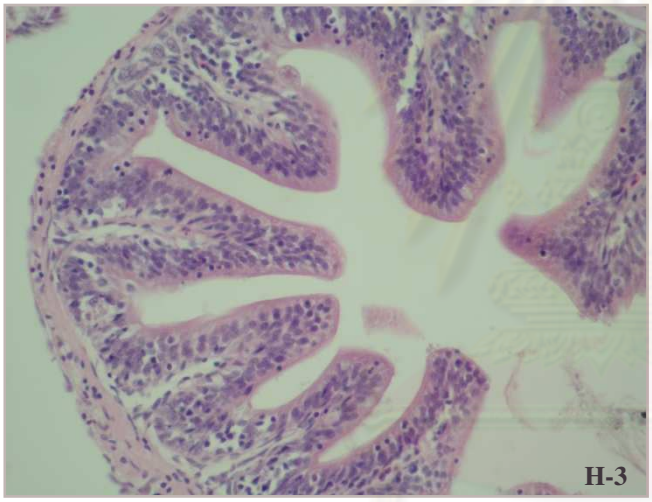
Week (date of collection)	Groups	Water quality parameter					
		pH	Ammonia (mg/l)	Nitrite (mg/l)	Hardness (mg/l)	Alkalinity (mg/l)	Water temperature (^{O}C)
Week1 (12/8/2551)	FKC	7.6	0.25	0.10	400	220	28.0
	FKC+ECP	7.6	0.25	0.10	400	220	28.0
	TSB	7.6	0.25	0.10	450	200	29.5
	None	7.6	0.25	0.10	400	200	29.0
Week2 (19/8/2551)	FKC	7.6	0.25	0.10	450	200	28.5
	FKC+ECP	7.6	0.25	0.05	400	200	28.5
	TSB	7.6	<0.25	0.00	500	200	29.0
	None	7.6	<0.25	0.00	450	200	29.0
Week 3 (26/8/2551)	FKC	7.6	0.25	0.05	500	220	28.5
	FKC+ECP	7.6	<0.25	0.10	400	290	28.0
	TSB	7.6	<0.25	0.00	500	200	29.0
	None	7.6	0.00	0.00	400	200	29.0

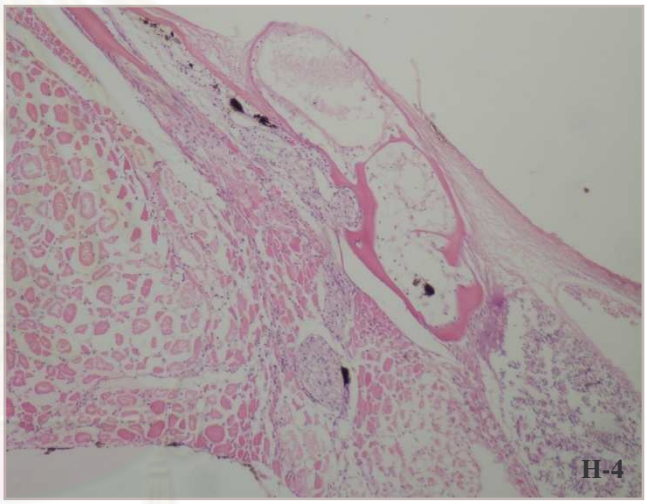
APPENDIX H

Vaccinated Survivors


H-1


H-2


H-3


H-4

All vaccinated survivors from the challenge were confirmed the streptococcal infection by conventional microbiological identification of the blood sample. Bacterial pathogens, including streptococcus, was not found in all cases (Table 4.22). The vaccinated survivors those presented skin abscess (Figure H-1) neither retained systemic streptococcal infection identified using the bacterial culture nor histological alteration of the visceral organs, spleen and intestine (Figure H-2 and H-3, H&E x100). Culture of the abscess content failed to recover streptococcal bacteria whilst the histological examination of the skin abscess revealed fibrous connective tissue surrounding debris (Figure H-4, H&E x40).

BIOGRAPHY

Miss Hathairat Maisak was born on June 8, 1981 in Nonthaburi, Thailand. She got the degree of Doctor of Veterinary Medicine (2nd Class Honours) from the faculty of Veterinary Medicine, Chulalongkorn University, Thailand in 2005. In 2006, she got the degree of Master of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Thailand. In 2007, she enrolled the degree of Doctor of Philosophy (Ph.D.) in the Department of Medicine, Faculty of Veterinary Science, Chulalongkorn University since 2010.