CHAPTER 4

MATERIALS AND METHODS

4.1 Hemoculture specimens collection

A total of 304 hemoculture specimens were collected from patients hospitalized with streptococcal infection at Maharaj Nakorn Chiang Mai Hospital (MNCH), Lamphun Hospital (LPH), Doi Saket Hospital (DSK) and Sanpatong Hospital (SPT) between September 2009 and August 2011 (Table 3). Streptococcal-positive hemocultures were cultured on blood agar and subjected to Gram-staining for morphology characterization. Then, these specimens were kept at -20 ^{o} C for DNA extraction. Four isolates of other streptococci, 2 isolates of Gram-positive bacteria, 1 isolate of Gram-negative bacteria, and *S. suis* P1/7 reference strains (serotype 2), MNCM 11 (serotype 1/2), and MNCM 07 (serotype 14) were included as control (Table 4).

4.2 Identification of *S. suis*

All streptococci isolates were streaked on bile esculin and esculin to observe hydrolytic and stabbed in trehalose, mannitol, raffinose, lactose, and sorbitol to observe acid production. Alpha-hemolytic streptococci that exhibited biochemical reactions as shown in Table 5 was suspected to be *S. suis*. Optochin disk diffusion test was performed by Bauer and Kirby method on 5% human blood agar (BA) to separate between *S. pneumoniae* and other α-hemolytic streptococci. Briefly, all streptococci isolates were streaked on BA and 5 µg optochin disc was placed on the medium. Blood agar plate was incubated at 37 ^{o}C for 24 hours in 5% CO_2

atmosphere. Moreover, all streptococci isolates were confirmed as *S. suis* by PCR assay based on 16S rRNA gene and *gdh* gene (Marois *et al*., 2004; Silva *et al*., 2006). The positive isolates were further characterized for serotype 2 and 1/2 or 1 and 14 by duplex PCR, monoplex PCR, and coagglutination test (Marois *et al*., 2004; Silva *et al*., 2006; Kerdsin *et al*., 2009; Gottschalk *et al*., 1989).

Table 3 Total of Specimen collection

Source	**Number of hemocultures**
Maharaj Nakorn Chiang Mai Hospital	135
Lamphun Hospital	167
Doi Saket Hospital	1
Sanpatong Hospital	1
Total	304

Table 4 List of bacteria investigated in this study

Bacterial strains (Number of isolates)			
Streptococcal-positive	**(304)**	***Streptococcus suis***	**(3)**
		S. suis P1/7 reference strain (serotype 2)	
		S. suis MNCM 11 (serotype 1/2)	
		S. suis MNCM 07 (serotype 14)	
Other species of streptococcus	**(4)**	**Gram-positive bacteria**	**(2)**
Streptococcus pneumoniae		*Staphylococcus aureus* ATCC 25923	
Streptococcus pyogenes		*Enterococcus faecalis*	
Streptococcus bovis		**Gram-negative bacteria**	**(1)**
Viridans streptococci		*Escherichia coli* ATCC 25922	

Table 5 Biochemical reactions for *S. suis* presumptive tests

Reagent	Reactions	Positive reactions (%)*
Bile esculin	-	4
Esculin	+	70
Trehalose	+	98
Mannitol	-	3
Raffinose	+	93
Lactose	+	99
Sorbitol	-	1
Optochin	Resistant	ND

ND = Not determine

*=Adapted from API 20 Strep bioMérieux (http://www.scribd.com/doc/10644/API20Strep; http://www.cdc.gov/ncidod/biotech/strep/strep-doc/section2.htm#table5; Gottschalk *et al*., 1991)

4.3 DNA preparation

Genomic DNA of *S. suis* was extracted directly from hemoculture using modified boiling-centrifugation method as described previously (Soumet *et al*., 1994). Briefly, one ml of hemoculture was centrifuged at 1,000 Revolutions Per Minute (rpm) for 3 minutes. The supernatant was removed to a new tube and centrifuged at 13,000 rpm for 1 minute. The pellet was washed at least 3 times or more with PBS pH 7.2 until it was colorless. The turbidity of the suspension was adjusted to equivalent to the McFarland number 3.0 standard with PBS pH 7.2, then it was kept in boiling water-bath for 5 minutes, cooled in ice and centrifuged at 13,000 rpm for 1 minute. This supernatant was ready for PCR assay.

For DNA preparation from cultured colonies, genomic DNA was extracted modified as described previously (Pruksakorn *et al*., 2000). Briefly, a single colony of overnight culture was transferred into 10 ml Todd-Hewitt broth (THB) and incubated overnight at 37 ^{o}C under 5% CO_2 atmosphere. Cells were centrifuged at 13,000 rpm for 1 minute and washed 3 times with PBS pH 7.2. Cell pellet was suspended in 0.2 ml lysozyme solution (10 mg/ml) and incubated at 37 ^{o}C for 1 hour. Sodium dodecyl sulphate (SDS) solution (20%, 20 µl) and Proteinase K (10 mg/ml, 10 µl) were added, and incubated at 55 ^{o}C for 3 hours. After incubation, 0.2 ml saturated NaCl solution was added, and the mixture was freezed at -20 ^{o}C for 30 minutes. The mixture was centrifuged at 12,000 rpm for 10 minutes to sediment any cell debris and proteins. The supernatant was transferred to a new tube, and the DNA was precipitated by adding 3 volumes of 95% ethanol. The tube was rocked gently until the DNA flocculation appeared. The DNA was washed twice with 70% ethanol. Finally, ethanol was discarded and DNA was dried at room temperature and resuspended in 40 µl of TE buffer and stored at -20 ^{o}C until used.

4.4 Detection of *S. suis* by polymerase chain reaction

DNA-extracted streptococci was used as template for PCR amplification using 16S rRNA specific primers as shown in Table 6 (Marois *et al*., 2004). The PCR mixture contained 10X PCR buffer$^{®Invitrogen}$, 2.5 mM $MgCl_2$$^{®Invitrogen}$, a 600 µM of each deoxynucleotide triphosphate$^{®Vivantis}$, 600 nM of 16S-195s forword primers and 16S-489as reverse primers, 0.2 µl of *Taq* DNA polymerase$^{®Invitrogen}$ (5 unit/µl), and 5 µl of the DNA template. DNase-RNase free water was used to replace DNA template for the negative-control monitor. The amplification process consisted of 40 cycles of

denaturation at 94 ^{o}C for 30 seconds, annealing at 58 ^{o}C for 30 seconds, and extension at 72 ^{o}C for 1 minute. To compare with 16S rRNA, gdh primers (Table 6) was used to amplify *gdh* specific gene of *S. suis* (modified from Silva *et al*., 2006). The amplification reaction components contained 16.05 µl of DNase-RNase-free water, 2.5 µl of 10X PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 0.05 µl of each gdh specific-primer pair, 0.1 µl of *Taq* DNA polymerase, and 5 µl of DNA template in a total volume of 25 µl. The amplification was performed for 30 cycles of PCR process under the following thermal cycling condition: 94°C for 3 minutes to initiate denaturation, 94°C for 1 minute, 50°C for 1 minute, 72°C for 1 minute, and the final extension step at 72°C for 10 minutes prior to holding at 4°C. All PCR products were analyzed by electrophoresis in 2.0% agarose gel, stained with 0.5 µg/ml ethidium bromide, and visualized under UV light. DNA ladder markers$^{®Vivantis}$, 100 bp were used to access the band size.

4.5 Serotyping of *S. suis* by polymerase chain reaction

Duplex PCR was performed using capsular polysaccharide (cps) specific primers to identify serotype 2 or 1/2 and serotype 1 or 14 as shown in Table 7 (Kerdsin *et al*., 2009). The amplification reaction components contained 12.4 µl of RNase-free water, 2.5 µl of 10X PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix, 1.25 µl of each cps specific-primer pair, 0.1 µl of *Taq* DNA polymerase, and 5 µl of DNA template in a total volume of 25 µl. The amplification used for initial denaturation was at 95°C for 3 minutes, followed by 35 cycles of 95°C for 45 seconds, 54°C for 45 seconds, 72°C for 1 minute, and the final extension

step at 72°C for 5 minutes prior to holding at 4°C. The *S. suis* isolates which their serotype 2 or 1/2 and serotype 1 or 14 by duplex PCR were comfirmed serotypes by monoplex PCR.

For monoplex PCR, cps2J and cps1J primers were used to comfirm serotype 2 or 1/2 and serotype 1 or 14, respectively as shown in Table 7 (modified from Marois *et al*., 2004; modified from Silva *et al*., 2006). The PCR mixture and reaction of PCR targeted to *cps*2 gene was prepared in a total volume of 25 µl, 9.05 µl of RNase-free water, 2.5 µl of 10X PCR buffer, 1.25 µl of 50 mM $MgCl_2$, 1.5 µl of 10 mM dNTP mix, 2.75 µl of each cps2J specific-primer pair, 0.2 µl of *Taq* DNA polymerase, and 5 µl of DNA template under the following thermal cycling condition of PCR targeted to 16S rRNA gene. PCR targeted to *cps*1 gene was prepared according to the procession and reaction of PCR targeted to *gdh* gene above, 1.1 µl of each cps1J specific-primer pair were replaced and performed at 53 °C for 1 minute of annealing. *S. suis* P1/7 reference strain (serotype 2), MNCM 11 (serotype 1/2), and MNCM 07 (serotype 14) were used as template for positive control of these PCR for serotyping.

4.6 Serotyping of *S. suis* by coagglutination test

Coagglutination test was performed to identify *S. suis* serotype 2 and 1/2. The reagents were prepared as described by previous study (Gottschalk *et al*., 1989). Briefly, *Staphylococcus aureus* Cowan1 was grew on tryptic soy agar at 37 °C overnight and cells were collected with 4 ml PBS (pH 7.4). After washing once with PBS, cells were re-suspended in 0.5% PBS-formalin and kept at room temperature for 3 hours. The mixture was heated at 80 °C for 30 minutes, and cooled in ice water immediately. Fifty microliter of Rabbit anti-streptococcal specific to *S. suis* serotype

2 (SSI Diagnostica, Denmark) was added to mixture in ratio 50:1, mixed, and stand at room temperature for 1 hour. After that, the mixture was washed with 8 ml of PBS, and re-suspended in 8 ml of PBS included 0.05% sodium azide and 0.1% bovine serum albumin.

For coagglutination testing, one drop of the reagent was mixed with bacterial suspension on a glass slide. Positive or clumping reaction was observed within 30-60 seconds. Capsular antigen of *S. suis* was used as positive control as well as *Staphylococcus* suspension coated with normal rabbit serum was used as negative control. (Gottschalk *et al*., 1989; Wongsawan K., 2008).

4.7 Sensitivity of PCR condition

The sensitivity of PCR was evaluated by using 10-fold serial dilutions of DNA extracted from *S. suis* P1/7 reference strain as a range of 1 ng to 0.1 fg. For hemoculture, sensitivity of PCR condition was determined by using overnight-simulated blood cultures of *S. suis* reference strain P1/7 and *S. suis* MNCM07 (adjusted to McFarland number 0.5). Then, viable plate count was performed by 10-fold serial dilutions to 10^{-10}. For each cell concentration, DNA was extracted by boiling-centrifugation method and amplified with the 16S rRNA and gdh primers.

4.8 Specificity of PCR condition

For specificity test, *S. pneumoniae, S. pyogenes,* Viridians streptococci, *S. bovis, E. faecalis, S. aureus* (ATCC 25923), and *E. coli* (ATCC 25922) were streaked on 5% blood agar plates, and a single colony was inoculated in 10 ml Todd-Hewitt broth. After overnight incubation at 37ºC, the DNA of gram-positive bacteria

was extracted as the above protocol. DNA of *E. coli* was extracted by using gram-negative bacteria protocol of Genomic DNA Mini Kit®Geneaid. Briefly, one ml of bacterial culture was centrifuged at 13,000 rpm for 1 minute and discard supernatant. Two hundred microliter of GT buffer was added to the sediment, mixed, and incubated at room temperature for 5 minutes. After incubation 200 µl GB buffer was added to the mixture, and incubated at 70 ^{o}C for 10 minutes. Two hundred microliter of ethanol was added to the sample lysate. The mixture was transferred to GD column and centrifuged at 13,000 rpm for 2 minutes. Four hundred microliter of W1 buffer was added to the column and centrifuged at 13,000 rpm for 30 seconds. Moreover, 600 µl of Wash buffer was added and centrifuged. After centrifugation, the flow-through was discarded and the column was dried by centrifuge again. Lastly, elution buffer was dropped on column matrix and centrifuged at 13,000 rpm for 30 seconds. The 100 ng of extracted DNA of these organisms were examined for the presence of a 294 bp and a 566 bp specific fragment corresponding to 16S rRNA and *gdh* gene of *S. suis*, respectively. In addition, 16S rRNA universal primers of bacterial cells as listed in Table 6 were used as PCR condition control. The expected fragment length of all gram-positive bacteria and gram-negative bacteria were shown only 996 bp of the 16S rRNA universal gene. For hemoculture, specificity of PCR condition to these bacteria was determined by using DNA extracted from each 10-fold serial dilution in a range of 10^{-3} to 10^{-9} as described in the above sensitivity of PCR.

Table 6 Oligonucleotide primers for the detection of *S. suis*

Target gene	Primers	Nucleotide sequences (5'-3')	Amplicon size (bp)	Reference
16S rRNA (*S. suis*)	16S-195(s)	CAGTATTTACCGCATGGTAGATAT	294	Marois *et al*., 2004
	16S-489(as2)	GTAAGATACCGTCAAGTGAGAA		
gdh (*S. suis*)	gdh-F	AAGTTCCTCGGTTTTGAGCA	566	Silva *et al*., 2006
	gdh-R	GCAGCGTATTCTGTCAAACG		
16S rRNA (universal)	U1	CCAGCAGCCGCGGTAATACG	996	Lu *et al*., 2000
	U2	ATCGGCTACCTTGTTACGACTTC		

Table 7 Oligonucleotide primers for the detection serotype of *S. suis*

Target gene	Primers	Nucleotide sequences (5'-3')	Amplicon size (bp)	Reference
cps (serotype 1 or 14	SS-cps1J-F	GATAGAGTTAGTTATTTGACTGA	217	Kerdsin *et al*., 2009
and 2 or 1/2)	SS-cps2J-F	GTTGAGTCCTTATACACCTG		
	SS-cpsJ-R	AACATTARTAAGCTATAATAAA	515	
*cps*2 (serotype 2 or	cps2J-s	GTTGAGTCCTTATACACCTGTT	459	Marois *et al*., 2004
1/2)	cps2J-as	CAGAAAATTCATATTGTCCACC		
*cps*1 (serotype 1 or 14)	cps1-F	TGGCTCTGTAGATGATTCTGCT	637	Silva *et al*., 2006
	cps1-R	TGATACGTCAAAATCCTCACCA		

4.9 Nucleotide sequence analysis of *S. suis*

4.9.1 Purification of PCR product

The *S. suis* isolates were selected to nucleotide sequencing and nucleotide sequence analysis. The PCR product of 16S rRNA and *gdh* genes were purified according to the Gel Extraction (Sequencing) protocol of Gel/PCR DNA Fragments Extraction Kit®Geneaid. Firstly, the PCR products were run in 2% agarose gel containing 0.5 μg/ml ethidium bromide and expected DNA fragment was excised under UV transilluminator. After weighting the gel slice, 500 μl of DF buffer was added, mixed and incubated at 55-60 °C for 10-15 minutes. The dissolved sample mixture was cooled at room temperature and 800 μl was transferred to DF column. Then, the mixture was centrifuged at 13,000 rpm for 30 seconds and discarded the flow-though. To wash DNA that binding to the column, 600 μl of W1 buffer was added and centrifuged for 30 seconds. The flow-through was discarded again, and 600 μl of Wash buffer (ethanol added) was added. After centrifuged at 13,000 rpm for 30 seconds and discarded the flow-though, the column matrix was dried by centrifuged again for 3 minutes. Next, the column was placed into a clean 1.5 ml microcentrifuge tube and then, 30 μl of Elution buffer was added into the center of column matrix. The column was stranded for 2 minutes and then centrifuged for 2 minutes to elute the purified DNA. Lastly, the purified DNA was assessed for quantity and quality by spectrophotometric at 260 nm and run by electrophoresis in 2% agarose gel.

4.9.2 Nucleotide sequencing

The purified PCR products were randomly sequenced and each sequencing was analyzed for similarity to each gene on nucleotide database by BLAST in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST).

4.10 Statistical analysis

The sensitivity and specificity values were calculated according to the following formula (Altman and Bland., 1994; http://www.rapid-diagnostics.org/accuracy.htm).

Sensitivity = TP/(TP+FN)

TP = the number of positive sample by PCR and culture method as a gold standard

FN = the number of negative sample by PCR but positive by culture

Specificity = TN/(FP+TN)

TN = the number of negative sample by PCR and culture method

FP = the number of positive sample by PCR but negative by culture