CHAPTER IV

MATERIALS AND METHODS

1. *Streptococcus* strains

1.1 Reference strains

1.1.1 Positive control

- Group A *Streptococcus* (R. C Lancefield, J17A4)
- Group C *Streptococcus* (M. Ciuca 'Chestle')
- Group G *Streptococcus* (PHLS, 'Valentle')

1.1.2 Negative control

- Group B *Streptococcus* (M. Ciuca 'Lewis')
- Group F *Streptococcus* (PHLS, 'O' s Mahoney')
- *Streptococcus pneumoniae* (ATCC49619)
- *Staphylococcus aureus* (ATCC25923)

They were obtained from *Streptococcus* Center in KANAKAWA Public Health Laboratory, Japan.

1.2 Clinical isolates

Sixty GGS and 52 GCS isolates were selected (table 5) from the isolated stored at –70 ^{0}C at the National Streptococcal Reference Center in Thailand (NSRT), Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. These organisms were obtained from patients admitted to King Chulalongkorn Memorial Hospital from 1995 to 2000. These isolates were divided into two group (non-invasive and invasive) by using the different site of infections. Non-invasive isolates was defined by the isolation of *Streptococcus* from non-sterile site such as throat, pus, and wound. Invasive isolates

were defined by the isolation of *Streptococcus* from normally sterile site, blood, body fluids, abscesses, or tissue.

Table 5: The number of GCS and GGS isolates used in this study from each year

	GCS		GGS	
	N*	n**	N	n
1995	18	18	36	11
1996	1	1	12	3
1997	0	0	44	10
1998	7	7	59	15
1999	9	9	66	12
2000	17	17	76	9

* N = Total number of isolates stored at NSRT

** n = Number of isolates used in this study

2. Characterization of *Streptococcus* species

2.1 Culture

These organisms were kept at –70 ^{0}C at NSRT and were thawed by using water bath at 37 ^{0}C and inoculate these organism on blood agar plate with incubation at 37 ^{0}C in an atmosphere with 5% CO_2 for 24 hour. When grown overnight, all isolates exhibited typical zones of β-hemolysis and large colony morphology and were selected one colony for pure subculture and incubation at 37 ^{0}C in an atmosphere with 5% CO_2 for 24 hour.

2.2 Identification of *Streptococcus* genus

2.2.1 Cellular morphology by gram stain

Apply a thin film of the specimen to a clean glass slide and allow it to air dry. Fix the slide by quickly passing the slide through a flame several times. Flood the slide with crystal violet stain for 30 seconds and rinse gently with running water. Flood the slide with Gram's iodine for 30 seconds and rinse gently under running water. Apply decolorizer so it runs over the stained area until no more color washes out and rinse gently with running water. Flood the slide with safranin counterstain for 30 seconds and rinse gently under running water and allow the slide to air dry. Examine the finished slide under a microscope (Larsen, 1995).

Interpretation: The streptococci are gram positive cocci, which the cell were stained in dark blue to purple color and they are spherical or ovoid cell, arrange in pairs or in chains.

2.2.2 Colony morphology

The organisms were streaked out on blood agar plate with incubation at 37 ^{0}C in an atmosphere with 5% CO_2 for 24 hour.

Interpretation: Group C streptococci are grayish white, glistening, which is wide zone of beta hemolysis

Group G streptococci are grayish white, matte, which is wide zone of beta hemolysis

2.3 Identification of *Streptococcus* species

2.3.1 Biochemical test

All isolates were confirmed as GGS and GCS by biochemical and enzymatic characteristics were determined with the API20 STREP (bioMerieux, Marcy-l'

Etoile, France). API20 STREP is a standardized method combining 20 biochemical tests. The enzymatic tests are inoculated with a dense suspension of organisms, made from a pure culture, which is used to rehydrate the enzymatic substrates. The metabolic end products produced during the incubation period are either revealed through spontaneous colored reactions or by the addition of reagents. The fermentation tests are inoculated with an enriched medium, which reconstitutes the sugar substrates. Fermentation of carbohydrates is detected by a shift in the pH indicator.

The API20 STREP strip consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars.

1. Acetoin production (VP test)
2. Hydrolysis (HIP test)
3. β-glucosidase (Esculin test)
4. Pyrrolidonyl arylamidase (PYRA test)
5. α-galactosidase (α-GAL test)
6. β-glucoronidase (β-GUR test)
7. β-galactosidase (β-GAL test)
8. Alkaline phosphatase (PAL test)
9. Leucine arylamidase (LAP test)
10. Arginine dihydrolase (ADH test)
11. Acidification (Ribose test)
12. Acidification (L-Arabinose test)
13. Acidification (Mannitol test)
14. Acidification (Sorbitol test)
15. Acidification (Lactose test)
16. Acidification (Trehalose test)
17. Acidification (Inulin test)
18. Acidification (Raffinose test)
19. Acidification (Starch test)
20. Acidification (Glycogen test)

Interpretation: After 4 hours of inoculation, the reactions were read by referring to the Analytical Profile Index. The percent of identity were accepted at 80%. If the identity is less than 80%, the reactions were reincubation at 24 hours.

2.3.2 Serological test (co-agglutination)

The organisms were confirmed as GGS and GCS by identification of their group carbohydrate antigen with a commercially available co-agglutination test (Phadebact, Boule Diagnostics AB, Huddinge, Sweden). All GGS and GCS isolates were cultured and were pick up ½-1 loopful of fresh growth and smear on paper for agglutinate reaction. One drop of antibody was mixed on smear and wait 1-2 min for read the result of reaction.

Interpretation: The result of co-agglutination test can be read when the GGS and GCS organisms were precipitated.

All GGS and GCS isolates were kept at –70 ^{0}C in tryptic soy broth supplemented with horse serum (GIBCO BRL, Eggentstein, Germany).

3. DNA isolation

The organisms were thawed and grew overnight on blood agar plates. One colony was selected for pure subculture. Then were pick up one loopful (perhaps half of a standard loopful) of fresh growth was resuspended in 100 µl of lysis buffer (0.85% NaCl, TE buffer, 1% Triton X) and heated for 15 min at 100 ^{0}C. Samples were briefly centrifuged for 5 min at 12,000 rpm to pellet debris and two microliters of supernatant were then used as template for each 50 µl PCR mixtures (Beall et al., 1996, 1997) protocol). DNA extraction of GGS and GCS were stored at –20 ^{0}C.

4. *Emm* gene amplification

4.1 Primers

The PCR primers employed were those described by Bernard Beall and his colleges from CDC (Beall, et al., 1996, 1997). Primer1, 5 ' TATTC (C/G) CTTAGAAAATTAA and primer2, 5' GCAAGTTCTTCAGCTTGTTT were used to amplify a large portion of the *emm* gene, which are highly conserved primers. MF2 5' GGATCCATAAGGAGCATAAAAATGGCTA and MR1 5' TGATAGCTTAGTTTTCTTCT TTGCGTTTT from publications by A. Podbielski and colleagues were used in this study (Podbielski et al., 1993).

4.2 Amplification of GCS and GGS by PCR

The PCR mixtures in a final volume of 50 µl comprised of 10x PCR buffer (Promega Corporation, Madison, WI), 15 mM $MgCl_2$, 10 mM deoxynucleotide triphosphates, 70 pmole of primer 1 and primer 2 (GIBCO BRL), 3U of Taq polymerase (Promega Corporation), and 2 µl of DNA extracted. Amplification was performed in a Perkin Elmer Gene Amp PCR 2400 model (PE Applied Biosystems, Foster City, CA). This amplification cycle was modified from Beall and his colleges's protocol by changing annealing temperature from 46^{0}C to 50^{0}C to get rid of non-specific band. The temperature program was as follows;

1 cycle

94 ^{0}C 1 min for initiation denaturation

10 cycle of

94^{0}C 15 sec for denaturation

50^{0}C 30 sec for annealing

72^{0}C 1 min 15 sec for extension

20 cycle of

94 ^{0}C 15 sec for denaturation

50^0C 30 sec for annealing

72^0C 1 min 15 sec for extension, with a 10 sec increment for each of the subsequent 19 cycles

And final extension

1 cycle of

72^0C 8 min and then at 4 ^{0}C storage

Stored PCR products –20 ^{0}C until use.

4.3 Analysis of amplified DNA

Ten microliter of amplified products were analysed by agarose 1.5% gel electrophoresis (GIBCO BRL) consisted of 2 μl/40 ml ethidium bromide, in Tris-acetate buffer at 100 volts for 30 min and visualized and photographed under UV light. The size of PCR product is approximately 1-1.5 kb. PCR product were stored at –20 ^{0}C until use.

4.4 Purification of PCR product

The PCR products were purified by QIAquick PCR purification kit as described by the manufacture (QIAGEN, Max-Volmer-StraBe4, Hilden, Germany). QIA quick PCR purification kits for direct purification of double stranded PCR products from amplification reactions and DNA cleanup from other enzymatic reactions. The QIA quick system combines of spin column technology with the selective were binding properties of a uniquely designed silica-gel membrane. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are efficiently washed away, the pure DNA is eluted with Tris buffer or water. Then measured of DNA concentration by spectrophotometer (BIO-RAD, SmartSpec ^{tm} 3000, U.S.A) and adjusted DNA concentration is approximately 100 ng/μl. for sequencing method.

5. Sequence analysis

Approximately 100 ng each of PCR products were analyzed. Two primers set,

: emmseq 2 5' TATTCGCTTAGAAAATTAAAAACAGG

: CR reverse 5' CCCTTACGGCTTGCTTCTGA

from Bernard Beall and his colleges in 1996 – 1997 and Sumaree Pruksakorn in 2000. They were sequenced with the Perkin Elmer Big Dye Terminator Cycle Sequencing Ready Reaction Kit in accordance with the manufacture's instructions and an ABI 310 automated sequencer (both from the Perkin Elmer Corporation, ABI PRISM, Applied Biosystem, Foster City, CA). The sequencing reaction in a final volume of 10 μl comprised 1μl of 5 pmole sequencing primer, 4μl Perkin Elmer Big Dye Terminator, 3 μl double distilled water and 2μl of 50 ng/μl of purify PCR product. Use the following cycling parameters:

25 cycle of

96^0C 30 sec for denaturation

55^0C 10 sec for annealing

60^0C 4 min for extension

Then sequencing reactions were purified by precipitating ethanol sodium acetate and then were stored at –20 ^{0}C until use. Before used, purify sequencing reaction were added with 15 μl template suppressor reagent (TSR) and then were heated 95 ^{0}C at 2 min and place on ice immediately.

6. Analysis

DNA sequences were analyzed by using programs Chromas. The BLAST 2 program from web site" http://www.ncbi.nlm.nih.gov/ BLAST " was used to determine levels of homology with published sequences in the GenBank and CDC, USA database.

Emm sequence type was identified with the following criteria. If they were greater than or equal to 95% identity over the first 160 bases of sequence to previously identified *emm* sequences, they are classified as the same type. Sequences were identified as novel *emm* type if they were less than 95% identity. However, if the sequences were not 100% identical to previously identified sequence but they were greater than 95% identity, sequences were identified as variant of the closely related *emm* type. The sequences starting with "*emm*" indicated that several reference laboratories besides the CDC, USA streptococcal laboratory have validated this type. Sequences starting with "*st*" (sequence type) have not yet been validated by all of the reference laboratories (http://www.cdc.gov/ncidod/biotech/strep/strepindex.html).

6.1 GenBank submission

Using sequin program that is a stand-alone software tool developed by the NCBI for submitting and updating sequences to the GenBank database. The process for submitting the sequences of *emm* gene by sequin program includes the preparation of the nucleotide to FASTA format that is the raw sequence with a definition line. The definition line begins with a>sign, and followed immediately by a name for the sequence and a title. When these nucleotides were prepared and then start to submit by insert the authors form, sequence format form, organism and sequences form. When these information of author, organism, and sequence were finished, we view in the GenBank form to check the error of this submission. The result of this submission was sending to GenBank by email address and attachment file and the accession number will be assigned for each submitted sequence.

6.2 Statistical analysis

The correlation between non-invasive and invasive specimens was calculated by Chi-square analysis. Chi-square analysis is the statistical for calculate the difference of two groups by using two by two table. The data will be considered significant when p value <0.05.