CHAPTER 3

MATERIALS AND METHODS

3.1 Setting

The study was carried out at 15 hospitals in Southern Thailand including a university hospital and 14 provincial hospitals. The university hospital was Songklanagarind Hospital and the provincial hospitals were tertiary care governmental hospitals. All hospitals possessed more than 330 beds. The provincial hospitals were Chumporn Hospital, Krabi Hospital, Maharaj-Nakhonsi Thammarat Hospital, Narathiwat-rajanagarind Hospital, Patthalung Hospital, Pattani Hospital, Vajira-Phuket Hospital, Pung-nga Hospital, Trang Hospital, Satul Hospital, Songkhla Hospital, Hat Yai Hospital, Suratthani Hospital and Yala Hospital. Three hospitals located in Songkhla province, including Songklanagarind Hospital, Songkhla Hospital and Had Yai Hospital. Besides, we also separated the region into 2 areas according to geography: upper Southern area and lower Southern area. Upper Southern area consisted of Chumporn Hospital, Krabi Hospital, Maharaj-Nakhonsi Thammarat Hospital, Vajira-Phuket Hospital, Pung-nga Hospital and Suratthani Hospital. Another 9 hospitals were in lower Southern area. Pneumococcal isolates were collected from each hospital during September 1, 2001 to May 31, 2002.

3.2 Sample Size Calculation

Based on the antimicrobial susceptibility of Thailand reported by National Antimicrobial Resistance Surveillance Center in year 2000, 1,748 isolates of pneumococci were included in the survey. The organism was isolated from sputum and

sterile sites 59% and 23%, respectively. Proportion formula was used to estimate sample size:

$$n = Z^2_{1-\alpha/2}\, \pi(1-\pi)/e^2$$

where n = sample size

$Z^2_{1-\alpha/2}$ = Z value or standard normal deviate, where 95% confidence interval, $Z^2_{1-\alpha/2} = 1.96$

π = 82%

e = acceptable error = 10%

thus

$$n = (1.96)^2(0.82)(0.18)/(0.1)^2$$
$$= 56.7$$

Of overall isolates, there was penicillin-nonsusceptible pneumococcal isolates 48%. Therefore,

$$n = (1.92)^2(0.48)(0.52)/(0.1)^2 \quad (\text{where } \pi = 48\%)$$
$$= 95.9$$

Hence, the sample size in the study was 96 pneumococcal isolates.

3.3 Ethics

The study protocol was approved by the Ethic Committees of Songklanagarind Hospital.

3.4 Inclusion and Exclusion Criteria

3.4.1 Inclusion Criteria

Pneumococcal isolates were from both sterile site such as blood and CSF and also sputum of patients who had community-acquired pneumococcal infections, including

pneumonia, bacteremia and meningitis. Therefore, pneumococci had to be isolated within 72 hours of hospital admission. Patient medical record had to be available.

3.4.2 Exclusion Criteria

Patient with hospital-acquired infection was excluded. Hospital-acquired infection is defined as either the infection that developed after 72 hours of hospitalization or the infection that developed within 2 weeks after previous hospital discharge.

3.5 Methods of Study

The study was divided into 2 parts:

Part 1: In vitro assessment of prevalence of drug-resistant *Streptococcus pneumoniae* in Southern Thailand

Part 2: Retrospective review of medical records of patient whose isolates were obtained in Part 1

3.5.1 Part 1: In vitro assessment of prevalence of drug-resistant *Streptococcus pneumoniae* in Southern Thailand

3.5.1.1 Materials and Instruments

The media, chemical and biological substances for culturing the isolates were as the following:

Mueller Hinton agar	Merck, Darmstadt, Germany
Mueller Hinton broth	Difco, Becton Dickinson, Sparks, MD, USA
Tryptic soy broth	Difco, Becton Dickinson, Sparks, MD, USA
Glycerine USP.	Srichand United Dispensing, Thailand

McFarland 0.5 standard (Appendix A)

Human blood and sheep blood

Instruments and kit-test for testing the drug sensitivity were as the following:

Microcentrifuge tube 1.5 ml

Petri dish 90 mm and sterile plastic plate 150 mm

E-test AB Biodisk North America, NJ, USA

(penicillin, cefotaxime, imipenem, levofloxacin, erythromycin)

Optochin disk Oxoid Limited, Hampshire, England

Incubator (35-37°C), CO_2 enriched chamber

Forceps, loop, swabs, test tubes, pipette

3.5.1.2 Isolation of *Streptococcus pneumoniae*

A prospective study was carried out by collecting isolates from studied patients who infected with pneumococci during September 1, 2001 to May 31, 2002 from 15 hospitals in Southern Thailand. Pneumococcal isolates were from blood, CSF and sputum of patients who developed pneumococcal infection within 72 hours of hospital admission.

The data collection for each isolate included hospital name, patient name, hospital number, age, sex, ward, specimen site and collection date (Appendix B). The types of collection tubes: tryptic soy broth and blood agar were prepared for collecting the organism. Tryptic soy broth consisted of 3 g of tryptic soy broth (Difco, Becton Dickinson, Sparks, MD, USA) and 20 ml of glycerine USP (Srichand United Dispensing, Thailand) made up to 100 ml in distilled water. The medium was autoclaved at 121°C for 15 minutes. Five ml of human blood was added to sterile medium at 40-45°C. Each 1 ml of the medium was dispensed aseptically into a microcentrifuge tube. The blood agar consisted of 3.4 g of Mueller Hinton agar (Merck, Darmstadt, Germany) in 100 ml of distilled water, and was autoclaved at 121°C for 15 minutes. Five percent of human blood (5 ml) was added to melted agar at 40-45°C. Each 1 ml of the blood agar was dispensed aseptically into a microcentrifuge tube. Those of data collection forms and

collection tubes were sent to each hospital. Pneumococcal isolate was kept in collection tubes by microbiologist of each hospital. After tapping the organism in tryptic soy broth tubes, the tubes were stored in freezer and were taken to laboratory by investigator. Additionally, after tapping pneumococci in blood agar tubes, the tubes were sent to laboratory by mail after incubating in 5% CO_2 atmosphere, 37°C, 18-20 hours.

After obtaining pneumococcal isolates from each hospital, the isolates were re-subculture to isolate pneumococci from other contaminated microorganisms. To prepare the blood agar plate, blood agar was dispensed aseptically into a 90 mm petri dish to give 20 ml of medium per plate. Pneumococcal isolate was streaked on blood agar plate in 4 separate directions. The plate was placed into 37°C incubator in 5% CO_2 atmosphere for 18-20 hours. Pure pneumococcal isolates were frozen in tryptic soy broth tubes at –70°C.

3.5.1.3 Identification of *Streptococcus pneumoniae*

Optochin disks were used to identify pneumococci. The Muller Hinton broth consisted of 2.1 g Mueller Hinton broth (Difco, Becton Dickinson, Sparks, MD, USA) in 100 ml of distilled water, and was autoclaved at 121°C for 15 minutes. Each 1 ml of Mueller Hinton broth was dispensed aseptically into each sterile test tube. The inoculum of pneumococci was emulsified in 1 ml of sterile Mueller Hinton broth. A 0.5 McFarland standard was used to adjust the density of the inoculum to achieve the approximated concentrations of $1\text{-}1.5 \times 10^8$ cfu/ml. The blood agar plates were prepared similarly to the procedure in 3.5.1.2. The inoculum was streaked the entire surface of blood agar in three separate directions by sterile cotton swab. After the surface of blood agar was dry, optochin disk (Oxoid Limited, Hampshire, England) was placed on the blood agar plate with a pair of forceps. The plate was incubated in 37°C incubator, 5% CO_2 atmosphere for 18-20 hours. The isolate was identified as pneumococci when diameter of clear zone is $\geq$ 16 mm.

3.5.1.4 Drug Susceptibility Testing

E test is a qualitative technique for determining the antimicrobial susceptibility. E test consists of a thin, inert and non-porous plastic strip. One side of the strip is calibrated with the MIC reading scale in μg/ml. The other surface of the strip covers with an exponential concentration gradient of antimicrobial agents. The continuous concentration range to 15 two-fold dilutions in a conventional MIC method. Five antibiotic E test (AB Biodisk North America, NJ, USA) included in this study were:

Penicillin, concentration ranges from 0.002 μg/ml to 32 μg/ml

Cefotaxime, concentration range from 0.002 μg/ml to 32 μg/ml

Imipenem, concentration range from 0.002 μg/ml to 32 μg/ml

Levofloxacin, concentration range from 0.002 μg/ml to 32 μg/ml

Erythromycin, concentration range from 0.016 μg/ml to 256 μg/ml

The sheep blood agar and Mueller Hinton broth were prepared as directed in the previous section (3.5.1.2 and 3.5.1.3). The sheep blood agar was dispensed aseptically into a 150 mm sterile plastic plate. Overnight culture of pneumococci were suspended in 1 ml of sterile Mueller Hinton broth to achieve 0.5 McFarlacd tubidity standard. A sterile cotton swab was dipped into suspension and squeezed out excess fluid against the sides of the tube and swabbed smoothly on the entire surface of Mueller Hinton agar supplemented with 5% sheep blood in three separate directions. Fifteen minutes later, E test strips of 5 drugs were placed into a plate with a pair of forceps (Appendix C). The plate was placed in 35°C incubator in 5% CO_2 atmosphere. After 20-24 hours of incubation, the MICs were determined (Appendix D). The National Committee for Clinical laboratory Standards 2002 criteria (NCCLS) was followed to interpret the susceptibility of tested organism. The MIC cutoff was classified into 3 categories: susceptible, intermediately susceptible and resistant (Table 8). Non-susceptible isolates were classified as isolates those were intermediately susceptible and resistant to the antibiotic tested. Pneumococci that resist to 3 or more antimicrobial agents of different classes

(penicillins, cephalosporins, macrolides, fluoroquinolones, co-trimoxazole, or carbapenems) are defined as multidrug-resistance (Crook and Spratt, 1998; Chenoweth, *et al*, 2000; Harwell and Brown, 2000).

The intermediate-susceptible pneumococcal strain, *Streptococcus pneumoniae* American type culture collection (ATCC) 49619 was used as a controlled organism in each of the susceptibility tests. The MICs of *Streptococcus pneumoniae* ATCC 49619 should be correlated with NCCLS guideline (Table 9).

Table 8 MICs interpretation for *Streptococcus pneumoniae*

Drugs	MICs, μg/ml		
	Susceptible	Intermediate	Resistant
Penicillin G	≤ 0.06	> 0.06 to < 2	≥ 2
Cefotaxime (meningitis)	≤ 0.5	> 0.5 to < 2	≥ 2
Cefotaxime (non-meningitis)	≤ 1	> 1 to > 4	≥ 4
Imipenem	≤ 0.12	> 0.12 to < 1	≥ 1
Levofloxacin	≤ 2	> 2 to < 8	≥ 8
Erythromycin	< 0.25	≥ 0.25 to < 1	≥ 1

Table 9 Quality control ranges of MICs for *Streptococcus pneumoniae* ATCC 49619

Drugs	MICs, μg/ml
Penicillin G	0.25-1
Cefotaxime	0.064-0.25
Imipenem	0.032-0.125
Levofloxacin	0.5-2
Erythromycin	0.032-0.25

Duplicated E test testing, and reading E test values by three persons were applied in this study in order to confirming the results. If MIC of the first and the second test were

different, the highest value had been selected. However, if the difference of the MIC between the first and the second test was more than 2-dilution, the MIC test was repeated.

3.5.1.5 Statistical Analysis

Descriptive statistics was used to analyze the prevalence of drug-resistant pneumococci as followed:

$$P = \frac{n}{N}$$

where: P = prevalence of drug-resistant pneumococci

n = total number of drug-resistant pneumococci

N = total number of pneumococcal isolates

The susceptibility of each antibiotic was described in percentages of sensitivity, and so were 50^{th} percentile (MIC_{50}) and 90^{th} percentile (MIC_{90}).

3.5.2 Part 2: Retrospective review of medical records of patient whose isolates were obtained in Part 1

3.5.2.1 Collection of Clinical Data

This part was a retrospective cohort study. The medical records of patients whose isolates obtained were reviewed. The data collection included demographics, clinical presentation of the infection, co-morbidity, diagnosis, prior antibiotic use, laboratory data (chemistry, hematology, microbiology) and antibiotic treatment (Appendix E).

3.5.2.2 Clinical Definitions

A community-acquired infection was defined by the presence of signs and symptoms of pneumococcal infection within 72 hours of hospitalization.

A disease was based on physician diagnosis, which documented in medical record.

Fever was defined as an axillary temperature $\geq$ 38°C, and resolution of fever was accepted when temperature remained below 38°C for at least 48 hours.

Death within 1 week of positive culture result was considered as related to that infection.

Co-morbidities were regarded as patients who had confirmed diagnosis of one or more of the following: cancer, cirrhosis or chronic liver disease, diabetes, chronic renal disease, HIV infection or AIDS, heart failure, chronic pulmonary disease, leukopenia (WBC < 5,000/mm^3), and hypertension.

3.5.2.3 Clinical Outcome Assessment

Simplified Acute Physician Score II (SAPS II) score (Appendix F) and Pediatric Risk of Mortality (PRISM) scores (Appendix G) were used to adjust illness severity in adults and children, respectively. The assessment was carried out by using patient parameters measured within the first 48 hours of hospital admission. The missing parameters were assumed to be within the normal limit.

Assessment of clinical outcome was based on medical record review. Patient clinical status was assessed on Days 2 (24-48 hours), 3 (48-72 hours), 7 and 14 after antibiotic therapy was initiated comparing with that on presentation. Those status were categorized into 5 categories as followed:

- Resolved: complete resolution of preexisting signs and symptoms of infection such as signs of respiratory distress on cases of pneumonia, patient's

responsiveness returned to normal in cases of sepsis and meningitis, or clinical laboratory parameters such as WBC count was within normal range.

- Improved: improvement of clinical status without complete resolution of clinical presentation of infection.
- Unimproved: no clinical improvement or deterioration.
- Worse: deterioration in clinical signs or overall condition, or need supportive care, for example oxygen requirement, mechanical ventilation, or alteration of antimicrobial based on poor clinical response even if there was no clear deterioration.
- Died: patient died during hospitalization.

Initial response within 72 hours of therapy was categorized into favorable or unfavorable response.

- Favorable: patient whose clinical status was resolved or improved within 72 hours after the initiation of antibiotic therapy for at least 48 hours.
- Unfavorable: patient whose clinical status was unimproved or worse or died within 72 hours after the initiation of antibiotic therapy for at least 48 hours.

Assessment of clinical response at the end of antimicrobial therapy (final clinical outcome) included only patients who received particular regimen at least 48 hours. Those were classified into 3 categories as followed:

- Successful: patient clinical status resolved or improved after antimicrobial therapy without other complication of infection or disease, or patient could be switched antibiotic therapy from intravenous to oral therapy within 7 days.
- Treatment failure: treatment regimen was changed due to poor clinical response except changing regimen due to adverse effect of that antimicrobial, or patient died from pneumococcal infection within 7 days.

- Undetermined: treatment regimen was changed based on susceptibility results and the patient's condition had not improved or deteriorated the response to treatment, or against therapy, or data from medical records was uncompleted.

3.5.2.4 Microbiology Outcome Assessment

Assessment of microbiological outcome was based on repeating of organism culture. Microbiological outcome was classified into 3 categories as followed:

- Eradicated: the organism was not found in subsequent culture at least 48 hours of initiated antimicrobial therapy.
- Persisted: the organism was found in subsequent culture at least 48 hours of initiated antimicrobial therapy.
- Undetermined: data from medical chart was uncompleted or no repeated culture.

3.5.2.5 Statistical Analysis

The univariate, χ^2 test, Fisher's exact test, Student's *t*-test and Mann-Whitney U test were used to analyze data. χ^2 test was used to compare the nominal data. Fisher's exact test was computed when a cell of the 2x2 table has an expected frequency of less than 5. Student's *t*-test was use to compare mean values of interval or ratio data, such as age, length of stay and severity score at presentation between patient with penicillin-susceptible and -nonsusceptible pneumococci. If data was not normally distribution, Mann-Whitney U test was used instead of Student's *t*-test.

Risk factors for infecting with penicillin-nonsusceptible pneumococci were analyzed by multiple logistic regression.

Statistically significant was accepted when $P < 0.05$.