CHAPTER III

MATERIALS AND METHODS

Materials

1. Media

1.1 Media for isolation and identification of *Staphylococcus aureus* (*S. aureus*)

a. Mannitol salt agar (MSA) (Difco Laboratories, USA)

b. Tryptose blood agar (Difco Laboratories, USA)

1.2 Media for antimicrobial susceptibility tests

a. Mueller-Hinton agar (MHA) (Difco Laboratories, USA)

b. Mueller-Hinton agar with 5% NaCl

c. Mueller-Hinton broth (MHB) (Difco Laboratories, USA)

1.3 Media for plasmid profile analysis

a. Tryptose blood agar (Difco Laboratories, USA)

b. 0.8% Agarose gel (Agarose type II:Medium EEO) (Sigma Chemical company, USA)

(The media formulas with the preparation method

were shown in the appendix.)

2. Reagents

2.1 Reagents for isolation and identification of *S. aureus*

a. 3% Hydrogen peroxide

b. Gram's stain reagents

c. Human plasma

d. Chromogenic cephalosporin (nitrocefin) (Glaxo, England)

2.2 Reagents for antimicrobial susceptibility tests

a. 1% Potassium phosphate buffer pH 6.0

b. 0.5 Mc Farland standard solution

c. Sterile normal saline

d. Methicillin sodium standard (895 μg/mg)

e. Antimicrobial paper disks

All antimicrobial disks were purchased from Difco Laboratories, USA. These included ampicillin 10 μg., cefotaxime 30 μg., cephalothin 30 μg., chloramphenicol 30 μg., clindamycin 2 μg., cloxacillin 1 μg., erythromycin 15 μg., gentamicin 10 μg., imipenem 10 μg., methicillin 5 μg., nafcillin 1 μg., neomycin 30 μg., norfloxacin 10 μg., oxacillin 1 μg., penicillin G 10 u., tetracycline 30 μg., trimethoprim 1.25 μg.and sulfamethoxazole 23.75 μg., and vancomycin 30 μg.

Disks were stored at 2°C to 8°C. Prior to use, disks were placed at room temperature for about 15 minutes to prevent condensation.

2.3 Reagents for plasmid profile analysis

a. Lysostaphin (Sigma Chemical company, USA)
b. Proteinase K (Amresco, USA)
c. RNase one (Promega, USA)
d. Lamda HindIII standard size of plasmid (Sigma Chemical company, USA)
e. Lysis buffer
f. Tris-borate buffer
g. 10 mM Tris, pH 8.0
h. 3 M Sodium acetate solution
i. TE buffer
j. Saturated phenol in 10 mM Tris, pH 8.0
k. Loading buffer
l. Chloroform (Merck, USA)
m. Isopropanol (Merck, USA)
n. 70% Ethanol
o. Ethidium bromide (Sigma Chemical company, USA)

(The reagent formulas with the preparation method were shown in the appendix. All reagents were made in bulk and stored at 4°C except the lysostaphin, proteinase K, RNase One and Lambda HindIII standard which were stored after dilution in aliquot at -20°C. Isopropanol, 70% ethanol, and ethidium bromide were stored at room

temperature.)

Methods

1. Study population

The study population was divided into 2 groups as followed:

1.1 Healthy personnel

a. Non-medical personnel

The non-medical personnel group consisted of 147 students from Faculty of Pharmaceutical Sciences, Chulalongkorn University. They did not achieve any kind of antibiotics 7 days before the time of specimen collection.

b. Medical personnel

The 115 medical personnel group consisted of both physicians and nurses from Siriraj Hospital and Police General Hospital.

1.2 Patients

a. Patients with wound infections

The 101 patients with wound infections who attended the out patient department, Siriraj Hospital were included in this study.

b. Burn patients

Burn patients consisted of 3 cases of in-patients from Bhumipol Adulyadej Hospital , 16 cases from

Police General Hospital and 32 cases from Siriraj Hospital. Number of these patients who used antibiotics were recorded.

2. Specimen collection

The specimen collection was done during November 1992 to February 1994. Sterile cotton swabs, which soaked with sterile normal saline immediately before used, were in the specimen collection. Nasal swabs were obtained from all non-medical personnel. Nasal and hand swabs were obtained from medical personnel while nasal, hand and wound swabs were obtained from patients.

3. Isolation and identification of *S. aureus*

Each type of swab was inoculated onto a tryptose blood agar plate and a mannitol salt agar plate. All plates were incubated overnight at 37°C. The identification of *S. aureus* strain was performed according to Bergey's manual (Krieg, 1984): Gram's staining, catalase and coagulase test (slide and tube method) are mentioned in the appendix.

4. Antimicrobial susceptibility test

4.1 Paper disk susceptibility test

a. Preparation of antimicrobial testing medium

Twenty-five millilitres of Mueller-Hinton agar (MHA) was dispensed into each of the sterile plates with internal diameter of 9 cm to yield a uniform depth of 4 mm. The agar plates were then stored at 4°C to 8°C and

wrapped in plastic bags if the storage period was exceed 5 days. Plates were stored and used within 2 weeks. Before performing the test, the plates were placed in an incubator at 35°C for 30 minutes with their lids slightly open to permit the evaporation of surface moisture.

b. Preparation of the inoculum

Four well-isolated colonies of each *S. aureus* isolate and *S. aureus* ATCC 25923 strain were selected from the culture agar plate and transferred to a tube containing 4 ml. of Mueller-Hinton broth (MHB).

c. Standardization of the inoculum

1. The inoculated broth culture was incubated at 37°C for 4 hour or until the turbidity of the culture was equal to the turbidity of 0.5 Mc Farland standard solution.

2. If the turbidity of the actively growing broth culture was exceed than that of 0.5 Mc Farland standard solution it would be adjusted with sterile normal saline to obtain an appropriate turbidity.

d. Streaking of the plate

A sterile cotton swab was dipped into the standardized inoculum. The excess inoculum was removed by rotating the swab several times against the inside wall of the tube above the fluid level. The surface of the MHA plate was inoculated by streaking the swab over the

surface. Streaking was repeated 3 times and for each time the plate was rotated 60° to ensure an even distribution of inoculum.

e. Application of disks

Immediately soon after the plates were streaked, the tested antimicrobial disks were applied with a sterile forcep. The disks were pressed down with slight pressure to ensure complete contact of the disk to the agar surface.

Disks were arranged at least 15 mm. from the edge of the plate and apart from each other by a distance of 15 to 20 mm. This arrangement reduced the likelihood of zones overlapping each other, which made quantification and interpretation difficult.

f. Incubation of plates

The inoculated plates were incubated aerobically at 37°C for 18 hour in an inverted position.

g. Interpretation of the disk susceptibility test

The diameter of each inhibition zone was measured with sliding vernier caliper. Zone diameter interpretative chart for staphylococci was shown in Table 4.

Table 4* Zone diameter interpretative chart for staphylococci (mm.)

Antimicrobial Agent	Zone diameter interpretative standard					Control zone diameter limits *S.aureus*
	Disc Potency	Resistant	Intermediate	Moderately susceptible	Susceptible	
Ampicillin	10 μg	≤28	-	-	≥29	27-35
Cefotaxime	30 μg	≤14	-	15-22	≥23	25-31
Cephalothin	30 μg	≤14	-	15-17	≥18	29-37
Chloramphenicol	30 μg	≤12	13-17	-	≥18	19-26
Clindamycin	2 μg	≤14	15-20	-	≥21	24-30
Cloxacillin	1 μg	≤10	11-12	-	≥13	
Erythromycin	15 μg	≤13	14-22	-	≥23	22-30
Gentamicin	10 μg	≤12	13-14	-	≥15	19-27
Imipenem	10 μg	≤13	-	14-15	≥16	-
Methicillin	5 μg	≤9	10-13	-	≥14	17-22
Nafcillin	1 μg	≤10	11-12	-	≥13	16-22
Neomycin	30 μg	≤12	13-16	-	≥17	18-26
Norfloxacin	10 μg	≤12	13-16	-	≥17	17-28
Oxacillin	1 μg	≤10	11-12	-	≥13	18-24
Penicillin	10 U	≤28	-	-	≥29	26-37
Tetracycline	30 μg	≤14	15-18	-	≥19	19-28
Trimethoprim/ Sulfamethoxazole	1.25 μg 23.75 μg	≤10	-	11-15	≥16	24-32
Vancomycin	30 μg	≤9	10-11	-	≥12	15-19

* Modified from Lorian, 1991

4.2 Determination of minimal inhibitory concentration (MICs) of methicillin against MRSA

Minimal inhibitory concentration of methicillin was obtained using the agar dilution method.

a. Cultures: *S. aureus* strain which resisted to methicillin by the disk susceptibility test were studied, including the *S. aureus* ATCC 25923.

b. Preparation of methicillin standard MHA plates: Methicillin sodium standard was weighed and dissolved in 1% phosphate buffer solution pH 6.0 . Methicillin solution was added in sterile melted Mueller-Hinton agar with approximately temperature of 45°C, to make the final methicillin concentration of 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg/ml, accordingly. A 25 ml volume of Mueller-Hinton agar containing methicillin were dispensed into a sterile plate with internal diameters of 9 cm. The antibiotic containing media were used on the same day.

c. Preparation of the inocula: A well-isolated colony of each tested organism was selected from a cultured agar plate and transferred into a tube containing of 4 ml of MHB. The method of inoculum preparation was performed a described in the section of the paper disk susceptibility test.

d. Standardization of the inocula: The method for the standardization of inoculum was similar to the method used in the inoculum preparation for the antimicrobial disk susceptibility test. After the broth cultures were adjusted, they were transferred into the replicator tray.

e. Plate inoculation: The organisms were replicated on Mueller-Hinton agar plates containing various concentration of methicillin sodium as prepared in **b.** The MHA plates without antibiotic were also incubated with all tested inoculum and used as control.

f. Incubation of plates: The inoculated plates were incubated aerobically at 37°C for 18 hour in an inverted position.

g. Determination of MICs: The MICs was determined by the lowest concentration of methicillin in the inoculated plate that could inhibit the growth of the organisms tested.

5. Detection for beta-lactamase

Beta-lactamase producing strains were detected using a rapid test for hydrolysis of a chromogenic cephalosporin [nitrocefin = {3-(2,4-dinitrosthyryl)(6R,7R)-7-(2-theinylacetamido)-cephem}] as recommended by O'Callaghan *et al.*, 1972.

5.1 Preparation of nitrocefin solution

a. The stock solution of nitrocefin was prepared to make a final concentration of 10 mg of nitrocefin per ml of dimethyl sulfoxide (DMSO).

b. Working solution was diluted with 0.05 M phosphate buffer, pH 7.0 to make a final concentration of 500 μg/ ml.

c. The solution was stored in a light protected container and kept at 4°C.

5.2 Procedure for beta-lactamase detection

Several colonies of tested *S. aureus* were emulsified in a volume of 0.1 ml of the above working solution of nitrocefin. The test was performed at room temperature. A positive reaction occurred when the color of the solution changed from yellow to red. The interpretation of the test was done within 10 minutes.

6. Plasmid profile analysis of MRSA

6.1 Cultivation of tested strains

The tested strains including *S. aureus* ATCC 25923 were cultivated on tryptose blood agar plates and incubated overnight at 37°C.

6.2 Plasmid DNA extraction

The plasmid DNA extraction was performed using the modified technique recommended by Goering and Duensing, 1990, Sambrook *et al.*, 1989 and Dunkle and

Slippel, 1984.

1. Several colonies of 2-3 mm in diameter were scraped from the plate by using a sterile loop. The culture was suspended in a sterile microtube containing a 50 μl volume of lysis buffer. A 70 μl volume of lysostaphin (75 μg/ml in 10 mM tris,pH 8.0) was then added.

2. The lysing mixture was then incubated at 37°C for 30 minutes and 1 μl of RNase One (1 mg/ml) was added. The mixture was then incubated at 37°C for 1 hour. Twenty μl of Proteinase K (50 μg/ml) was added into the mixture and the mixture was then incubated at 37°C for 1 hour.

3. The lysis process was completed by boiling for 3 minutes and immediately cooled in ice bath.

4. The mixture was then centrifuged at 10,000 g for 15 minutes at room temperature (about 24°C) and the supernatant which contained plasmid DNA was recovered.

5. The supernatant was extracted with equal volume of phenol-chloroform (1:1). The mixture was vortexed for 5 minutes and centrifuged at 10,000 g for 20-30 minutes at room temperature. The supernatant was then again recovered and reextracted twice with equal volume of chloroform.

6. DNA was precipitated from the cleared lysate by the addition of 420 μl of cool isopropanol and 1:10 volume of 3 N sodium acetate solution and freezing at -20°C for 30 minutes.

7. The mixture was immediately centrifuged at 10,000 g for 15 minutes, and the supernatant was aspirated with a micropipet.

8. The residual which supposed to contain plasmid DNA was washed by addition 400 μl of 70% ethanol. The suspension was then centrifuged at 10,000 g for 2 minutes,and the supernatant was removed by a micropipet.

9. The microtube containing DNA precipitate was then dried in a cool silica gel box and stored at -20°C for overnight.

10. The residual DNA was resuspended with 20 μl volume of TE buffer, and 3 μl volume of loading buffer and then ready to be separated by using an electrophoresis.

6.3 Plasmid profile analysis

Each of 20 μl volume of plasmid DNA samples included 15 μl volume of the Lambda HindIII (700 ng), as standard size of plasmid were analyzed on a 0.8% agarose gel sized 10x15 cm, in tris-borate-EDTA (TBE) buffer at 70 V/cm for 2.5 hour. The gel was then stained with ethidium bromide (0.5 μg/ml in TBE) and viewed by UV transillumination. The photograph was taken with the 667 polaroid film size 3¼ x 4¼ inches. The size or profile of plasmid DNA was determined by calculating comparatively to the Lambda HindIII.