CHAPTER IV

MATERIALS AND METHODS

1. Subjects All the specimens in this study were obtained at Siriraj hospital. The details of the specimen were shown in Table 4-1 :

1.1. Population

The study population was divided into 2 groups as follow:

1.1.1. Healthy medical personnel

a. The Burn unit : The medical personnel group consisted of 26 physicians, 11 nurses, 11 assistants and 6 workers who worked in burn unit.

b. The Traumatic intensive care unit (TICU) : This group consisted of 24 medical personnel included physicians and nurses.

1.1.2. Patients

a. The Burn Unit : The 30 patients who were admitted in the burn unit Siriraj Hospital during the period of specimen collection.

b. TICU : The 11 patients were also included in this study.

2.4. Medical equipment in the burn unit : This included bed rails, enteral pumps and bath tubs.

2. Specimen collection

The specimen collection was done in the 32 weeks period. All specimen were collected by using sterile cotton swabs which were moistened with 0.85% sterile normal saline solution (NSS) prior used from the population as shown in Table4-1.

2.1 Patients in the burn unit: Specimen collection was performed under this following criteria. Nasal cavities, hands and wounds swabs were taken twice a week from the time that patients were admitted until discharge or move out from the burn unit

2.2. Medical personnel in the burn unit : Specimen collection was performed under this following criteria : nasal cavities swabs were taken weekly throughout the period of study.

2.3. Patients and medical personnel in the TICU : Types of specimen were the same as obtained from the burn unit except the specimen collection was obtained only one time on the day that MRSA emergence was reported.

2.4. Medical equipment : The medical equipment that were used for individual patients ; the bed rails, the enteral pumps particularly area of control panel which were operated by medical personnel were swab twice a week. The bath tubs swabs were taken twice a week before they were being used.

3. Isolation and Identification of *Staphylococcus aureus*

Swabs were inoculated onto a tryptose blood agar and a mannitol salt agar (Difco, USA), then incubated overnight at 37°C. The identification of *S. aureus* was performed according to Bergey's Manual of Systemic Bacteriology (104) including Gram's staining, catalase test, O-F glucose fermentation, and slide and tube coagulase test. Catalase test was performed in order to distinguish staphylococci from streptococci and micrococci. *Staphylococcus* species could ferment glucose and form acid which was indicated by turning color of O-F medium to yellow and no gas forming. The organisms with positive coagulase test were identified as coagulase-positive staphylococci.

MRSA were identified based on the resistance to oxacillin (1 µg) on Mueller-Hinton agar with 4% NaCl after the 24 hours incubation at 35°C. If the zone size was 10 mm or less, the culture was methicillin-resistant *S. aureus* (MRSA). *S. aureus* ATCC 25923 was also included in this test as a reference strain , and the zone size of the reference strain must be in the range of 18-24 mm.

4. Antibiotic Susceptibility test : Paper disk susceptibility test was performed according to disk diffusion method by Kirby-Bauer (105) and NCCLS (106) as followed :

Table 4-1 The number of specimen which taken from the 30 burn patients, the 54 medical personnel, and the medical equipment.

Code	Number of specimen which taken from			Total
	Nasal cavity	Hand	Wound	
A	3	3	3	9
B	9	9	9	27
C	12	12	11	35
D	15	15	15	45
E	2	2	2	6
F	8	8	8	24
G	1	1	-	2
H	9	9	9	27
I	6	6	6	18
J	7	7	7	21
K	5	5	5	15
L	8	8	8	24
M	1	1	1	3
N	7	6	7	20
O	4	4	4	12
P	1	1	1	3
Q	13	13	13	39
R	4	4	4	12
S	1	1	1	3
T	10	10	10	30
U	10	10	10	30
V	3	3	3	9

A, B,...,DD = the burn patients, a1-a11 = nurses , b1-b11 = assistant nurses, c1-c6 = workers, d1-d24 = Residents, d25-d26 = assigned doctors at the burn unit

Table 4-1 (cont.) The number of specimen which taken from the 30 burn patients, the 54 medical personnel, and the medical equipment.

Code	Number of specimen which taken from			Total
	Nasal cavity	Hand	Wound	
W	1	1	1	3
X	14	14	14	42
Y	14	14	14	42
Z	6	6	6	18
AA	2	2	2	6
BB	5	5	5	15
CC	4	4	4	12
DD	6	6	6	18
a1	31	-	-	31
a2	30	-	-	30
a3	29	-	-	29
a4	30	-	-	30
a5	29	-	-	29
a6	29	-	-	29
a7	28	-	-	28
a8	21	-	-	21
a9	29	-	-	29
a10	30	-	-	30
a11	30	-	-	30
b1	30	-	-	30
b2	30	-	-	30
b3	29	-	-	29

A, B,...,DD = the burn patients, a1-a11 = nurses , b1-b11 = assistant nurses, c1-c6 = workers, d1-d24 = Residents, d25-d26 = assigned doctors at the burn unit

Table 4-1 (cont.)The number of specimen which taken from the 30 burn patients, the 54 medical personnel, and the medical equipment.

Code	Number of specimen which taken from			Total
	Nasal cavity	Hand	Wound	
b4	30	-	-	30
b5	16	-	-	16
b6	31	-	-	31
b7	29	-	-	29
b8	30	-	-	30
b9	30	-	-	30
b10	29	-	-	29
b11	30	-	-	30
c1	31	-	-	31
c2	31	-	-	31
c3	31	-	-	31
c4	7	-	-	7
c5	2	-	-	2
c6	22	-	-	22
d1	1	-	-	1
d2	4	-	-	4
d3	4	-	-	4
d4	5	-	-	5
d5	4	-	-	4
d6	4	-	-	4
d7	4	-	-	4
d8	4	-	-	4

A, B,...,DD = the burn patients, a1-a11 = nurses , b1-b11 = assistant nurses, c1-c6 = workers, d1-d24 = Residents, d25-d26 = assigned doctors at the burn unit

Table 4-1 (cont.)The number of specimen which taken from the 30 burn patients, the 54 medical personnel, and the medical equipment.

Code	Number of specimen which taken from			Total
	Nasal cavity	Hand	Wound	
d9	4	-	-	4
d10	4	-	-	4
d11	4	-	-	4
d12	7	-	-	7
d13	7	-	-	7
d14	6	-	-	6
d15	6	-	-	6
d16	5	-	-	5
d17	2	-	-	2
d18	3	-	-	3
d19	9	-	-	9
d20	12	-	-	12
d21	7	-	-	7
d22	7	-	-	7
d23	5	-	-	5
d24	1	-	-	1
d25	27	-	-	27
d26	28	-	-	28
Enteral pump	-	-	-	191
Bed rails	-	-	-	191
Bath tub No.1	-	-	-	64
Bath tub No.2	-	-	-	64

A, B,...,DD = the burn patients, a1-a11 = nurses , b1-b11 = assistant nurses, c1-c6 = workers, d1-d24 = Residents, d25-d26 = assigned doctors at the burn unit

a. Preparation of media

Twenty-five millimeters of Mueller-Hinton agar (MHA)(Difco, USA) were poured into each 10 cm-diameter petri dish to yield a agar depth of a 4 mm. The mediums were then stored at 4°C and used within 2 weeks. Before performing the test, the petri dishes were placed in an incubator at 35°C for 30 minutes with their lids slightly open to permit the evaporation of surface mixture.

b. Preparation of inoculum and standardization of inoculum

The well-isolated colonies of each 18 hours MRSA culture and *S. aureus* ATCC 25923 strain were selected from blood agar plates and transferred to a tube containing 5 ml normal saline solution (NSS). The turbidity of culture was adjusted to 0.5 Mc Farland standard solution to obtain approximately 1.5×10^8 cells/ml.

c. Inoculation of standard inoculum

A sterile cotton swab was dipped in the adjusted inoculum and rotated several times against the inside wall of the tube to remove excess liquid. The entire surface of the MHA plate was inoculated by streaking the swab over the surface. Streaking was repeated 3 times and for each time the plates were rotated 60° to ensure an even distribution of inoculum.

d. Application of disks

Immediately or not later than 15 minutes after the inoculation of the plates, the antibiotic disks were applied to the surface of the medium with sterile forceps in order that diffusion and growth proceeded stimulously. The disks were then slightly pressed down to ensure complete contact of the disks to the agar surface.

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

e. Incubation of plates

The inoculated plates were incubated aerobically at 35°C for 24 hours in an inverted position.

f. Interpretation of the disk susceptibility test

The diameter of each zone of inhibition was measured with digital sliding vernier caliper. Zone diameter interpretation chart for staphylococci according to the standard of NCCLS (106) was used as shown in Table 4-2.

5. Detection for beta-lactamase

Beta-lactamase producing MRSA strains were detected according to the method recommended by Schoenknecht *et. al.*(1985) (107) as followed :

a. Preparation of nitrocefin substrate

Ten mg of nitrocefin was suspended in 1 ml of dimethyl sulfoxide. The solution was then diluted with 0.1M phosphate buffer (pH 7.0) to the concentration of 500 μg/ml. This solution would be yellow to light orange and stable for many weeks at 4 to 10°C.

b. Detection for beta-lactamase by rapid chromogenic cephalosporin method

One drop of the nitrocefin substrate was added onto a clean glass slide. One colony of the 18 hours MRSA culture was picked and then mixed with the substrate on the glass slide. The nitrocefin substrate would turn red within 10 minutes at room temperature (usually 1-2 minutes) if the culture was beta-lactamase producing strain.

6. Analysis of restricted fragments of chromosomal DNA by Pulsed-Field Gel Electrophoresis (PFGE)

Chromosomal DNA analysis by Pulsed-Field Gel Electrophoresis was performed according to the method recommended by Maslow *et.al.* (108) as followed :

a. Sample preparation

Each strain of MRSA was streaked onto Tryptic Soy Agar (TSA) (Merck, Germany) to yield single colonies. A single colony was isolated into 0.5 ml Tryptic Soy Broth (TSB) (Merck, Germany) and was grown for 2 hours or until turbid. The culture was streaked out onto a TSA plate and incubated overnight at 37°C. A single colony was picked to inoculate into 5 ml TSB and was then incubated overnight at 37° C. About 1 x 10^8 CFU/ml of the organisms were obtained and was then centrifuged at

1100xg for 15 minutes at 4°C in 5 ml PIV buffer. The PIV buffer was decanted from the cell pellet. The cell was resuspended and mixed thoroughly in 1.2 ml cold PIV buffer and was placed on ice.

The low melting point agarose (Difco, USA) was prepared by suspending 0.15g agarose in 10 ml PIV buffer in 25-ml flask. The agarose was melted by placing the flask into a beaker of boiling water. One ml of melted agarose was dispensed into each tube. The tube containing agarose was placed in 50°C waterbath. One ml of MRSA cell in PIV buffer was added. The tube was then slightly vortex. Three hundred microliters of the mixture was immediately dispensed into each well of the plug mould that had already placed in the ice-tray for 15 minutes before used. The mould was then placed at 4°C for 30 minutes to solidify the agarose plug.

Fresh lysis solution was made by adding 400 μl lysostaphin (Sigma, USA) and 800 μl lysozyme (Amresco,USA) into 40 ml lysis buffer. Four ml lysis solution were dispensed into each 15 ml round-bottom tube. When the plugs were solidified, they were pushed out from the mould into the tube with lysis solution and were incubated overnight at 37°C on a roller. The tubes were then chilled on ice for at least 15 minutes to harden the plugs. The lysis solution was carefully aspirated and 4 ml of ESP solution was dispensed into each tube. The tubes with each plug were incubated overnight at 50°C with gently shaking. The tubes were again chilled and the fresh ESP solution was changed one more time. The tubes were again incubated overnight at 50°C.

b. Restriction enzyme digestion

The plugs were washed in 5 ml 1xTE buffer at 37°C on a roller for four times for 1 hour, 1 hour, 2 hours and overnight. A labeled microcentrifuged tube containing restriction enzyme *Sma*I (Biorad, USA), 1x restriction buffer , bovine serum albumin (final concentration 100 μg/ml) and water to final volume of 250 μl were prepared for each strain. Each washed plug was sliced into a small piece about 1 mm thick using a glass coverslip. A sliced plug was added to the labeled

microcentrifuged tube contained restriction enzyme solution, and was then incubated overnight at 30°C. Each sliced plug was washed 3 times using 300 μl sterile water, the labeled microcentrifuged tube was then slowly rotated to make sure the sliced plug was washed perfectly. The sliced plug was repeatedly washed 3 times.

c. Gel preparation and loading

The running gel was made by dissolving 1 gram of Ultrapure high-melting temperature agarose (Gibco BRL,Spain) (1%wt/vol) in 90 ml 0.5xTBE buffer. The agarose was melted by autoclaving at 121°C, 15 pound/inch^2 for 2 minutes. The agarose was cool to the appropriate temperature (approximately 50°C), and then poured into the mould. The 10-well comb was placed in the gel to make 10-well running agarose gel. The gel was placed in the mould until solidified and then transferred to the electrophoresis tank (CHEF-DRIII system, BioRad, USA) where the gel was preelectrophoresed for 1 hour in 0.5xTBE buffer to improve the clarity and resolution of the gel using this following condition ; V = 6 v/m , initial switch time = 1 s , final switch time = 40 s and the temperature was 14°C . Each sliced plug was then loaded into each well of the gel. All the wells of the gel were filled with 2% low-melting point agarose to protect the sliced plug in the well. The gel was then placed in a PFGE tank with 0.5xTBE buffer and electrophoresed using the same condition as the preelectrophoresis condition except for the running time which was 24 hours.

d. Gel visualization

The gel was stained for 30 minutes with 0.025% ethidium bromide solution. After that it was rinsed with 200 ml tap water for 1-2 minutes. The gel was then photographed under UV illumination by using Image analysis Gel Doc 1000.

Table 4-2 Zone size interpretative chart for staphylococci (mm)

Antimicrobial agents	Disk potency	Zone diameter interpretative standard				Control zone diameter limits ATCC25923
		Resistance	Intermediate	Intermediate susceptible	Susceptible	
Amoxycillin/ Clavulanic acid	30 μg	≤19	-	-	≥20	28-36
Ampicillin/ Sulbactam	20 μg	≤13	-	14-16	≥17	29-37
Cefoperazone/ Sulbactam	75 μg/ 30 μg	-	-	-	≥21	-
Imipenem	10 μg	≤13	-	14-15	≥16	-
Vancomycin	30 μg	≤9	10-11	-	≥12	15-19
Teicoplanin	30 μg	-	-	-	≥12	-
Fosfomycin	50 μg	-	-	-	≥15	-
Chloramphenicol	30 μg	≤12	13-17	-	≥18	19-26
Erythromycin	15 μg	≤13	14-22	-	≥23	22-30
Clarithromycin	15 μg	-	-	-	≥18	-
Clindamycin	2 μg	≤14	15-20	-	≥21	24-30
Gentamycin	10 μg	≤12	13-14	-	≥15	19-27
Netilmicin	30 μg	≤12	13-14	-	≥15	22-31
Ciprofloxacin	5 μg	≤15	-	16-20	≥21	22-30
Co-trimoxazole	1.25μg/ 23.75μg	-	-	-	≥21	-

μg = microgram