ANALYSIS OF THREE PHENOTYPING METHODS AND PULSED-FIELD GEL ELECTROPHORESIS FOR DIFFERENTIATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATED FROM TWO HOSPITALS IN THAILAND

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Abstract. Methicillin resistant *Staphylococcus aureus* (MRSA) is an important hospital and community-acquired pathogen. Rapid and reliable epidemiologic typing is necessary for controlling the spread of MRSA outbreak. The objective of this study was to compare the phenotyping with the genotyping method to differentiate MRSA isolates obtained from the two hospitals in Thailand (central and northeastern). Seventy-four MRSA isolates were randomly collected and confirmed by the presence of *mecA* gene. Antibiogram, phage typing and enterotoxin production were used for the phenotyping analysis. Pulsed-field gel electrophoresis (PFGE) with *SmaI* digestion of chromosomal DNA was used for the genotyping analysis. We found 17 distinct profiles by the 3 phenotypic typing methods and 18 PFGE types designated as 5 major types (A - E) and 13 subtypes. The most frequent PFGE types and their related subtypes found in both hospitals were A and C, comprising 54 and 27%, respectively. The antibiogram could differentiate 6 different types. All isolates were resistant to the majority of antimicrobial agents tested, but were susceptible to vancomycin and fosfomycin. Ten (13.5%) MRSA isolates produced enterotoxin A. Nontypable phage and phage type 77 were found predominantly in MRSA isolated from the northeast and central hospital, respectively. A significant correlation was found between the phenotyping and the genotyping methods and there was a good correlation between antibiogram and PFGE. Antibiogram typing alone can be used as a useful epidemiological marker for practical purposes. PFGE types A and C were the common endemic MRSA clones in both hospitals in Thailand.

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is an important hospital and community-acquired pathogen. In Thailand, MRSA has also emerged at an alarming rate with severe morbidity and mortality (National Antimicrobial Resistance Surveillance Center, 1999). Some MRSA strains, also known as epidemic clones, can spread quickly (Montesinos *et al*, 2002). Therefore, specific and rapid epidemiologic typing is necessary for tracking interhospital spread and evolution of MRSA strains so that any outbreak can be controlled and eradicated.

Several methods, including both phenotyping and genotyping, have been used for discriminating MRSA strains (Tenover *et al*, 1994; Na'was *et al*, 1998; Sawai *et al*, 1998). Phenotyping methods, such as antibiogram, phage typing and enterotoxin productions, have been used by some investigators (Adesiyun *et al*, 1992; Sawai *et al*, 1998). Antibiogram is the most widely used typing tool in many hospital outbreaks, as the technique is simple to perform, inexpensive and easy to interpret (Adesiyun *et al*, 1992; Rossney *et al*, 1994; Essawi *et al*, 1998; Sawai *et al*, 1998). The genotyping method of

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pulsed field gel electrophoresis (PFGE) has been shown to have a great discriminative power for MRSA typing but the technique is costly, time-consuming, and requires electrophoresis equipment and skill (Blanc *et al*, 2002; Montesinos *et al*, 2002). Therefore, the aims of our study were to compare PFGE typing with the combination of three phenotyping methods, and to differentiate MRSA clones found in two hospitals in Thailand.

MATERIALS AND METHODS

Bacterial strains

Seventy-four methicillin resistant *S. aureus* (MRSA) isolates were randomly collected from clinical samples and carriers from two hospitals in Bangkok (BK) and in Khon Kaen (KK), Thailand, between 1997-2001. All isolates were confirmed as *S. aureus* based on coagulase production and standard microbiological procedures (Kloss and Bannerman, 1995). MRSA was confirmed by disk agar diffusion, broth microdilution and the presence of *mecA* gene by PCR.

Oxacillin disk agar diffusion (ODD) technique

A disk diffusion test was performed following National Committee for Clinical Laboratory Standards (NCCLS, 2000) using 1 μg of oxacillin disk on Muller-Hinton agar. The inhibition zone diameter for oxacillin ≤10 mm was considered as showing resistance.

Broth microdilution (MIC) technique

All 74 *S. aureus* isolates were also confirmed as MRSA by the broth microdilution method according to NCCLS protocol (NCCLS, 2000). Minimum inhibitory concentrations (MIC) were determined using a two-fold dilution of oxacillin in Muller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% NaCl. The MIC ≥16 mg/l was determined as resistance.

Detection of *mecA* gene

DNA was prepared following the method of Weller (1999). PCR was performed using the primers *mecA*1 (5′- AAAATCGATGGTAAAGG TTGGC) and *mecA*2 (5′ AGTTCTGCAGTACCGG ATTTTGC) (Murakami *et al*, 1991). The reaction was conducted in 50 μl of a reaction mixture containing DNA (10-200 ng), 200 μM each of deoxynucleoside triphosphates (dNTP) (Gibco BRL), 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 nM of each primer and 1.5 units of *Taq* polymerase (Gibco BRL). The thermal cycler (Perkin-Elmer, Gene Amp, PCR 2400) was programed for 30 amplification cycles consisting of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 1 minute. Positive PCR result showed a 533 bp fragment of *mecA* gene.

Antibiogram

Antimicrobial susceptibility test of 74 MRSA isolates was performed by disk agar diffusion method (NCCLS, 2000) using commercially manufactured disks (Oxoid). All isolates were examined for susceptibility pattern against a panel of 13 antimicrobial agents : cephalothin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), cotrimoxazole (25 μg), gentamicin (10 μg), penicillin (10 U), lincomycin (30 μg), tetracycline (30 μg), kanamycin (30 μg), oxacillin (1 μg), ciprofloxacin (5μg), fosfomycin (50μg) and vancomycin (30 μg).

Bacteriophage typing

Phage typing was performed using the international phage typing set issued by the International Center, Colindale, UK. The phage typing set consisted of lytic group I : 29, 52, 52A, 79, 80; lytic group II : 3A, 3C, 55, 71; lytic group III : 6, 42E, 47,53, 54, 75, 77, 83A, 84, 85; lytic group V : 94, 96; and miscellaneous group : 81, 95. Susceptibility to phages was determined by standard routine test dilution (RTD) at 100 x RTD and 1,000 x RTD.

Enterotoxin detection

Production of enterotoxin A, B, C, D and TSST-1 was determined by a reverse passive latex agglutination kit (SET-RPLA, OXOID) according to the manufacturer' s instructions.

Pulsed field gel electrophoresis (PFGE) analysis

Extracted chromosomal DNA was digested with the *Sma*I restriction enzyme according to Smith *et al* (1988) with the following modifications. In brief, MRSA cells were grown overnight in 10 ml of brain heart infusion at 35ºC. Cells were harvested by centrifugation and resuspended in 1ml of TES buffer (2M Tris-HCl pH 8.0, 0.5 M EDTA pH 8, 2M NaCl) to achieve a

turbidity at Mc Farland No.4. This suspension was then mixed with equal volume of 1.6% low melting agarose (Bio-Rad Laboratories, Richmond, CA, USA) and poured into a gel mould block to solidify (plugs). Cells in plugs were treated overnight with lysis solution (1% sarkosyl, 0.5 mM EDTA, 2 mg/ml of protease). The DNA in a plug then was digested overnight with 60 units of *SmaI* (New England Biolabs, USA). PFGE was performed with CHEF DR II system (Bio-Rad Laboratories) employing 1% of ultra pure high melting temperature agarose (Bio-Rad Laboratories). Running condition was 6V/cm, with switching times of 10-20 seconds for 22 hours in Tris-borate-EDTA running buffer. The gel were stained with ethidium bromide and photographed under UV light. Chromosomal DNA of *Saccharomyces cerevisiae* (Bio-Rad Laboratories) was used for molecular standard marker. The band patterns were interpreted according to the guidelines of Bannerman *et al* (1995).

Statistical analysis

Chi-square and Fisher' s exact tests with SPSS program were used for analysis of the correlation between the various MRSA typing methods.

RESULTS

Antibiograms and MIC

All 74 MRSA isolates were analyzed for the antimicrobial susceptibility patterns of 13 antimicrobial agents. All isolates were resistant to penicillin, tetracycline, erythromycin, cephalothin, gentamicin and kanamycin. Resistance to vancomycin and fosfomycin was not found. There was variable resistance to lincomycin (43 %), ciprofloxacin (90%), chloramphenicol (95%) and cotrimoxazole (97%). Most of MRSA showed high MIC values (Table 1). Six patterns (a, b, c, d, e and f) were identified (Table 2): pattern "a" was susceptible to lincomycin, fosfomycin and vancomycin; "b" to fosfomycin and vancomycin; "c" to chloramphenicol, lincomycin, fosfomycin and vancomycin; "d" to cotrimoxazole, lincomycin, fosfomycin and vancomycin; "e" to lincomycin (intermediate sensitive), fosfomycin and vancomycin; and "f" to lincomycin, ciprofloxacin, fosfomycin and vancomycin.

Table 1
Comparison of PFGE patterns with phenotypes of 74 MRSA isolates[a].

PFGE		Antibiogram	Phage type	Enterotoxin	MICs
No. of isolates	pattern				
11	A0	A(7),c(2),e(1),f(1)	77(1), NT(10)	A(2)	128(2),256(5),512(4)
7	A1	a(1),b(6)	77(3), NT(4)		128(1),256(2),512(3),>512(1)
2	A2	a(2)	79(1), NT(1)		32(2)
2	A3	a(1),e(1)	79(1), NT(1)	A(1)	512(2)
5	A4	a(1),b(2),d(2)	77(3), NT(2)		256(1),512(2),>512(2)
5	A5	a(5)	NT(5)		256(4),512(1)
8	A6	a(4),b(4)	77(4),85(1),NT(3)		128(2),256(4),512(2)
1	B0	c(1)	NT(1)		512(1)
3	C0	b(3)	77 (3)	A(1)	512(2),>512(1)
12	C1	a(4),b(8)	77(5),85(2),NT(5)		256(4),512(5),>512(3)
1	C2	b(1)	NT(1)		256(1)
1	C3	b(1)	NT(1)		512(1)
1	D0	b(1)	85(1)		256(1)
3	D1	a(2),f (1)	85(1),96(1),NT(1)	A(1)	128(1),256(1),512(1)
1	D2	b(1)	NT(1)		128(1)
3	D3	a(1),f(2)	77(1),96(1),NT(1)	A(2)	128(1),256(2)
5	E0	b(1),c(1),f(3)	NT(5)	A(3)	128(1),256(3),512(1)
3	E1	a(1),b(2)	NT(3)		256(1),512(2)

[a]Figures in parentheses are numbers of MRSA isolates; NT = non-typeable phage typing

Table 2
PFGE major pattern and subtype of 74 MRSA isolates.

PFGE pattern	PFGE subtype							Total (%)
	0	1	2	3	4	5	6	
A	11	7	2	2	5	5	8	40 (54)
B	1	0	0	0	0	0	0	1 (1)
C	4	11	1	1	0	0	0	17 (23)
D	1	3	1	3	0	0	0	8 (11)
E	5	3	0	0	0	0	0	8 (11)

Table 3
Distribution of phenotypic group designated by phenotyping methods in comparison with PFGE pattern of 74 MRSA isolates.

Phenotypic groups	Antibiogram	Antimicrobial susceptibility	Phage type	Enterotoxin	PFGE patterns[a]	Total (%)
1	a	L,FOS,V	77		A(4), C(1), D(1)	6 (8)
2	a	L,FOS,V	79	A	A(1)	1 (1)
3	a	L,FOS,V	79		A(1)	1 (1)
4	a	L,FOS,V	85		C(2), D(1)	3 (4)
5	a	L,FOS,V	NT	A	A(1)	1 (1)
6	a	L,FOS,V	NT		A(14), C(1), D(1), E(1)	17 (23)
7	b	FOS,V	77	A	C(1)	1 (1)
8	b	FOS,V	77		A(7), C(6)	13 (18)
9	b	FOS,V	85		A(1), D(1)	2 (3)
10	b	FOS,V	NT		A(4), C(6), D(1), E(3)	14 (19)
11	c	C,L,FOS,V	NT	A	A(1), E(1)	2 (3)
12	c	C,L,FOS,V	NT		A(1), B(1)	2 (3)
13	d	SXT,L,FOS,V	NT		A(2)	2 (3)
14	e	L(IS),FOS,V	NT		A(2)	2 (3)
15	f	L,Cl,FOS,V	96	A	D(2)	2 (3)
16	f	L,Cl,FOS,V	NT	A	D(1), E(2)	3 (4)
17	f	L,Cl,FOS,V	NT		A(1), E(1)	2 (3)
						74 (100)

C = chloramphenicol, L= lincomycin, FOS = fosfomycin, Cl= ciprofloxacin, SXT = cotrimoxazole, V = vancomycin, NT= nontypeable phage typing

[a] Figures in parentheses are numbers of MRSA isolates

[p-value of the three phenotyping methods (antibiogram, phage typing and enterotoxin production) and PFGE was 0.01]

Bacteriophage typing

Of the 74 MRSA isolates, 45 (60%) could not be typed with the phages by RTD test. Eleven of the 23 (47%) isolates from the hospital in Bangkok (HBK) were phage type 77 and 10 of 23 (43 %) could not be typed, whereas 35 of the 51 (69%) MRSA isolates from the hospital in Khon Kaen (HKK) were non-typable. Therefore phage type 77 was predominant in the HBK whereas nontypable phages were predominant in the HKK.

Enterotoxin productions

Enterotoxin A was found in 10 of 74 (13.5%) MRSA isolated from both hospitals (Table 1) . Nine of 51 (17.6%) and 1 of 23 (4.3%) MRSA isolated from HKK and HBK produced enterotoxin A, respectively.

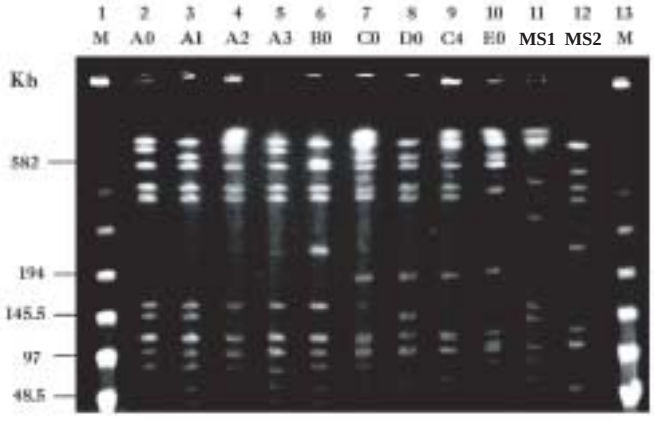

Fig 1–PFGE pattern of *Sma*I-digested chromosomal DNA of MRSA strains. Lanes 1 and 13, DNA size marker (Kb); lanes 2-5, type A and subtype A0 (A1-A3); lanes 6-10, other types and subtypes (B0, C0, D0, C4, E0); lanes 11 and 12, methicillin sensitive *S. aureus* (MSSA).

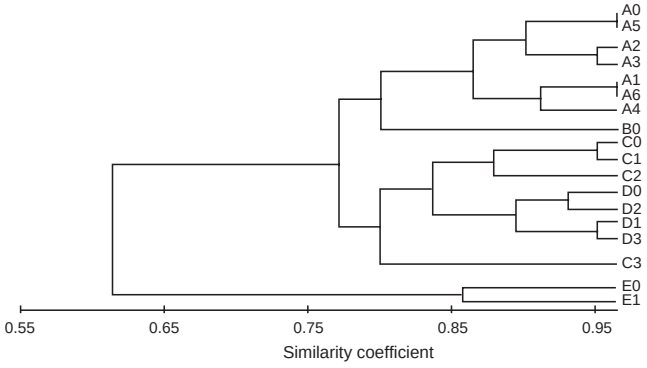

Fig 2–Dendrogram of similarities (obtained by the mismatch coefficient based on the presence or absence of bands) among 18 PFGE types obtained from 74 MRSA isolates.

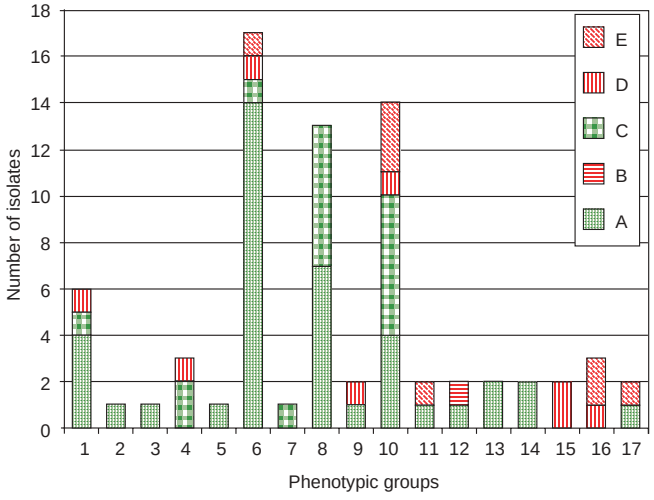

Fig 3–Distribution of 5 PFGE types in 17 phenotypic groups of 74 MRSA isolates.

PFGE analysis

MRSA isolates were analyzed by PFGE of *Sma*I digested chromosomal DNA. Approximately 10-13 fragments ranging from 45-550 kb were found. PFGE type B and C showed differences of 4-6 bands compared with PFGE type A (Fig 1). The differences can be explained by simple insertions or deletions of DNA or the gain or loss of restriction sites (Tenover, 1994). Eighteen distinct PFGE types comprising of 5 major patterns, designated as type A0, B0, C0, D0 and E0, and 13 subtypes were found (Table 2). Of the 74 isolates, 54% were PFGE type A, 23% type C, 11% type D, 11% type E, and 1% type B. PFGE type A was predominant in HKK whereas PFGE type C was predominant in HBK. The dendrogram generated with standard clustering software (the unweighted pair-group method with arithmetic means, UPGMA) showed that PFGE types A and A5, and types A1 and A6 were most closely related with 96% similarity followed by PFGE A2 and A3, C and C1, and D1 and D3 with 95% similarity (Fig 2). The 74 MRSA isolates could be classified into 3 major groups classified by genetic variation, revealing 80% similarity (Fig 2).

Correlation of PFGE with phenotyping methods

Many MRSA isolates showed identical phenotypes but with different PFGE patterns (Table 3). However, there was significant correlation between PFGE and the phenotyping methods (p= 0.01), and antibiogram alone (p=0.03). PFGE type A was highly correlated to the phenotypic group 6 (Table 3 and Fig 3) and related to antibiogram type a (Fig 4).

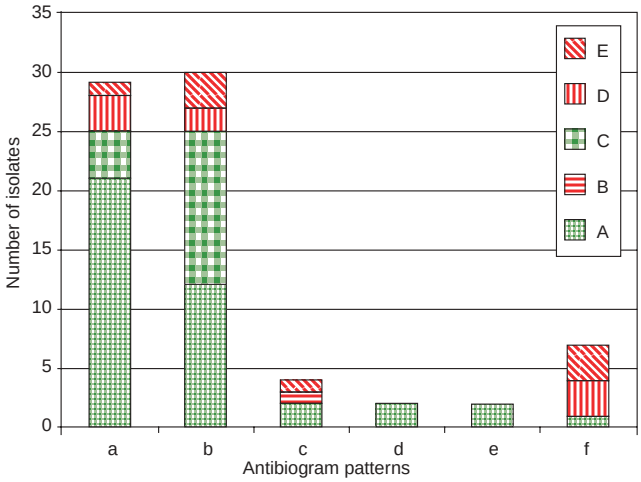

Fig 4–Distribution of 5 PFGE types and 6 antibiogram types of 74 MRSA isolates (p-value of antibiogram patterns compared with PFGE patterns was 0.03).

DISCUSSION

Several techniques are available for differentiating MRSA. Phenotypic methods such as antibiogram, phage typing and enterotoxin productions have been widely used by clinical microbiologists (Adesiyun *et al*, 1992; Sawai *et al*, 1998; Murchan *et al*, 2004). Antibiogram is a routine standard procedure in all microbiological laboratories because it is readily available, easy to determine, and relatively inexpensive (Adesiyun *et al*, 1992; Rossney *et al*, 1994; Essawi *et al*, 1998). The identification of new unusual patterns of antibiogram among MRSA may raise the suspicion of an outbreak or the presence of a new strain (Rossney *et al*, 1994). However, some reports have shown that antibiogram has relatively limited use in epidemiological studies because of phenotyping variation, antimicrobial resistance affected by environmental factors, and instability due to horizontal transmission which may not provide a high discriminatory power (Tenover *et al*, 1994; Maslow *et al*, 1995). Several reports have shown that the antibiogram typing can be used for MRSA typing (Blanc *et al*, 1994; Na'was *et al*, 1998; Montesinos *et al*, 2002).

Phage typing, by contrast, is time-consuming, labor-intensive, requires standardized phage sets, and increases the problem of untypeable phage types but it is still accepted as a phenotyping method (Schlichting *et al*, 1993; Tenover *et al*, 1994; Jorgensen *et al*, 1996). Some researchers have described that antibiogram and phage typing may not be able to distinguish between MRSA strains (Shopsin and Kreiswirth, 2001). When comparing between phage typing method and PFGE, some researchers have shown that MRSA isolates, which were untypeable by phage typing, were identical by PFGE and RAPD (Tambic *et al*, 1997). Enterotoxin production has been used less frequently, but some researchers have used it for MRSA typing (Schmitz *et al*, 1997; Sawai *et al*, 1998).

For the genotyping, PFGE has become the most trusted epidemiologic marker for MRSA as it is a highly discriminatory, stable and reproducible method (Nada *et al*, 1996; Essawi *et al*, 1998; Na'was *et al*, 1998; Montesinos *et al*, 2002). We found 17 distinct profiles by the combination of the three phenotypic methods (antibiogram, phage typing, and enterotoxin productions) and 18 PFGE types designated as 5 major types (A0 through E0) and 13 subtypes (Table 1). PFGE types A and C, and their related subtypes, were predominant in Bangkok and Khon Kaen hospitals. We showed that antibiogram differentiated 6 different types. Santos *et al* (1999) have shown that all of 62 MRSA isolates, collected from two hospitals in Brazil, were identified into 10 PFGE profiles and PFGE type A was the predominant clone in both hospitals (Santos *et al*, 1999).

The significant correlation between PFGE and the antibiogram in our study agreed with the previous reports (Montesinos *et al*, 2002). Blanc *et al* (1994) showed a good correlation among antibiogram (using a selection of antibiotics and inhibition zones), ribotyping and epidemiological data. However, a number of investigators showed no correlation between PFGE and the antibiogram (Leski *et al*, 1998; Santos *et al*, 1999). As the antibiogram typing can show good

correlation with PFGE, it indicates that the antibiogram typing is still appropriate for the routine laboratories as an epidemiological marker. Although PFGE is a very effective typing method, it is not practical for routine laboratory use. It may be appropriate for epidemiological research for investigating outbreaks.

When the combination of three phenotyping methods (antibiogram, phage typing and enterotoxin production) was compared with PFGE, the correlation is higher than that of antibiogram typing alone. Phage typing and enterotoxin production are not available in routine laboratories and are not suitable for differentiating MRSA strains. However, they can be used in combination with other methods to increase the discriminatory power and also in research.

S. aureus producing enterotoxin A was found most frequently among cooks working in HKK (Chomvarin *et al*, 1993) and it was found most frequently in the both MRSA and MSSA isolates (data not shown). This indicated that staphylococcal producing enterotoxin A is widely spread among the general population. Nontypeable phage type *S. aureus* was also found the most frequently in the both MRSA and MSSA (data not shown).

The most frequent PFGE type and its related subtypes found in HBK and HKK was A (54%) and C (27%), respectively. These findings indicated that the existence of the common epidemic MRSA clones was widely spread in two hospitals located approximately 500 km apart. The spread of MRSA strains found in our study was similar to those reported by other investigators in other countries. The reasons for the spread may be due to the prolonged carrier status and the increased mobility of the population around the country (de Lencastre *et al*, 1997; Roman *et al*, 1997).

In conclusion, significant correlation between 3 phenotypic methods and PFGE typing for discrimination of MRSA strains was found in our study. Antibiogram typing might still be an appropriate method for MRSA typing in microbiological laboratories. MRSA strains of PFGE types A and C were the epidemic strains predominant in both Bangkok and Khon Kaen hospitals.

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