METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*: 1-YEAR COLLECTION AND CHARACTERIZATION FROM PATIENTS IN TWO TERTIARY HOSPITALS, SOUTHERN THAILAND

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Abstract. Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen causing nosocomial and other types of infections worldwide. In a one-year survey of patients in two tertiary hospitals, namely, Maharaj Nakhon Si Thammarat and VachiraPhuket Hospitals, southern Thailand, 64 MRSA strains (prevalence of 8.1%) were obtained mainly from the elderly (> 45 years of age). PCR-based assay revealed high prevalence of virulence genes, *femB* (30%) and *spa* (34%), and of SCC*mec* type II from VachiraPhuket (36%) and Maharaj Nakhon Si Thammarat (38%) Hospitals. The majority of MRSA strains were resistant to clindamycin (98%), erythromycin (95%), gentamicin (84%), and tetracycline (80%), while still sensitive to chloramphenicol, cotrimoxazole, fusidic acid, and vancomycin. These data are important for effective treatment of MRSA-infected patients and for implementing control strategies to prevent spread of MRSA within hospitals.

Keywords: methicillin-resistant *Staphylococcus aureus*, SCC*mec*, virulence gene, Thailand

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) first emerged in the United Kingdom in late 1960s (Jevon, 1961). MRSA strains possess *mecA* encoding PBP2a, a low affinity penicillin-binding protein, which counteracts the function of β-lactam antibiotics (Utsui and Yokota, 1985). This bacterial species also is equipped with various virulence factors, *viz*, coagulase causing plasma clot (coded by *coa*) (McAdow *et al*, 2011), aminoacyltransferase FemB (coded by *femB*) catalyzing pentaglycine interpeptide bridge of *S. aureus* peptidoglycan (Kobayashi *et al*, 1994), panton valentine leukocidin (coded by *luk-PV*) for destroy-

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ing skin and soft tissue (Chetchotisakd *et al*, 2007), staphylococcal enterotoxin A-D (coded by *sea-sed*) including toxic shock syndrome toxin (coded by *tst*) that lead to non-specific massive release of cytokines from T-cells and macrophages, resulting in excessive cellular immune response and toxic shock (Marrack and Kappler, 1990), and von Willebrand factor binding protein (coded by *vWbp*), a coagulase (Ruggeri, 1999; McAdow *et al*, 2011). In 2003, approximately 400,000 in-patients were reported to be infected in the United States (Noskin *et al*, 2007), and 19,000 hospital mortalities were documented in 2005 (Klevens *et al*, 2007).

Although MRSA is a nosocomial pathogen responsible for outbreaks in hospitals and health care institutions [known as hospital-acquired (HA)-MRSA], in the late 1990s MRSA was noticed to be responsible for skin and soft tissue infections (SSTIs) in the community (Herold *et al*, 1998) and currently is referred to as community-acquired (CA)-MRSA. Incidences of CA-MRSA are increasing world-wide but HA-MRSA still plays a crucial role (Song *et al*, 2011; Tavares *et al*, 2013).

MRSA is able to resist various antimicrobial agents. The resistance phenotype involves a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*), which contains *mecA* and is flanked by cassette chromosome recombinase genes that enables SCC*mec* to be transferred intra- and interspecies. HA-MRSA has spread globally from disseminated clones (Gordon and Lowy, 2008) and this frequently is associated with SCC*mec* types I to III (Bartlett, 2008). CA-MRSA often possesses PVL and SCC*mec* type IV, a smaller SCC*mec* type that is easier for horizontal transmission (Bartlett, 2008). Unfortunately, CA-MRSA has spread to health care facilities, replacing traditional HA-MRSA and causing nosocomial infections in hospitals (Valsesia *et al*, 2010; Cole and Popovich, 2013).

MRSA are recorded in Thailand, but reports from southern Thailand are few in number (Lulitanond *et al*, 2013). This scarcity of information is a crucial drawback for appropriate patient care and for formulating control and prevention measures in this region of the country. This study, therefore, focused on investigating the prevalence and virulence characteristics, such as the presence of virulence genes and antimicrobial susceptibility profiles, of MRSA strains in two tertiary hospitals in southern Thailand.

MATERIALS AND METHODS

MRSA collection

In the course of a year (May 2014 to June 2015), MRSA isolates were obtained from patients attending VachiraPhuket Hospital, Phuket Province and Maharaj Nakhon Si Thammarat Hospital, Nakhon Si Thammarat Province, Thailand. Samples from wards throughout the hospitals were screened for *S. aureus*. Each sample was inoculated on blood agar and a typical golden-yellow colony was selected. *S. aureus* was identified by Gram staining and standard biochemical tests (Murray *et al*, 2003) and by amplification of its specific nuclease gene (*nuc*) (Zhang *et al*, 2004). MRSA was identified using a disk diffusion method employing cefoxitin (30 μg) (Oxoid, Hampshire, UK) (CLSI, 2014). The presence of *mecA* also was monitored by PCR (Bunnueang *et al*, 2015).

The research protocols were approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University,

Thailand (approval no. REC57-0134-19-2).

Identification of *S. aureus* virulence genes

Detection of 10 *S. aureus* virulence genes (*coa*, *femB*, *luk-PV*, *sea*, *seb*, *sec*, *sed*, *spa*, *tst*, and *vWbp*) was performed using uniplex PCR employing primers listed in Table 1. An individual colony was inoculated into 3 ml of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) and incubated at 37ºC for 6 hours with aeration. Bacterial culture was boiled for 10 minutes, immediately immersed on ice for 5 minutes and centrifuged at 11,000*g* for 10 minutes. Boiled supernatant was diluted 10-fold in sterile deionized water and used as a PCR template. Amplification was carried out in a 25-µl reaction Go*Taq* Flexi system mixture containing 3.0 mM $MgCl_2$, 0.1 mM dNTPs, 0.4 µM each primer pair, 0.5 U Go*Taq*® DNA polymerase (Promega, Madison, WI), 1X Go*Taq*® Flexi buffer, and 2 µl of DNA template. Thermocycling (conducted in T100™ Thermal Cycler; Bio-Rad, Hercules, CA) conditions were as follows: 95ºC for 3 minutes; followed by 35 cycles of 94ºC for 1 minute, 48ºC for 1 minute (for *femB*) or 50ºC for 1 minute (*coa*, *sea*, *seb*, *sec*, *sed*, *tst*, and *vWbp*) or 55ºC for 1 minute (*spa*) or 57ºC for 1 minute (*luk-PV*), and 72ºC for 1 minute (1.5 minutes for *spa*); and a final heating at 72ºC for 5 minutes. Amplicons were analyzed using 1.0% agarose gel-electrophoresis and stained with ethidium bromide before image captured by a WSE-5200 Printpraph 2M gel imaging system (ATTO, Tokyo, Japan).

SCC*mec* typing

Staphylococcal cassette chromosome *mec* (SCC*mec*) (type I to VI) was identified by a PCR-based assay as previously described (Milheiriço *et al*, 2007). In brief, PCR was carried out in a 25-µl mixture composed of the same reagents as mentioned above except for the inclusion of primer pairs specific for each of the SCC-*mec* types (Table 1) and changes to the annealing temperatures (annealing temperature was 50ºC for primers kdp-F1 and R1, and 53ºC for CIF-F2 and R2). Amplicons were analyzed as described above, except that 1.2% agarose gel-electrophoresis was employed.

Antimicrobial susceptibility test

MRSA samples were determined for their antimicrobial susceptibility by disk diffusion method (CLSI, 2014), using cefoxitin (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), fosfomycin (50 µg), fusidic acid (10 µg), gentamicin (10 µg), penicillin G (10 µg), tetracycline (30 µg), and trimethoprim/sulfamethoxazole (23.75/1.25 µg). Vancomycin susceptibility was performed using an E-test strip (0.015-256 µg) (Oxoid, Hamshire, UK), with a susceptibility breakpoint of ≤ 4 µg/ml.

Statistical analysis

Data were analyzed using SPSS for Windows, version 11.0 (SPSS, Chicago, IL). Prevalence of MRSA in patients between the two sexes was compared by *t*-test. One-way ANOVA was used to compare the prevalence of MRSA among age groups, hospital units, and specimen types. Significant difference among virulence gene types and antimicrobial agents were analyzed by one-way ANOVA. Significance is set at p-value < 0.05.

RESULTS

Bacterial collection

Twenty-two MRSA out of 92 *S. aureus* isolates were collected from Vachira-Phuket Hospital, and 42/698 from Maharaj Nakhon Si Thammarat Hospital within the one year study period. MRSA strains that found more frequently in males than

Table 1
Oligonucleotide primers used in the study.

Target gene	Name	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
luk-PV	luk-PV1	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	Lina *et al*, 1999
	luk-PV2	GCATCAASTGTATTGGATAGCAAAAGC		
coa	COA1	CGAGACCAAGATTCAACAAG	730	Wichelhaus *et al*, 2001
	COA2	AAAGAAAACCACTCACATCAGT		
vWbp	vWbp-F	GCTGGATTAAATGGTGAAAGTCATG	320	Bunnoeng *et al*, 2014
	vWbp-R	GTTTATTAAAACGTTTTTGATGACC		
femB	FemB1	TTACAGAGTTAACTGTTACC	651	Kobayashi *et al*, 1994
	FemB2	ATACAAATCCAGCACGCTCT		
spa	SPA1	ATCTGGTGGCGTAACACCTG	1,500	Wichelhaus *et al*, 2001
	SPA2	CGCTGCACCTAACGCTAATG		
sea	SEA-F	GCAGGGAACAGCTTTAGGC	520	Monday and Bohach, 1999
	SEA-R	GTTCTGTAGAAGTATGAAACACG		
seb	SEB-SEC-F	ATGTAATTTTGATATTCGCAGTG	643	Monday and Bohach, 1999
	SEC-R	TGCAGGCATCATATCATACCA		
sec	SEC-F	CTTGTATGTATGGAGGAATAACAA	283	Monday and Bohach, 1999
	SEC-R	TGCAGGCATCATATCATACCA		
sed	SED-F	GTGGTGAAATAGATAGGACTGC	384	Monday and Bohach, 1999
	SED-R	ATATGAAGGTGCTCTGTGG		
tst	tst-F	GCTTGCGACAACTGCTACAG	559	Monday and Bohach, 1999
	tst-R	TGGATCCGTCATTCATTGTTAA		
nuc	nuc1	GTAGGTGGCAAGCGTTATCC	279	Zhang *et al*, 2004
	nuc2	CGCACATCAGCGTCAG		
J1 region (type II)	CIF F2	TTCGAGTTGCTGATGAAGAAGG	495	Milheiriço *et al*, 2007
	CIF R2	ATTTACCACAAGGACTACCAGC		
ccr complex (type II, IV)	*ccr*C F2	GTACTCGTTACAATGTTTGG	449	Milheiriço *et al*, 2007
	*ccr*C R2	ATAATGGCTTCATGCTTACC		
J3 region (type III)	RIF5 F10	TTCTTAAGTACACGCTGAATCG	414	Milheiriço *et al*, 2007
	RIF5 R13	GTCACAGTAATTCCATCAATGC		
J1 region (type III)	SCCmecVJ1F	TTCTCCATTCTTGTTCATCC	377	Milheiriço *et al*, 2007
	SCCmecVJ1R	AGAGACTACTGACTTAAGTGG		

Table 1 (Continued).

Target gene	Name	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
J3 region (type I, II, IV, VI)	dcs F2	CATCCTATGATAGCTTGGTC	342	Milheiriço *et al*, 2007
	dcs R1	CTAAATCATAGCCATGACCG		
ccr complex (type II, IV)	*ccr*B2 F2	AGTTTCTCAGAATTCGAACG	311	Milheiriço *et al*, 2007
	*ccr*B2 R2	CCGATATAGAAWGGGTTAGC		
J1 region (type II)	kdp F1	AATCATCTGCCATTGGTGATGC	284	Milheiriço *et al*, 2007
	kdp R1	CGAATGAAGTGAAAGAAAGTGG		
J1 region (type III)	SCCmecIIIJ1F	CATTTGTGAAACACAGTACG	243	Milheiriço *et al*, 2007
	SCCmecIIIJ1R	GTTATTGAGACTCCTAAAGC		
mec complex (type II and III)	mecI P2	ATCAAGACTTGCATTCAGGC	209	Milheiriço *et al*, 2007
	mecI P3	GCGGTTTCAATTCACTTGTC		

females at Maharaj Nakhon Si Thammarat Hospital ($p = 0.003$), was found to be comparable between the two sexes at VachiraPhuket Hospital (Table 2). The majority of MRSA strains were from elderly groups, ≥ 61 and ≥ 46 years of age at Maharaj Nakhon Si Thammarat and VachiraPhuket Hospital, respectively ($p < 0.05$). Most of the samples were from the Medicine wards in both hospitals and sputum was the most frequent source. One MRSA isolate was collected from an HIV-infected patient.

S. aureus virulence genes

In addition to *mecA*, both *spa* and *femB* were detected in 33% and 50% of MRSA strains from Maharaj Nakhon Si Thammarat and VachiraPhuket Hospital, respectively. Detection rates of *coa* and *vWbp* were found to be comparable ranging from 12% to 19% (Table 3). The MRSA strain from the HIV-infected patient contained only *femB*, *mecA* and *spa*. Overall, MRSA in both hospitals carried a higher rate of *femB* and *spa* than the other 9 virulence genes ($p < 0.05$). In addition, among staphylococcal enterotoxin genes, *sea* was the only type that carried by MRSA of 19% (Table 3).

SCC*mec* typing

The majority of MRSA strains from both hospitals could not be classified into any SCC*mec* type, and were placed into the untypeable group (UT). Thirty-eight percent and 36% of MRSA strains from Maharaj Nakhon Si Thammarat and VachiraPhuket Hospitals, respectively belonged to SCC*mec* type II, including the strain from a HIV-infected patient; 9% and 14% from VachiraPhuket Hospital belonged SCC*mec* type I and III, respectively; and 10% from Maharaj Nakhon Si Thammarat Hospital belonged to SCC*mec* type VI (Fig 1).

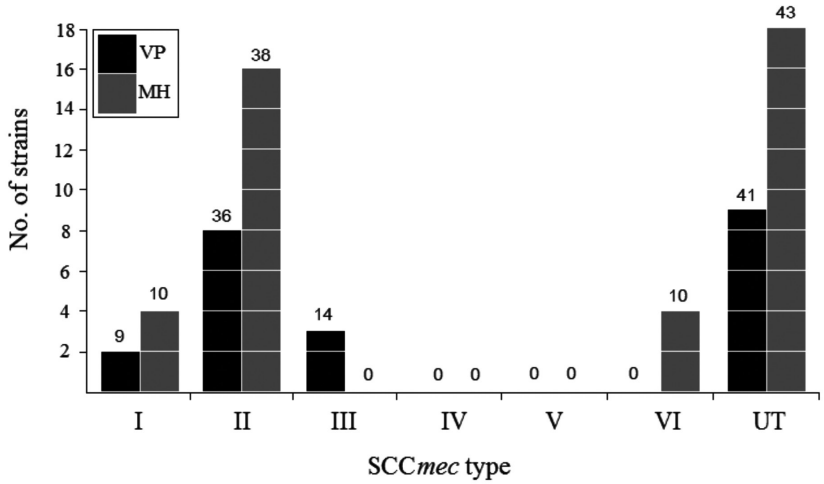

Fig 1–Prevalence of MRSA SCC*mec* types from patients, Maharaj Nakhon Si Thammarat (MH) and VachiraPhuket (VP) Hospitals. SCC*mec* types were determined by PCR-based assay as described in Materials and Methods. Number above each type is percent SCC*mec* type in each hospital. UT, untypeable.

Antimicrobial susceptibility

All MRSA strains from both hospitals were resistant to cefoxitin and penicillin and most strains from the two hosptials were also resistant to clindamycin, erythromycin, gentamicin, and tetracycline (Table 4). However, most MRSA strains were susceptible to chloramphenicol, trimethoprim/sulfamethoxazole, fusidic acid and vancomycin (MIC of 0.5-2.0 μg/ml).

DISCUSSION

MRSA infection is a public health cencern as the bacterium is resistant to a number of antibiotics in clinical use and can cause severe pathologies. Thus, identification of the presence of MRSA, its antibiotic sensitivity profile and presence of virulence genes, is of vital importance in providing effective treatment and preventing its spread among other patients in the same hospital setting. In the current study, the prevalence of MRSA (8.1%) in two hospitals in southern Thailand was comparable to that (9.2%) of Rajavithi Hospital, Bangkok in 2006 (Jariyasethpong *et al*, 2010). These prevalence rates in Thailand are high compared to the two other reports, one from Paris, France and another one from Geneva, Switzerland, which revealed rate of 7.9% and 3.3%, respectively (Lucet *et al*, 2005; Harbarth *et al*, 2006). These differences in prevalence of MRSA infections among hospitals in Thailand and Europe may depend on various risk factors, namely, age, sex, history of MRSA infection, and previous usage of broad spectrum cephalosporins (Harbarth *et al*, 2006; Jariyasethpong *et al*, 2010). The latter factor initially was observed in 1988 in a tertiary hospital in southern Thailand (Jamulitrat *et al*, 1988). Thus, surveillance and monitoring of MRSA infection should be paid much attention as Thailand has been shown to have high antibiotic prescription rates, as well as transition of the Thai population to an aged society in near future.

All MRSA strains from both Maharaj Nakhon Si Thammarat and VachiraPhuket Hospitals were not CA-MRSA because they did not possess *luk-PV* and SCC*mec* type IV typically found in such strains (Bartlett, 2008). These findings are in agreement with previous reports of very rare CA-MRSA cases in a hospital setting in Thailand (Mekviwattanawong *et al*, 2006; Lulitanond *et al*, 2013).

Table 2
Demographic data of MRSA-infected patients at VachiraPhuket and Maharaj Nakhon Si Thammarat Hospitals, Thailand, May 2014 - June 2015.

Variable	VachiraPhuket Hospital Number (%)	Maharaj Nakhon Si Thammarat Hospital Number (%)
Sex		
Male	12 (54)^{A}	28 (67)^{B}
Female	10 (45)^{A}	14 (33)^{A}
Age (years)		
≤ 15	1 (4)^{A}	4 (9)^{A}
16-30	1 (4)^{A}	6 (14)^{A}
31-45	0 (0)^{A}	5 (12)^{A}
46-60	10 (45)^{B}	5 (12)^{A}
≥ 61	10 (45)^{B}	22 (52)^{B}
Hospital unit		
Out-patient	0 (0)^{A}	4 (9.5)^{A}
In-patient		
Medicine	14 (64)^{C}	21 (50)^{C}
Surgery	7 (32)^{B}	6 (14)^{AB}
Intensive care unit	1 (4)^{A}	10 (24)^{B}
Emergency unit	0 (0)^{A}	1 (2)^{A}
Specimen		
Pus	6 (27)^{AB}	5 (12)^{A}
Bile	1 (4)^{A}	0 (0)^{A}
Blood	1 (4)^{A}	6 (14)^{A}
Urine	1 (4)^{A}	0 (0)^{A}
Tissue	2 (9)^{A}	0 (0)^{A}
Sputum	10 (45)^{B}	31 (74)^{B}
Dialysis fluid	1 (4)^{A}	0 (0)^{A}
HIV-associated infection		
Positive	1 (4)^{A}	0 (0)^{A}
Negative	21 (96)^{B}	42 (100)^{B}

Different uppercase letters indicate significant differences among groups ($p < 0.05$).

Antimicrobial susceptibility profiles of MRSA in our study corresponded to previous reports from Siriraj Hospital, Bangkok, which indicated that the majority of MRSA strains are resistant to clindamycin, trimethoprim/sulfamethoxazole, erythromycin, gentamicin, and tetracycline, (Mekviwattanawong *et al*, 2006; Kiratisin, 2010). However, only a small proportion of MRSA strains in the our survey were resistant to trimethoprim/sulfamethoxazole. In the Siriraj Hospital surveys, a minority of MRSA samples are resistant to fosfomycin compared to over half in our study. There are several explanations for fosfomycin resistance in clinical isolates, *viz*, decreased drug uptake, modification of drug target

Table 3
Prevalence of virulence genes detected in MRSA from patients at VachiraPhuket and Maharaj Nakhon Si Thammarat Hospitals Thailand, May 2014 - June 2015.

Hospital	Number of positive strains (%)										
	mecA	*coa*	*femB*	*luk-PV*	*spa*	*vWbp*	*sea*	*seb*	*sec*	*sed*	*tst*
MH	42	5	8	0	14	5	5	0	0	0	0
(n = 42)	(100)	(12)	(19)	(0)	(33)	(12)	(12)	(0)	(0)	(0)	(0)
VP	22	4	11	0	8	3	7	0	0	0	0
(n = 22)	(100)	(18)	(50)	(0)	(36)	(14)	(32)	(0)	(0)	(0)	(0)
Total	64^{D}	9^{B}	19^{C}	0^{A}	22^{C}	8^{B}	12^{B}	0^{A}	0^{A}	0^{A}	0^{A}
(n = 64)	(100)	(14)	(30)	(0)	(34)	(13)	(19)	(0)	(0)	(0)	(0)

MH, Maharaj Nakhon Si Thammarat Hospital; VP, VachiraPhuket Hospital. Different uppercase letters indicate significant differences among groups ($p < 0.05$).

Table 4
Antimicrobial susceptibility profiles of MRSA strains from patients at Maharaj Nakhon Si Thammarat and VachiraPhuket Hospitals, Thailand, May 2014 - June 2015.

Hospital	Number of resistant strains (%)										
	C	DA	E	FOX	FOS	FD	CN	P	TE	SXT	Va
MH	1	42	41	42	27	2	34	42	31	0	0
(n = 42)	(2)	(100)	(98)	(100)	(64)	(5)	(81)	(100)	(74)	(0)	(0)
VP	3	21	20	22	11	0	20	22	20	3	0
(n = 22)	(14)	(96)	(91)	(100)	(50)	0	(91)	(100)	(91)	(14)	(0)
Total	4^{A}	63^{C}	61^{C}	64^{C}	38^{B}	2^{A}	54^{BC}	64^{C}	51^{BC}	3^{A}	0^{A}
(n = 64)	(6)	(98)	(95)	(100)	(59)	(3)	(84)	(100)	(80)	(5)	(0)

MH, Maharaj Nakhon Si Thammarat Hospital; VP, VachiraPhuket Hospital. C, chloramphenicol; CN, gentamicin; DA, clindamycin; E, erythromycin; FD, fusidic acid; FOS, fosfomycin; FOX, cefoxitin; P, penicillin; SXT, trimethoprim/sulfamethoxazole; TE, tetracycline; Va, vancomycin. Different uppercase letters indicate significant differences among groups ($p < 0.05$).

and inactivation of drug itself. The main mechanism for development of fosfomycin resistance is thought to be a reduction in cell permeability (Castañeda-García *et al*, 2013).

Virulence gene detection also is important. Our study focused on MRSA virulence genes as different numbers of virulence gene carriage can influence the degree of severity. Among the most crucial virulence genes in *S. aureus*, Panton-Valentine leukocidin (PVL), a pore-forming toxin, plays an important role in necrotizing diseases. It can destroy skin and soft tissue including neutrophils. PVL was shown to induce rapid activation and death of human and rabbit neutrophils but not simian or murine cells (Löffler *et al*, 2010). Thus, besides investigation of bacterial epidemiology and antimicrobial resistant profiles, surveillance of the types of virulence genes harbored by MRSA is encouraged to be

carried out, thereby assisting in the prognosis of the course of illness.

In immune-compromised patients, either as a result of drug treatment or through acquired immunodeficiency syndrome (AIDS), there are higher rates of CA-MRSA colonization and infection (Hidron *et al*, 2010). In the present study, HA-MRSA, inferred from the presence of SCC*mec* type II, was detected in an HIV-infected patient. This HA-MRSA sample appeared to have similar virulence profile to that from non-HIV patients in the same hospital. Chetchotisakd *et al* (2007) reported a fatal case of a Thai HIV-infected patient with *luk-PV*-positive methicillin-susceptible *S. aureus* (MSSA) during the recovery period when CD4 cells are increasing. It is important to recognize that both MRSA and MSSA infections are able to cause mortality depending on the set of virulence factors carried by the pathogen and the immune status of the patient.

In conclusion, based on data gathered in this study, we conclude that infections caused by MRSA in VachiraPhuket and Maharaj Nakhon Si Thammarat Hospitals, southern Thailand were caused by HA-MRSA strains that approximately 50% of the samples carried at least two virulence genes. HA-MRSA strains in both hospitals were resistant to antimicrobials: clindamycin, erythromycin, gentamicin, and tetracycline. Elderly patients were more likely to become infected. Regular surveillance of MRSA infection in hospitals all over Thailand should be conducted to obtain up-to-date information useful not only for patient's treatment and welfare but also for implementing control measures.

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