MOLECULAR CHARACTERIZATION AND RELATIONSHIP OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* AMONG STRAINS FROM HEALTHY CARRIERS AND UNIVERSITY HOSPITAL PATIENTS, SOUTHERN THAILAND

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Abstract. Methicillin-resistant *Staphylococcus aureus* (MRSA) plays an important role in nosocomial infections including those in communities. MRSA enables colonization in the nares and throats of healthy people. In this study, investigation of MRSA prevalence from the throats of healthy subjects in southern Thailand revealed that among 153 isolates, 2 showed *mecA*⁺ genotype by PCR. One *mecA*⁺ isolate was susceptible to methicillin, indicating a cryptically methicillin-resistant strain. Antimicrobial susceptibility test demonstrated that 43% were resistant to erythromycin. More importantly, two isolates had the propensity of reduced susceptibility to vancomycin. Other virulence genes harbored by 2 and 8 MRSA from healthy carriers and hospitals, respectively, exhibited that 3 clinical strains possessed coagulase gene while von Willebrand factor binding protein gene was present in one clinical MRSA strain. Staphylococcal enterotoxin A gene was found in 2 clinical MRSA isolates and Panton-Valentine leukocidin gene in 3 *S. aureus* isolates. However, all MRSA in this study lacked Panton-Valentine leukocidin gene, suggesting that they were belonged to hospital-associated MRSA lineage. MRSA typing by repetitive-sequence PCR revealed distinguishable fingerprint patterns among the MRSA isolates from both healthy carriers and hospital patients, indicating the heterogeneity of their genetic elements and that the infections caused by MRSA in this area resulted from different clones. This demonstrated a wide variety of MRSA strains in the population of southern Thailand.

Keywords: healthy carrier, *mecA*, MRSA, rep-PCR, Thailand

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INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium that plays a role in causing human diseases ranging from skin and soft tissue infections to life-threatening diseases (David and Daum, 2010). Since the first

emergence of methicillin-resistant *S. aureus* (MRSA), there have been a continuous increase of MRSA cases throughout the world (de Sousa *et al*, 2003; Johnson *et al*, 2005; Witte *et al*, 2007). This organism is one of the most common bacterial species that engenders serious problem in medical institutions. Resistance to methicillin is mediated through *mec* operon, which is a part of the Staphylococcal cassette chromosome *mec* (SCC*mec*) (El Karamany *et al*, 2013). *MecA* encodes an altered penicillin-binding protein (PBP2a or PBP2′) that has a lower affinity for binding β-lactam antibiotics (Mostafa, 2013).

The pathogenicity of *S. aureus* is related to various bacterial virulence factors, such as coagulase (encoded by *coa*), clumping factor and fibronectin-binding protein, and toxic-shock syndrome toxin (TSS), which are involved with the host attachment and tissue invasion. Coagulase binds and activates prothrombin and converts fibrinogen to fibrin, contributing to the plasma clot (McAdow *et al*, 2011). These factors are responsible for tissue invasion, and γ-hemolysin and Panton-Valentine leukocidin (PVL) play important roles in destroying host defense system (Gordon and Lowy, 2008). This includes von Willebrand factor (vWF), a glycoprotein synthesized by endothelial cells and megakaryocyte and is essential in the maintenance of hemostasis, especially in the circumstance of a rapid blood flow (Ruggeri, 1999). This glycoprotein can be reacted with *S. aureus* virulence factor, von Willebrand factor binding protein, vWbp.

PVL is a bi-component exotoxin transmitted by bacteriophage and is encoded by two genes, *lukF-PV* and *lukS-PV* (Watkins *et al*, 2012). PVL belongs to the family of synergohymenotropic toxins (Supersac *et al*, 1993), which are cytotoxic to human peripheral blood mononuclear cells (PMNs) and monocytes (Genestier *et al*, 2005). PVL production is associated with furuncles, cutaneous abscesses and severe necrotic skin infection (Cribier *et al*, 1992; Couppié *et al*, 1994; Prévost *et al*, 1995). In hospitals, less than 5% of *S. aureus* isolates were found to produce PVL (Prévost *et al*, 1995). In addition, there is a strong epidemiological correlation between the presence of PVL and a community-acquired methicillin-resistant *S. aureus* (CA-MRSA) infection (Gordon and Lowy, 2008).

S. aureus protein A, encoded by *spa*, is a 40 kDa antiphagocytic protein that covalently anchors to the peptidoglycan of bacterial cell wall. Approximately 90% of *S. aureus* protein A are present in the cell wall, and the remaining 10% located in the bacterial cytoplasm (Shakeri *et al*, 2010).

Besides *mecA*, *femA* and *femB* have been demonstrated to code for proteins affecting levels of methicillin resistance in *S. aureus* (Berger-Bächi *et al*, 1989; Kobayashi *et al*, 1994). The presence of *mecA* together with the absence of *femB* are used as markers to differentiate between coagulase-positive MRSA and methicillin-resistant coagulase-negative *S. aureus* (R-CNS) (Kobayashi *et al*, 1994; Jonas *et al*, 2002).

In Thailand, MRSA also plays a role in nosocomial infections (Jamulitrat *et al*, 1988; Hortiwakul *et al*, 2004). However, the situation of MRSA in healthy population has not been widely studied. Kitti *et al* (2011) studied the prevalence of MRSA strains among university students in Thailand and found 1% (2 of 200) of MRSA in nasal carriage. However, in southern Thailand, there has been no report of MRSA in healthy carriers. In order to gain informations regarding the status of the prevalence of MRSA strains in the healthy carriers in the area of a university hospital in southern Thailand and to assess the potentiality of the bacteria to confer disease, this study

investigated the presence of MRSA and characterized the virulence pattern in strains from healthy carriers, including the investigation of antibiogram patterns of MRSA strains. In addition, molecular relationship of the strains was performed using repetitive-sequence PCR (rep-PCR) in order to elucidate the clonal relatedness among MRSA in the study area.

MATERIALS AND METHODS

Bacterial strains

A total of 153 throat swab samples from healthy students in medical institutes associated with Songklanagarind Hospital, Hat Yai, Thailand and hospital canteens, were screened for $mecA^+$ *S. aureus* by culturing on Mannitol salt agar and incubating at 37ºC for 18 hours. Yellow colonies were selected and subjected to standard microbiological methods, including catalase assay, coagulase tube test (using 1:4 dilution of rabbit plasma), Gram staining and subsequently investigation for *mecA* by PCR method. Clinical MRSA samples were obtained from Microbiology Unit, Songklanagarind Hospital and were used to compare with strains from healthy carriers. Unrelated MRSA strains from different areas were obtained from Vachira Phuket Hospital (approximately 450 km north-west of Songklanagarind Hospital) and included in this study. The research protocols were approved by the ethics committee of the Faculty of Medicine, Prince of Songkla University, Thailand (EC code 56-365-19-2).

Antimicrobial susceptibility test

All *S. aureus* samples were determined for antimicrobial susceptibility using the disk diffusion method (CLSI, 2011). Antimicrobial agents (Oxoid, Hamshire, UK) used in this experiment were as follows: amikacin (30 µg), erythromycin (15 µg), methicillin (5 µg), gentamicin (10 µg), chloramphenicol (30 µg), cephalothin (30 µg), ceftazidime (30 µg), and vancomycin (30 µg). Vancomycin susceptibility was based on the criteria of CLSI (2007). Clear zones were measured using a Vernier caliper.

Detection of virulence genes

PCR-based detection of *mecA* was performed by amplifying the highly conserved region of the 2,456 bp *mecA* sequence using primers, mecA1 (5'-GTAGAAATGACT-GAACGTCCGATAA-3') and mecA2 (5'-CCAATTCCACATTGTTTCGGTC-TAA-3') as described previously (Geha *et al*, 1994). *FemB* was amplified using primers, femB1 (5'-TTACAGAGTTAACT-GTTACC-3') and femB2 (5'-ATACAAATC-CAGCACGCTCT-3') as described by Kobayashi *et al* (1994); Panton-Valentine leukocidin-producing (*luk-PV*) gene with primers, *luk-PV*-1 (5'-ATCATTAG-GTAAAATGTCTGGACATGATCCA-3') and *luk-PV*-2 (5'-GCATCAASTGTATT-GGATAGCAAAAGC-3') as described by Lina *et al* (1999); and *coa* and *spa* using the primers COA1 (5'-CGAGAC-CAAGATTCAACAAG-3') and COA2 (5'-AAAGAAAACCACTCACAT-CAGT-3'), and SPA1 (5'-ATCTGGTGGC-GTAACACCTG-3') and SPA2 (5'-CGCT-GCACCTAACGCTAATG-3'), respectively, as described by Wichelhaus *et al* (2001). The primers used for amplification *vWbp* [vWBP-F (5'-GCTGGATTAAATGGT-GAAAGTCATG-3') and vWBP-R (5'-GTT-TATTAAAACGTTTTTGATGACC-3')], were designed based on the consensus sequences of *vWbp*s obtained from the National Center for Biotechnology Information (NCBI). Staphylococcal enterotoxin A (*sea*) and toxic shock syndrome toxin genes (*tst*) were amplified using primers SEA-F (5'-GCAGGGAACAGCTTTAGGC-3') and SEA-R (5'-GTTCTGTAGAAGTAT-

Table 1

Antimicrobial resistance of *S. aureus* isolates from throat swabs of healthy carriers.

Institution/year (number)	Antimicrobial resistance			
	Vancomycin (30 µg) (%)	Amikacin (30 µg) (%)	Erythromycin (15 µg) (%)	Methicillin (5 µg) (%)
1/2010 (*n* = 29)	0/29 (0)	0/29 (0)	20/29 (69)	0/29 (0)
2/2012 (*n* = 17)	0/17 (0)	0/17 (0)	9/17 (53)	0/17 (0)
3/2012 (*n* = 15)	0/15 (0)	0/15 (0)	2/15 (13)	1/15 (7)
4/2012 (*n* = 7)	0/7 (0)	0/7 (0)	1/7 (14)	0/7 (0)
5/2012 (*n* = 85)	[a]2/85 (2)	0/85 (0)	33/85 (39)	0/35 (0)
Total	2/153 (1)	0/153 (0)	65/153 (42)	1/153 (1)

[a]Clear zone diameter for vancomycin of 14 mm.

GAAACACG-3'), and TST-F (5'-GCTT-GCGACAACTGCTACAG-3') and TST-R (5'-TGGATCCGTCATTCATTGTTAA-3'), respectively, as described by Monday and Bohach (1999). PCR was carried out using Go*Taq*® Flexi system (Promega, Madison, WI) and amplicons were analyzed by 1.0% agarose gel-electrophoresis and stained with ethidium bromide before visualization and documentation.

Identification of *Staphylococcus aureus* by 16S rDNA analysis

All *mecA*[+] *S. aureus* from healthy carriers and patients identified by standard biochemical tests were further confirmed as *S. aureus* by PCR amplification of *S. aureus*-specific 16S rDNA using primers, 16S rRNA-F (5'-GTAGGTGGCAAGC-GTTATCC-3') and 16S rRNA-R (5'-CG-CACATCAGCGTCAG-3') (Monday and Bohach, 1999). The 228 bp amplicon was detected by 1.0% agarose gel-electrophoresis as described above.

MRSA typing by rep-PCR

Rep-PCR genotyping of MRSA strains was performed using primers RW3A (5'-TCGCTCAAAACAACGACACC-3') (Delvecchio *et al*, 1995). In brief, 100 ng of DNA template were added in a total of 30 µl reaction volume comprising of 0.4 mM each dNTP, 0.85 µM primer, 1X Go*Taq* Green buffer, 2.5 mM MgCl$_2$, and 5.0 U Go*Taq* DNA polymerase. Thermocycling (T100™ Thermal cycler; Bio-Rad, Hercules, CA) reactions were as follows: 95°C for 3 minutes; 60 cycles of 1 minute at 94°C, 1 minute at 54°C, and 2 minutes at 72°C at a ramp rate of 0.5°C/second for the 2nd heating step. Amplicons were analyzed by 1.5% agarose gel-electrophoresis as described above. Dendrogram was constructed using unweighted pair-group method of arithmetic average (UPGMA) (BioNumerics software version 7.0, Applied Maths, Sint-Martens-Latem, Belgium).

RESULTS

Bacterial isolation and antimicrobial susceptibility test

In order to investigate the antimicrobial resistances of *S. aureus* isolates, 4 antimicrobial agents were preliminarily employed, namely, amikacin (30 µg), erythromycin (15 µg), methicillin (5 µg), and vancomycin (30 µg). A total of 153 *S. aureus* isolates demonstrated susceptibility

Table 2
Antimicrobial resistance of 2 *mecA*⁺ *S. aureus* isolates from healthy carriers and
8 MRSA strains from hospitals.

Strain	Source	Antimicrobial resistance pattern
PSU19	H	AK, E, MET, CN, C, KF, CAZ
PSU20	H	E, MET, CN, KF, CAZ
PSU21	H	E, MET, CN, KF, CAZ
PSU22	H	AK, E, MET, CN, KF, CAZ
PSU23	H	E, MET, KF, CAZ
PSU24	C	E, MET, CAZ
PSU83	C	E, CAZ
PSU84	H	E, MET, CN, KF, CAZ
PSU85	H	AK, E, MET, CN, C, KF, CAZ
PSU86	H	E, MET, CN, KF, CAZ

H, hospital; C, healthy carrier; AK, amikacin 30 µg; CAZ, ceftazidime 30 µg; KF, cephalothin 30 µg; C, chloramphenicol 30 µg; E, erythromycin 15 µg; CN, gentamicin 10 µg; MET, methicillin 5 µg; VA, vancomycin 30 µg.

to amikacin (Table 1). One isolate (0.6%) from Institute 3 was resistant to methicillin and 65 isolates (42%) were resistant to erythromycin. More importantly, 2 isolates (1%) from Institute 5 exhibited a clear zone of vancomycin of 14 mm (Table 1) which probably suggesting that these strains with reduced susceptibility to vancomycin.

Detection of *S. aureus* virulence genes

Among 153 throat swab samples, 2 (1%) isolates (PSU24 and PSU83) were positive for *mecA*. Five representative clinical MRSA strains from Songklanagarind Hospital (PSU19-23) and 3 unrelated MRSA strains from Vachira Phuket Hospital (PSU84-86) were positive for *mecA* (Fig 1) and analysis of 16S rDNA confirmed that these isolates were *S. aureus* (Fig 1). PSU24 and PSU83 as well as clinical MRSA strains PSU21-23, PSU84 and PSU86 exhibited negative results for *femB* and *coa*, suggesting that they were methicillin-resistant coagulase-negative Staphylococci (Fig 1). Nevertheless, phenotypic characteristics of coagulase production determined by coagulase tube test were inconsistent with the genotyping results. The strains that were *coa* negative demonstrated plasma clotting property, and only MRSA strain PSU85 possessed von Willebrand factor binding protein gene, encoding another coagulase (Fig 1). *Luk-PV* was examined in all *S. aureus* isolates and although 3 (2%) contained *luk-PV* they were *mecA*⁻ strains. In addition, *mecA*⁺ *S. aureus* isolates PSU24 and PSU83, and all clinical MRSA strains lacked *luk-PV* (Fig 1). None of the strains possessed toxic shock syndrome toxin gene while staphylococcal enterotoxin A was found in clinical MRSA, PSU20 and PSU85, isolated from Songklanagarind and Vachira Phuket Hospital, respectively.

Antimicrobial susceptibility test of *mecA*⁺ *S. aureus* and clinical MRSA isolates

Two *mecA*⁺ *S. aureus* from healthy carriers and 8 MRSA strains from hospitals were subsequently investigated for antimicrobial activity against gentamicin (10 µg),
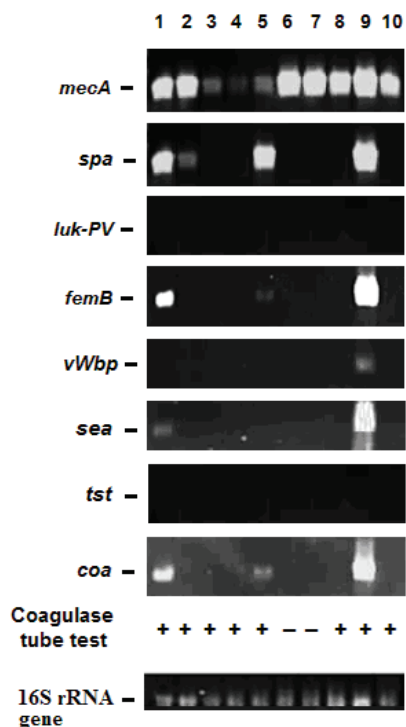
Fig 1–Detection of *mecA*, *spa*, *luk-PV*, *femB*, *vWbp*, *sea*, *tst*, *coa* and 16S rDNA by PCR. Experimental protocols are in Materials and Methods. Lanes 1-5, clinical MRSA strains PSU20, PSU21, PSU22, PSU23 and PSU19, from Songklanagarind Hospital; lanes 6 and 7, MRSA strains PSU24 and PSU83 from healthy carriers; lanes 8-10, unrelated clinical MRSA strains PSU84-PSU86 from Vachira Phuket Hospital.
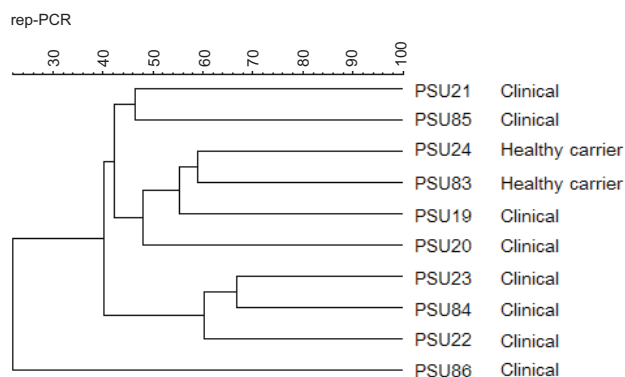

Fig 2–rep-PCR-based dendrogram of MRSA strains among healthy carriers and patients.

chloramphenicol (30 µg), cephalothin (30 µg), and ceftazidime (30 µg). Two *mecA*+ *S. aureus* from healthy carriers were susceptible to gentamicin, chloramphenicol and cephalothin but resistant to erythromycin and ceftazidime, a third generation cephalosporin (Table 2). Furthermore, these 2 *mecA*+ *S. aureus* were resistant to a lower number of antimicrobials compared to strains from patients (Table 2). All clinical MRSA strains were resistant to erythromycin, methicillin, cephalothin and ceftazidime. In addition, the majority of MRSA hospital isolates were resistant to gentamicin. Two (PSU19 and PSU85) of 8 clinical strains were resistant to chloramphenicol (Table 2).

MRSA typing by rep-PCR

Based on rep-PCR-based dendrogram, there were distinguishable fingerprint pattern in all strains (Fig 2). All MRSA strains, except clinical isolate PSU86, were related at least at 40% similarity. MRSA PSU24 and PSU83 from healthy carriers showed the genetic relationship at approximately 60% similarity. Clinical MRSA strains from hospitals appeared to have been caused by divergent strains. However, MRSA strains from healthy carriers also clearly showed that they were distantly related to the clinical strains. Thus, MRSA in hospitals and those from healthy carriers originated from different clones.

DISCUSSION

MRSA infections are of great concern worldwide as they cause serious problems in both hospitals and communities at large. The presence of *mecA* is an important molecular marker to identify MRSA in clinical and environmental samples. Uemura *et al* (2004) reported MRSA from nose and throat of 12% of volunteers, comprising of nursing students (*n* = 104), medical

students (*n* = 18) and staff members (*n* = 35) in the Faculty of Medicine, University of Ryukyus, Japan. Rohde *et al* (2009) reported that among 203 Texas University students, 60 carried *S. aureus* and 15 were identified as MRSA. In this study, the incidence of MRSA in 153 healthy carriers in a hospital setting in southern Thailand was only 1%, in agreement with a previous study of MRSA nasal carriage from university students in other parts of Thailand (Kitti *et al*, 2011). The factors that led to the low level of MRSA colonization in healthy carriers in this area are still obscure. The infrequency of medical institutions visitations, good hygiene in medical institutions and canteens, hospitalization before MRSA detection and antibiotics usage, including the presence of underlying chronic illnesses of the population in this area, can be factors affecting the scarceness of MRSA colonization in humans (Jernigan *et al*, 2003; Hidron *et al*, 2005).

S. aureus with reduced susceptibility to vancomycin has been reported worldwide (Trakulsomboon *et al*, 2001; Song *et al*, 2004; Lulitanond *et al*, 2009). In Thailand, there have been reports of vancomycin-intermediate *S. aureus* (VISA) MRSA strains in various hospitals during the previous half decade and from VISA screening between 2002 and 2003 and between 2006 and 2007, it was implied that the reduction of MRSA vancomycin susceptibility in the hospitals has gradually increased (Lulitanond *et al*, 2009). For *S. aureus* isolated in the present study, although the MIC breakpoints for vancomycin should be confirmed to define the being of VISA, we used disk diffusion method from CLSI 2007 criteria to initially attribute the reduction of vancomycin susceptibility strains. These strains were detected in 1% of isolates from healthy volunteers, suggesting the presence of reduced vancomycin susceptibility strains

in people associated with healthcare institution in southern Thailand. Marques *et al* (2010) found MRSA that were resistant to oxacillin and vancomycin in 60 nursing and pharmacy students from a Portuguese higher health school. More recently, Onanuga and Temedie (2011) found among 120 asymptomatic inhabitants of Amassoma community in Nigeria, comprising of 60 university students and 60 villagers, 1 and 2 *S. aureus* isolates with vancomycin resistance, respectively.

In this study, it was noted that a large proportion (42%) of *S. aureus* isolated from Thai healthy carriers were resistant to erythromycin, in agreement with the recent report of 26.7% (Kitti *et al*, 2011). Resistance of erythromycin can occur by a number of means. Some phages have the capability of transferring antibiotic resistance traits by transduction of plasmids or plasmid elements previously incorporated into the genome, such as pI258 containing erythromycin resistance transduced by phages p11 and p11de (Jensen and Lyon, 2009). Alternatively, the J region of SCC*mec* can be incorporated by plasmids, transposons or insertion of sequences, which bring antibiotic resistant genes with them. For example, Tn*554* encoding erythromycin-resistant gene *ermA* is incorporated into J2 region in SCC*mec* type II of *S. aureus*. Furthermore, the erythromycin-resistant *S. aureus* might be colonized in the throat of humans. These strains may be transmitted from person-to-person or by contaminated food consumption (Boost *et al*, 2013).

One isolate (PSU83) obtained from healthy carriers in this study demonstrated the presence of *mecA*. Nonetheless, it phenotypically demonstrated methicillin susceptibility. This phenomenon can occur in MRSA strains that contain *mecA* but do not produce PBP2', referred as cryptically methicillin-resistant strains (Murakami

et al, 1991). Although the determination of MRSA by genotype and phenotype can be inconsistent, the detection of *mecA* is still considered to be indispensable for the precise identification of MRSA.

Faria *et al* (2008) found that 99% of *S. aureus* strains can be classified by *spa* typing, also reported by Strommenger *et al* (2008). Shakeri *et al* (2010) reported that 8 of 208 (4%) of *S. aureus* lacked *spa*, but could be found in 3% of MRSA. In this present study, *spa* typing could not be employed because only 4 (40%) of MRSA strains demonstrated positive amplification of *spa* fragment. Thus, in the circumstance that *spa* is absent, other molecular tools can be applied for studying the genetic relationship among MRSA strains.

In this study, 7 of 10 MRSA strains lacked *coa*, but some of those strains have plasma clotting phenotype. Bjerketorp *et al* (2002) found a novel *S. aureus* virulence factor called von Willebrand factor binding protein (vWbp) that functions as a coagulase. vWbp displays sequence homology to *coa* D12 domain (Bjerketorp *et al*, 2004). Its C-terminal domain lacks the L and R domains of *coa*, which are replaced by a unique vWF and fibrinogen binding sites (Bjerketorp *et al*, 2002). In this study, among the *coa*-negative MRSA strains, vWbp was detected in only one strain. Thus, other virulence factors responsible for the clotting phenomenon, such as clumping factor, may play a role.

S. aureus is able to produce several types of staphylococcal enterotoxins (SEs) including toxic-shock syndrome toxin (TSST), responsible for food poisoning in humans (Balaban and Rasooly, 2000). In this study, clinical MRSA, PSU20 and PSU85, displayed the presence of a number of virulence genes, including that of staphylococcal enterotoxin A. Thus, these strains might also be able to participate in causing severe diseases in human due to the presence of this superantigen.

Although hospital-acquired MRSA (HA-MRSA) has played a pivotal role in causing human diseases, community-acquired MRSA (CA-MRSA) appears to be spreading and gradually increasing, resulting in such cases in many areas throughout the world (Coombs *et al*, 2004; Tavares *et al*, 2010; Rolo *et al*, 2012). D' Agata *et al* (2009), employing a mathematical model, predicted that CA-MRSA will be predominant and displace the traditional HA-MRSA. Gordon and Lowy (2008) showed a strong correlation between CA-MRSA infection and presence of Panton-Valentine leukocidin. In this study, only 2% of methicillin-susceptible *S. aureus* isolated from throats contained *luk-PV*. Among MRSA strains from hospital-associated healthy carriers and clinical MRSA strains, all lacked *luk-PV*. Thus, without the characterization of SCC*mec* type, this might suggest the lack of CA-MRSA in *S. aureus* healthy carriers in southern Thailand.

Rep-PCR identifies repeated sequences throughout the bacterial chromosome. Its reliability and reproducibility are much higher than arbitrarily primed-PCR (AP-PCR) because it targets specific sequences allowing the use of high stringency PCR conditions (Weller, 2000), and in a number of studies, it showed comparable resolution power to pulsed-field gel electrophoresis (Northey *et al*, 2005; Ross *et al*, 2005). In this study, the clinical MRSA strains from Songklanagarind Hospital, which were thought to be related to the MRSA strains from healthy carriers isolated from the same area, including the unrelated strains from Vachira Phuket Hospital, surprisingly exhibited high heterogeneity using rep-PCR, indicating that in southern Thailand, there existed a wide variety of MRSA strains.

In conclusion, despite the fact that there is a low prevalence of MRSA in healthy university population in southern Thailand, the epidemiology of MRSA and the surveillance of MRSA infection should be frequently evaluated in order to prevent outbreaks of this highly pathogenic micro-organism in this region of the country.

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