DISTRIBUTION AMONG THAI CHILDREN OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* LACKING *CNA*, *FNBA* AND *ICAAD*

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Abstract. *Staphylococcus aureus* is a bacterium causing infections in both community and hospitals. *S. aureus* nasal colonization increases the risk of acquiring *S. aureus* infection. In this study, the prevalence of *S. aureus* nasal colonization was determined in Thai children, showing that nasal colonization was found in 78 of 217 subjects and the methicillin-resistant *S. aureus* (MRSA) carriage rate was present in 5 of 217 children. Among 78 *S. aureus* isolates, resistance to penicillin, erythromycin, clindamycin, chloramphenicol, trimethoprim/sulfamethoxazole, oxacillin and cefoxitin was found in 99%, 8%, 1%, 4%, 1%, 8% and 6% of the isolates, respectively. The presence of 3 adhesin genes, investigated by PCR, revealed that among 78 isolates, *fnbA*, *icaAD* and *cna* was detected in 73, 72 and 48 isolates, respectively but not in the 5 MRSA isolates. Ninety-five percent and 31% of *S. aureus* isolates were able to form strong and weak biofilms, respectively. Four MRSA isolates that lacked *icaAD* were non-biofilm-forming strains. These results revealed a high prevalence of *S. aureus* nasal colonization in healthy Thai children with 2% being MRSA. Effective strategies to prevent *S. aureus* transmission and infection are therefore needed in the Thai community.

Keywords: *Staphylococcus aureus,* nasal colonization, biofilm, methicillin resistance Staphylococcus aureus (MRSA), *cna* , *fnbA*, *icaAD*

INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium that causes a variety of diseases ranging from minor skin infections to life-threatening diseases. *S. aureus* is a human commensal bacterium that commonly inhabits the nasal cavity and skin surface. Adherence of *S. aureus* to human tissue is mediated by a number of genes encoding microbial surface components, such as fibronectin-binding protein (Fnb) and collagen-binding protein (Cna) that recognize host cell proteins adhesive matrix molecules (Sivaraman *et al*, 2009), and Fnb and Cna contribute to *S. aureus* tissue colonization and pathogenesis (Elasri *et al*, 2002; Jett and Gilmore, 2002). *S. aureus* is also capable of producing biofilm and

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anchoring to medical devices, which play relevant roles both in colonization and infection. Biofilm formation in *S. aureus* involves polysaccharide intercellular adhesin (PIA) or polymeric N-acetyl-glucosamine (PNAG) encoded by *icaADBC* operon (O'Gara, 2007).

Most *S. aureus* strains have developed antibiotic resistance, posing a major and immediate threat to public health and the spread of methicillin-resistant *S. aureus* (MRSA) causes problems in hospitals worldwide (Ippolito *et al*, 2010). In the past decade, the epidemiology of community *S. aureus* disease is rapidly changing with the spread of community-onset MRSA strains (CA-MRSA), which is usually associated with children and the incidence is increasing worldwide (Mollaghan *et al*, 2010; Mollema *et al*, 2010). The colonization rate of *S. aureus* in healthy subjects worldwide has been reported to range from 15% to 52% and colonization rate of MRSA of up to 9% (Creech *et al*, 2005; Chatterjee *et al*, 2009; Lozano *et al*, 2011; Kitti *et al*, 2011; Ho *et al*, 2012).

To date, the causes and factors for the increasing incidence of CA-MRSA remain incompletely understood. Nasal carriers of *S. aureus* have an increased risk of acquiring *S. aureus* infections in both community and hospital settings (Wertheim *et al*, 2005). The prevalence of MRSA nasal colonization has increased significantly among healthy individuals, especially children who can disseminate the bacteria to other children, usually through close contact with contaminated hands or surfaces (Creech *et al*, 2005; Pathak *et al*, 2010).

In order to protect against transmission of *S. aureus* and MRSA in the community, it is necessary to identify the genetic determinants of virulence which are important in adherence in the nasal niche. As there are few studies on virulence determinants in community isolated methicillin-sensitive *S. aureus* (MSSA) and MRSA, this study determined adhesin genes, *icaAD*, *fnbA* and *cna*, from MSSA and MRSA isolated from children in a Thai community.

MATERIALS AND METHODS

Isolation and identification of *S. aureus*

Samples were collected from nasal swab samples of 217 healthy children from 3 primary schools in 3 different districts in Phitsanulok Province, Thailand (107 males and 110 females; ages between 3-12 years) over a 12-month period (2010-2011). Swab samples were streaked onto mannitol salt agar (HiMedia, Mumbai, India) plates and incubated at 35ºC for 24 hours. Cultures with yellow colonies were selected and evaluated using Gram stain, catalase, DNase, mannitol fermentation and coagulase tests. Isolates were identified as being *S. aureus* if they were positive for catalase, DNase, and coagulase. All *S. aureus* isolates were confirmed by PCR using specific 16 S rRNA *Staphylococcus* primers (Table 1). The protocol of this study was approved by Naresuan University Ethics Committee.

Identification of MRSA

MRSA strains were screened using oxacillin salt method (Kitti *et al*, 2011) and confirmed using cefoxitin disk (30 g) on Mueller-Hinton agar.

Determination of antimicrobial susceptibility

Susceptibility to chloramphenicol, erythromycin, gentamicin, oxacillin, penicillin, vancomycin, trimethoprim/sulfamethoxazole (TMP-SMX), rifampicin, clindamycin, linezolid, cefoxitin, mupiro-

Table 1
List of primers used for detecting *S. aureus* genes.

Target gene	Primer	Size	Tm	Reference
icaAD	TATTCAATTTACAGTCGCAC			
	GATTCTCTCCCTCTCTGCCA	407	58	Yazdani *et al*, 2006
fnbA	GATACAAACCCAGGTGGTGG			
	TGTGCTTGACCATGCTCTTC	191	55	Zmantar *et al*, 2008
cna	AAAGCGTTGCCTAGTGGAG			
	AGTGCCTTCCCAAACCTTTT	192	52	Arciola *et al*, 2005
mecA	TGGCTATCGTGTCACAATCG			
	CTGGAACTTGTTGAGCAGAG	310	58	Ryffel *et al*, 1990
16S	CGAAAGCCTGACGGAGCAA			
	AACCTTGCGGTCGTACTCCC	597	57	Palazzo *et al*, 2005

cin, ciprofloxacin and fusidic acid (Oxoid, Hampshire, UK) was determined according to the antibiotic disk diffusion method (CLSI, 2010). The plates were incubated at 35ºC for 24 hours. Zones of inhibition were determined based on whether the microorganism was susceptible, intermediate, or resistant to each antibiotic.

Detection of *S. aureus mecA, icaAD, fnbA* and *cna* by PCR

The presence of *mecA*, *icaAD*, *fnbA* and *cna* were detected as described previously (Ryffel *et al*, 1990; Arciola *et al*, 2005; Yazdani *et al*, 2006; Zmantar *et al*, 2008; Kitti *et al*, 2011) using primers listed in Table 1, with *S. aureus* strain COL as positive control. Each PCR was performed in triplicate in a thermocycler (Thermo PCR sprint, Fisher Scientific, Waltham MA) and PCR amplicons were analyzed by electrophoresis in 1% agarose gel containing 0.5 g/ml ethidium bromide (Fig 1).

Detection of biofilm formation

Quantitative microtiter plate assay for biofilm formation were performed as described by Bekir *et al* (2012). In brief, *S. aureus* isolates were cultivated overnight in 96-well polystyrene tissue culture microtiter plates (Nunc, Roskilde, Denmark) at 37ºC with trypticase soy broth supplemented with 0.25% glucose as the growth medium. After incubation, the culture medium was removed and adherent cells were fixed with 95% ethanol and stained

Table 2
Drug resistance patterns of *S. aureus* isolated from nasal carriers.

Antibiotic	MRSA (%) (n = 5)	MSSA (%) (n = 73)
Cefoxitin (30 g)	5 (100)	-
Oxacillin (1 g)	5 (100)	1 (1)
Penicillin (10 UI)	5 (100)	72 (99)
Erythromycin (15 g)	2 (40)	4 (5)
Clindamycin (2 g)	1 (20)	-
TMP-SMX (25 g)	1 (20)	-
Chloramphenicol (30 g)	-	3 (4)

MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; TMP-SMX,trimethoprim/sulfamethoxazole.

Table 3
Presence of *icaAD*, *fnbA* and *cna* in 78 *S. aureus* isolates.

fnbA	*icaAD*	*cna*	MSSA (%) (n = 73)	MRSA (%) (n = 5)
-	-	-	0	5 (100)
+	-	-	1 (1)	0
+	+	-	24 (31)	0
+	+	+	48 (61)	0

MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*.

Table 4
Biofilm formation and incidence of *icaAD* in 78 *S. aureus* isolates.

Incidence of *icaAD* (%)	Biofilm negative (%) ($OD_{570nm} < 0.1$)	Low grade biofilm positive (%) ($0.1 \leq OD_{570nm} < 1$)	Highly biofilm positive (%) ($OD_{570nm} \geq 1$)
Present 72 (92)	0	26 (33)	46 (59)
Absent 6 (8)	4 (5)	2 (3)	0

with 1% crystal violet. Absorbance at 570 nm was determined. Isolates are considered biofilm-positive if they have an $OD_{570\,nm} > 0.1$. Each isolate was tested in triplicate.

RESULTS

Nasal colonization of MSSA and MRSA in Thai children

The incidence of coagulase-negative staphylococci (CNS) nasal colonization was 44% (95/217) and the isolation rate of *S. aureus* was 36% (78/217). There were 73 children (34%) harboring MSSA, and 5 (2%) MRSA.

Antimicrobial susceptibility

All *S. aureus* isolates were tested for their antimicrobial susceptibility towards 14 antimicrobials. The majority of isolates were resistant to penicillin (99%), but there were resistance to cefoxitin (6%), chloramphenicol (4%), clindamycin (1%), erythromycin (8%), oxacillin (8%) and TMP-SMX (1%) (Table 2). All 5 MRSA isolates were multi-drug resistant (MDR), *ie*, resistant to ≥ 3 antibiotics. The MRSA isolates showed resistance to cefoxitin (100%), oxacillin (100%), penicillin (100%), erythromycin (40 %), clindamycin (20%) and TMP-SMX (20%), but were sensitive to ciprofloxacin, fusidic acid, gentamicin, linezolid, mupirocin, rifampicin, and vancomycin.

Detection of *S. aureus mecA, icaAD, fnbA* and *cna*

Amplicons (597 bp) of 16 S rRNA were present in all *S. aureus* isolates (data not shown). Amplicons (310 bp) of *mec*A were detected in all 5 MRSA isolates (Fig 1A), but not those of *icaAD*, *fnbA,* and *cna*. Amplicons of *fnbA, cna* and *icaAD* are shown in Fig 1B. Among the 78 *S. aureus* isolates, *fnbA*, *icaAD* and *cna* was

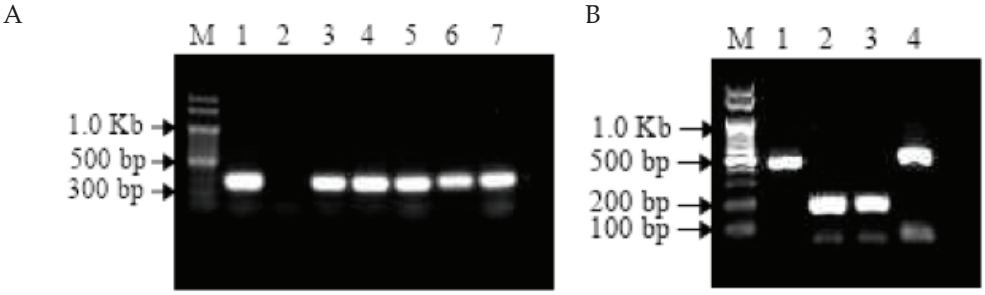

Fig 1–Amplification of *mecA* (A) from 5 MRSA isolates and *S. aureus icaAD, fnbA, cna* and 16 S rRNA (B). Amplicons were separated by 1% agarose gel-electrophoresis and visualized by ethidium bromide staining. Panel A. Lane M, DNA markers; lane1, positive control *S. aureus* strain COL; lane 2, negative control; lanes 3-7, MRSA isolates. Panel B. Lane M, DNA markers; lane 1, *icaAD* (407 bp); lane 2, *fnbA* (191 bp); lane 3, *cna* (192 bp); lane 4, 16 S rRNA (597 bp).

present in 73 (93%), 72 (92%) and 48 (61%) strains, respectively and all 3 virulence genes were present in 48 (61%) of MSSA isolates (Table 3).

Biofilm formation

Of the 78 strains of *S. aureus* tested for the ability to form biofilm, 46 (59%) were able to form a strong biofilm and 28 (36%) formed a weak biofilm. However, 4 MRSA isolates (80%) were non-biofilm-forming. The presence of *icaAD* was observed in 72 (92%) of *S. aureus* strains that were able to form biofilm (Table 4).

DISCUSSION

A recent study in healthy subjects identified 1% MRSA carriers in Thai adults (Kitti *et al*, 2011). Our study in Thai children showed higher rates of *S. aureus* and MRSA colonization. High rates of MRSA colonization have been reported in children worldwide (Creech *et al*, 2005; Chatterjee *et al*, 2009). The associated risk factors or contacts with high risk persons were not evaluated in this study. Persistence of MRSA in anterior nares has been observed (Verkade *et al*, 2013). Our study showed that all 5 MRSA carriers in the first investigation were transient carriers as MRSA strains were not detected after 1 year in these individuals.

Drug resistance patterns of *S. aureus* nasal isolates in different countries are different. *S. aureus* isolated strains are typically resistant to penicillin (96-100%), erythromycin (8-42%), tetracycline (22%), clindamycin(5-25 %) and TMP-SMX (14%) (Chatterjee *et al*, 2009; Ramana *et al*, 2009; Kitti *et al*, 2011; Ho *et al*, 2012). MSSA strains in this study exhibited penicillin, erythromycin and chloramphenicol resistance. Two of the MRSA isolates showed resistance to clindamycin and TMP-SMX which are effective drugs used against CA-MRSA skin infection (McMullen *et al*, 2009). This may be explained by the wide use of these antibiotics for treatment of bacterial infection in children. In addition, antibiotics (penicillin, erythromycin, clindamycin and TMP-SMX) can be purchased without prescription in Thailand. The uncontrolled administration of antibiotics is likely to drive the emergence of the resistance strains in the community.

In this study, *fnbA* was present in all

MSSA isolates consistent with previous reports (Arciola *et al*, 2005; Wiśniewska *et al*, 2008; Zmantar *et al*, 2008). This gene is highly prevalent in *S. aureus* strains isolated from human nasal epithelium (Nashev *et al*, 2004). The other adhesin gene that is important in *S. aureus* colonization is *cna*. The incidence of *cna* in the 78 *S. aureus* (both MRSA and MSSA) was 61%, higher than that observed by Montanaro *et al* (1999) (29%), Nashev *et al* (2004) (46.7%) and Arciola *et al* (2005) (46%) in Italy and Bulgaria, respectively. Difference of the *cna* gene prevalence in this study is probably due to different techniques in PCR detection or difference in the distribution of *S. aureus* genotype in different countries.

In *S. aureus*, the ability to form biofilm helps the bacteria to survive adverse environment within the host. *IcaAD* is an important genetic determinant for biofilm formation (O'Gara, 2007). Among 78 *S. aureus* isolates, *icaAD* was detected in most MSSA strains, all of which were able to form biofilm *in vitro*. Prevalence of *icaAD* in *S. aureus* has been reported to range from 35.29 to 78.26% (Zmantar *et al*, 2008; Dhanawade *et al*, 2010). The presence of *ica*AD gene in MSSA was strain variation (O' Neil *et al*, 2007). The high frequency of *ica* operon detection is associated with clinical *S. aureus* strains (Zmantar *et al*, 2008; Esteban *et al*, 2010).

Interestingly, all 5 MRSA isolates lacked *icaAD, fnbA* and *cna*. Taneike *et al* (2006) reported that all MRSA isolated from nosocomial outbreaks in Japan lacked *fnbB*. This is in contrast with other studies in which *fnbA* and *cna* was found in 90% and 63%, respectively, of MRSA strains (Wiśniewska *et al*, 2008). O'Neill *et al* (2007) showed that a higher percentage of MSSA (14%) than MRSA (0%) was found positive for slime-producing (biofilm) ability. Biofilm development in MRSA is *ica* independent and involves other adhesin proteins (Croes *et al*, 2009).

In conclusion, our study showed that the *S. aureus* carrier rate in Thai children was high. Most of them contained the adhesin genes, *icaAD*, *fnbA* and *cna*. MRSA carrier rate was increased to 2%. From this data, school sanitation and hygiene education programs are needed to minimize transmission of MRSA. In addition, the genetic variation of adhesin genes and mechanisms for nasal colonization need further investigation.

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