Tropical Biomedicine 35(2): 442–452 (2018)

Molecular characteristics of infection and colonization isolates of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA)

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Received 16 October 2017; received in revised form 7 February 2018; accepted 9 February 2018

Abstract. *Staphylococcus aureus* is a gram-positive coccus that colonizes the skin and mucous membranes, particularly the anterior nares. Recently, community-acquired MRSA (CA-MRSA) has emerged as a cause of skin and soft-tissue infections in healthy individuals. These strains are sensitive to antimicrobials, carry genes for Panton-Valentine leukocidin (PVL) toxin, and feature the staphylococcal cassette chromosome *mec* (SCC*mec*) type IV or V. The suspected mode of transmission involves close contact with carriers, leading to skin or nasal colonization that results in subsequent active infection. This study was undertaken to determine the molecular characteristics of CA-MRSA isolates in children presenting with wound infections at Likas Hospital, Sabah, Malaysia, and the possible mode of transmission. The results showed that the majority of CA-MRSA infection isolates were from scalp abscesses (49%) in 1–5-year-old children (70%) in the Filipino (54%) community. The presence of the *mec* gene was detected in all isolates and the PVL virulence factor was found in 92% of the isolates. SCC*mec* typing revealed that 57% of the isolates were untypable, 35% harbored the SCC*mec*IVa element, and one each had SCC*mec*IVc, SCC*mec*V, or SCC*mec*II. Sixteen *S. aureus* strains were isolated from nasal swabs in 19 family members of index patients. Fourteen of these cultures were positive for catalase, coagulase, and DNAase. All of the colonization isolates carried the *mecA* gene and only a third were positive for the PVL toxin. SCC*mec* typing showed that 79% of the isolates were untypable and two had SCC*mec*IVa element and one had SCCmecV element. When five pairs of infection and colonizing isolates were compared by *spa* typing, only two pairs showed identical *spa* type with possible transmission between the patient and family contact. Further studies are necessary to establish CA-MRSA transmission by performing multiple-site cultures multiple times instead of one-time nares-only sample collection.

INTRODUCTION

Staphylococcus aureus is a gram-positive coccus that colonizes the skin and mucous membranes, particularly the anterior nares (Williams, 1963). It is a causative agent of suppurative diseases, metastatic infections, food poisoning, and certain fatal infections (Lowy, 1998). The first methicillin-resistant *S. aureus* (MRSA) strains emerged two years after methicillin was introduced; these were primarily associated with nosocomial infections and were therefore termed hospital acquired-MRSA (HA-MRSA) (Rosenthal *et al.*, 2012). In the 1990s, MRSA infections were reported in healthy individuals without healthcare-associated risk factors; these are known as community-acquired MRSA (CA-MRSA) (CDC, 1999). CA-MRSA is defined as MRSA isolated from an outpatient or within 48 hours of hospital admission in a patient who was not recently associated with any healthcare facilities and had not received antibiotic therapy (Millar *et al.*, 2007). The

common clinical symptoms of CA-MRSA in children are abscesses, cellulitis, and pneumonia (CDC, 1999; Herold 1998). These isolates are more susceptible to antimicrobials compared to nosocomial isolates (Shopsin & Kreiswirth, 2000). Molecular analysis of CA-MRSA isolates from different regions have revealed that they share the same genetic characteristics, such as harboring SCC*mec*IV or V elements and the Panton-Valentine leukocidin (PVL) virulence factor (Vandenesch *et al.*, 2003). The suspected mode of transmission involves close contact with carriers, leading to skin or nasal colonization that results in subsequent active infection (Wertheim *et al.*, 2005; Eko *et al.*, 2015). Conventional methods, such as culture and antibiotic susceptibility testing, are used to identify MRSA, while molecular typing methods, such as pulse-field gel electrophoresis (PFGE), multi-locus sequence typing (MLST), staphylococcal cassette chromosome (SCC*mec*) typing, and Staphylococcus protein A (*spa*) typing, are used to determine the sources and modes of transmission of CA-MRSA (Struelens *et al.*, 2009).

The global prevalence of CA-MRSA shows that the ST80-MRSA-IV strain (European clone) is widespread in Europe, while the ST8-MRSA-IV strain (USA300 clone) is most prevalent in North America (Otter & French, 2010; Nimmo, 2012). The ST59-MRSA-IV clone is commonly seen in East Asia (Chuang & Huang, 2013). In Malaysia, ST30-MRSA strains carrying the SCC*mec* IV or V elements and the PVL toxin gene have been reported (Sam *et al.*, 2008; Ahmad *et al.*, 2009). The present study was undertaken to determine the molecular characteristics of CA-MRSA strains in children presenting with wound infections at the Women and Children's Hospital in Likas, Sabah, Malaysia, and to compare them with nasal colonizing isolates from their family members. To characterize CA-MRSA isolates, PCR was done for the *mecA* gene, the PVL virulence factor, the SCC*mec* element, and the *spa* gene. To determine colonization, nasal swabs were collected from family contacts of the index patients for culture and molecular characterization.

MATERIALS AND METHODS

Clinical samples and CA-MRSA isolates

The study was approved by the Medical Research Ethical Committee (MREC), Malaysia (National Medical Research Register [NMRR] 14-1552-23197 [IIR]). From September 2015 to February 2017, a total of 37 *S. aureus* isolates were obtained from the pathology laboratory at Hospital Wanita Dan Kanak-Kanak Sabah (HWKKS) in Likas, Sabah, Malaysia, which is a government referral hospital for women and children. All isolates were identified by the hospital laboratory using culture characteristics on sheep blood agar, gram stain, and standard biochemical methods, such as catalase, slide and tube coagulase, DNAase, and cefoxitin susceptibility. The patients' records were reviewed to determine whether they fulfilled the epidemiological criteria for CA-MRSA, namely isolation of MRSA within 48 hours of hospitalization. The exclusion criteria were no history of antibiotic intake, no hospitalizations or admissions to health care facilities within the previous year, and no medical devices or indwelling catheters permanently placed through the skin. Demographic data, such as age, gender, ethnicity, isolation site, and sample type, were all retrieved from the hospital clinical case notes. Nasal swabs from 19 family contacts of CA-MRSA index cases were collected using sterile swabs, along with a questionnaire, after consent was obtained from the parent/guardian. Family contacts were usually father or mother of the patient. Twelve samples were collected from 12 different households while five samples were from one household (parents, two sisters and brother) and two samples were from another household (both parents). The samples were transported to Universiti Malaysia Sabah. The MW2 (ATCC BAA-1707) strain was used as the positive control for microbiological and molecular assays.

Microbiological cultures

The nasal swabs from the family contacts of the index cases were cultured on sheep blood agar plates and incubated at 37°C for 24–48 hours. Beta hemolytic colonies were

isolated and subjected to gram staining and biochemical tests including catalase, slide and tube coagulase, and DNAse. All CA-MRSA isolates were then stored at -80°C in Luria-Bertani broth supplemented with 20% glycerol for molecular characterization.

Detection of *mecA*, SCC*mec*, and PVL toxin genes

Chromosomal DNA was extracted from CA-MRSA isolates using Qiagen Mini Blood DNA extraction kit. The primer sequences for mecA, SCC*mec*I-V and PVL toxin (*luk-PV-1*, *luk-PV-2*) and the methods used for PCR were according to Zhang *et al*. (2005) and Lina *et al*. (1999). For *mecA* gene amplification and SCC*mec* typing, PCR was performed using 10 pmol/µL of primers and 1 µl of DNA template in a ready-to-go PCR tube (GE, USA). The thermocycling conditions were as follows: initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 2 minutes, and the final extension at 72°C for 10 minutes (Zhang *et al*., 2005). For detection of the PVL toxin gene, the thermal cycling conditions used for PCR were an initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and the final extension at 72°C for 10 minutes (Lina *et al*., 2005). When more than one set of primers was used in multiplex PCR, the thermal cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, 10 cycles of 94°C for 45 seconds, annealing at 65°C for 45 seconds, extension at 72°C for 1.5 minutes and continued for 25 cycles of 94°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1.5 minutes, and the final extension at 72°C for 10 minutes. Gel electrophoresis was done to visualize the PCR products in 2% agarose gel (Zhang *et al*., 2005).

***Spa* gene sequencing**

PCR for the *spa* gene was performed with specific primers 1095F and 1517R (Harmsen *et al*., 2003). The thermal cycling conditions were as follows: initial denaturation for 10 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 60°C for annealing, 45 seconds at 72°C for the extension, and a final extension at 72°C for 10 minutes. Gel electrophoresis was done using 1.5% agarose gel (Shopsin *et al*., 1999). The PCR products were purified and sent to the First BASE Sdn. Bhd. Kuala Lumpur, Malaysia for sequencing. The *spa* gene sequences were entered in the Spa Type Finder software to determine the spa types of CA-MRSA isolates (http://spatyper.fortinbras.us/).

Statistical analysis

The frequency for each variable in the research questionnaire was calculated by chi-square or Fisher's exact tests. A *P* value of <0.05 indicated statistical significance.

RESULTS

Demographic characteristics of CA-MRSA isolates

A total of 37 isolates of CA-MRSA were collected for molecular characterization, all of which met the CA-MRSA criteria. When the data were classified according to ethnic groups, more than half of the CA-MRSA index patients were of Filipino ethnicity (n=20, 54%). The second most common group of CA-MRSA patients were Bajau (n=7, 19%) followed by Dusun (n=3, 8%), Malay (n=2, 5%), and one each of Chinese, Rungus, Lundayeh, Sungai, and Brunei (Figure 1). All patients were divided into three groups according to age. The infant group age range was 1–11 months, the child group age range was 1–5 years, and the older-child group age range was 6–10 years. As shown in Figure 2, the highest prevalence of CA-MRSA was seen in the 1–5-year-old children (n=26, 70%) followed by infants (n=9, 24%) and older children (n=2, 5%). The majority of the index cases were females (n=22, 60%), although this was not statistically significant. All CA-MRSA isolates were obtained from abscesses from various body sites; the distribution of sites is summarized in Figure 3. The most common infection site was the scalp (18 samples, 49%), followed by the arm and foot (4 samples each, 11%), two each from the eyelid, neck, axilla, and thigh, and one each from the lip, scrotum, and inguinal area.

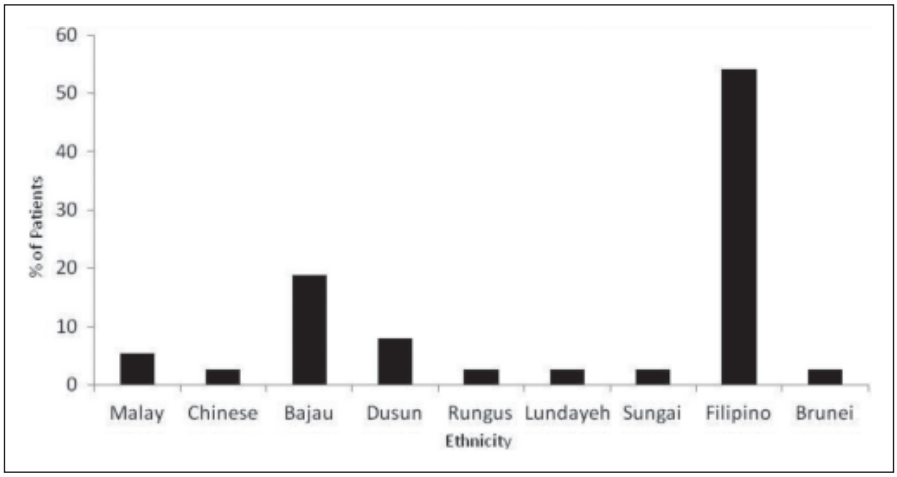

Figure 1. CA-MRSA isolates from patients in different ethnicity.

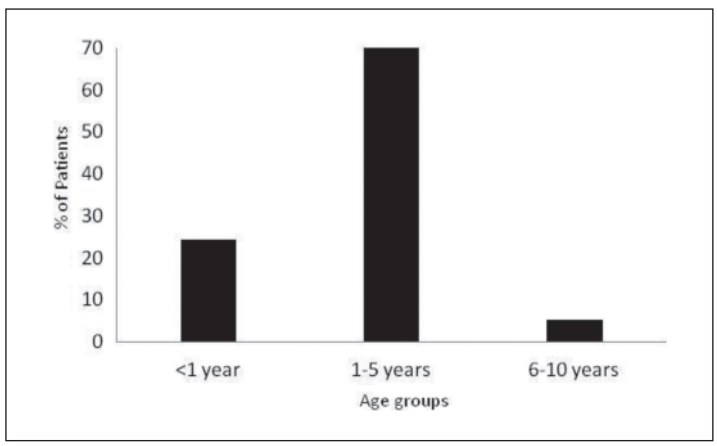

Figure 2. Distribution of CA-MRSA isolates in different age groups.

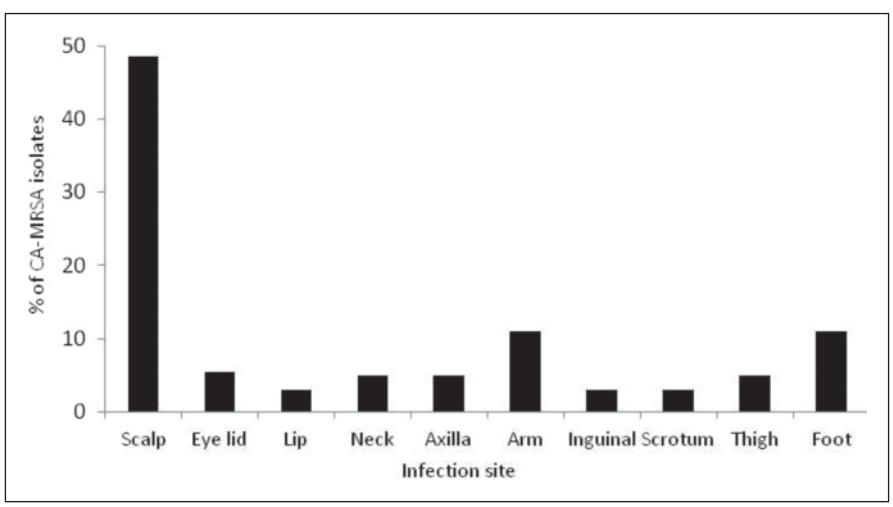

Figure 3. CA-MRSA isolates cultured from different body sites.

Eight out of 9 infants and 10 out of 26 (38%) children in the age group of 1–5 years had scalp infections. In addition, 49% (18/37) of the index cases reported contact with children other than family members. Some of the risk factors for CA-MRSA transmission observed in this study were shared towels (5/15), shared bar soaps (6/15), boils in a family member (9/15), cuts or scars (5/15), and low socioeconomic status (13/15, <1000 MYR).

Molecular characteristics of infection and colonization isolates of CA-MRSA

As shown in Table 1, the presence of *mecA*, the methicillin-resistance gene, was detected in all infection isolates. Of these 37 isolates, the PVL virulence factor was detected in 34 (92%) isolates. Twenty-one (57%) MRSA isolates had untypable SCC*mec* elements. Thirteen (35%) isolates harbored SCC*mec*IVa elements and one each had SCC*mec*IVc, SCC*mec*V, or SCC*mec*II elements. From these 37 index cases, 19 family members from 14 households were contacted for nasal swab collection to determine CA-MRSA transmission. Sixteen MRSA cases were excluded from the study since the parents did not give consent, while seven were unregistered and there was no contact number or address recorded. From 19 nasal swab samples, beta hemolytic colonies were isolated from 16 (84%) samples that showed gram-positive cocci. Fourteen of these isolates were also positive for catalase, coagulase, and DNAase, and all 14 of these carried the *mec*A gene while only five (36%) were positive for the PVL virulence factor. SCC*mec* typing revealed that 11 (79%) of these isolates were untypable, while two (14%) had the SCC*mec*IVa element and one (7%) had SCCmecV element. When the SCC*mec* types of five PVL-positive colonization isolates were compared with the corresponding index-case infection isolates, we could not find any associations as six out of ten isolates were untypable. Since SCC is a mobile genetic element, we determined *spa* types for five pairs of infection and colonization isolates. As shown in Table 1, four different *spa* types were detected. Six out of 10 isolates were typed as t019, two were typed as t122 and one each as t186 and t975. Only two pairs of infection and colonization isolates had identical *spa* type (t019) while the other three pairs had different *spa* types.

Table 1. Comparison of molecular characteristics of infection and colonization isolates of CA-MRSA

Characterization/Typing method		Infection isolates	Colonization isolates
Presence of *Mec*A gene		37 (100%)	14 (100%)
Presence of PVL virulence factor		34 (92 %)	5 (36%)
SCC*mec* typing	Untypable	21 (57%)	11 (79%)
	IVa	13 (35%)	2 (14%)
	IVc	1 (3%)	–
	V	1(3%)	1 (7%)
	II	1(3%)	–
Spa typing	Isolate Number	*Spa* Types	
	A/F1167	t019	t019
	A/F2301	t122	t019
	A/F5413	t019	t019
	A/F0204	t186	t122
	A/F7429	t975	t019

A – Index case; F – Family contact.

DISCUSSION

It has been reported that MRSA colonization is associated with increased risk for MRSA infection (Davis *et al.*, 2004). Hence, we screened family members of CA-MRSA index patients with wound infections to determine whether there was possible CA-MRSA transmission within families in the local community. Some of the risk factors for CA-MRSA infections identified in this study were ethnicity, younger age, infection site, prior infection among close contacts, overcrowding, and lower socioeconomic status. Although Filipinos are not indigenous to Sabah, the majority of the index patients in this study were of Filipino ethnicity. Several studies showed that the immigrants contributed to the importation of CA-MRSA (Bartels *et al.*, 2009). The MRSA infection rate was reported to be higher among the Indian community in west Malaysia (Ghaznavi-Rad *et al.*, 2010). CA-MRSA skin infections in returned travelers or long-term residents from the Philippines have been reported (Bochet *et al.*, 2008; Bartels *et al.*, 2009; Ko *et al.*, 2013).

The most common MRSA infection site reported in this study was scalp abscesses in children between the ages of 5 months to 5 years. Although these children showed no predisposing risk factors, it is possible that they were exposed to contaminated household surfaces through physical activities, such as rolling and playing with other children (Uhlemann *et al.*, 2011). It has been shown that *S. aureus* strains can persist from 7 days to 7 months on environmental surfaces (Kramer *et al.*, 2006). In addition, contamination from their mothers' forearms might also play a role in *S. aureus* colonization in children before infection has occurred (Wertheim *et al.*, 2005). Culturing swabs from other body sites, such as hands, fingertips, and forearms, could be considered when screening family members of index patients (Champion *et al.*, 2014). Based on the questionnaire, half of the respondents stated that the index patients had interactions with other children at school or their younger siblings. *S. aureus* infection and MRSA colonization in healthy young children who are persistent carriers has been reported in several studies (Oguzkaya-Artan *et al.*, 2008). Many studies have also shown that younger children are more likely to have CA-MRSA infections (Centers for Disease Control and Prevention, 1999; Herold *et al.*, 1998). CA-MRSA transmission in two different households involving young children was reported previously (Urth *et al.*, 2005). Thus, screening children other than family members is necessary to determine the transmission of CA-MRSA. Similar to other reports, the prevalence of CA-MRSA in the present study did not vary by gender (Ghaznavi-Rad *et al.*, 2010).

According to the questionnaire used in this study, 60% of respondents reported that family members had a history of pre-existing boils before the index patient developed the MRSA infection. Recurrent CA-MRSA infections can occur 1–3 months after the initial CA-MRSA case involving 2–4 people in the same household (Crum *et al.*, 2006). Overcrowding was also observed in this study, which may cause the bacteria to spread easily among family members (Ragan, 2006). A correlation between a greater number of family members and *S. aureus* carriage was reported in India and Taiwan (Dey *et al.*, 2013, Chen *et al.*, 2011). Another factor that may have contributed to CA-MRSA infections in this study is the socioeconomic status of patients living in poverty, especially in urban areas (Rashid & Ghani, 2007).

All of the CA-MRSA infection isolates and 14 of the *S. aureus* colonization isolates from family members in this study were positive for *mecA*. The *mecA* gene codes for methicillin resistance and is located on SCC, the mobile genetic element. The m*ecA* PCR is considered to be the gold standard for the detection of methicillin resistance and is used for characterizing MRSA isolates (Asghar, 2014; Pereira *et al.*, 2014; Sun *et al.*, 2013). Most (92%) of the CA-MRSA infection isolates and one-third (36%) of the CA-MRSA colonization isolates obtained from family contacts had the PVL toxin gene. PVL is a cytotoxin that causes leukocyte destruction and tissue necrosis, and is considered a constant marker for CA-MRSA (Boyle-Vavra

et al., 2007). PVL-positive CA-MRSA isolates are usually associated with skin infections, including furuncles, abscesses, and cellulitis, as well as severe necrotizing pneumonia and sepsis (Lina *et al*., 1999; Bocchini *et al*., 2006). Thus, the PVL-positive CA-MRSA colonization isolates may have the capacity to cause infection in the presence of CA-MRSA risk factors. The presence of the PVL virulence factor has increasingly been shown in HA-MRSA isolates in recent years (Sun *et al*, 2013; Valle *et al*., 2016).

Fifty-seven percent and 79% of the CA-MRSA infection and colonization isolates, respectively, harbored untypable SCC*mec* elements when primers to detect SCC*mec* types I, II, III, IVa, IVb, IVc, IVd, and V were used (Zhang *et al*., 2005). Untypable SCC*mec* types have been reported by many others (Asghar, 2014; Sun *et al*., 2013; Yao *et al*., 2010; Zhang *et al*., 2005). In addition to retesting with the primer sequences reported by Oliveira *et al*. (2002) and Milheirico *et al*. (2007), SCC*mec* typing can be extended to other types, such as SCC*mec*IVe, IVg, IVh, IVi, and IVj and to the recently discovered types SCC*mec*VI and VII, for a complete assessment (Turlej *et al*., 2011). The untypable MRSA isolates may also belong to a novel SCC*mec* element, and further improvements in SCC*mec* typing might aid the detection of newer SCC*mec* types and subtypes. SCC*mec*IVa was the most common type found in index cases and family contacts in this study, similar to other reports (Berglund *et al*., 2005; Sun *et al*., 2013). The presence of both the PVL-toxin gene and the SCC*mec* type IV element has been reported among CA-MRSA isolates in several studies (Asghar 2014; Champion *et al*., 2014; Sun *et al*., 2013). One CA-MRSA isolate harbored the SCC*mec*IVc element, and MRSA carrying the SCC*mec*IVc element was also reported in Japan and Sweden (Ito *et al*., 2001; Berglund *et al*., 2005). The presence of the SCC*mec*V element has been reported by others in both CA-MRSA and HA-MRSA isolates (Sun *et al*., 2013; Yao *et al*., 2010). Staphylococcus clones carrying SCC*mec* elements IV and V may spread more easily in the community as well as in the hospital due to the small size of the cassette chromosome that enhances its mobility (Pereira *et al*., 2014). Although the presence of SCC*mec* types I, II, and III was reported in HA-MRSA isolates, one of the infection isolates in our study harbored the SCC*mec*II element, showing that SCC*mec* types are not exclusive as CA strains become "domesticated" to healthcare settings and HA strains become "feral" and are established in the community (Miller *et al*., 2010; Yao *et al*., 2010). Additional genetic markers are necessary for differentiating CA and HA isolates. In the present study, based on SCC*mec* typing, no association was found between the five PVL-positive colonization isolates and their corresponding infection isolates from the index cases. However, two pairs of infection and colonization (A/F1167, A/F5413) isolates had similar *spa* types showing possible CA-MRSA transmission between the patient and the family member. Whole genome sequencing of these isolates is necessary to confirm transmission of CA-MRSA among family members. The frequency of *spa* types of infection and colonization isolates are 1.04%, 0.16%, 0.20%, and 0.02% for t019, t122, t186, and t975 respectively, according to the Ridom Spa Server database (http://www.spaserver.ridom.de). Six out of ten isolates in this study belong to *spa* type t019 which belongs to ST30, and two of these colonizing isolates had SCC*mec* IV element. PVL-positive, ST30-MRSA-SCC*mec*IV with *spa* type t019 is known to be the most successful pandemic clone and has been widely found in the Philippines, Singapore, Taiwan, Hong Kong, Malaysia, and Japan (Song *et al*., 2011). It has been demonstrated previously that *S. aureus* colonizing strain types are diverse, complex, and frequently not concordant with the infecting isolate (Miller *et al*., 2012).

Studies that directly compared colonizations and infections with *S. aureus* have shown that paired colonization and infection isolates were phenotypically and genetically indistinguishable (Eko *et al*., 2015). We were able to observe possible transmission of CA-MRSA in only two families based on molecular characteristics and this could be due to the transient nature of colonization (Champion *et al*., 2014). MRSA carriage was

not found in nasal swab cultures after outbreaks of MRSA among college football players, suggesting that the participants were carrying MRSA in sites other than the anterior nares (Kazakora *et al.*, 2005). Miller *et al.* (2012) reported that a nares-only survey would have missed 48% of *S. aureus* and 51% of MRSA-colonized individuals, and assessing only nasal colonizations may underestimate the true burden of *S. aureus* carriage in individuals and in the community. Higher colonization rates of MRSA in athletes and their support staff were reported after continuous sample collection from three anatomic sites over a 12-week period as opposed to the one-time nares-only collection (Champion *et al.*, 2014; Miller *et al.*, 2012).

In summary, the majority of the CA-MRSA isolates in this study were from scalp abscesses in Filipino children. The *mecA* gene was present in all isolates and the PVL virulence factor was present in the majority of the isolates from the index cases. All of the colonization isolates carried the *mecA* gene and only a third were positive for the PVL toxin. SCC*mec* typing revealed that the majority of the infection and colonization isolates were untypable, and the most common type was SCC*mec*IVa. *Spa* typing showed an association between colonization and infection isolates, with possible transmission between patients and family contacts. More detailed studies are necessary to define the link between colonization and infection by performing multiple-site cultures multiple times.

Acknowledgments. The authors thank the staff of infectious disease unit, pathology laboratory and pediatric wards, Hospital Wanita Dan Kanak-Kanak Sabah (HWKKS), Likas, Sabah, Malaysia for providing CA-MRSA isolates and helping with nasal swab collection.

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