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# Population structure of colonizing and invasive *Staphylococcus aureus* strains in northern Vietnam

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*Staphylococcus aureus* is an important global health problem worldwide. There is still scarce information on the population structure of *S. aureus* strains in Asia, where the majority of the world population lives. This study characterized the diversity of *S. aureus* strains in northern Vietnam through multilocus sequence typing (MLST). Eighty-five carriage isolates from the community and 77 invasive isolates from the clinical setting were selected and tested for meticillin resistance and the presence of Panton–Valentine leukocidin (PVL). MLST was performed on these isolates, of which CC59 (25.4 %), CC188 (17.3 %) and CC45 (16.7 %) were the predominant clonal complexes (CCs). CC59 carriage isolates had significantly lower rates of meticillin-resistant *S. aureus* (MRSA) than their corresponding clinical group isolates (32 vs 83 %). There were no significant differences in rates of MRSA between carriage isolates and clinical isolates of CC45 and CC188. CC59 carriage isolates were significantly lower in rates of $PVL^+$ than CC59 clinical isolates (32 vs 83 %), but the converse was shown in CC45 isolates (14 vs 0 %, respectively). This study revealed vast differences in the molecular epidemiology and population structure of *S. aureus* in community and clinical settings in Vietnam. Nevertheless, the data underline the spread of virulent and/or resistant strains (MRSA and/or $PVL^+$) in the community, suggesting the necessity for further surveillance to determine the mechanism of transmission of these strains (i.e. MRSA/$PVL^+$) outside clinical settings.

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## INTRODUCTION

*Staphylococcus aureus* is a common bacterium that colonizes about 20–30 % of the human population but can also cause a variety of infections from localized to systemic, including skin infections, deep abscesses, endocarditis, pneumonia and sepsis (Wertheim *et al.*, 2005a; van Belkum *et al.*, 2009; Gonzalez *et al.*, 2005). In many cases, carriage of specific *S. aureus* strains is associated with subsequent infection when the opportunity arises (Wertheim *et al.*, 2005a). Worldwide there has also been an increase in meticillin-resistant *S. aureus* (MRSA), with a shift from being mainly a hospital-acquired pathogen to a common cause of community-acquired skin and soft tissue infections (Centers for Disease Control and Prevention, 1999; Witte *et al.*, 1997; Udo *et al.*, 1993). In Vietnam, outbreaks of severe community-acquired MRSA (CA-MRSA) infections have occurred, as reported among

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Abbreviations: CA, community-acquired; CC, clonal complex; MLST, multilocus sequence typing; MRSA, meticillin-resistant *S. aureus*; MSSA, meticillin-sensitive *S. aureus*; NHTD, National Hospital of Tropical Diseases; PVL, Panton–Valentine leukocidin; ST, sequence type.

nine children in 2006 (Tang *et al.*, 2007). Another report revealed that 19 % of *S. aureus* bloodstream infections from Vietnam were methicillin resistant and often not managed well (Song *et al.*, 2011). This is relevant because significantly heightened mortality has been associated with MRSA bloodstream infection compared with meticillin-sensitive *S. aureus* (MSSA) infection (Cosgrove *et al.*, 2003).

In order to understand the clinical epidemiology of *S. aureus* and the spread of antibiotic resistance, it is important to investigate the overall population structure of *S. aureus* for both colonizing and invasive isolates. Considering that the majority of the global population lives in Asia and that *S. aureus* is a common pathogen throughout this continent, more data are needed from this region. Several molecular methods have been developed to compare *S. aureus* populations in various regions, of which multilocus sequence typing (MLST) and *S. aureus* protein A (spa) typing are the most commonly used (Tenover *et al.*, 2006; Enright *et al.*, 2000; Kondo *et al.*, 2007). Through MLST, different clonal complexes (CCs) have been found to predominate in a variety of geographical regions. Clinical studies conducted in Asian countries have found that sequence type 59 (ST59), ST30 and ST72 dominate over MRSA in both community- and hospital-acquired isolates (Song *et al.*, 2011). In the current Vietnamese study, we aimed to identify and describe the major *S. aureus* CCs, the meticillin resistance and the presence of the virulence determinant, Panton–Valentine leukocidin (PVL) in isolates from invasive bloodstream infections and compare these with carriage isolates in urban and rural healthy individuals in the catchment area of the hospital.

## METHODS

**Sample collection.** From February to May 2012, samples were collected from healthy individuals living in the Dong Da and Ba Vi districts in northern Vietnam (community setting). Dong Da district is an urban district with a population of 352 000 people and a typical urban Vietnamese socioeconomic structure. Ba Vi is a rural district with farming as the main occupation. Nasal swabs of the anterior nares and throat swabs of the posterior pharynx and tonsillar areas were collected using sterile Dacron swabs (Copan, Italy). Subsequently, all swabs were transported in bacterial transport medium to the microbiological laboratory of the National Hospital of Tropical Diseases (NHTD) within 24 h of collection time and were plated on agar for pathogen identification as described previously (Van Nguyen *et al.*, 2014). Clinical invasive *S. aureus* isolates were obtained from positive blood cultures in the period from November 2009 to December 2012 from patients admitted to NHTD as described elsewhere (Thwaites, 2010). A total of 591 *S. aureus* isolates [216 nasal swabs (36.5 %); 293 throat swabs (49.5 %); 82 blood samples (13.9 %)] were collected, from which 162 *S. aureus* isolates, one per individual, were randomly selected for this study (85 carriage isolates and 77 invasive ones).

**Microbiology.** Blood cultures were performed at NHTD using the automated Bactec system (Becton Dickinson). In the case of a positive blood culture, Gram staining and a subculture on Columbia blood agar were performed. Nasal and throat swabs were cultured on phenyl mannitol agar plates (Oxoid). Suspected *S. aureus* colonies from blood and swab cultures were identified by colony morphology, Gram stain, and coagulase and catalase testing. Meticillin resistance was determined by cefoxitin disk diffusion on Mueller–Hinton agar plates according to Clinical and Laboratory Standards Institute criteria (CLSI, 2012).

**Molecular testing and typing.** *S. aureus* identity and meticillin resistance were confirmed by PCR amplification of the coagulase gene and *mecA* gene, as described elsewhere (Sabet *et al.*, 2007). We also tested for the presence of the PVL gene by PCR according to a previously described method (McClure *et al.*, 2006).

MLST was performed on the 162 selected isolates using the protocol and primers described on the MLST website for *S. aureus* (http://saureus.mlst.net) (Enright *et al.*, 2000). The allelic profiles were identified based on this database, and the STs were then classified into CCs by the eBURST version 3 program (http://saureus.mlst.net/eburst/, accessed 20 July 2014). STs that shared at least six identical alleles of seven MLST loci were grouped in a CC according to the most stringent definition in the eBURST program.

**Data analysis.** Univariate analysis was performed using Pearson's $\chi^2$ test and Fisher's exact test where appropriate. A two-sided $P$ value of $<0.05$ was considered significant. All calculations were performed using R (version 3.0.1).

**Phylogenetic analysis.** We examined phylogenetic relationships among the 162 isolates using the data from all seven genetic regions, concatenated into a single sequence (no partitioning between regions). The sequences were aligned using CLUSTAL Omega, as implemented in SeaView (Galtier *et al.*, 1996; Sievers *et al.*, 2011). Maximum-likelihood phylogenetic analysis was implemented with PhyML (version 3.0), using a generalized time reversible mutation model (Guindon *et al.*, 2010). Bootstrap support was determined using 100 replicates. The resulting tree was then viewed in SeaView for subsequent analysis.

## RESULTS

### Study population and *S. aureus* population structure

Of the 162 isolates on which molecular typing (MLST) was performed, 27 (16.7 %) were from females and 111 (68.6 %) were from males [there were no data on gender for 24 isolates (14.8 %)]. Samples were from participants ranging in age from 3 to 87 years. The median age was 29 years (interquartile range 13.5–41.5; Table 1). Fifty-three of the 162 MLST typed isolates were MRSA (32.7 %) and 109 strains were MSSA (67.3 %). The number of MRSA isolates was 28 (32.9 %) and 23 (29.9 %) in the carriage group (Dong Da and Ba Vi) and invasive group (NHTD), respectively. The PVL gene was present in 19 (22.4 %) of 85 carriage isolates and in 25 (32.5 %) of 77 invasive isolates ($P=0.16$).

Overall, 29 different STs were detected, including 12 singleton STs (Table 2, Fig. 1). The most dominant CCs were CC59 (43 isolates, 26.5 %), CC188 (28 isolates, 17.3 %), CC45 (27 isolates, 16.7 %), followed by CC88 (11 isolates, 6.8 %), CC398 (12 isolates, 7.4 %), CC25 (seven isolates, 4.3 %), CC121 (eight isolates, 4.9 %) and CC8 (three isolates, 1.9 %). Singletons consisted of 23 isolates (14.2 %).

**Table 1.** Population profiles by study site

| Characteristic | Study site | | |
|---|---|---|---|
| | Dong Da ($n$=31; nasopharyngeal swabs) | Ba Vi ($n$=54; nasopharyngeal swabs) | NHTD ($n$=77; blood isolates) |
| Female/male (%) | 25.8/54.8 | 24.1/59.3 | 7.8/80.5 |
| Age [median (interquartile range)] | 13 (9–23) | 17 (11–37) | 36 (28–49) |
| MRSA (%) | 19.4 | 40.7 | 32.5 |
| $PVL^+$(%) | 19.4 | 22.2 | 33.8 |

CC45 isolates were more common across carriage isolates compared with invasive isolates (24.7 % for carriage isolates vs 7.8 % for invasive isolates; $P$=0.002). No significant difference was found between the rate of CC59 carriage (29.4 %) and CC59 invasive (23.4 %) isolates. However, relatively more CC59 isolates were found in the rural (i.e. Ba Vi) carriage group (67.7 %), as compared to the urban (i.e. Dong Da) carriage group (7.4 %; $P<0.001$). CC188 isolates displayed significantly higher rates in invasive isolates (29.4 %) compared with carriage isolates (7.7 %; $P<0.001$).

Both carriage and invasive strains broadly co-occurred in our phylogenetic analysis (Fig. 1). Nevertheless, the tree clearly showed that some CCs were more present in invasive samples such as CC398, CC188 and CC88, whereas CC59 and CC45 were more present in the carriage samples. Both meticillin resistance and the presence of PVL were also distributed broadly across the phylogeny but with a distribution depending on the CC and on the invasive versus carriage status. For example, MRSA/$PVL^+$ isolates were more prevalent in invasive CC59 samples, whereas CC188 isolates did not have any samples that were $PVL^+$.

## Distribution of MRSA among CCs

Of the total number of MRSA isolates, CC59 had the most MRSA isolates (23/53 isolates, 43.4 %), and of MSSA isolates, CC188 was the most predominant (25/109 isolates, 22.9 %) (Table 2, Fig. 1). There were differences between invasive and carriage strains within specific CCs. MRSA was more common among invasive CC59 isolates ($n$=15,

**Table 2.** Molecular attributes of the *S. aureus* isolates

| CC | ST ($n$) | Total isolates [$n$ (%)] | Carriage isolates | | Invasive isolates | |
|---|---|---|---|---|---|---|
| | | | MRSA/MSSA | $PVL^+/PVL^-$ | MRSA/MSSA | $PVL^+/PVL^-$ |
| CC188 | ST188 (27), ST2393 (1) | 28 (17.3) | 1/8 | 0/9 | 2/17 | 0/19 |
| CC8 | ST8 (1), ST239 (2) | 3 (1.9) | 0/1 | 0/1 | 2/0 | 0/2 |
| CC25 | ST25 (6), ST1029 (1) | 7 (4.3) | 1/5 | 2/4 | 0/1 | 0/1 |
| CC45 | ST45 (26), ST546 (1) | 27 (16.7) | 11/10 | 3/18 | 5/1 | 0/6 |
| CC59 | ST59 (39), ST338 (1), ST537 (2), ST3069 (1) | 43 (26.5) | 8/17 | 8/17 | 15/3 | 15/3 |
| CC88 | ST88 (10), ST2141 (1) | 11 (6.8) | 1/3 | 1/3 | 0/7 | 0/7 |
| CC121 | ST121 (7), ST1964 (1) | 8 (4.9) | 2/2 | 1/3 | 0/4 | 3/1 |
| CC398 | ST398 (1), ST1232 (11) | 12 (7.4) | 1/3 | 1/3 | 0/8 | 6/2 |
| Singleton | | 23 (14.2) | | | | |
| | ST5 | 1 | 0/1 | 0/1 | 0 | 0 |
| | ST6 | 7 | 0/5 | 1/4 | 0/2 | 0/2 |
| | ST9 | 1 | 0 | 0 | 0/1 | 0/1 |
| | ST7 | 1 | 0 | 0 | 0/1 | 0/1 |
| | ST15 | 5 | 1/1 | 0/2 | 0/3 | 0/3 |
| | ST72 | 2 | 0 | 0 | 0/2 | 0/2 |
| | ST97 | 1 | 0 | 0 | 0/1 | 0/1 |
| | ST285 | 1 | 0 | 0 | 0/1 | 1/0 |
| | ST406 | 1 | 1/0 | 1/0 | 0 | 0 |
| | ST834 | 1 | 0 | 0 | 1/0 | 0/1 |
| | ST942 | 1 | 1/0 | 1/0 | 0 | 0 |
| | ST1281 | 1 | 0/1 | 0/1 | 0 | 0 |
| Total | | 162 (100 %) | 28/57 | 19/66 | 25/52 | 25/52 |

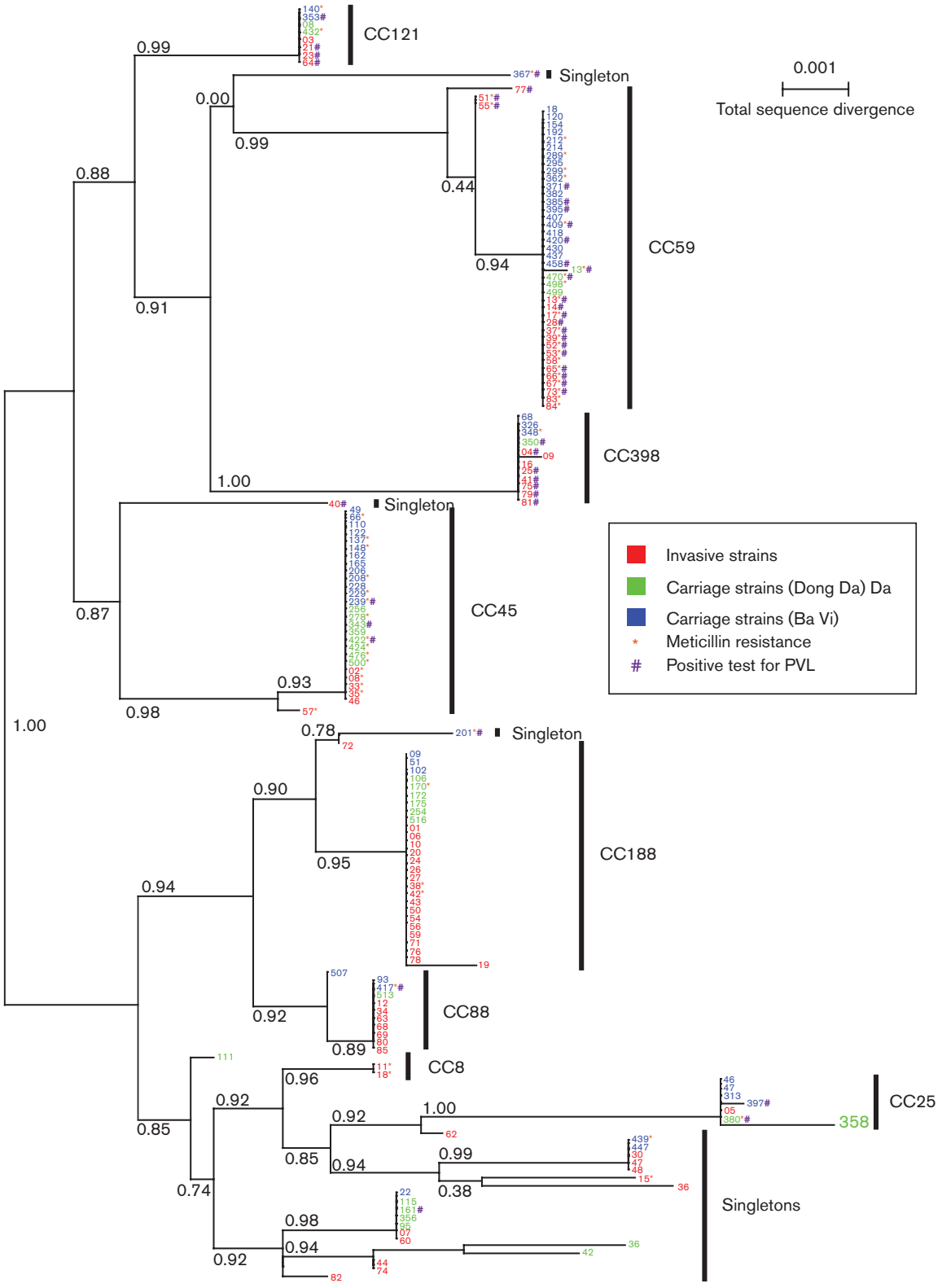


**Fig. 1.** Maximum-likelihood phylogenetic tree for the 162 samples with full datasets. Samples labelled in blue are carriage strains from rural Ba Vi and in green are from urban Dong Da; samples labelled in red are invasive hospital strains. An asterisk indicates meticillin resistance and # indicates presence of PVL. Numbers to the right of the vertical black bars indicate clonal complex designation. Numbers along the branches indicate branch support based on 100 bootstrap replicates. Bar, 0.1 % total sequence divergence.

83.3 %) compared with CC59 carriage group isolates ($n$=8, 32.0 %; $P<0.001$). There were no significant differences in number of MRSA isolates between invasive and carriage CC45 and CC188 isolates ($P>0.05$). Nevertheless, five out of the six CC45 invasive isolates were MRSA (83.3 %) whereas 11 out of 21 CC45 carriage isolates were MRSA (52.4 %; $P$=0.2). To characterize the population structure of invasive MRSA and MSSA, we tested for differences among isolates from CC45, CC59 and CC188. Invasive MRSA and MSSA isolates displayed significant differences between CC45, CC59 and CC188 ($P<0.001$). There were five (83.3 %) invasive MRSA and one (16.7 %) invasive MSSA CC45 isolates, 15 (83.3 %) invasive MRSA and three (16.7 %) invasive MSSA CC59 isolates, and finally two (10.5 %) invasive MRSA and 17 (89.5 %) invasive MSSA CC188 isolates.

### Distribution of PVL among CCs, MRSA and invasive versus carriage isolates

The prevalence of PVL$^+$ isolates differed among CCs (Table 2, Figs 1 and 2). PVL in the invasive strains was most commonly found in CC59 (15 isolates, 19.4 %), CC398 (six isolates, 7.8 %) and CC121 (three isolates, 3.9 %). PVL distribution in the carriage isolates was found mainly in CC59 (eight isolates, 9.8 %). The prevalence of PVL$^+$ and PVL$^-$ isolates was significantly different across CC45, CC59 and CC188 ($P<0.001$).

There was a statistical difference in the proportion of PVL$^+$ isolates between MSSA and MRSA: 21/53 (39.6 %) MRSA isolates and 24/109 (22.0 %) MSSA isolates were positive for the *luk* gene encoding PVL ($P$=0.005; Fig. 2). Of the 21 PVL$^+$ MRSA isolates, 15 (71.4 %) belonged to CC59, and the remaining six isolates were distributed among a total of four CCs, comprising ST406, CC25, CC45 and CC88. The PVL$^+$ MRSA isolates in the carriage group were not significantly higher than in the invasive group.

There were also significant differences in the proportion of PVL$^+$ isolates between invasive and carriage groups according to the CC. There were significantly higher rates of invasive PVL$^+$ CC59 isolates (15 isolates, 83.3 %) than carriage PVL$^+$ CC59 isolates (eight isolates, 32.0 %) ($P<0.001$). The same trend ($P<0.001$) was shown in CC398 : six invasive PVL$^+$ CC398 isolates (75.0 %) and one carriage PVL$^+$ CC398 isolate (25.0 %). On the other hand, there were significantly lower rates of invasive PVL$^+$ CC45 isolates (0 isolates, 0 %) than carriage PVL$^+$ CC45 isolates (three isolates, 14.3 %) ($P<0.001$). The same trend ($P<0.001$) was shown in CC88: no invasive PVL$^+$ CC88 isolates (0 %) and one carriage PVL$^+$ CC88 isolate (33.3 %).

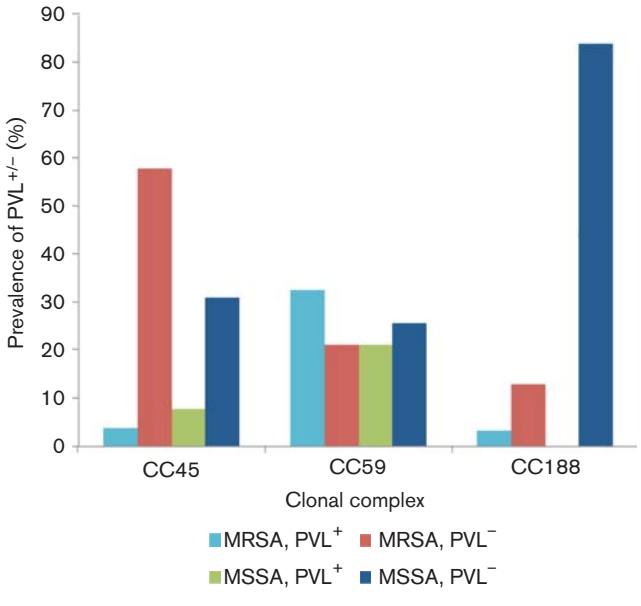


**Fig. 2.** Distribution of PVL among MRSA and MSSA CC45, CC59 and CC188 isolates.

## DISCUSSION

Here, we have provided data on the population structure of MRSA and MSSA in both carriage and invasive disease isolates in northern Vietnam. Most previous studies conducted in East Asian countries on *S. aureus* have focused on MRSA infections and either clinical or carriage isolates. We found a high level of genetic diversity in colonizing and invasive *S. aureus* strains. The most dominant *S. aureus* CCs detected in our collection were CC59, CC45 and CC188. All invasive CCs were also represented in carriage isolates, showing that community-acquired invasive *S. aureus* will be challenging to prevent. CC59 was particularly prevalent among both carriage and clinical isolates. CC45 and CC25 were more common among carriage compared with invasive isolates. CC188 was more common among invasive strains.

In other Asian studies, CC30, CC59 and ST239 have been reported to be dominant CCs (Sheng *et al.*, 2009; Song *et al.*, 2011; Feil *et al.*, 2008; Chen *et al.*, 2013). In Europe, ST80, ST398 and ST152 are the predominant STs in CA-MRSA. In North America, USA300 (ST8) and USA400 (ST1) prevail. Interestingly, we did not find any ST30, ST80 or USA400 isolates in our study. A report from Japan has shown several cases of ST8 (USA300) CA-MRSA infection in Asia (Shibuya *et al.*, 2008). Only one ST8 isolate was detected in our study, which was susceptible to meticillin.

A multinational study in Asia revealed that MRSA infections in the community are increasing in Asian countries (Song *et al.*, 2011). In 2006, there was an outbreak of severe CA-MRSA infections of ST59 in southern Vietnam following routine vaccination injections (Tang *et al.*,

2007). This is in agreement with our results, as CC59 was predominant in our study with a high proportion of MRSA and PVL$^+$ isolates in invasive but also in carriage isolates. These data suggest that CC59 is widely spread, invasive in nature and commonly resistant in Vietnam, and thus appears as the most worrying CC in this country. This CC was also recently reported as a predominant clone associated with *S. aureus* infections in Chinese children as well as adults in Taiwan and Sri Lanka (Geng *et al.*, 2010; Li *et al.*, 2013; Du *et al.*, 2011). It is possible that CC59 is currently spreading between adjacent regions, supporting its dominance in the Asian region as a whole.

CC45 was the second most frequent CC in our study, particularly among carriage isolates, with a high proportion of MRSA. Throughout Germany, the Netherlands and Canada, CC45 is common in both carriage and disease (Wannet *et al.*, 2004; Witte *et al.*, 1997; Simor *et al.*, 2002; Wertheim *et al.*, 2005b). In a report from the USA in 2010, USA600 (ST45) MRSA caused bloodstream infection with a high rate of mortality but with low carriage rates in community. The study suggested that unique virulent characteristics might be involved in USA600 bloodstream infections, which were not assessed in our study (Moore *et al.*, 2010). The relative low proportion of invasive CC45 isolates in our study and the absence of PVL suggest that CC45 is predominantly a colonizer in Vietnam. Nevertheless, it is worth noting that the majority of the CC45 invasive strains were MRSA, and thus the spread of this CC in Vietnam needs to be followed.

The third main CC in our study, CC188, is a double locus variant of ST1. In contrast to CC45, 19/27 (70.3 %) of CC188 isolates were found in the invasive clinical group with low levels of meticillin resistance. This CC seems to weakly spread in the community in Vietnam, suggesting the source of transmission is clinical settings. CC188 has not been reported as a dominant CC in most other countries, such as Malaysia, Europe, America and Australia (Ghaznavi-Rad *et al.*, 2010; Otter and French, 2010; Wertheim *et al.*, 2005b; Rolo *et al.*, 2012; Nimmo and Coombs, 2008). Studies in Korea, Hong Kong and Malaysia only sporadically found ST188 isolates in the MRSA population (Ghaznavi-Rad *et al.*, 2010; Peck *et al.*, 2009; Monecke *et al.*, 2011). Only in Taiwan has CC1 been reported as one of the four major CCs (Wang *et al.*, 2012).

Concerning CC398, we detected ST1232, which is a single locus variant of ST398. CC398 MRSA is considered a zoonotic pathogen, mainly affecting people who work with pigs and veal calves (van Loo *et al.*, 2007). Nevertheless, in numerous countries, MSSA CC398 has now been identified in healthcare workers and patients without exposure to livestock but also in the environment of a French intensive care unit, indicating the capacity for emergence of this clone in hospitals (Brunel *et al.*, 2014). Thus, this clone may cause a significant public health problem if it can spread successfully from human to human (de Neeling *et al.*, 2007; Cuny *et al.*, 2009; Wassenberg *et al.*, 2011). Eight out of 12 (66.7 %) CC398 MSSA isolates were from clinical disease, suggesting that zoonotic CC398 *S. aureus* is also causing human disease in Vietnam, an agricultural country. In addition, 75 % of the CC398 MSSA clinical isolates tested positive for the presence of PVL, emphasizing their virulence.

Previous studies have reported the presence of the PVL gene in many different *S. aureus* CCs: CC59, CC88, CC30, CC45, CC1, CC80, CC22 and CC398 (Monecke *et al.*, 2007; Otter and French, 2010). The prevalence of MRSA containing the PVL gene is also very diverse according to the region. In Asia, the prevalence of the PVL gene in CA-MRSA and hospital-acquired MRSA was found to be 14.3 and 5.7 %, respectively (Song *et al.*, 2011). In Taiwan, 28 (11.1 %) of 253 MRSA strains from bloodstream infection harboured PVL, while nine (7.1 %) of 126 community-onset MRSA isolates were PVL$^+$ (Wang *et al.*, 2010a, b). In a study of 100 *S. aureus* isolates from diverse cases of skin and soft tissue infection at a university hospital in Germany, 30 isolates were positive for the genes encoding PVL and three of them were MRSA (Monecke *et al.*, 2007). In this study, the proportion of isolates containing PVL was 22.3 and 32.4 % in the carriage and clinical groups, respectively. These proportions are higher than in previous reports and than in a recent study carried out in China (Li *et al.*, 2013). Indeed, Li *et al.* (2013) reported 55.5 % of CC59 MRSA strains to be PVL$^+$ in mainland China, whereas we detected 61.9 % of CC59 MRSA as PVL$^+$ in northern Vietnam.

It is worth noting that this study had several limitations. The *S. aureus* isolates used for this study were obtained from two health centres and a hospital in Hanoi in northern Vietnam, and thus no generalization can be made at a national scale. Further studies should offer a more comprehensive analysis of the molecular epidemiology of *S. aureus* throughout the country and possibly other Asian countries. In addition, further studies in Vietnam should examine the drivers of the emergence of invasive CCs and develop strategies to control the spread of virulent clones. Additionally, it would be of interest to include the clinical outcome of patients with invasive *S. aureus* disease in future studies.

In conclusion, we found that the population structure of *S. aureus* in northern Vietnam is different from that in other Asian countries and also from high-income countries such as Europe and the USA. The predominant CCs of *S. aureus* in our study were CC59, CC45 and CC188 MSSA. CC59 was widespread among both carriage and invasive isolates with a high proportion of MRSA and PVL virulence. It is thus essential to continue *S. aureus* surveillance with genotyping in Asia on a larger scale and to report trends in invasiveness and drug resistance over time.

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