A persistent antimicrobial resistance pattern and limited methicillin-resistance-associated genotype in a short-term Staphylococcus aureus carriage isolated from a student population

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Summary The aim of the present study was to assess and compare the antimicrobial susceptibility pattern against a panel of antibiotics and molecular and methicillin resistance-associated genotypes of 120 carriage S. aureus isolates previously isolated from a student population at two isolation events within a one-month interval. The antibiotic susceptibility of isolates was determined using the Kirby-Bauer disc-diffusion method (cefoxitin by Etest). The MRSA was screened using polymerase chain reaction for the presence of the meca gene. The meca-positive isolates were subjected to staphylococcal cassette chromosome (SCC) mec
typing, multilocus sequence typing (MLST) and eBURST analysis. All isolates were characterized for the presence of the Panton–Valentine leukocidin (PVL) gene, an enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) pattern and the spa type. For the two occasions where S. aureus was isolated, the highest frequency of resistance was observed for penicillin (70% and 65%, respectively), with a lower rate against erythromycin and tetracycline (<12%). All isolates were susceptible to ciprofloxacin and gentamycin. As for methicillin resistance, eight isolates had minimum inhibitory concentrations (MIC) of resistant categories, but 10 isolates (8.33%) were positive for the meca gene. The meca-positive isolates belonged to SCCmec types I (n = 9) and V (n = 1). MLST was resolved for only three MRSAs, ST508 (n = 1), ST88 (n = 1) and ST96 (n = 1). The results of the eBURST analysis showed that the MRSA isolates analyzed in the present study were potentially related to MRSA identified in other countries. Approximately half of the persistent S. aureus carriers harbored S. aureus of a similar spa type in the respective individuals during both isolation events. A persistent antimicrobial pattern and limited distinct MRSA were observed over the short study period. The latter frequently exhibited SCCmec type I, commonly associated with hospital-acquired (HA) characteristics, but further delineation is needed to justify the origins of these bacteria.

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Introduction

Staphylococcus aureus is a well-known opportunistic pathogen that frequently colonizes the skin and nasal mucosa of humans. Although multiple body sites can be colonized in human beings, the anterior nares have been identified as the primary reservoirs of S. aureus, with carrier rates ranging between 20 and 30% in healthy populations [1]. It has been reported that the colonization of S. aureus in the nose is a potential risk factor for acquiring subsequent staphylococcal infections [2,3]. In addition, this opportunistic pathogen shows increasing resistance to certain antimicrobial agents, causing difficulties in managing staphylococcal infections [4]. In recent years, methicillin-resistance S. aureus (MRSA) strains have emerged as a major cause of health care-associated and community-associated infections worldwide. The incidence of S. aureus and MRSA is now growing at an alarming rate, with a high incidence of community-associated MRSA infections in both children and adult populations [5]. Therefore, the continuous surveillance of S. aureus and evaluation of the antibiotic susceptibility patterns of these bacteria are crucial to monitor the emerging trend. Moreover, analysis of the phenotypic and genotypic characteristics of S. aureus isolates carried in a population over a period of time might also provide further insight into the persistent pattern of antimicrobial resistance and associated genotypes.

The present study utilized a collection of S. aureus isolates recently isolated from the anterior nares of a health science student population obtained over a one-month period [6] to compare the antimicrobial susceptibility pattern against a panel of commonly used antibiotics and identify the molecular characteristics pertaining to methicillin resistance-associated genotypes, sequence types (ST), ERIC-based fingerprint patterns and surface protein A (Spa) types. This comparative analysis might provide a model for understanding the dissemination and epidemiological patterns of S. aureus over a short-term transition in a population.

Materials and methods

Isolates

In a recent study [6], 192 health science undergraduate students at the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, participated in a nasal swab collection, with 60 students (31.3%) isolated for S. aureus. In the following month, 180 of these students participated in a second sampling, with 60 students (33.3%) positive for S. aureus. During the one-month period, 39 participants were defined as persistent carriers of S. aureus during both first and second sampling events. Methicillin resistance was preliminarily screened using the cefoxitin-disc diffusion method and polymerase chain reaction (PCR) for the presence of the 147-bp meca gene. All 120 isolates were preserved at −80°C in Luria Bertani broth supplemented with 20% glycerol.

A persistent antimicrobial resistance pattern and limited methicillin-resistance

In the present study, the bacterial stocks were revived on blood agar and re-identified based on Gram staining, growth on Mannitol Salt Agar (MSA) (Merck KGaA, Frankfurter Straße, Darmstadt, Germany) and coagulase reactions. All isolates were subjected to genomic DNA extraction for molecular analysis using the Exgene™ Cell SV Extraction Kit (AIBiotech, Science Park Drive, Singapore). *S. aureus* American Type Culture Collection (ATCC) 700699 and 25923 were used as reference strains in all analyses.

### Antibiotic susceptibility of *S. aureus*

The susceptibility of *S. aureus* isolates to various antibiotics was determined according to Clinical and Laboratory Standards Institute guidelines [7]. The Etest method was used to examine susceptibility against cefoxitin, while the disc diffusion method was used for erythromycin (15 μg), gentamicin (10 μg), ciprofloxacin (5 μg), rifampin (5 μg), penicillin (10 units) and tetracycline (30 μg) (all from Oxoid, Altrincham, Cheshire, England).

### Detection of methicillin-resistance gene

The detection of the *mecA* gene was re-certified through PCR using the following primers: *mecA*-F (5’-AAA ACTAGGTTGGTGGTAAGATATACC-3’) and *mecA*-R (5’-GAAAGGATCGTACTGG GTTATCAG-3’), according to Suhaili et al. [8]. Isolates with amplification of the expected size (147 bp) were categorized as MRSA strains. A few representatives of the PCR products were sequenced for gene identity confirmation (data not shown).

### SCCmec typing for methicillin-resistant *S. aureus* (MRSA)

All MRSA isolates were subjected to SCCmec typing through multiplex PCR according to Boye et al. [9], targeting genes *ccrA2-B, ccrC, IS1272 and mecA–IS431* to differentiate SCCmec types I (415 bp), II (937 bp), III (518 bp), IV (937 and 415 bp) and V (518 and 359 bp).

### Multilocus sequence typing (MLST)

Only *mecA*-positive isolates were subjected to MLST analysis. The nucleotide sequences of seven housekeeping genes, carbamate kinase (arc) (456 bp), shikimate dehydrogenase (aroE) (456 bp), glyceral kinase *(glp F)* (465 bp), guanylate kinase *(gmk)* (429 bp), phosphate acetyltransferase *(pta)* (474 bp), triosephosphate isomerase *(tpi)* (402 bp) and acetyl coenzyme A acetyltransferase *(ygl L)* (516 bp), were amplified through PCR according to Enright et al. [10]. The PCR products were sequenced at the 1st BASE lab (Serang, Selangor, Malaysia). The allelic profiles and sequence types (ST) were determined using the MLST database at http://www.mlst.net. The eBURST algorithm was also generated at http://eburst.mlst.net/.

### Detection of Panton–Valentine Leukocidin (PVL) gene

All isolates were screened for PVL gene through PCR using the following primers: *luk-PV-1* (5’-ATCATAGTAAATGTCTGGACATGCCA-3’) and *luk-PV-2* (5’-GCTCAASTGTATTTGGATGAAAAAGC-3’) and running parameters according to Lina et al. [11]. Isolates with a band fragment of 433 bp were sequenced for gene identity confirmation (data not shown) and reported as positive for the PVL gene.

### Spa typing

The polymorphic X region of the *spa* gene in the genomic DNA of *S. aureus* was amplified through PCR using the following primers: 1095F (5’-AGACGATCTGCTGGTGCAG-3’) and 1517R (5’-GCTTTTGCAATGTCATTACTG-3’) and running parameters according to Japoni-Nejad et al. [12]. The PCR products were sequenced at the 1st BASE lab (Serang, Selangor, Malaysia) using the same primers. Spa types were determined using DnaGear software at http://w3.ualg.pt/hshah/DNAGear/.

### Enterobacterial repetitive intergenic consensus (ERIC)-PCR

ERIC-PCR was performed according to Ye et al. [13], using primers ERIC1R (5’-ATGTAAGCTCCCTGGGATTAC-3’) and ERIC2 (5’-AAGTAAGTGGACTGGGTTAGCG-3’). Images of the DNA band patterns were analyzed to generate a dendrogram using GelComparII version 6.6.11 software (Applied Maths, Sint-Martens-Latem, Belgium) and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

### Results and discussion

#### Antimicrobial susceptibility pattern

All isolates survived preservation and matched the characteristics of *S. aureus*. Upon antimicrobial assay, the highest frequency of resistance was observed for penicillin in both isolations (70%
and 65%, respectively) (Table 1). This finding is consistent with other studies involving healthy communities showing a high resistance to penicillin (>60%) [14—17]. Penicillin is one of the most commonly used antibiotics to treat bacterial infections. Since the 1950s, penicillin has become the most useful therapeutic agent in treating staphylococcal infections [18]. Reflecting the wide usage of this antibiotic, a high level of resistance of S. aureus toward penicillin can be expected. In addition, a low resistance rate of the S. aureus isolates to tetracycline and erythromycin (<12%) in both isolation events was also observed. These data are consistent with other studies reporting a low rate of tetracycline resistance (<12%) among healthy individuals [14,17,19]. A low resistance rate to erythromycin (<12%) was also reported in other local surveillance studies [14,20,21]. However, the efficacy of ciprofloxacin, gentamycin, and rifampin remains exceptional, and the S. aureus isolates examined in the present study remained susceptible to these antibiotics. Similarly, a study at the same locality in 2006 also reported a high sensitivity of S. aureus nasal isolates to ciprofloxacin (86.4%), gentamycin (97.5%) and rifampin (98.8%) [14]. These findings indicate that certain antibiotics remain effective over time for treating S. aureus infections. Therefore, the use of these drugs should be properly managed to minimize the emergence of resistance.

Cefoxitin was used to infer methicillin resistance at the phenotypic level, and eight isolates (four each from first and second isolation, respectively) were identified based on the Etest. Four MRSA isolates (two each from first and second sampling, respectively) were isolated from two persistent carrier individuals. One of the MRSA isolates (from second sampling of persistent carrier) was also multidrug-resistant (MDR), showing co-resistance to three antibiotics of different classes [22,23]: erythromycin (macrolide), penicillin (β-lactam) and tetracycline (tetracycline). There were two other MDRs, but these isolates were characterized as MSSA strains from the first sampling. Although a low prevalence of MRSA was observed in the study population, previous findings at the same locality in 2009 and 2012 reported no individuals screened for S. aureus in those studies were colonized by MRSA [21,24]. In addition, the results of the present study indicate the persistence of MRSA over a short period, with one strain associated with MDR. This result might represent the current status, likely indicating a turning point for further dissemination if no precautionary measures are taken.

### Distribution of mecA gene and SCCmec type

Among the 120 isolates of S. aureus, 10 (8.33%) isolates (four and six isolates from first and second isolation events, respectively) were positive for the mecA gene, as these isolates showed the expected amplicon size. The mecA-positive detection was consistent with the eight isolates showing resistance toward cefoxitin in the Etest, while the other two mecA-positive isolates (from second event: one persistent carrier and one non persistent carrier) were cefoxitin-susceptible according to Etest methods. This result potentially reflects the presence of phenotypic heterogeneity among the drug resistant (heteroresistance) strains of S. aureus in the present study, therefore screening S. aureus isolates for the presence of mecA is warranted to avoid the misclassification of MRSA as MSSA, highlighting the need for PCR screening to monitor the dissemination of potential MRSA. SCCmec typing of the 10 mecA-positive isolates identified SCCmec type I for nine isolates and SCCmec type V for one isolate. SCCmec type I has been previously reported to be carried by hospital acquired-methicillin resistant S. aureus (HA-MRSA) [9,25]. The presence of MRSA with HA-associated SCCmec type I in this population might suggest that the isolates originated from

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**Table 1** Antimicrobial susceptibility patterns of S. aureus isolated from the first and second isolation events.

<table>
<thead>
<tr>
<th>Types of antibiotics</th>
<th>The first isolation event (n = 60)</th>
<th>The second isolation event (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I (0%)</td>
<td>R (6.67%)</td>
</tr>
<tr>
<td>Cefoxitin (30 μg)</td>
<td></td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Erythromycin (15 μg)</td>
<td>2 (3.33%)</td>
<td>4 (6.67%)</td>
</tr>
<tr>
<td>Gentamicin (10 μg)</td>
<td>0 (0%)</td>
<td>7 (11.67%)</td>
</tr>
<tr>
<td>Ciprofloxacin (5 μg)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Rifampin (5 μg)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Penicillin (10 units)</td>
<td>0 (0%)</td>
<td>42 (70%)</td>
</tr>
<tr>
<td>Tetracycline (30 μg)</td>
<td>0 (0%)</td>
<td>7 (11.67%)</td>
</tr>
</tbody>
</table>

R, resistant; I, intermediate.

* Etest method.
hospital settings. Considering that the participants in the present study were students of the health science program living in the vicinity of the hospital and mixing with colleagues from the medical program, such exposure might result in the spread of MRSA from the hospital to the student community, or vice versa.

ST and eBURST in comparison with SCCmec type and presence of PVL gene

Only the 10 mecA-positive isolates were subjected to MLST, whereby the respective amplicons from the isolates matching the expected DNA band size were successfully sequenced. Nevertheless, submission in the MLST database only assigned three isolates for ST, while the remaining isolates could not be typed (Table 2). The three MRSA were ST508 (1st sampling), ST88 (1st sampling) and ST96 (2nd sampling), and all MRSA were SCCmec type I. In the eBURST analysis, the MRSA isolate with ST508 belonged to a single-locus variant [SLV] of clonal complex (CC) 45 (Fig. 1A). CC45 was previously reported as one of the major lineages of MRSA clones [26,27]. Interestingly, this MRSA isolate was PVL negative, consistent with a report that PVL positivity was less detected among CC45-MRSA isolates [27]. Previous studies from Malaysia reported that MLST type ST508 of CC45 was present among MSSA strains isolated from the hospital [28] and community [26]. However, in the present study, ST508 of CC45 was identified in a MRSA isolate. The presence of ST508-MRSA in the present study might infer that the MRSA strain evolved from ST508-MSSA strains after acquiring the SCCmec mobile element. However, the ST508-MRSA isolate examined in the present study carried SCCmec type I and might also be related to ST45-MRSA sporadically detected in Hong Kong, which also carried SCCmec type I [27,29]. Another MRSA isolate examined in the present study resides in CC88 (Fig. 1B). This isolate had ST88 and was PVL negative. Fan et al. [25] reported that ST88 was largely identified among community isolates in China. The other MRSA in the present study clustered in CC96, as shown in Fig. 1C. This isolate had ST96 and was positive for the PVL gene. This isolate was the only MRSA (from second isolation) detected among the five isolates with the PVL gene, in the present study but the latter were all MSSAs (two isolates from each isolation). Nevertheless, ST96 has not been widely reported. Recently, one strain of CC96-MRSA with SCCmec type III was reported in Russia [30]. The presence of a PVL-positive MRSA strain associated with SCCmec type I (commonly carried by HA) is troublesome, as PVL has been associated with high mortality and morbidity, reflecting the virulence property of this gene.

ERIC-PCR, spa type and comparative analysis

Considering that the sampling was performed twice in a same population over a short period of time, there could be dissemination of the related isolates within the population, particularly in persistent carriers in which the same isolate could have persisted over the one-month period in the respective individuals, particularly the MRSA and MDR strains. The ERIC assay yielded two to eight bands ranging approximately 100–4000 base pairs, which were subsequently subjected to dendrogrammatic analysis. Based on the generated dendrogram, the majority of the isolates were genetically distinct (Fig. 2), with only a limited potential related dissemination. All isolates were segregated into eight clusters: clusters A to H, at only 29% similarity. Spa typing was also performed to correlate the

Table 2 Results of the MLST analysis.

<table>
<thead>
<tr>
<th>Isolates code</th>
<th>Sequence type</th>
<th>arcC</th>
<th>aroeE</th>
<th>glpF</th>
<th>gmk</th>
<th>pta</th>
<th>tip</th>
<th>yqiL</th>
</tr>
</thead>
<tbody>
<tr>
<td>A04_P03</td>
<td>ST508</td>
<td>10</td>
<td>40</td>
<td>8</td>
<td>6</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>B23_P03</td>
<td>NT</td>
<td>3</td>
<td>36</td>
<td>1</td>
<td>14</td>
<td>141</td>
<td>170</td>
<td>13</td>
</tr>
<tr>
<td>A35_P24</td>
<td>ST88</td>
<td>22</td>
<td>1</td>
<td>14</td>
<td>23</td>
<td>12</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>B13_P24</td>
<td>NT</td>
<td>31</td>
<td>31</td>
<td>1</td>
<td>14</td>
<td>12</td>
<td>170</td>
<td>11</td>
</tr>
<tr>
<td>B58_NP</td>
<td>ST96</td>
<td>12</td>
<td>1</td>
<td>15</td>
<td>11</td>
<td>1</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>A02_NP</td>
<td>NT</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>15</td>
<td>161</td>
<td>170</td>
<td>40</td>
</tr>
<tr>
<td>A38_NP</td>
<td>NT</td>
<td>20</td>
<td>20</td>
<td>23</td>
<td>6</td>
<td>161</td>
<td>170</td>
<td>40</td>
</tr>
<tr>
<td>B16_NP</td>
<td>NT</td>
<td>12</td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>44</td>
<td>57</td>
<td>40</td>
</tr>
<tr>
<td>B06_NP</td>
<td>NT</td>
<td>31</td>
<td>31</td>
<td>1</td>
<td>1</td>
<td>12</td>
<td>170</td>
<td>11</td>
</tr>
<tr>
<td>B55_NP</td>
<td>NT</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>15</td>
<td>233</td>
<td>99</td>
<td>40</td>
</tr>
</tbody>
</table>

NT - non-typable (isolate does not have a sequence type).
a Isolates A04_P03 and B23_P03 were obtained from a single individual, while isolates A35_P24 and B13_P24 were obtained from another individual.
distribution of the isolates in the dendrogram, but only 76 (63.3%) isolates were successfully typed. Nineteen (48.72%) of the 39 persistent carriers carried S. aureus of a single spa type in the respective individual in the two sampling events; four persistent carriers carried isolates of spa type t701 \( (n=8) \), while other individuals carried types t267 \( (n=4) \), t008 \( (n=4) \) and t127 \( (n=4) \). Isolates of spa types t505 \( (n=2) \), t1236 \( (n=2) \), t164 \( (n=2) \), t050 \( (n=2) \), t002 \( (n=2) \), t024 \( (n=2) \), t2883 \( (n=2) \), t4720 \( (n=2) \) and t346 \( (n=2) \) were each carried by a single different individual, respectively, in both isolation events. A majority of isolates of a single spa type carried in the respective persistent carriers \( (15/19) \) also showed similar antibiotic susceptibility patterns. Furthermore, there were five persistent carriers, each with isolates of a similar spa type, antibiotic susceptibility pattern and ERIC based-DNA fingerprint in the respective individual, suggesting that the respective carriers potentially carried related strains over the one-month period; two of these persistent carriers had isolates \( (A39\_P26 \text{ and } B28\_P26; \text{ A45\_P29 and B36\_P29}) \) grouped in cluster E, while the remaining carriers had isolates \( (A22\_P14 \text{ and } B7\_P14; \text{ A24\_P15 and B3\_P15; A50\_P32 and B10\_P32}) \) grouped in cluster F. To a certain extent, these findings are consistent with the statement that persistent carriers might carry one type of strain over time [31]. Nonetheless, most of the MDR and all 10 mecA-positive strains were non-typable, and one isolate failed in the PCR detection for the spa gene. Two (one MRSA and one MSSA) out of the three MDR isolates were positioned in the same cluster (cluster F), while the other MDR (MSSA) isolate was in cluster A. In addition, the MRSA isolates were distinctly distributed among the clusters: two MRSA isolates were in cluster A (A02\_NP and A04\_P03), three MRSA isolates in clusters E (A35\_P24, A38\_P25 and B16\_P05) and F (B06\_P17, B13\_P24 and B55\_P11), respectively, and one MRSA isolate was in clusters C (B23\_P03) and G (B58\_NP), respectively. For MRSA from persistent carriers, two MRSA from a single individual harbored a similar SCCmec type I, with a non-typable spa type and an antibiotic susceptibility pattern, but both isolates were not clustered in the same group (A04\_P03; Cluster A and B23\_P03; Cluster C). Another pair of MRSA in a persistent carrier (A35\_P24; Cluster E and B13\_P24; Cluster F), showed similar patterns, except for susceptibility against erythromycin (the isolate from the first isolation event was resistant, while the isolate from the second isolation event showed an intermediate result). These results suggest that most of the MRSA were potentially of a diverse genetic background.

Figure 1 eBURST diagram of the three clonal complexes (CC), CC45, CC88 and CC96, associated with the three MRSA isolates (in red) examined in the present study, respectively.
Figure 2. Dendrogram of the ERIC-based DNA fingerprint analysis using GelcomparII. *MRSA and Multi-drug resistance (MDR) are shown with abbreviations. 1st column: Isolate Code; 2nd column: Isolation event (1: 1st event; 2: 2nd event); 3rd column: Resistance pattern (P: Penicillin; T: Tetracycline; E: Erythromycin); and 4th column: Spa type. The identity of the isolates is labeled with 'A' for isolates from the first isolation and 'B' for isolates from the second isolation, followed by numbers. Additional labeling includes 'NP' for isolates from non-persistent carriers and 'P' from persistent carriers, followed by a number. The latter will have the same number for isolates obtained from the same individual.
Conclusion

As a whole, a persistent antimicrobial pattern and limited methicillin resistance-associated genotypes were observed over the short study period, with the potential persistence of similar isolates in some of the respective carriers. The number of MRSA strains was low, but those strains identified in different individuals were largely distinct. Interestingly, some strains had genotypes associated with a clonal complex comprising isolates identified in other countries. The source of these MRSAs is of interest, but whether the MRSAs in the present study are of community or hospital origin cannot be discriminated. Although most of the isolates carried SCCmec type I, a previously prominent characteristic of HA-MRSA, these bacteria could not be easily differentiated, as both HA- and community-acquired (CA) MRSA have merged and evolved and the earlier genotype associated with either HA- or CA-MRSA is disappearing [32].

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Competing interests

None declared.

Ethical approval

None required.

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