Mutations in \textit{rpoB} and \textit{fusA} cause resistance to rifampicin and fusidic acid in methicillin-resistant \textit{Staphylococcus aureus} strains from a tertiary hospital in Malaysia

King-Ting Lim\textsuperscript{a,b}, Cindy Shuan Ju Teh\textsuperscript{b,c}, Mohd Yasim Mohd Yusof\textsuperscript{c} and Kwai-Lin Thong\textsuperscript{a,b,*}

\textsuperscript{a}Institute of Biological Science, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia; \textsuperscript{b}Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya, Kuala Lumpur; \textsuperscript{c}Department of Medical Microbiology, University of Malaya Medical Centre, Kuala Lumpur

\*Corresponding author: Tel: +603-79674437; Fax: +603-79675908; E-mail: thongkl@um.edu.my

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\textbf{Background:} The prevalence of resistance to rifampicin and fusidic acid among Malaysian strains of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) is increasing. This study aimed to determine the mechanisms of rifampicin and fusidic acid resistance and the genetic diversity of MRSA strains from a Malaysian tertiary hospital.

\textbf{Methods:} Minimum inhibitory concentrations (MIC) for 21 MRSA strains were determined by agar dilution test and Etest. The resistance genes, \textit{staphylococcal chromosome cassette mec} (SCC\textit{mec}) types, multilocus-sequence typing (MLST) types and \textit{spa} types, were determined by PCR and DNA sequencing.

\textbf{Results:} MIC for rifampicin and fusidic acid resistance ranged from <1 to 8 \textmu g/ml and from <1 to 256 \textmu g/ml, respectively. A double mutation (484Arg/His and 517Glu/Gln) in \textit{rpoB} causes high rifampicin resistance while a mutational change (461Leu/Lys) in \textit{fusA} was observed in seven strains highly resistant to fusidic acid. Five of the seven were also resistant to rifampicin (MIC 8 \textmu g/ml) and carried a mutated \textit{rpoB} gene (484Arg/His). No other acquired fusidic acid resistance gene (\textit{fusB, fusC} or \textit{fusD}) was detected. Most (14/21) of the strains belonged to clone ST239-III-t037. Three belonged to ST22-IV-t1378 and the remaining four to ST239-III-t2029, ST239-III-t421, ST1178-IV-t1107 and ST241-III-t363, respectively.

\textbf{Conclusions:} The study showed that both rifampicin and fusidic acid resistance was associated with mutational change in \textit{rpoB} and \textit{fusA}, respectively. All rifampicin-resistant strains were from the same clone ST239-III-t037 whereas strains resistant to fusidic acid were genetically more diverse.

\textbf{Keywords:} MLST, mutation, rifampicin fusidic acid, spa, \textit{Staphylococcus aureus}

\textbf{Introduction}

Rifampicin, a semisynthetic derivative of rifamycin, is an important antibiotic used in combination therapy for treatment of deep-seated staphylococcal infections and tuberculosis.\textsuperscript{1} Rifampicin resistance is often associated with mutations in the \textit{\beta}-subunit of RNA polymerase encoded by the gene \textit{rpoB}. This mutation is related to amino acid changes found in two particular regions of \textit{rpoB}, namely cluster I (amino acids 462–488) and cluster II (amino acids 515–530).\textsuperscript{2} Fusidic acid is often used topically to treat staphylococcal infection.\textsuperscript{3} The drug binds to elongation factor G (EF-G) on the ribosome and inhibits protein synthesis.\textsuperscript{4} Resistance to fusidic acid is often associated with mutations in the gene encoding EF-G (\textit{fusA}) or RpfF (\textit{fusE}) or acquisition of the gene \textit{fusB}, \textit{fusC} or \textit{fusD}.\textsuperscript{4,5}

In Malaysia, rifampicin is used with fusidic acid as an alternative therapy to vancomycin to combat MRSA infections.\textsuperscript{6} Forest and Tamura\textsuperscript{7} reported that rifampicin, when combined with fusidic acid, daptomycin or linezolid, is effective against MRSA infections. O’Neill et al.\textsuperscript{8} also indicated that a rifampicin and fusidic acid combination is effective against MRSA and vancomycin-intermediate \textit{S. aureus} infections. However, little is known about the mechanism of rifampicin and fusidic acid resistance in \textit{S. aureus} in Malaysia. Rohani et al.\textsuperscript{9} observed rifampicin and fusidic acid resistance rates of 3.3% and 3.8%, respectively, in Malaysia in 1996, using the disk diffusion test, while Norazah et al.\textsuperscript{6} found a 5% rifampicin and fusidic acid resistance rate over the period 1997 to 1999. However, in 2012 the resistance rates of rifampicin and fusidic acid among MRSA isolates in Malaysia had increased to 9.5% and 13.8%, respectively.\textsuperscript{10}
The purpose of this study was to determine the expression of rifampicin and fusidic acid resistance, and the rpoB and fusA gene mutations conferring that resistance, in MRSA strains isolated from a local teaching hospital in Kuala Lumpur. The genetic diversity of these strains resistant to rifampicin and fusidic acid was determined by SCCmec (staphylococcal chromosome cassette mec) typing, spa typing and multilocus-sequence typing.

Materials and methods

Bacterial strains

In this study we used all the available MRSA strains (n=188) resistant to rifampicin or fusidic acid from the culture collection of the Laboratory of Biomedical Science and Molecular Microbiology (LBSMM), Institute of Graduate Studies, University of Malaya.

The MRSA strains (isolated in 2003, 2004, 2007 and 2008) were previously recovered from inpatients in a tertiary hospital in Klang Valley, Malaysia. The tertiary hospital has departments for orthopaedics, paediatrics, surgery, obstetrics and gynaecology, oncology and radiotherapy, and intensive care units.

Among the 21 MRSA strains with rifampicin or fusidic acid resistance, nine were associated with infections and had been cultured from blood (n=2), pus (n=2), tissue (n=3), graft (n=1) or wound (n=1). The remaining 12 strains were associated with colonisation and had been cultured from nasal swabs (n=3), central venous catheter tip (n=1), nasal secretion (n=1), sputum (n=3), chest tube (n=1) and tracheal secretion (n=3). The strains were isolated during 2003 (n=6), 2007 (n=1) and 2008 (n=14).

Minimum inhibitory concentration determination

Minimum inhibitory concentration (MIC) for rifampicin was determined using the agar microdilution method described by the Clinical Laboratory and Standards Institute (CLSI; Wayne, PA, USA). MIC for fusidic acid was confirmed by Etest (AB Biodisk, Solna, Sweden). An isolate is considered susceptible to rifampicin if the MIC is ≤1 μg/ml and susceptible to fusidic acid if the MIC is ≤1 μg/ml.

PCR detection of rifampicin and fusidic acid resistance genes

PCR amplifications of rpoB, fusA to fusE were performed using primers as previously described. Repeated PCR tests for fusA using the published primers failed to achieve any amplification. Hence, new primers were designed in this study. Briefly, the fusA sequence from S. aureus (GenBank accession no. NC_003923.1) was retrieved from GenBank. A pair of primers [5′-CGGTATCATGGCTCACATTG-3′ and 5′-GTACCAGCACCTTGAGTT-3′] were designed based on the sequence, using the Primer 3 program (http://frodo.wi.mit.edu/primer3). The specificity of the primers was evaluated using the In silico PCR program (http://insilico.ehu.eus/PCR/) and a 1962 bp product was observed for S. aureus strains.

Genomic DNA from S. aureus was used as the DNA template. The PCR conditions used for detection of genes fusA to fusE were as follows: initial denaturation (94°C for 5 min) followed by 30 cycles of denaturation (94°C for 1 min), extension (72°C for 1 min) and final extension (72°C for 5 min); the parameters used for detection of rpoB was as described by Mick et al. PCR amplicons were purified using a Qiagen DNA purification kit (Qiagen GmBH, Hilden, Germany) and sequenced to validate the product. Nucleotide sequences of rpoB and fusA obtained were compared to the rpoB wild-type sequence from the S. aureus genome (GenBank accession no. X64172) and the fusA sequence from S. aureus MW2 (GenBank accession no. NC_003923.1), using Mega 4 software (Biodesign Institute, Tempe, AZ, USA).

Results

Bacterial strains

We previously reported the rifampicin and fusidic acid resistance rates for 18 MRSA strains, based on the disk diffusion test. We extended the study to determine the MIC for rifampicin and fusidic acid with the aim of correlating these values to the mutational change in the respective genes.

Except for the strain MRSA0307-23, which was isolated from a patient who had been treated with fusidic acid for 4 days, all the other strains were recovered from patients with no history of rifampicin and fusidic acid usage.

Minimal inhibitory concentrations of rifampicin and fusidic acid

The MICs for rifampicin and fusidic acid ranged from <1 to 8 μg/ml and from <1 to 256 μg/ml, respectively. Five MRSA strains (24%) had co-resistance to both rifampicin and fusidic acid (Tables 1 and 2).

Characterisation of rifampicin and fusidic acid resistance determinants

Six rifampicin-resistant strains with MIC 8.0 μg/ml had the mutational change 484Arg/His in cluster 1 of rpoB. Among them, five strains had an additional amino acid substitution change 517Glu/Gln in cluster II that had not been reported previously. Amino acid alteration of 477Ala/Asp was observed in an MRSA strain with MIC 4 μg/ml, whereas a mutational change 481His/Asn was observed in another isolate with MIC 2 μg/ml. Silent mutation Ala (GGC→GCT) at 325′ was present in all the rifampicin-resistant strains while silent mutation Gly (GGT→GGA) at 462′ was exhibited by isolate MRSA0308-23.

In this study, none of the fusidic acid-resistant strains harboured fusB, fusC or fusD genes and showed no mutation in fusE. Amplification of fusA was successful for all 18 fusidic acid-resistant strains.
<table>
<thead>
<tr>
<th>Strain (MRSA0)</th>
<th>Ward</th>
<th>Year</th>
<th>Specimen</th>
<th>Rifampicin resistance</th>
<th>Fusidic acid resistance</th>
<th>MLST type</th>
<th>spa type</th>
<th>SCCmec type</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>308-23 ICU</td>
<td>2003</td>
<td>Blood</td>
<td>8</td>
<td>CGT-CAT GAA-CAA</td>
<td>484Arg→His 517Glu→Gln</td>
<td>256 TTA-AAA 461Leu→Lys</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
</tr>
<tr>
<td>310-26 ICU</td>
<td>2003</td>
<td>Nasal swab</td>
<td>8</td>
<td>CGT-CAT GAA-CAA</td>
<td>484Arg→His 517Glu→Gln</td>
<td>256 TTA-AAA 461Leu→Lys</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
</tr>
<tr>
<td>809-32 Other</td>
<td>2008</td>
<td>Pus</td>
<td>8</td>
<td>CGT-CAT GAA-CAA</td>
<td>484Arg→His 517Glu→Gln</td>
<td>256 TTA-AAA 461Leu→Lys</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
</tr>
<tr>
<td>811-25 Surg</td>
<td>2008</td>
<td>Tip</td>
<td>8</td>
<td>CGT-CAT GAA-CAA</td>
<td>484Arg→His 517Glu→Gln</td>
<td>256 TTA-AAA 461Leu→Lys</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
</tr>
<tr>
<td>812-33 ICU</td>
<td>2008</td>
<td>Nasal swab</td>
<td>8</td>
<td>CGT-CAT GAA-CAA</td>
<td>484Arg→His 517Glu→Gln</td>
<td>256 TTA-AAA TGT-TGG GAA-AAA ATG-TGG</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
</tr>
<tr>
<td>705-13 Med</td>
<td>2008</td>
<td>Blood</td>
<td>8</td>
<td>CGT-CAT GAA-CAA</td>
<td>484Arg→His 517Glu→Gln</td>
<td>&lt;1 -a</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
</tr>
<tr>
<td>308-10 Ortho</td>
<td>2003</td>
<td>Tissue</td>
<td>4</td>
<td>GCT-GAT</td>
<td>477Ala→Asp 517Glu→Gln</td>
<td>&lt;1 -a</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
</tr>
<tr>
<td>809-1 Surg</td>
<td>2008</td>
<td>Graft</td>
<td>2</td>
<td>CAT-AAT</td>
<td>481His→Asn</td>
<td>&lt;1 -a</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
</tr>
</tbody>
</table>

No mutations observed.

ICU: intensive care unit; med: medical; MIC: minimum inhibitory concentration (µg/ml); MLST: multilocus sequence typing; ortho: orthopaedic; surg: surgical.
Table 2. Characteristics of 13 of 21 rifampicin- and fusidic acid-resistant strains of methicillin-resistant Staphylococcus aureus (MRSA) isolated in a tertiary hospital in Malaysia

<table>
<thead>
<tr>
<th>Strain (MRSA0)</th>
<th>Ward</th>
<th>Year</th>
<th>Specimen</th>
<th>Rifampicin resistancea MIC</th>
<th>Fusidic acid resistance</th>
<th>MLST type</th>
<th>spa type</th>
<th>SCCmec type</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>805-15</td>
<td>Ortho</td>
<td>2008</td>
<td>Tissue</td>
<td>&lt;1</td>
<td>256 TTA-AAA 461Leu→Lys</td>
<td>ST241</td>
<td>t363</td>
<td>III</td>
<td>JN597296</td>
</tr>
<tr>
<td>812-30</td>
<td>Medical</td>
<td>2008</td>
<td>Tracheal secretion</td>
<td>&lt;1</td>
<td>256 TTA-AAA 461Leu→Lys</td>
<td>ST239</td>
<td>t2029</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>810-6</td>
<td>Medical</td>
<td>2008</td>
<td>Sputum</td>
<td>&lt;1</td>
<td>96 GCA-ACA 67Ala→Thr</td>
<td>ST22</td>
<td>t1378</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>810-10</td>
<td>Other</td>
<td>2008</td>
<td>Tracheal secretion</td>
<td>&lt;1</td>
<td>96 GCA-ACA 67Ala→Thr</td>
<td>ST22</td>
<td>t1378</td>
<td>IV</td>
<td>JN597301</td>
</tr>
<tr>
<td>307-23</td>
<td>Ortho</td>
<td>2003</td>
<td>Wound</td>
<td>&lt;1</td>
<td>96 CAC-TAC 457His→Tyr</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
<td>JN597292</td>
</tr>
<tr>
<td>0810-7</td>
<td>Ortho</td>
<td>2008</td>
<td>Pus</td>
<td>&lt;1</td>
<td>64 GCA-ACA 67Ala→Thr</td>
<td>ST239</td>
<td>t421</td>
<td>III</td>
<td>JN597300</td>
</tr>
<tr>
<td>807-8</td>
<td>Surgical</td>
<td>2008</td>
<td>Swab</td>
<td>&lt;1</td>
<td>64 CAC-TAC 457His→Tyr</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
<td>JN597298</td>
</tr>
<tr>
<td>810-17</td>
<td>Medical</td>
<td>2008</td>
<td>Sputum</td>
<td>&lt;1</td>
<td>64 GCA-ACA 67Ala→Thr</td>
<td>ST22</td>
<td>t1378</td>
<td>IV</td>
<td>JN597302</td>
</tr>
<tr>
<td>805-17</td>
<td>Paed</td>
<td>2008</td>
<td>NS</td>
<td>&lt;1</td>
<td>16 CAC-TAC 457His→Tyr</td>
<td>ST1178</td>
<td>t1107</td>
<td>IV</td>
<td></td>
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<tr>
<td>805-17</td>
<td>Medical</td>
<td>2008</td>
<td>Sputum</td>
<td>&lt;1</td>
<td>6 TTA-TCA 461Leu→Ser</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
<td>JN597297</td>
</tr>
<tr>
<td>801-26</td>
<td>Ortho</td>
<td>2008</td>
<td>Tracheal secretion</td>
<td>&lt;1</td>
<td>6 TTA-TCA 461Leu→Ser</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
<td>JN597295</td>
</tr>
<tr>
<td>312-35</td>
<td>Surgical</td>
<td>2003</td>
<td>Chest tube</td>
<td>&lt;1</td>
<td>6 CCA-CTA 404Pro→Leu</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>302-4</td>
<td>Ortho</td>
<td>2003</td>
<td>Tissue</td>
<td>&lt;1</td>
<td>6 CCA-CTA 404Pro→Leu</td>
<td>ST239</td>
<td>t037</td>
<td>III</td>
<td></td>
</tr>
</tbody>
</table>

a No mutations observed.

MIC: minimum inhibitory concentration (µg/ml); MLST: multilocus sequence typing; NS: nasopharyngeal secretion; ortho: orthopaedic; paed: paediatric; SCCmec: staphylococcal chromosome cassette mec
by using the pair of primers designed in this study. Analysis of the DNA sequence in the fusA amplicon revealed several mutations in 15 strains. The amino acid alteration 461Leu/Lys was common among the resistant strains (n = 7, 31.3%) with a high level of fusidic acid resistance (MIC 256 μg/ml), whereas amino acid alteration 461Leu/Ser was associated only with MIC 6 μg/ml (Tables 1 and 2). In contrast, a strain (MRSA0812-33) that had multiple mutations, including three novel substitutions (317Met/Trp, 596Cys/Trp and 602Glu/Lys) exhibited high levels of fusidic acid resistance (MIC 256 μg/ml). However, the resistance mechanism caused by these mutational changes in fusA is unknown and needs further investigation.

Silent mutation Val (GTC→GTG) at 553 was present in 12 fusidic acid-resistant strains whereas silent mutation Leu (TTA→TTG) at 325 was present in two fusidic acid-resistant strains (MRSA0810-7 and MRSA0810-17). Two silent mutations, Pro (CCG→CCA) at 342 and Ala (GCC→GCG) at 325 were exhibited by strain MRSA0805-1. Five MRSA strains in this study exhibited high resistance to both rifampicin (MIC 8 μg/ml) and fusidic acid (256 μg/ml). All five strains with both rifampicin and fusidic acid resistance have multiple mutations in rpoB (484Arg/His and 517Glu/Gln) and the mutational change 461Leu/Lys in fusA.

Genetic relatedness of rifampicin and fusidic acid-resistant strains

SCCmec, spa and MLST typing of 21 MRSA strains gave two SCCmec types [SCCmec type III (n = 17) and SCCmec type IV (n = 4)], six distinct spa types [t037 (n = 14), t363 (n = 1), t421 (n = 1), t1107 (n = 1), t1378 (n = 3) and t2029 (n = 1)] and four different ST types [ST239 (n = 16), ST22 (n = 3), ST241 (n = 1) and ST1178 (n = 1)].

Discussion

All 21 MRSA strains studied were multidrug resistant (resistant to more than two classes of antimicrobial agents). Previously, we found that resistance to linezolid, teicoplanin and vancomycin was low.19

No amplification was observed for fusA using published primers14 despite three repeated PCR tests using the recommended annealing temperatures. In order to rule out the possibility of amplification failure, internal control primers (primer mecA P4 and mecA P7; also used in SCCmec typing) were added in the third repeat (results not shown). Therefore, we postulate that inhibition of PCR amplification could be attributable to mutations occurring in the primers binding sites. Such an observation has been reported previously by Liu et al.20

Isolates with mutational change 484Arg/His and 477Ala/Asp had MICs of 4–8 μg/ml. This is in contrast to the study by Wichelhaus et al.,21 who reported that strains with the similar mutation change 484Arg/His and 477Ala/Asp had a high level of resistance (MIC 256 μg/ml).

Although Wichelhaus et al.21 reported that double mutations in rpoB can cause a high level of resistance to rifampicin, our results showed that the double mutations in rpoB in five MRSA strains resulted in a MIC of 8 μg/ml. This indicates that the additional mutational change of 517Glu/Gln might not be associated with a high level of resistance to rifampicin as mutational change 484Arg/His itself was associated with higher rifampicin resistance.21

No fusB, fusC or fusD gene was detected among fusidic acid resistant strains despite repeated experiments being carried out using three different sets of published primers.3,4,14 Absence of fusB and fusC genes among strains resistant to fusidic acid is uncommon, as previous reports in Taiwan showed that 73.5% of fusidic acid resistant strains harbour fusC22 and more than 10% of fusidic acid resistant strains in 13 European countries harboured either fusB or fusC.23 Unfortunately, no positive control was available for the detection of fusB, fusC and fusD resistance genes, so negative results should be treated with caution.

Although fusE was present in all the strains, no mutation was detected. The absence of fusE-class mutation is not surprising as its occurrence is rare.5,23

The mutational change 461Leu/Lys, which was associated with a high level of fusidic acid resistance (MIC 256 μg/ml),5,24 was observed in six MRSA strains. Similarly, the non-synonymous change 461Leu/Ser associated with low-level fusidic acid resistance (4–8 μg/ml)5 was observed in only two MRSA strains (MIC 6 μg/ml).

Six other strains with MIC 64–96 μg/ml showed amino acid alteration at 67Ala/Thr (n = 1), 457His/Tyr (n = 2) and a combination of 67Ala/Thr and 457His/Tyr (n = 3). This differed from the report by Besier et al.25 that indicated that mutation change 67Ala/Thr did not contribute to fusidic acid resistance, and is unable to compensate for the fitness loss because of resistance-mediating amino acid. Interestingly, no mutation was observed for fusA gene in three strains with a MIC of 6 μg/ml (n = 2) or 16 μg/ml (n = 1).

Combined analysis of MLST-SCCmec-spa types gave six different combined types with ST239-III-t037 being predominant (n = 14, 67%). Clone, ST239-III-t037 was detected among strains cultured from 2003 (n = 6), 2007 (n = 1) and 2008 (n = 7). This clone was also reported in other tertiary hospitals in Malaysia.26

The remaining seven 2008 strains were grouped in five different combined MLST-SCCmec-spa types, including ST22-IV-11378 (n = 3), ST239-III-t421 (n = 1), ST1178-IV-t1107 (n = 1), ST241-III-t363 (n = 1) and ST239-III-t2029 (n = 1). Both ST239-III-t421 and ST239-III-t2029 might have derived from clone ST239-III-t037 as they shared five spa-type repeats succession (15-12-02-25-17, kreiswirth ID:WGAOM). De novo generation of mutants conferring resistance could also be possible as clone ST239-III-t2029 was found to have similar fusidic acid mutational change (461Leu/Lys) to that in clone ST239-III-t037 reported in 2003.

Five strains (four from 2003 and one from 2008) that were resistant to both rifampicin and fusidic acid originated from the same clone ST239-III-t037 and shared an identical amino acid alteration at 484 (Arg/His) in rpoB and 461 (Leu/Lys) in fusA. This indicates persistence of this clone from 2003 to 2008.

The presence of ST239-III-t037 MRSA strains with rifampicin and fusidic acid resistance could signal the potential loss of efficacy of these drugs in treating MRSA infections. This is a cause for concern, because the combination of rifampicin and fusidic acid is used for treatment of complicated S. aureus infections.7,27 The 2012 National Surveillance Antibiotic Resistance report from the Malaysian Ministry of Health indicated that the current rifampicin resistance rates among Malaysian methicillin-sensitive S. aureus (MSSA) and MRSA were 0.8% and 9.5%, respectively.10 Meanwhile, the current fusidic acid resistance rate among
Malaysian MSSA and MRSA in 2012 were 12.7% and 14.8%, respectively. The rates of resistance to both rifampicin and fusidic acid were found to be higher in 2012 than the 5% reported by Norazah et al.\textsuperscript{6} from 1997 to 1999.

Limitations of this study were the low number of MRSA strains with rifampicin and fusidic acid resistance in the different study periods (2003, 2007 and 2008) and the fact that no MRSA strains from 2004, 2005 and 2006 were available for analysis.

In conclusion, different amino acid alterations were responsible for rifampicin and fusidic acid resistance among Malaysian MRSA strains. Presence of rifampicin and fusidic acid resistance in MRSA strains signals the potential loss of the use of these drugs against MRSA. Therefore, good infection control procedures are essential in order to monitor and control the prevalence of MRSA strains with rifampicin and fusidic acid resistance.

Authors' contributions: KTL and KLT designed the study protocol; KTL carried out the experiments; KTL and CSJT analysed the data. KTL drafted the manuscript; CSTJ, MYMY and KLT critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. KTL is guarantor of the paper.

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