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Novel mutations in penicillin-binding protein genes in clinical Staphylococcus aureus isolates that are methicillin resistant on susceptibility testing, but lack the mec gene

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Objectives: Methicillin-resistant Staphylococcus aureus (MRSA) is an important global health problem. MRSA resistance to β-lactam antibiotics is mediated by the mecA or mecC genes, which encode an alternative penicillin-binding protein (PBP) 2a that has a low affinity to β-lactam antibiotics. Detection of mec genes or PBP2a is regarded as the gold standard for the diagnosis of MRSA. We identified four MRSA isolates that lacked mecA or mecC genes, but were still phenotypically resistant to penicilinase-resistant β-lactam antibiotics.

Methods: The four human S. aureus isolates were investigated by whole genome sequencing and a range of phenotypic assays.

Results: We identified a number of amino acid substitutions present in the endogenous PBPs 1, 2 and 3 that were found in the resistant isolates but were absent in closely related susceptible isolates and which may be the basis of resistance. Of particular interest are three identical amino acid substitutions in PBPs 1, 2 and 3, occurring independently in isolates from at least two separate multilocus sequence types. Two different non-conservative substitutions were also present in the same amino acid of PBP1 in two isolates from two different sequence types.

Conclusions: This work suggests that phenotypically resistant MRSA could be misdiagnosed using molecular methods alone and provides evidence of alternative mechanisms for β-lactam resistance in MRSA that may need to be considered by diagnostic laboratories.

Keywords: β-lactams, MRSA, mecA, mecC

Introduction

β-Lactam antibiotics work by acylation of the transpeptidase domain active site of penicillin-binding proteins (PBPs), blocking access to their substrate and preventing cross-linking of peptidoglycan strands during cell wall synthesis. Initially, resistance to penicillin in Staphylococcus aureus was mediated by the expression of a β-lactamase enzyme, which breaks down penicillin. Since the introduction of methicillin, a semi-synthetic β-lactamase-resistant penicillin, in 1961, methicillin-resistant S. aureus (MRSA) has emerged that is resistant to the majority of β-lactam antibiotics. Resistance to β-lactams in MRSA is mediated by the acquisition of the mecA gene, which encodes an alternative PBP2a, which has low affinity for β-lactam antibiotics and enables the bacteria to assemble the cell wall in the presence of the drug. More recently, a divergent form of the mecA gene, known as mecC (previously mecA_LGA251), was identified in isolates of S. aureus from both animals and humans. S. aureus isolates with other types of resistance to β-lactams have been described and are known as borderline oxacillin-resistant S. aureus (BORSA) or intrinsically resistant S. aureus. Resistance in some BORSA isolates is attributed to the presence or overexpression of β-lactamase enzymes, while in intrinsically resistant and some BORSA isolates it is mediated by chromosomal mutations. Furthermore, in vitro studies generating β-lactam-resistant isolates under the selection of β-lactam antibiotics have also identified a number of other genes able to mediate intrinsic β-lactam resistance. Here, we report four clinical MRSA isolates from three multilocus...
Results

We identified four mecA-negative isolates (XB84, 85, 86 and 87) from the Scottish MRSA Reference Laboratory that exhibited resistance to penicillinase-resistant β-lactam antibiotics, and were misclassified as methicillin susceptible based on detection of mecA and mecC genes.

### Methods

#### Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using disc susceptibility testing and Etest as previously described. 

#### Genetic analysis

Genomic DNA was extracted from overnight cultures grown in TSB at 37°C. A list of the isolates used in this study is shown in Table 1.

#### Whole genome sequencing

Four isolates underwent whole genome sequencing. This confirmed that none of the isolates harboured a mecA gene and that XB84, 85 and 86 were resistant to penicillinase-resistant β-lactam antibiotics, but lack both mecA and mecC, that are responsible for β-lactam resistance. This demonstrates that mecC-negative MRSA isolates with resistance to multiple β-lactam antibiotics, but lack both mecA and mecC, may be misclassified as methicillin susceptible based on detection of mecA or mecC genes.

#### Results

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Country</th>
<th>Origin</th>
<th>Isolated from</th>
<th>Year of isolation</th>
<th>MLST</th>
<th>spa type</th>
<th>OXA (mm)</th>
<th>FOX Etest (mg/L)</th>
<th>FOX Etest (mg/L)</th>
<th>PEN G (mm)</th>
<th>CRO Etest (mg/L)</th>
<th>β-Lactamase</th>
<th>blaZ gene</th>
<th>blaZ type</th>
</tr>
</thead>
<tbody>
<tr>
<td>XB84</td>
<td>Scotland</td>
<td>human</td>
<td>wound</td>
<td>2010</td>
<td>15</td>
<td>t048</td>
<td>11</td>
<td>2</td>
<td>12</td>
<td>9</td>
<td>2</td>
<td>&gt;32</td>
<td>+</td>
<td>C</td>
</tr>
<tr>
<td>XB85</td>
<td>Scotland</td>
<td>human</td>
<td>wound</td>
<td>2010</td>
<td>1</td>
<td>t127</td>
<td>0</td>
<td>18</td>
<td>4</td>
<td>12</td>
<td>1</td>
<td>&gt;32</td>
<td>+</td>
<td>C</td>
</tr>
<tr>
<td>XB86</td>
<td>Scotland</td>
<td>human</td>
<td>wound</td>
<td>2010</td>
<td>15</td>
<td>t067</td>
<td>0</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>1.5</td>
<td>&gt;32</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>XB87</td>
<td>Scotland</td>
<td>human</td>
<td>wound</td>
<td>2010</td>
<td>8</td>
<td>t008</td>
<td>0</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>1</td>
<td>&gt;32</td>
<td>+</td>
<td>A</td>
</tr>
</tbody>
</table>

MLST, multilocus ST; OXA, oxacillin; FOX, cefoxitin; PEN G, penicillin G; CRO, ceftriaxone; CLA, clavulanic acid.

For oxacillin: Etest MIC breakpoint is >2 mg/L; disc diffusion—susceptible ≤15 mm diameter, resistant ≥14 mm diameter.

For cefoxitin: Etest MIC breakpoint is >4 mg/L; disc diffusion—susceptible ≥22 mm diameter, resistant <21 mm diameter.

For penicillin G: Etest MIC breakpoint is >0.12 mg/L; disc diffusion—susceptible ≥25 mm diameter, resistant ≤24 mm diameter.

### Table 1. Relevant phenotypic and genotypic characteristics of resistant isolates
antibiotic: clavulanic acid. No major reduction was seen in the zones of inhibition of oxacillin, cefoxitin or penicillin in combination with clavulanic acid at either 2:1 or 1:1, suggesting that β-lactamase production was not mediating resistance (Table 1).

In addition to PBP2a and β-lactamase, five other proteins (PBP2, PBP4, GdpP, YjbH and AcrB) have been reported previously to be associated with β-lactam resistance in S. aureus. The transpeptidase domains of PBP2, PBP4, GdpP, YjbH and AcrB have been shown previously to reduce the acylation efficacy of PBP2β-lactams and thus provide some degree of resistance. Given the small number of isolates in this study and that our targeted search for mutations might have excluded other genes involved in resistance, further experimental work is necessary to characterize the contribution of the novel PBP substitutions to β-lactam resistance. Mutations in PBP1 and PBP3 have not been implicated previously in S. aureus resistance; however, in Staphylococcus lugdunensis, a tetrapeptide duplication in the transpeptidase domain of PBP1 has been shown to be associated with increased β-lactam resistance. Currently, mec gene-negative MRSA isolates are not widely reported, but it is important to characterize the basis for resistance in these isolates, especially for clinical laboratories that rely on the molecular detection of mecA/C and/or PBP2a as the gold standard for MRSA detection. Furthermore, PBP2a has been highlighted as an attractive target for drug development and should PBP2a-targeted inhibitors become available, it is important to understand alternative mechanisms for S. aureus to develop resistance to β-lactams. Finally, it is clear that there are multiple distinct mechanisms for β-lactam resistance in S. aureus and these need to be taken into consideration by diagnostic laboratories.

### Table 2. Locations of amino acid substitutions identified in methicillin-resistant isolates that were absent in susceptible isolates of the same ST

<table>
<thead>
<tr>
<th>Protein</th>
<th>PBP1</th>
<th>PBP2</th>
<th>PBP3</th>
<th>YjbH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td>MLST</td>
<td>Y336C</td>
<td>Y336N</td>
<td>T371I</td>
</tr>
<tr>
<td>XB84</td>
<td>15</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>XB85</td>
<td>1</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>XB86</td>
<td>15</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>XB87</td>
<td>8</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

MLST, multilocus ST.

### Discussion

In this work we have identified MRSA isolates belonging to three STs, which unlike BORSA strains are resistant to both oxacillin and cefoxitin. This resistance does not appear to be mediated by hyperproduction of a β-lactamase and we have identified a number of novel substitutions in the transpeptidase domains of PBPs 1, 2 and 3 that we hypothesize could mediate this resistance. The transpeptidase domains of PBPs are the target of β-lactam antibiotics and substitutions in the transpeptidase domain of PBP2 have been shown previously to reduce the acylation efficacy of PBP2β-lactams and thus provide some degree of resistance. Given the small number of isolates in this study and that our targeted search for mutations might have excluded other genes involved in resistance, further experimental work is necessary to characterize the contribution of the novel PBP substitutions to β-lactam resistance. Mutations in PBP1 and PBP3 have not been implicated previously in S. aureus resistance; however, in Staphylococcus lugdunensis, a tetrapeptide duplication in the transpeptidase domain of PBP1 has been shown to be associated with increased β-lactam resistance. Currently, mec gene-negative MRSA isolates are not widely reported, but it is important to characterize the basis for resistance in these isolates, especially for clinical laboratories that rely on the molecular detection of mecA/C and/or PBP2a as the gold standard for MRSA detection. Furthermore, PBP2a has been highlighted as an attractive target for drug development and should PBP2a-targeted inhibitors become available, it is important to understand alternative mechanisms for S. aureus to develop resistance to β-lactams. Finally, it is clear that there are multiple distinct mechanisms for β-lactam resistance in S. aureus and these need to be taken into consideration by diagnostic laboratories.

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mec gene-negative MRSA

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Transparency declarations
None to declare.

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