Missense mutations of PBP2a are associated with reduced susceptibility to ceftaroline and ceftobiprole in African MRSA

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Introduction

Ceftaroline and ceftobiprole are recently introduced cephalosporins, which are active against MRSA by inhibiting PBP2a. Recently, high rates of resistance to ceftaroline were reported from Ghana. The objective of this study was to assess rates of resistance to ceftaroline and ceftobiprole in MRSA from Africa and to describe potential missense mutations of PBP2a.

Methods: MRSA isolates derived from Staphylococcus aureus colonization (n = 37) and infection (n = 23) and were collected in Côte d’Ivoire (n = 17), DR Congo (n = 6), Gabon (n = 21) and Nigeria (n = 16). The MICs were determined by the broth microdilution method. The mecA gene was sequenced and missense mutations were associated with the corresponding MLST ST.

Results: In total, 16.7% (n = 10) and 15% (n = 9) of isolates were resistant to ceftaroline and ceftobiprole, respectively. The corresponding MICs of ceftaroline and ceftobiprole correlated significantly (r = 0.92). Isolates belonging to ST241 harboured a triple mutation of PBP2a (N146K–N204K–G246E), which was associated with high rates of resistance to ceftaroline (90.9%) and ceftobiprole (81.8%).

Conclusions: Resistance to ceftaroline and ceftobiprole were only detected in Nigeria and were associated with ST241 and a triple mutation of PBP2a.

Materials and methods

Bacterial isolates

The MRSA isolates (n = 60) derived from studies on S. aureus colonization (n = 37) and infection (n = 23) and were collected in Côte d’Ivoire (n = 17, in 2012), DR Congo (n = 6, in 2011–13), Gabon (n = 21, in 2009–13) and Nigeria (n = 16, in 2007–12).9 All isolates were spa and SCCmec typed, and MLST was performed particularly for one isolate per spa type per study.5,6 The lukS-PV/lukF-PV gene encoding the Panton–Valentine leucocidin was detected by PCR.8

Antimicrobial susceptibility testing

The MICs of ceftaroline and ceftobiprole as well as nine other antibiotics (cefotaxin, oxacillin, vancomycin, teicoplanin, daptomycin, linezolid, fosfomycin, tigecycline and rifampicin) were determined by the
broth microdilution method using customized 96-well plates (Merlin, Bornheim-Hersel, Germany) in accordance with the ISO 20776-1 guidance. MIC values were interpreted applying available EUCAST breakpoints (ceftaroline, susceptible ≤1 mg/L and resistant >1 mg/L; and ceftobiprole, susceptible ≤2 mg/L and resistant >2 mg/L; Version 5.0). Additionally, MICs of ceftaroline, ceftobiprole and cefoxitin were determined by the gradient diffusion method (Etest, bioMérieux, Marcy-Étoile, France) according to the manufacturer's instructions. All tests were performed in triplicate and the median MIC value was used for analysis. S. aureus ATCC 29213 served as a quality control strain. MICs for the quality control strain were within acceptable limits throughout testing.

**mecA sequencing**

Genomic DNA was extracted with QiAamp according to the manufacturer's instructions (Qiagen, Hilden, Germany). The mecA gene was amplified using a previously published forward primer (MecA_Seq R 5′-ACCTTTCACACCTCATTACAC-3′). The amplicon (2190 bp) was sequenced using several nested primers: Mec_180_R (5′-ACTGGCAAGATGTTGCG-3′), MEC2 (5′-CCACCGAC TGGTCTGGCCAGT-3′), Mec_1217_R (5′-TTTGATGGAACCTGTGA-3′), Mec_1697_R (5′-ACTACGGTAACATTGATCGCA-3′), Mec_721_F (5′-GAGGTCGTGATTGTTGCA-3′) and Mec_180_F (5′-CCACCGAGTGGTCTGGCAGT-3′). Sequences were aligned with MUSCLE as implemented in Mega 6 (www.megasoftware.net) using the mecA reference sequences of N315 (SA0038) and strain 4977 (JQ58212), which displays four missense mutations of mecA (N146K, E150K, N204K, G246E). The GenBank accession numbers of the detected mutant types are KR936059–KR936061.

**Statistical analysis**

MIC50 and MIC90 were calculated as the 50th and 90th percentiles of the MIC values. The correlation between MICs of ceftaroline and ceftobiprole was tested with Pearson’s correlation coefficient. MICs from Etest were rounded to the next doubling dilution step for comparison of the MICs between Etest and microdilution. The significance level was set at 0.05. All analyses were performed with 'R' (https://cran.r-project.org, Version 2.13.1).

**Results**

In total, 16.7% (n=10) and 15% (n=9) of isolates were resistant to ceftaroline and ceftobiprole, respectively, according to the broth microdilution method. The correspondent MICs of ceftaroline and ceftobiprole correlated significantly (r=0.92 (95% CI 0.87–0.95), P<0.001; Figure 1).

The proportion of resistant isolates as determined by Etest was 10% (n=6) and 15% (n=9) for ceftaroline and ceftobiprole, respectively. There was a trend for lower MICs by Etest compared with microdilution, which resulted in four very major errors and a 93.3% categorical agreement (Table S1, available as Supplementary data at JAC Online) for ceftaroline. In contrast, the categorical agreement between Etest and microdilution was 100% for ceftobiprole.

In total, 28 isolates had a WT PBP2a amino acid sequence and belonged to ST5, ST8 and ST88. These isolates were predominantly found in Côte d’Ivoire and Gabon (Table 1). All were susceptible to ceftaroline and ceftobiprole.

Isolates displaying the combination of three missense mutations of PBP2a (N146K –N204K –G246E) were solely detected in Panton–Valentine leucocidin-positive isolates.

**Table 1.** PBP2a missense mutations, genotypes and susceptibility to ceftobiprole and ceftaroline of African MRSA isolates

<table>
<thead>
<tr>
<th>Mutation (no. of isolates)</th>
<th>ST (spa types)</th>
<th>Country (no. of isolates)</th>
<th>Ceftaroline (mg/L)</th>
<th>Ceftobiprole (mg/L)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MIC50</td>
<td>MIC90</td>
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<tr>
<td>WT (28)</td>
<td>ST5 (t653), ST8 (t112, t121, t451, t12858), ST88 (t186, t786), ST2947 (t186)</td>
<td>Côte d’Ivoire (17), Gabon (10), Nigeria (1)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>N146K–N204K–G246E (11)</td>
<td>ST15 (t084), ST7241 (t037, t1152)</td>
<td>Nigeria (11)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>S225R (11)</td>
<td>ST6 (t1476), ST8 (t064, t1476), ST45 (t437, t8860)</td>
<td>DR Congo (5), Gabon (3), Nigeria (3)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>G246E (10)</td>
<td>ST15 (t084), ST88 (t186, t786, t1603, t2253, t3202 t195), ST241 (t037, t7152)</td>
<td>DR Congo (5), Gabon (4), Nigeria (3)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
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</table>

Panton–Valentine leucocidin-positive isolates are in bold.
Nigeria between 2007 and 2013 and belonged to ST15 (n = 1) and ST241 (n = 10), which both cluster in the MLST clonal complex, CC5. Resistance rates were high in this group for ceftaroline (90.9%, n = 10) and ceftobiprole (81.8%, n = 9). Noteworthy, all but one isolates (ST241) were resistant to ceftaroline and ceftobiprole. Triple mutants also had markedly higher MICs of cefoxitin compared with isolates carrying the WT mecA or single mutations (Table S2). This was confirmed by a gradient diffusion method, which covers a broader concentration range (MIC\textsubscript{50} >256 mg/L versus MIC\textsubscript{50} 96 mg/L, respectively).

Isolates that harboured either the S225R (n = 11) or G246E (n = 10) missense mutation were fully susceptible to ceftaroline and ceftobiprole.

All isolates were susceptible to vancomycin (MIC\textsubscript{50} 0.75 mg/L, MIC\textsubscript{90} 0.75 mg/L), teicoplanin (MIC\textsubscript{50} 0.5 mg/L, MIC\textsubscript{90} 1 mg/L), daptomycin (MIC\textsubscript{50} 0.5 mg/L, MIC\textsubscript{90} 0.5 mg/L), linezolid (MIC\textsubscript{50} 2 mg/L, MIC\textsubscript{90} 2 mg/L) and fosfomycin (MIC\textsubscript{50} ≤1 mg/L and MIC\textsubscript{90} 4 mg/L; Table S2). Three isolates (5%) were resistant to rifampicin.

Discussion

We analysed resistance rates and the MICs for African MRSA isolates of new cephalosporins ceftaroline and ceftobiprole. The main findings are high rates of resistance to both agents in a clone belonging to ST241, which were associated with a combination of three missense mutations of PBP2a (N146K – N204K – G246E).

Overall, the ceftaroline resistance rate in our MRSA collection from four African countries (Côte d’Ivoire, DR Congo, Gabon, Nigeria) is similar to the resistance rates reported from Ghana based on broth microdilution (20%), but was markedly higher compared with large MRSA collections from Europe (0.05%–4.5%).

Similarly, ceftobiprole resistance was also higher in our collection (15%) compared with a large study from Europe, Turkey and Israel (1.7%).

In contrast to our findings, ceftaroline resistance was not associated with ceftobiprole resistance in the report from Ghana. It is possible that the isolates in our study have different PBP2a mutations compared with Ghanaian MRSA. One might also speculate, that ST241 MRSA harbour mutations in additional genes, which might mediate resistance to ceftobiprole (i.e. pbp4, gdpP). Our data demonstrate an underestimation of ceftaroline MIC and high rate of very major errors using the Etest method. Similarly, Strommenger et al. have also reported generally lower Etest MICs compared with broth microdilution results.

We detected four different missense mutations; three of them have been reported from other countries: N146K (Germany, Greece, Spain, Switzerland, Thailand), N204 (Germany, Thailand) and G246E (Germany, Thailand, Switzerland). To the best of our knowledge, the S225R missense mutation has not been reported yet, but seems to be present in West and Central Africa (Table 1). However, this mutation does not interfere with ceftaroline or ceftobiprole binding to PBP2a, as all isolates carrying this mutation were susceptible to these agents.

In contrast, the combined mutation N146K–N204K–G246E was associated with high rates of resistance to ceftaroline (90.9%) and ceftobiprole (81.8%). However, as not all isolates with this triple mutation were resistant, one might argue that additional mutations in other genes are necessary to mediate full resistance.

The N146K mutation is located in the non-penicillin binding domain of PBP2a and confers resistance to ceftaroline through an alteration of the salt bridge network at the allosteric site. Binding of ceftaroline to this allosteric site causes a conformational change in the WT, which enhances access of a second molecule to the active site. While N146K is associated with MICs in the range 1–2 mg/L (ceftaroline) and 2–4 mg/L (ceftobiprole), other mutations such as Y446N and E447K are required for high-level resistance to ceftaroline (MIC >32 mg/L).

N146K was always co-detected with G246E, but G246E does not seem to confer resistance to ceftaroline or ceftobiprole as single mutants of G246E had MICs within the susceptibility range (Table 1). In our study, resistance to ceftaroline and ceftobiprole was only found in isolates belonging to ST241 and ST15 (Table 1). To the best of our knowledge, resistance to ‘fifth-generation cephalosporin’ in these clones has not been described outside of Africa yet. In contrast, ceftaroline-resistant MRSA was mostly found in isolates belonging to ST228 and ST239 in Germany, Switzerland, Spain and Thailand. The triple mutation (N146K–N204K–G246E) seems to have a selective advantage towards cefoxitin as the MIC of cefoxitin was markedly higher in triple mutants compared with other isolates without this mutation pattern (Table S2).

A few limitations of this study need to be addressed: First, we were unable to perform SCCmec typing, as only two of 60 isolates were typeable. Second, the sample size was too small to draw a clear epidemiological picture of the distribution of ceftaroline- and ceftobiprole-resistant ST241 MRSA in Africa. It is possible that we will underestimate the resistance rates, as the ST239/ST241 MRSA lineage is widespread in North and West Africa.

In conclusion, resistance to ceftaroline and ceftobiprole in Africa is associated with ST241 and the triple mutation N146K–N204K–G246E.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).
References


