Analysis of *Staphylococcus aureus* clinical isolates with reduced susceptibility to ceftaroline: an epidemiological and structural perspective

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**Objectives:** Ceftaroline, approved in Europe in 2012, has activity against methicillin-resistant *Staphylococcus aureus* (MRSA), with MIC90 values of 1–2 mg/L depending on geographical location. During a global 2010 surveillance programme, conducted prior to the European launch, 4 *S. aureus* isolates, out of 8037 tested, possessing ceftaroline MIC values of >2 mg/L were identified. The objective of this study was to characterize these four isolates to elucidate the mechanism of ceftaroline resistance.

**Methods:** MIC determinations were performed using broth microdilution and whole genome sequencing was performed to enable sequence-based analyses.

**Results:** The only changes in proteins known to be required for full expression of methicillin resistance that correlated with the ceftaroline MIC were in penicillin-binding protein 2a (PBP2a). Isolates with a ceftaroline MIC of 2 mg/L had a Glu239Lys mutation in the non-penicillin-binding domain whereas the four isolates with ceftaroline MIC values of 8 mg/L carried an additional Glu 447Lys mutation in the penicillin-binding domain. The impact of these mutations was analysed using the known X-ray structure of *S. aureus* PBP2a and a model for ceftaroline resistance proposed. Analysis of the core genomes showed that the isolates with reduced susceptibility to ceftaroline were epidemiologically related.

**Conclusions:** Mutations in PBP2a can affect the activity of ceftaroline against MRSA. Although a rare event, based on surveillance studies, it appears a first-step change in the non-penicillin-binding domain together with a second-step in the penicillin-binding domain may result in elevation of the ceftaroline MIC to >2 mg/L.

**Keywords:** resistance, MRSA, penicillin-binding proteins

**Introduction**

The increasing level of resistance to multiple classes of antibacterial agents coupled with the paucity of new agents in clinical development has caused worldwide concern regarding the spread of multidrug-resistant bacterial pathogens causing serious infections that are unable to be adequately treated. Methicillin-resistant *Staphylococcus aureus* (MRSA) still represents a significant cause of healthcare-associated infections and the increased prevalence of community-associated MRSA infections is of particular concern. Ceftaroline, the active metabolite of the prodrug ceftaroline fosamil, is a new cephalosporin that was approved in Europe in 2012 for the treatment of community-acquired pneumonia and complicated skin and soft tissue infections. It was approved in the USA in 2010 for the treatment of acute bacterial skin and skin structure infections and community-acquired bacterial pneumonia. Ceftaroline has excellent *in vitro* activity against *Streptococcus* spp., but the distinguishing feature from the majority of other *β*-lactam antibacterial agents is the activity against MRSA. The anti-MRSA activity of ceftaroline is due to its increased affinity for penicillin-binding protein (PBP) 2a (MecA), the PBP that is acquired through the horizontal acquisition of the staphylococcal cassette chromosome *mec* (SCCmec) in multiple epidemiological lineages of *S. aureus* and confers resistance to other *β*-lactam antibiotics. However, multiple genes have been implicated in the maximal expression of methicillin and *β*-lactam resistance in *S. aureus*. These include the four PBPs (PBP1–4) as well as PBP2a. Further, the absolute level of resistance also is dependent on other chromosomally encoded factors that are involved in cell wall precursor formation and turnover. These include the *fem*-encoded proteins (factors essential for methicillin resistance), including FemA and FemB, involved in the
isolates involved in infection outbreaks and study transmission
develop over time and can be used to accurately link multiple
nucleotide polymorphisms (SNPs) and other minor variations still
eus isolates has been found to be relatively stable. However, single
isms of decreased ceftaroline activity.

S. aureus acquisition and loss of mobile genetic elements in the genomic
eus. Furthermore, the resistant MRSA isolates with ceftaroline MIC values of
2 mg/L along with ceftaroline-susceptible MSSA isolates all were inhibited by
MIC 90 value of 0.25 mg/L. However, there was a geographical bias observed with the MRSA isolates
were classified as intermediate following EUCAST interpretive
criteria. The resistant MRSA isolates with ceftaroline MIC values of
≥ 2 mg/L were also included for comparison and would be classified as intermediate following
CLSI interpretive criteria and resistant following EUCAST interpretive
criteria. The reference S. aureus isolates were obtained from the
ATCC (Manassas, VA, USA), the Network on Antimicrobial Resistance in
Staphylococcus aureus (NARS, Chantilly, VA, USA) and the University of
Chicago (Chicago, IL, USA).

Antimicrobial susceptibility testing

The MIC for each isolate was determined using the broth microdilution
method following the guidelines of CLSI document M07-A9. All com-
pounds were tested in accordance with CLSI recommendations. S. aureus
ATCC 29213 was used as the quality control isolate. Ceftaroline was
obtained from Cerexa, Inc. (Oakland, CA, USA) and all other reference com-
pounds used were obtained from US Pharmacopeial Convention (Rockville,
MD, USA).

Whole genome sequencing and analysis

Genomic DNA purified on the Maxwell 16 platform (Promega, Madison, WI, USA) and quantified using the Qubit Fluorometer (Invitrogen Life
Technologies, Grand Island, NY, USA) was used as input material for library
construction. DNA libraries were prepared using the Nextera library con-
struction protocol (Illumina, San Diego, CA, USA) following the manufac-
turer’s instructions and sequenced on a MiSeq Sequencer (Illumina). For
each isolate, ~2.5 million 150 bp paired-end sequence reads were de
novo assembled and analysed using the CLCBio suite of software tools
(Cambridge, MA, USA). The SCCmec types of the MRSA isolates were deter-
dined by comparison to reference sequences and confirmed by PCR using
type-specific oligonucleotides as previously described. The MLST profiles of the isolates were determined by comparison of the seven allele
sequences against the curated public database available at http://
saureus.mlst.net. The CLCbio assemblies were then annotated using
Prodigal and orthologous groups were clustered using OrthoMCL. The
nucleotide sequence of the shared orthologous genes between all of
the isolates, the core genomes, were used to build the phylogenetic tree using FastTree.
The reference genomes used for this
analysis were from the ST239 strains KJD60082 and T0131, the ST5
strain N315 and eight ST228 isolates from a longitudinal study.

Molecular modelling

The structures of S. aureus PBP2 (PDB:2OLU and 2OLV) and S. aureus PBP2a,
in the apo form (PDB:1MW1) and in complex with ceftobiprole (PDB:4OKI),
were used to support the structural interpretations. The PBP2a mutations
identified in this study were mapped onto the known PBP2a structure

Results and discussion

Ceftaroline surveillance against S. aureus

During the 2010 global surveillance programme, a total of 8037 S.
aureus isolates were acquired and tested for their susceptibility to
ceftaroline. The overall S. aureus MIC50 was determined to be
1 mg/L, although there appear to be different population distrib-
utions between the methicillin-resistant and methicillin-
susceptible isolates, with the MSSA isolates all being inhibited by
≤ 1 mg/L ceftaroline and having an MIC90 value of 0.25 mg/L (Figure 1). The population distribution of MSSA isolates was iden-
tical between the major geographic regions, with both the cef-
taroline MIC50 and MIC90 values being 0.25 mg/L. However, there was a geographical bias observed with the MRSA isolates
where both the MIC50 and MIC90 values for the USA were one
dilution lower than for the other geographic regions (Figure 1). There were four MRSA isolates (0.05%) with a ceftaroline MIC of
> 2 mg/L (Figure 1). Three of these isolates came from a single
medical centre in Thailand, whereas the last isolate was isolated in
Spain. These four isolates, along with ceftaroline-susceptible
MRSA and MSSA as well as MRSA isolates that exhibited ceftaroline
MIC values of 2 mg/L isolated from the same medical centres, were
further characterized (Table 1).

Susceptibility profiles

The antimicrobial susceptibility profiles of the S. aureus isolates from
Thailand and Spain along with S. aureus reference isolates are shown in Table 1. Three MRSA isolates from Thailand
(ARC3828, ARC3829 and ARC3830) and a single isolate from

Materials and methods

Collection of bacterial isolates

The resistant MRSA isolates with ceftaroline MIC values of ≥ 4 mg/L follow-
ing CLSI interpretive criteria were obtained from separate patients at
different medical centres in Bangkok, Thailand (n = 3) and Madrid, Spain (n = 1) as
part of the 2010 AWARE global surveillance programme. Furthermore, the
additional ceftaroline-susceptible MRSA and methicillin-susceptible S. aureus
(MSSA) isolates (ceftaroline MIC of ≤ 1 mg/L) from the same medical centres were selected for comparative purposes (Table 1). MRSA isolates
from Bangkok and Madrid with a ceftaroline MIC of 2 mg/L were also
inclusion criteria and would be classified as intermediate following
CLSI interpretive criteria and resistant following EUCAST interpretive
criteria. The reference S. aureus isolates were obtained from the
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Susceptibility profiles

The antimicrobial susceptibility profiles of the S. aureus isolates from
Thailand and Spain along with S. aureus reference isolates are shown in Table 1. Three MRSA isolates from Thailand
(ARC3828, ARC3829 and ARC3830) and a single isolate from
Spain (ARC3824) had reproducible ceftaroline MIC values of 8 mg/L (four measurements per isolate).

**Genetic analysis of loci involved in full expression of β-lactam resistance**

The genome sequences of the surveillance isolates included in this study were determined and the loci implicated in the full expression of β-lactam resistance were compared with the equivalent proteins from the 11 reference strains. Although some polymorphisms were identified in several of the loci known to be involved in maximal expression of β-lactam resistance, the majority of the amino acid substitutions were not uniquely found in isolates with decreased susceptibility to ceftaroline and were therefore felt unlikely to be directly involved in the observed phenotype. Indeed, the only changes that correlated with the ceftaroline MIC values were located in the mecA gene of the MRSA isolates, which encodes the alternative PBP, PBP2a. Variations in PBP2a have previously been reported in several MRSA isolates from Greece that resulted in ceftaroline MIC values of 2 and 4 mg/L. Variations in PBP2a have previously been reported in several MRSA isolates from Greece that resulted in ceftaroline MIC values of 2 and 4 mg/L. A summary of the differences in the alignment of the PBP2a proteins is presented in Table 2. The PBP2a sequences from the eight MRSA reference strains were identical except for a single Gly246Glu substitution in strain COL and all of these strains were inhibited by a ceftaroline concentration of 1 mg/L except the MRSA strain Mu3, which displays heterogeneous resistance to vancomycin and produces an antagonistic phenomenon between vancomycin and β-lactam drugs. Furthermore, this Gly246Glu

### Table 1. Susceptibility profiles of S. aureus surveillance isolates and reference strains to ceftaroline and comparator compounds

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CPT, ceftaroline; MET, methicillin; OXA, oxacillin; LVX, levofloxacin; VAN, vancomycin; LZD, linezolid; FEP, cefepime; CAZ, ceftazidime.

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substitution was also found in the MRSA strain BAA-39 and strain ARC3826, both of which had a ceftaroline MIC of 1 mg/L, suggesting that Gly 246 contributes little, if anything, to the ceftaroline susceptibility. Strain ARC3826 also carries Asn 146Lys and Asn204Lys substitutions and the lack of direct involvement of the Asn204 residue in reduced ceftaroline susceptibility is also supported by the fact that this substitution was found in strain BAA-39, a representative of the ST239 Hungarian clone (Table 2). While the Asn 146Lys mutation is found in strain ARC3826, which has a ceftaroline MIC of 1 mg/L, several other MRSA isolates with this substitution were identified that exhibited a ceftaroline MIC of 2 mg/L (Table 2).

The four surveillance isolates that resulted in ceftaroline MIC values of 8 mg/L all had the same two glutamic acid-to-lysine substitutions: Glu239Lys in the non-penicillin-binding or dimerization domain and Glu447Lys in the penicillin-binding or transpeptidase domain. Furthermore, the Glu 239Lys substitution was also present in numerous isolates that had a ceftaroline MIC of 2 mg/L that were isolated from the same medical centres in Spain and Thailand (Table 2). Taken together, these data suggested that a single substitution at key residues in the non-penicillin-binding domain (nPBD) of PBP2a is sufficient to give a slight elevation in the ceftaroline MIC value and that an additional substitution in the penicillin-binding domain (PBD) gives rise to a further elevation in the ceftaroline MIC value. Of the six MRSA isolates from Thailand that exhibited a ceftaroline MIC of 2 mg/L, three of them carried the Glu 239Lys substitution in PBP2a, whereas the remaining three isolates carried the Asn146Lys substitution. In addition, four of the five MRSA isolates from Spain carried the Glu239Lys substitution in PBP2a, whereas one possessed the Asn146Lys substitution (Table 2).

Epidemiological linkage between isolates

A comparison of the strains’ relatedness was performed using MLST analysis. All of the four ceftaroline-resistant isolates with MIC values of 8 mg/L, irrespective of their geographical origin, as well as three of the isolates from Thailand and all five of the isolates from Spain with a ceftaroline MIC of 2 mg/L belonged to ST228 (Table 2). In addition, all of these isolates contained the type I SCCmec, giving the designation ST228-MRSA-I (Table 2). S. aureus isolates with the ST228 allelic profile, also known as the ‘Southern German clone’, are disseminated throughout Western Europe and are commonly isolated in Spain. However, ST228 isolates have not been isolated in Thailand (according to the MLST database), where ‘Brazilian’ ST239 SCCmec III isolates predominate and contain only one MLST allele in common.
centre in Thailand that had a ceftaroline MIC of 1 mg/L was confirmed to be ST239-MRSA-III (Table 2). The remaining three isolates from Thailand that exhibited a ceftaroline MIC value of 2 mg/L belonged to ST764, which carry four MLST alleles in common with ST228 but have not been reported outside of Japan.

The de novo-assembled genome sequences of the ST228 MRSA isolates from this study were analysed to identify SNPs as well as insertions and deletions and compared with the full genome sequences of eight ST228-MRSA-I isolated from Switzerland over several years. While all the ST228 isolates are related, they can be clearly differentiated into two clades (Figure 2). As expected, ARC3826 clusters closely with the ST239 reference strains T0131 and JKD6008 (Figure 2). The three ST764 isolates from Thailand, which share six MLST alleles in common with ST5 as well as the SCCmec type II, cluster with the S. aureus N315 isolate (Figure 2). Using the core genome of the ST5 isolate, S. aureus N315, as the baseline reference, the number of SNPs, including small insertions/deletions was enumerated. The core genomes of the two ST239 isolates contained a large number of common SNPs, >10400, when compared with the core genome of S. aureus N315. There were 302 SNPs in common between all 21 ST228-MRSA-I isolates and N315 (Figure 2). The eight ST228-MRSA-I from Switzerland contained 337 SNPs in common and the unique ST228 isolate from Spain, ARC5498, which carries the Asn146Lys substitution in MecA, grouped with the Swiss

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Table 2. Summary of molecular typing and PBP2a residue changes in S. aureus isolates

<table>
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<tr>
<th>Strain</th>
<th>Molecular type</th>
<th>CPT MIC (mg/L)</th>
<th>PBP2a residue number</th>
<th>nPBD</th>
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<td></td>
<td></td>
<td>146</td>
<td>150</td>
<td>204</td>
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CPT, ceftaroline.

aCombination of MLST (ST) type and SCCmec type (I–IV). ARC3823 has a new MLST type due to novel arcC and aroe alleles.

bData extracted from Mendes et al.26

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Characterization of ceftaroline-non-susceptible S. aureus isolates

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ST228 isolates (Figure 2). A similar analysis observed 343 SNPs in common between the five and six ST228-MRSA-I isolates that carry the Glu 239Lys mutation from Spain and Thailand, respectively (Figure 2), when compared with S. aureus N315. In addition, the ST228-MRSA-I isolates could clearly be separated into two distinct subgroups. There were 446 SNPs that differentiated the core genomes of the ST228-MRSA-I isolates from Spain/Thailand (which contain the Glu 239Lys substitution in PBP2a) from the core genomes of the ST228-MRSA-I isolates from Switzerland. Whereas many of these SNPs were randomly distributed across the core genome, there are two loci with a higher density of changes that represent transposase insertions that are distinct to each ST228 subgroup. This, taken together with the presence of discriminating polymorphisms found only in each ST228 subgroup, provides further evidence of an epidemiological linkage between the surveillance isolates from Thailand and the majority of the Spanish isolates. Further discrimination within the surveillance isolates was also possible, as there are 126 SNP differences found between the six isolates from Thailand (strain ARC3827, ARC5500 and ARC5503 with a ceftaroline MIC of 2 mg/L and ARC3828, ARC3829 and ARC3830 with a ceftaroline MIC of 8 mg/L) and the Spanish isolates with either a ceftaroline MIC of 8 mg/L (ARC3824) or a ceftaroline MIC of 2 mg/L (ARC5493, ARC5495, ARC5496 and ARC5497). There are a few additional unique SNPs that are common to the three Thailand isolates with ceftaroline MIC values of 8 mg/L that are not seen in the strains from Thailand with a ceftaroline MIC of 2 mg/L, providing supportive evidence that once the second PBP2a mutation (Glu447Lys) was introduced, raising the ceftaroline MIC to 8 mg/L, there was a clonal spread rather than this mutational event occurring three separate times. Pairwise comparisons of the core genomes of the three Thailand isolates with ceftaroline MIC values of 8 mg/L showed that the number of differences range from 15 to 44, providing additional evidence of their epidemiological relatedness and supporting a clonal spread within the hospital in Thailand rather than three independent mutational events, consistent with the low frequency of resistance emergence with ceftaroline.33

Given the confirmed evolutionary lineage of these strains determined by whole genome sequencing, as well as the ST228
Characterization of ceftaroline-non-susceptible *S. aureus* isolates

Clan being more commonly found in southern Europe and here-fore never reported in Thailand, a likely epidemiological path can be established. The most likely scenario is that an ancestral ST228-MRSA-I isolate with a ceftaroline MIC of 2 mg/L carrying the Glu239Lys mutation in PBP2a was transmitted to Thailand and representatives of the descendants were isolated as strains ARC3827, ARC5500 and ARC5503 in Thailand. Both the ancestral MRSA isolate in Spain (whose descendants were isolated as ARC5493, ARC5495, ARC5496 and ARC5497) and a similar isolate in Thailand then independently acquired the second mutation in PBP2a, Glu447Lys, which elevated the ceftaroline MIC to 8 mg/L (Figure 3). It is also possible that a ‘wild-type’ ST228 *S. aureus* MRSA isolate was transmitted from Spain to Thailand and that the wild-type isolate in both countries independently acquired the Glu239Lys and the Glu447Lys PBP2a mutations, but given the reported low emergence of resistance after serial passage with ceftaroline, this scenario seems less likely. However, it is clear that the Spanish strain ARC3824 with a ceftaroline MIC of 8 mg/L was not the strain that was transmitted to Thailand given the distinct polymorphisms seen in this isolate compared with both the three 2 mg/L and the three 8 mg/L isolates from Thailand.

**Structurally based perspective of PBP2a mutations on ceftaroline susceptibility**

The residues in Table 2 were mapped onto the PBP2a structure bound to ceftobiprole, a closely related anti-MRSA cephalosporin that is also a potent inhibitor of PBP2a. The Glu447Lys change is the only mutation that is located near the cephalosporin-binding pocket, while many of the remaining residues are located in the dimerization interface of the nPBD (Figure 4a). The Glu447Lys mutation has also been observed as one of several mutated residues in laboratory mutants generated after serial passage with ceftaroline. While Glu447 is not directly involved in binding based on the ceftobiprole crystal structure, it is located adjacent to the critical Tyr444 in the binding pocket that provides a hydrophobic stack to the R2 groups of ceftobiprole (Figure 4b). Cefaroline and ceftobiprole have subtle differences in their R2 groups, but given that the same mutations affect susceptibility it strongly suggests that the key interacting residues and mechanisms of resistance are similar between these anti-MRSA cephalosporins. The Glu447Lys change is a charge alteration from negative to positive that likely creates a new salt bridge with the neighbouring Glu446 residue. This results in a structural rearrangement of the loop and influences the flexibility to efficiently bind and accommodate large inhibitors and will impact ceftaroline susceptibility, similar to that proposed for ceftobiprole (Figure 4b).

Other mutations that result in elevated ceftaroline MIC values are all located in the nPBD. Of these, Glu239 appears most significant and has been previously observed in ceftobiprole-resistant laboratory mutants. The Glu239 residue is located in the charged protein interface of the crystallographic dimer (Figure 4c) and in the crystal structure it interacts with the positively charged Lys317 and Lys318 residues from the opposite monomer (Figure 4d). This pattern of dimer interface interaction formed by residues with opposite charges is also observed for Glu150, which mutates to a Lys in previously observed *S. aureus* strains with ceftaroline MIC values of at least 2 mg/L. It has been established that PBP2a-mediated resistance to β-lactams in MRSA is imparted by the weaker binding of β-lactams to the more occluded PBP2a transpeptidase pocket. PBP2a does not carry any transglycosylation function and hence it allows the cell to maintain peptidyltransferase function in the presence of β-lactams but relies on PBP2 for the transglycosylase activity. Experimental evidence has demonstrated a cooperative functioning between PBP2 and PBP2a (and PBP4) for *S. aureus* cell wall biogenesis and susceptibility to antimi-crobial agents. However, it remains unclear if this functional synergy requires a direct protein–protein interaction or if these functions could operate independently. Expression of PBP2a in the cefoxime-resistant PBP2b phenotype reversed the decrease in cross-linked muropeptides, suggesting that PBP2a functions cooperatively with PBP2 targeted by the selective action
A recent study on the inhibition of wall teichoic acid biogenesis has further supported the hypothesis that the colocalization of PBP2 and PBP2a is necessary for manifestation of the β-lactam resistance phenotype. The consistently observed mutations on one face of the PBP2a nPBD in the ceftaroline- and ceftobiprole- (the only two PBP2a inhibitors) resistant isolates, along with studies highlighting the importance of close interaction with PBP2, suggest that a likely partner for PBP2a protein–protein interaction in the S. aureus cell is PBP2.

Structural information on S. aureus PBP2 is available, which allowed for analysis of the surface electrostatic potential. A structural overlay of PBP2 and PBP2a shows that the similar nPBD interface of PBP2 is lined with positively charged residues allowing a similar heterodimeric contact interface (Figure 4e). Taken together, a generalized model of ceftaroline (and likely ceftobiprole) resistance mediated by PBP2a in S. aureus MRSA can be proposed. Cellular inhibition by ceftaroline can be overcome via two mechanisms. A direct change in the binding pocket conformation can result in reduced ability of the inhibitor to bind and inhibit the peptidyltransferase activity in cells. A second mechanism of inhibition is disruption of the protein–protein interaction between PBP2a and PBP2 that is needed for cooperative bridging of the transpeptidase and transglycosylase functions in PBP2a-containing MRSA strains (Figure 5). This would enable an alternative protein partner, such as PBP4, with transpeptidase activity to cooperatively interact with PBP2, thus bypassing the PBP2a inhibitory roadblock caused by ceftaroline. PBP4 has been shown to not be inhibited by ceftaroline but has been implicated as a PBP2
partner protein that provides transpeptidase activity in *S. aureus* cell wall biogenesis. While this second mechanism does not elevate the MIC as significantly as seen with the binding site mutation, it would influence the physiological pentapeptide function in the MRSA cell in the absence of any inhibitor. Indeed, in the well-controlled set of laboratory-generated ceftobiprole-resistant mutants containing nPBD mutations, the doubling times of the mutants were observed to be significantly reduced compared with the parent strains, indicating a compromised function of the mutated protein. It should be noted that these nPBD mutations were found in clinical isolates prior to the launch of the anti-MRSA cephalosporins and so it is unlikely they emerged due to specific selective pressure. This raises the possibility that there is some biologically relevant functional pressure that has allowed selection and maintenance of these mutations. Further experimentation is needed to fully differentiate the impact of the nPBD mutations from the PBD mutations to understand the impact of each on cell wall biogenesis in the *S. aureus* cell as well as whether there is an additional mechanism of ceftaroline non-susceptibility (MIC values of 2 mg/L), which can be further elevated by the accumulation of additional mutations in the TP domain.

**Figure 5.** Model of PBP2a-mediated ceftaroline resistance in *S. aureus*. (a) Methicillin (black square) binds and inhibits the transpeptidase (TP) domain of PBP2 in MSSA, preventing transglycosylation (TG) activity. However, in MRSA the acquisition of PBP2a, whose TP domain is not inhibited by methicillin, can dimerize with PBP2 through electrostatic charges (horizontal black lines) and provide the TP activity necessary to complement the TG activity of PBP2 required for cell wall biogenesis. (b) Ceftaroline (black circle) can bind to the TP domains of both PBP2 and PBP2a in the MRSA cell, thus preventing cooperative TP-TG activity needed for cell wall biogenesis. However, in strains that carry mutations in the dimerization domain of PBP2a, the association between PBP2a and PBP2 is weakened and allows the association of other proteins to dimerize with PBP2 and provide the TP activity and lead to ceftaroline non-susceptibility (MIC values of 2 mg/L), which can be further elevated by the accumulation of additional mutations in the TP domain.

**Conclusions**

MRSA isolates with ceftaroline MIC values of ≥2 mg/L in the 2010 surveillance set were quite rare. The characterization of the small number (n=4) of ceftaroline-resistant MRSA isolates identified (following CLSI interpretive criteria) from medical centres in Thailand and Spain has identified mutations in PBP2a that correlate with the increased ceftaroline MIC. Although additional chromosomal factors are known to influence β-lactam resistance, the potent affinity of ceftaroline for PBP2a suggests this is likely the primary mechanism of resistance and this is supported by the genomic similarity of the isolates. The PBP2a mutations identified in this study fall into two broad categories: changes that are proposed to directly influence the binding of ceftaroline to the transpeptidase domain of PBP2a; and changes in the nPBD domain that potentially destabilize a protein–protein interaction. Assimilation of evidence from this study as well as recent literature has enabled a hypothesis that the physiological partner of PBP2a in the MRSA cell is PBP2. Destabilization of the PBP2a–PBP2 interface requires that another protein, one that is recalcitrant to ceftaroline inhibition, provides the transpeptidase activity to PBP2 to ensure the correct cell wall biogenesis. Ceftaroline has low affinity for PBP4 and with the previously reported association of PBP4 with PBP2 make this a viable candidate worthy of additional experimentation. The use of whole genome sequencing provided a high level of discrimination between these MRSA strains and the ceftaroline-non-susceptible isolates in this study were shown to be epidemiologically related in a sublineage of ST228-MRSA-I. Furthermore, the three strains with ceftaroline MIC values of 8 mg/L isolated from the medical centre in Thailand have core genomes that are closely related enough to suggest a clonal spread. Further global epidemiological studies, especially of MRSA isolates with ceftaroline MIC values of ≥2 mg/L, are warranted to understand the transmission and emergence dynamics of ceftaroline-non-susceptible MRSA isolates.

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Transparency declarations

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