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Genotypic analysis of Italian MRSA strains exhibiting low-level ceftaroline and ceftobiprole resistance

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ABSTRACT

The aim of this study was to address the involvement of PBP mutations in the bactericidal activity to novel cephalosporins, alone and in combination with daptomycin, in not-related multidrug-resistant methicillin-resistant *Staphylococcus aureus* strains isolated during a nationwide Italian survey. MICs determination and time-killing assays were performed and *mec*A, *pbp*1, *pbp*2, *pbp*3, *pbp*4, and *gdp*P genes were sequenced. Ten strains showed low-level resistance to ceftaroline and ceftobiprole. PBP2a sequence analysis identified four different mutations (N146K; N204K; T235I; E239K) uniquely present in the non-penicillin-binding domain (nPBD). Epidemiologically, this resistance was associated with the most widespread MDR Italian clone ST228-SCCmecI-t001/t041, confirming its proclivity to accumulate mutations, and it is also associated to substitutions in the GdpP signaling protein, involved in the maintenance of di-AMP balance, recently associated with resistance to beta-lactams. Despite these mutations, both drugs retained their potent *in vitro* bactericidal activity and showed a synergistic effect towards difficult-to-treat isolates.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is still one of the most important pathogens isolated in invasive and non-invasive infections in some South-European countries, showing an incidence of 33.9% in Italy (European Centre for Disease Prevention and Control (ECDC) 2015).

Its inclusion in the WHO list as a worrisome multidrug-resistant (MDR) organism is due to its ability to infect a wide range of human hosts and its resistance to multiple antibiotic classes (Tacconelli et al. 2017). The development of resistance to linezolid is still rare in this species, while rifampicin resistance is increasing in our isolates with respect to Europe (Bongiorno et al. 2017).

The introduction of novel cephalosporins into the recent therapeutic pipelines has opened new ways for overcoming the numerous challenges created by *S. aureus* in adapting its survival behavior *versus* the host (Purrello et al. 2016).

As well known, ceftaroline (CPT) and ceftobiprole (BPR) are new novel β-lactam broad-spectrum cephalosporins with anti-MRSA activity (Barbour et al. 2009; Biek et al. 2010). In particular, their novel structure, resistance to degradation by some β-lactamases and high affinity binding not only to PBP2a, but also to PBP1, 2 and 3 (Kosowska-Shick et al. 2010), inhibit them at therapeutically useful concentrations.

Despite the recent approval of novel cephalosporins, isolates with reduced susceptibility levels to CPT and BPR have been identified worldwide (Chan et al. 2015; Argudín et al. 2017).

To date, resistance has mainly been mediated by amino acid (AA) substitutions in PBP2a, located in the primary ceftaroline-binding pocket in the transpeptidase active site [penicillin-binding domain (PBD)], giving rise to high-level resistance (>32 mg/L) (Lahiri and Alm 2016).

Additionally, PBP2a substitutions can be found in a groove in the non-penicillin-binding domain (nPBD), and have been linked to low level (<1–8 mg/L) resistance to ceftaroline (Alm et al. 2014).

A potential role in tolerance and resistance to β-lactams has also been ascribed to the alteration of c-di-AMP phosphodiesterase (GdpP) function, an hydrolyzing enzyme; mutations in the *gdp*P gene resulted in abnormal c-di-AMP levels involving a complex signaling cascade as well as the blocking of peptidoglycan biosynthesis (Griffiths and O'Neill 2012).

Apart from the single center survey by Morroni et al. (Morroni et al. 2018), no data on the national resistance scenario, underlying mechanisms of resistance and their implication in a possible bactericidal loss, are available for ceftaroline in Italy. From a collection of MRSA strains isolated during an Italian nationwide surveillance study (Campanile et al. 2015), ten not-epidemiologically-related strains were found to

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be resistant to many antibiotics, including heteroresistance to vancomycin (hVISA), daptomycin, rifampicin (Bongiorno et al. 2017) and also to novel cephalosporins (Campanile et al. 2018).

Our experimental plan started with the idea to:

Investigate the mechanism of resistance to ceftaroline and ceftobiprole by searching potential missense mutations in genes encoding PBP2a, native PBPs (PBP1, PBP2, PBP3, PBP4) and GdpP.

Verify the role of these mutations in influencing the cidal activity displayed by these drugs, both alone and in combination with daptomycin.

2. Materials and methods

2.1. Strains

The study sample included 10 unrelated Italian multidrug-resistant methicillin-resistant *S. aureus* (MDR-MRSA) isolates, recovered in 2012 during a national Italian survey molecularly characterized (Campanile et al. 2015; Bongiorno et al. 2017) and showing a ceftobiprole resistance profile.

2.2. Antibacterial and bactericidal activity

All strains were further analyzed for their *in vitro* antibacterial and bactericidal activity to novel cephalosporins. The antimicrobial susceptibility profiles of the MRSA isolates were evaluated according to EUCAST guidelines (The European Committee on Antimicrobial Susceptibility Testing 2018), and vancomycin-intermediate isolates were defined according to CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI) 2017).

In vitro activity of vancomycin (Sigma Chemical, St. Louis, MO, USA), daptomycin (Novartis, Basel, Switzer-land), linezolid (Pfizer Inc., New York, NY, USA), ceftaroline (Pfizer Inc., New York, NY, USA) and ceftobiprole (Basilea Pharmaceutica Ltd., Basel, Switzerland) was carried out by the broth microdilution method (BMD), following standard criteria (The European Committee on Antimicrobial Susceptibility Testing 2018).

Time-killing experiments were performed in duplicate in 20 mL tubes containing Cation-adjusted Mueller-Hinton broth (CA-MHB) (Difco, Detroit, MI) using a starting inoculum of 10^5–10^6 CFU/mL, with ceftobiprole (1×, 2× and 4× MIC) alone or in combination (second antibiotic at 1× MIC). All experiments were repeated at least three times; data points are averages from duplicate CFU/ml determinations within an experiment.

Bactericidal activity was defined as a ≥ 3 log10 decrease in bacterial count at 24 h. Synergy was measured at 24 h and was defined as a ≥ 2 log10 decrease in CFU/mL by the combination compared with its most active constituent and a ≥ 2 log10 decrease in the CFU/mL below the starting *inocula*.

For the screening of VISA and hVISA phenotypes the MIC Test Strip GRD (Glycopeptide Resistance Detection) was used, comprising a double-sided predefined gradient of vancomycin (VA) and teicoplanin (TEC), following the instructions provided by the manufacturer (Liofilchem®, Italy). Interpretation criteria: hVISA was defined as GRD either VA or TEC ≥8 mg/L, with VA MIC <4 mg/L; VISA was defined as GRD either VA or TEC ≥8 mg/L, with VA MIC ≥4 mg/L. The results obtained were confirmed by using the population analysis PAP/AUC as a standard method, as already published (Campanile et al. 2015).

2.3. Molecular characterization

All isolates were molecularly characterized for SCC*mec* typing, PVL and ACME locus, MLST, *spa*-type and mutations responsible for linezolid resistance, as previously described (Campanile et al. 2015).

2.4. Gene amplification and sequence analysis

The *mec*A, *pbp*1, *pbp*2, *pbp*3, *pbp*4 and *gdp*P genes of ceftaroline-resistant isolates were amplified by PCR using primers designed for this study (table 1 suppl.). Sequencing was performed using the Dye Terminator DNA sequencing kit V1.1 (Applied Biosystems™) followed by purification using the DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany). The sequences obtained were corrected and analyzed using the Chromas Lite 2.1 program; the sequences were exported in FASTA format. Sequence alignments were performed by using BLAST (Basic Local Alignment Search Tool; (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and UniProt programs (www.uniprot.org). N315, COL and MW2 MRSA strains were used as reference.

2.5. Structural analysis of PBP2a mutations

The bioinformatic program Swiss-Model ExPASy (http://swissmodel.expasy.org) was used to obtain the Protein Data Bank (PDB) format of PBP2a protein from the FASTA format. The structures of WT PBP2a (PBD:3ZG0), available in the public PDB database, were used for the structural analyses. The molecular surface of the protein and the aminoacidic mutations identified in this study were mapped using the graphics program PyMOL (Schrödinger; www.pymol.org).

3. Results

In agreement with the EUCAST breakpoints, all 10 MDR-MRSA strains showed resistance to ceftobiprole and ceftaroline with MIC values of 4 mg/L and 4–8 mg/L, respectively. 8/10 strains showed an hVISA phenotype, 2/10 were linezolid-resistant (G2576 T in 23S rRNA) (data not shown), and 2/10 were daptomycin-nonsusceptible (DNS) (table 1).

In time-kill experiments, single-drug exposures to both cephalosporins resulted in a potent bactericidal activity at 24 h and achieved a greater log reduction (3–4 $\log_{10}$ reduction) at the diverse concentrations tested, *i.e.* 1×, 2× and 4× MIC. Synergism was exhibited in combination with daptomycin 1× MIC only against DNS isolates, at the 4× MIC of ceftobiprole and ceftaroline, and enhanced killing at the 1× and 2× MICs, with a 2 $\log_{10}$ reduction. Results of representative experiments are presented in fig. 1.

PCR and sequencing analysis of *mec*A, *pbp*1, *pbp*2, *pbp*3 and *pbp*4 genes revealed diverse mutations only in PBP2a, underlying the moderate MIC increase to both cephalosporins in the 10 strains. Sequence analysis of PBP2a in CPT-BPR resistant isolates identified four different missense mutations. The N146K mutation was the most widespread in our sample (6/10) and was located in the PBP2a non-binding penicillin domain, but confers resistance to ceftobiprole and ceftaroline, and other β-lactams through an alteration of the salt bridge network at the allosteric site. The second most widespread mutation was the E239K substitution (3/10), due to the change of a single base in the codon GAG that encodes for glutamic acid, leading to the formation of an AAG codon that encodes a lysine. This mutation occurs again in the domain of the PBP2a protein that does not bind penicillin. These two more represented mutations were found in the major Italian epidemic clone ST228-SCC*mec*I-t001/t041 (9 strains). The only isolate belonging to ST239-SCC*mec*III-t037, showed the combination of two missense mutations, without affecting the MICs of both drugs (4 mg/L): N204K and the T235I substitution, in which the mutation of a single base in the ACU codon that codes for threonine, leads to the formation of an AUU codon coding for an isoleucine.

The structure of *S. aureus* WT PBP2a (PBD:3ZG0) was used to support the structural interpretations. By using PyMOL, the PBP2a mutation identified in this study mapped uniquely into the known non-penicillin-binding domain (nPBD) of PBP2a (fig. 2).

All BPR/CPT-R strains belonging to ST228 (CC5) carried mutations (D105, P392S) in the *gdp*P gene, coding for a c-di-AMP hydrolyzing

Table 1
Phenotypic and molecular characteristics of the 10 multidrug-resistant methicillin-resistant *Staphylococcus aureus* (MDR-MRSA) isolates in study.

Strains	Location	Clone	MDR phenotype	BPR MIC (mg/L)	CPT MIC (mg/L)	PBP2a	GdpP
SA 01	Fano	ST228-SCC*mec*I-t041	hVISA/DNS	4	8	N146K	D105N-P392S
SA 02	Milano	ST228-SCC*mec*I-t041	hVISA/DNS	4	8	N146K	D105N-P392S
SA 03	Siena	ST228-SCC*mec*I-t001	hVISA	4	4	E239K	D105N-P392S
SA 04	Napoli	ST228-SCC*mec*I-t001	hVISA	4	4	N146K	D105N-P392S
SA 05	Modena	ST228-SCC*mec*I-t041	VSSA	4	4	N146K	D105N-P392S
SA 06	Rome	ST228-SCC*mec*I-t041	VSSA/LNZ-R	4	8	N146K	D105N-P392S
SA 07	Cosenza	ST228-SCC*mec*I-t001	hVISA	4	4	N146K	D105N-P392S
SA 08	Milano	ST228-SCC*mec*I-t041	hVISA	4	4	E239K	D105N-P392S
SA 09	Rome	ST228-SCC*mec*I-t041	hVISA/LNZ-R	4	4	E239K	D105N-P392S
SA 10	Piacenza	ST239-SCC*mec*III-t037	hVISA	4	4	N204K; T235I*	-

hVISA = Heteroresistant to vancomycin; DNS = daptomycin-nonsusceptible; LNZ-R = linezolid-resistant; MDR = multi-drug resistant; BPR = ceftobiprole; CPT = ceftaroline; PBP2a = penicillin-binding protein 2a; GdpP = c-di-AMP phosphodiesterase; MIC = minimum inhibitory concentration.
All mutations were analyzed *versus* N315, MW2, COL and Mu50.
* This study.

enzyme, unlike MW2 and COL control strains. These amino acidic substitutions were only in common with N315 and Mu50 control strains (belonging to CC5). We hypothesized that these mutations are clonally related. No mutations in the other *pbp* genes (*pbp1, pbp2, pbp3, pbp4*) were detected.

4. Discussion

We studied the occurrence of low-level resistance to the novel cephalosporins in a sample of MDR-MRSA strains, addressing the involvement of PBP mutations in the bactericidal activity of ceftobiprole and ceftaroline, alone and in combination with daptomycin. The strains showed a complex profile of resistance to the most anti-Gram-positive drugs, *i.e.* heteroresistance to vancomycin (8/10), daptomycin (2/10), and linezolid (2/10), isolated from different parts of Italy from severe and invasive infections.

Ceftaroline and ceftobiprole interact with PBP2a in similar modes: the former binds to the allosteric domain of PBP2a, leading to a conformational change that allows a second molecule to bind to the active site, blocking its activity (Lovering et al. 2012); the latter forms a hydrophobic stacking interaction between its R2 residue and the PBP2a active site (Lahiri and Alm 2016).

The N146K mutation is the most widespread in our sample (6/10) followed by the E239K substitution (3/10), both represented the most reported in the literature (Alm et al. 2014;Kelley et al. 2015 ; Morroni et al. 2018).

All detected mutations fall uniquely in the non-penicillin-binding domain (nPBD) and have been linked to a small decrease in CPT/BPR

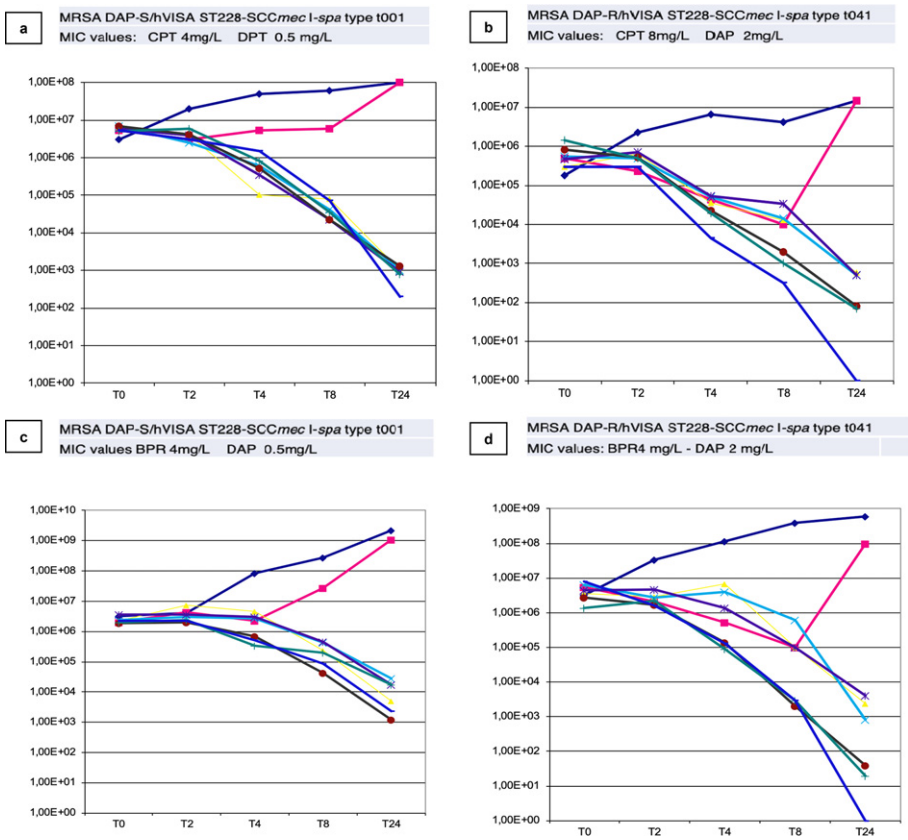

Fig. 1. Examples of the bactericidal activity of ceftaroline (CPT) and ceftobiprole (BPR) alone and in association with daptomycin (DAP) by time-kill curve analysis. Representative assays of daptomycin-susceptible (DAP-S) and daptomycin-resistant (DAP-R) methicillin-resistant *Staphylococcus aureus* (MRSA), tested with CPT/DAP (a; b) and BPR/DAP (c; d), were shown. Dark blue, free; pink, DAP 1×; yellow, CPT or BPR 1×; light blue, CPT or BPR 2×; purple, CPT or BPR 4×; black, DAP1×/CPT or BPR 1×; green, DAPT1×/CPT or BPR 2×; blue DAP1×/CPT or BPR 4×.

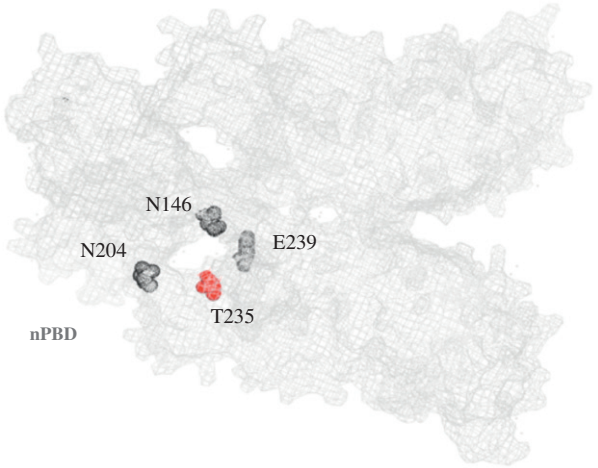

Fig. 2. Structural analysis of PBP2a mutations in ceftaroline-resistant isolates. The residues mutated in the non-penicillin-binding domain (nPBD) of PBP2a protein are depicted as spheres.

susceptibility (Kelley et al. 2015; Lahiri and Alm 2016). This region has been hypothesized to be an important protein – protein interface, proposed to be functionally important for cell-wall biogenesis, either by interaction with other PBPs (Fishovitz et al. 2014) or other ligands, such as cell-wall fragments, which may regulate the trans-peptidase pocket *via* allostery (Otero et al. 2013).

Epidemiologically, this resistance was associated with the most widespread MDR Italian clone ST228-SCC*mec*I-t001/t041, confirming the proclivity of this clone to accumulate mutations (Kelley et al. 2015; Morroni et al. 2018). In a single isolate belonging to ST239-SCC*mec*III-t037 CPT/BPR resistance was due to PBP2a substitutions at positions different to those found for ST228 (N204K; T235I). In this strain, also the T235I substitution, first reported in Italy, exists in the nPBD, confirming this region as a hot-spot of mutations, even if its role in ceftaroline/ceftobiprole resistance has to be experimentally demonstrated by further investigations.

All strains showed D105 and P392S substitutions in the GdpP signaling protein, involved in the maintenance of di-AMP balance (Corrigan et al. 2011). The role of this gene on ceftobiprole and ceftaroline resistance has been recently described, and it is still under investigation. We hypothesized that these mutations in our isolates could be clonally related to CC5.

Moreover, *in vitro* data showed a sustained bactericidal activity of ceftaroline and ceftobiprole against all the MRSA isolates despite their MDR phenotype and PBP2a alterations, and the combination of both drugs with daptomycin exhibited a synergistic effect against the DNS isolates analyzed. About that, we could explain this observation with the see-saw effect occurring when there is increased daptomycin resistance, leading to enhanced cidal activity (Shafiq et al. 2017).

Even if some limitations, two important observations emerged from our study. Firstly, the confirmation that novel cephalosporins still retain their *in vitro* bactericidal activity against Italian MDR-MRSA clinical isolates, and overcome resistance to daptomycin, by enhancing its binding to the cell membrane through changes in membrane fluidity and charge. Secondly, the spreading of rare low-level novel cephalosporins resistance isolates in Italy associated to different single amino acid changes in the same or different MRSA STs; this phenomenon should be monitored, given the increasing use of these molecules in the clinical practice.

Nevertheless, it is possible to assess that ceftaroline and ceftobiprole, as single drugs and in association with daptomycin, could be suggested as an effective therapy in the treatment of serious infections sustained by MDR-MRSA isolates, also novel cephalosporin resistant.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2019.06.004.

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