AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

MECHANISMS OF RESISTANCE

High Rate of Ceftobiprole Resistance among Clinical Methicillin-Resistant *Staphylococcus aureus* Isolates from a Hospital in Central Italy

Gianluca Morroni,[a] Andrea Brenciani,[b] Lucia Brescini,[a] Simona Fioriti,[b] Serena Simoni,[b] Antonella Pocognoli,[c] Marina Mingoia,[b] Eleonora Giovanetti,[d] Francesco Barchiesi,[a] Andrea Giacometti,[a] Oscar Cirioni[a]

[a]Infectious Diseases Clinic, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy

[b]Microbiology Unit, Department of Biomedical Sciences and Public Health, Polytechnic University of Marche, Ancona, Italy

[c]Microbiology Unit, Clinical Pathology Laboratory Ospedali Riuniti, Ancona, Italy

[d]Microbiology Unit, Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy

ABSTRACT Ceftobiprole is a fifth-generation cephalosporin with activity against methicillin-resistant *Staphylococcus aureus* (MRSA). One-year surveillance at the Regional Hospital of Ancona (Italy) disclosed a 12% ceftobiprole resistance rate (12/102 isolates; MIC, ≥4 mg/liter). Epidemiological characterization demonstrated that the resistant isolates all belonged to different clones. Penicillin-binding protein (PBP) analysis showed substitutions in all PBPs and a novel insertion in PBP2a. The *mecB* and *mecC* genes were not detected. Ceftobiprole susceptibility screening is essential to avoid therapeutic failure and the spread of ceftobiprole-resistant strains.

KEYWORDS MRSA, cephalosporins, ceftobiprole, penicillin-binding protein, *mecA* gene

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a pathogen with a wide diffusion in Europe (1), as well as in Italy, accounting for about 30% of all invasive *S. aureus* strains described in Italy to date (2). Resistance to β-lactamases is often due to the *mecA* gene, which encodes the low-affinity penicillin-binding protein 2a (PBP2a). Ceftobiprole, a fifth-generation broad-spectrum cephalosporin, shows activity against Gram-positive and Gram-negative bacteria and is also active against MRSA (3); in particular, it has demonstrated high affinity not only for the common PBPs but also for PBP2a (4). The antibiotic which has recently been approved has been shown to display relative stability against β-lactamases and a low propensity to develop resistance (3), as confirmed by the low rates of resistant staphylococcal isolates found in surveillance studies (5–8). Nonetheless, some papers have described ceftobiprole resistance among MRSA strains (5, 6, 9–11). This resistance is probably due to mutations in *pbp* genes, especially *mecA* or *pbp4* (9–12). During a recent MRSA survey, we decided to test ceftobiprole against 102 strains isolated from February 2017 to February 2018 from a variety of specimens collected at Ospedali Riuniti in Ancona, Italy. MIC determination by broth microdilution (13) showed that 88% of MRSA strains ($n = 90$) were susceptible to ceftobiprole, with MICs ranging from 0.03 to 2 mg/liter. The resistance rate was 12% (12 isolates; MIC, ≥4 mg/liter), which is considerably higher than the rates (1.7 to 3.5%) detected in surveillance studies in Europe (5–6). Similar resistance rates (15%) have been found only in an African surveillance study (11). The 12 ceftobiprole-resistant MRSA strains were recovered from wounds ($n = 4$) and pulmonary secretions ($n = 8$) of patients admitted to different departments, except for 3 strains, which were col-

Received 7 August 2018 **Returned for modification** 21 August 2018 **Accepted** 15 September 2018

Accepted manuscript posted online 1 October 2018

Citation Morroni G, Brenciani A, Brescini L, Fioriti S, Simoni S, Pocognoli A, Mingoia M, Giovanetti E, Barchiesi F, Giacometti A, Cirioni O. 2018. High rate of ceftobiprole resistance among clinical methicillin-resistant *Staphylococcus aureus* isolates from a hospital in central Italy. Antimicrob Agents Chemother 62:e01663-18. https://doi.org/10.1128/AAC.01663-18.

Address correspondence to Andrea Brenciani, a.brenciani@univpm.it.

lected from general medicine patients. The MIC_{50} and MIC_{90} of the 102 isolates were 1 mg/liter and 4 mg/liter, respectively. The resistant isolates were further characterized for *pbp* gene mutations and to determine the epidemiological relationships among them (Table 1). According to *spa* typing (14) and multilocus sequence typing (MLST) (15), 8 isolates shared the same *spa* type (t041) and MLST (sequence type 228 [ST228], clonal complex 5 [CC5]) and were classified as "South German" or "Italian" clones, i.e., nosocomial strains that are widespread in central and southern Europe (16). SmaI–pulsed-field gel electrophoresis (SmaI-PFGE) analysis (17) demonstrated that the 8 strains showed 4 different PFGE patterns (B, D, E, and F), with three identical strains (pattern F2) and two closely related isolates (patterns F1 and F3) belonging to pulsotype F (Fig. 1). These correlations suggested the possibility of an intrahospital outbreak of a ceftobiprole-resistant clone. The remaining 4 isolates showed different PFGE pulsotypes (A, C, G, and H, respectively) and molecular types, as follows: MRSA 350990 was assigned to a novel *spa* type (t18014) and to ST22 (CC22), MRSA 354432 was assigned to *spa* type t1476 and ST8 (CC8), MRSA 420822 was assigned to *spa* type t5948 and the new ST4873 (CC59), and MRSA 422665 was assigned to *spa* type t002 and ST5 (CC5) (Table 1). CC22, CC8, and CC5 are associated with nosocomial infections (18–19), whereas CC59 is associated with community-acquired infections (20). PBP sequences of the resistant isolates were obtained (21–22) and compared with the susceptible strain *S. aureus* Mu50. The PBP mutations explained the epidemiological context in the following ways. First, *spa* type t041 and ST228 strains harbored the N146K mutation in PBP2a. This mutation, which has been reported in association with two other substitutions, as N146K-N204K-G246E (11), is found in the non-penicillin-binding domain (non-PBD), which mediates resistance through interactions with other proteins (23). Three of these 4 strains, MRSA 351138, 365325, and 366780, also bore the C197Y mutation in PBP2. Mutations in PBP1 (S194N) and PBP4 (N337D) were also detected in MRSA 365325. Second, MRSA 350990 (*spa* type t18014, ST22) exhibited mutations in all PBPs, with S225R in PBP2a; S629T in PBP1; C197Y, L256V, P285A, and T439V in PBP2; R504K and K584F in PBP3; and D98E, S189T, and E398A in PBP4. Despite the numerous mutations, the ceftobiprole MIC was only slightly above the breakpoint, suggesting that they do not all affect resistance. Third, MRSA 354432 (*spa* type t1476, ST8) lacked the *mecA*, *mecB,* and *mecC* genes and showed mutations in all the PBPs tested (D118N in PBP1, C197Y in PBP2, P233L and S438T in PBP3, and S189T in PBP4), which are probably responsible for cefoxitin resistance; in contrast, ceftobiprole resistance may be due to substitutions in PBP4, which seems to play a key role in ceftobiprole-resistant strains lacking PBP2a (24). Fourth, MRSA 420822 (*spa* type t5948, ST4873) harbored a wild-type *mecA* gene and carried several mutations in the other *pbp* genes: 3 mutations in PBP1 (A329V, E499D, and G515S), 4 mutations in PBP2 (C197Y, P285A, Q358H, and T439V), a mutation in PBP3 (A330S), and 2 mutations in PBP4 (S189T and V210I). Notably, this community-associated strain had an elevated MIC (32 mg/liter), which to the best of our knowledge is the highest ceftobiprole MIC reported so far in clinical strains, despite its wild-type PBP2a. Moreover, it showed the highest number of PBP mutations, which may be responsible for the high MIC, even though the involvement of other resistance mechanisms cannot be excluded (15). Fifth, MRSA 422665 (*spa* type t002, ST5) showed a 5-amino-acid insertion (VQHED) in the non-PBD at 259 to 260 in PBP2a, as well as a mutation in PBP2 (V607M). The insertion has the potential to affect interactions with other proteins (23), inducing ceftobiprole resistance, but it does not affect β-lactamase resistance. This is the first report of an amino acid insertion in PBP2a in a ceftobiprole-resistant strain. None of the strains carried the *mecB* or *mecC* gene. The present surveillance study, although limited to isolates recovered from a single center, showed a high ceftobiprole resistance rate and PBP mutations that were not confined to amino acid substitutions. Notably, since ceftobiprole became available at Ospedali Riuniti only in early 2017, selective pressure can be excluded. This suggests that mutations conferring ceftobiprole resistance can be induced not only by selective pressure but also arise independently. The present findings highlight the need to perform ceftobiprole

TABLE 1 Characteristics of the ceftobiprole-resistant MRSA strains analyzed in this study

MRSA strain	Isolation date	Department[a]	Infection type[b]	CFB MIC (mg/liter)[c]	Mutation(s) by PBP[d]					Molecular typing[e]		
					PBP2a	PBP1	PBP2	PBP3	PBP4	Pulsotype	*spa* type	MLST (CC)
350990	Feb 2017	Spinal surgery	HAI	4	S225R	S629T	C197Y, L256V, P285A, T439V	R504K, K584F	D98E, S189T, E398A	A	t18014	ST22 (CC22)
351138	Feb 2017	Dermatology	HAI	4	N146K	WT	C197Y	WT	WT	B	t041	ST228 (CC5)
354432	Feb 2017	Infectious diseases	HAI	4	ND	D118N	C197Y	P233L, S438T	S189T	C	t1476	ST8 (CC8)
365325	Apr 2017	General medicine	HAI	8	N146K	S194N	C197Y	WT	N337D	D	t041	ST228 (CC5)
366780	Apr 2017	Neurosurgery	HAI	4	N146K	WT	C197Y	WT	WT	E	t041	ST228 (CC5)
415469	Dec 2017	Subintensive medicine	HAI	4	N146K	WT	WT	WT	WT	F1	t041	ST228 (CC5)
420822	Jan 2018	Rehabilitation	CAI	32	WT	A329V, E499D, G515S	C197Y, P285A, Q358H, T439V	A330S	S189T, V210I	G	t5948	ST4873 (CC59)
421322	Jan 2018	ICU	HAI	4	N146K	WT	WT	WT	WT	F2	t041	ST228 (CC5)
421786	Jan 2018	Otolaryngology	HAI	4	N146K	WT	WT	WT	WT	F2	t041	ST228 (CC5)
422253	Jan 2018	Subintensive medicine	HAI	4	N146K	WT	WT	WT	WT	F2	t041	ST228 (CC5)
422665	Jan 2018	LTC	HAI	4	INS 259 VQHED 260	WT	V607M	WT	WT	H	t002	ST5 (CC5)
423591	Jan 2018	Subintensive medicine	HAI	8	N146K	WT	WT	WT	WT	F3	t041	ST228 (CC5)

[a]ICU, intensive care unit; LTC, long-term care.
[b]HAI, hospital-associated infection; CAI, community-associated infection.
[c]CFB, ceftobiprole.
[d]WT, wild type; ND, not detected; INS, insertion.
[e]MLST, multilocus sequence type; CC, clonal complex.

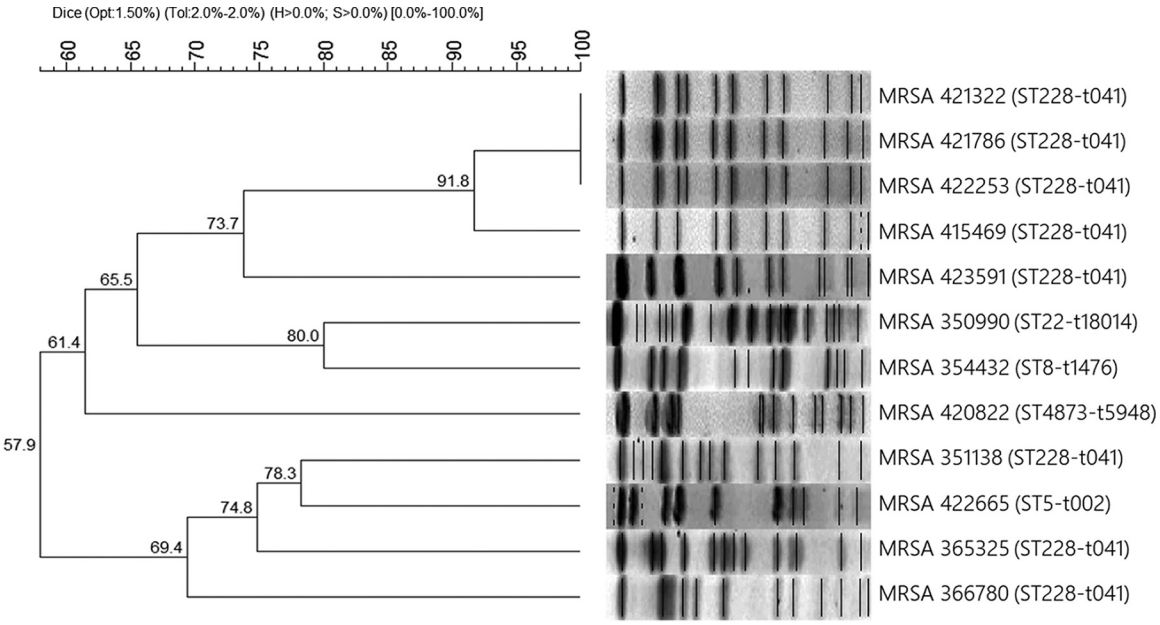

FIG 1 SmaI-PFGE pattern and dendrogram of the ceftobiprole-resistant MRSA strains. Profiles were analyzed with BioNumerics software version 7.0 (Applied Maths Scientific Software Development, Sint-Martens-Latem, Belgium). The dendrogram was built by applying the Dice similarity coefficient, with 1.5% optimization and 2.0% tolerance. Clustering was obtained using the unweighted pair group method with arithmetic mean. Opt, optimization; Tol, tolerance; H, minimum height; S, minimum surface.

screening before treatment to avoid therapeutic failure and the spread of resistant strains.

Data availability. The nucleotide sequences of mutated PBPs were deposited in GenBank under accession numbers MH798847 to MH798870.

ACKNOWLEDGMENTS

We are grateful to Daniela Bencardino and Elisa Ponzio for their technical assistance.

This work was financially supported by Progetto Strategico di Ateneo 2016–Università Politecnica delle Marche, "Study of new compounds and innovative strategies to control complicated bacterial skin infections."

REFERENCES

1. Köck R, Becker K, Cookson B, Van Gemert-Pijnen JE, Harbarth S, Kluytmans J, Mielke M, Peters G, Skov RL, Struelens MJ, Tacconelli E, Navarro Torné A, Witte W, Friedrich AW. 2010. Methicillin-resistant *Staphylococcus aureus* (MRSA): burden of disease and control challenges in Europe. Euro Surveill 15:pii=19688.
2. European Centre for Disease Prevention and Control. 2017. Surveillance of antimicrobial resistance in Europe. 2016. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). European Centre for Disease Prevention and ControlSolna, Sweden. https://ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/ears-net.
3. Zhanel GG, Lam A, Schweizer F, Thomson K, Walkty A, Rubinstein E, Gin AS, Hoban DJ, Noreddin AM, Karlowsky JA. 2008. Ceftobiprole: a review of a broad-spectrum and anti-MRSA cephalosporin. Am J Clin Dermatol 9:245–254. https://doi.org/10.2165/00128071-200809040-00004.
4. Davies TA, Page MGP, Shang W, Andrew T, Kania M, Bush K. 2007. Binding of ceftobiprole and comparators to the penicillin-binding proteins of *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Streptococcus pneumoniae*. Antimicrob Agents Chemother 51:2621–2624. https://doi.org/10.1128/AAC.00029-07.
5. Farrell DJ, Flamm RK, Sader HS, Jones RN. 2014. Ceftobiprole activity against over 60,000 clinical bacterial pathogens isolated in Europe, Turkey, and Israel from 2005 to 2010. Antimicrob Agents Chemother 58:3882–3888. https://doi.org/10.1128/AAC.02465-14.
6. Pfaller MA, Flamm RK, Duncan LR, Streit JM, Castanheira M, Sader HS. 2018. Antimicrobial activity of ceftobiprole and comparator agents when tested against contemporary Gram-positive and -negative organisms collected from Europe (2015). Diagn Microbiol Infect Dis 91:77–84. https://doi.org/10.1016/j.diagmicrobio.2017.12.020.
7. Hischebeth GTR, Gravius S, Molitor E, Kohlhof H, Hoerauf A, Hilgers C, Randau TM. 2018. Activity of ceftobiprole against *Staphylococcus* spec. isolates derived from foreign body associated infections. Diagn Microbiol Infect Dis 91:175–178. https://doi.org/10.1016/j.diagmicrobio.2018.01.010.
8. Henriksen AS, Smart J, Hamed K. 2018. Comparative activity of ceftobiprole against coagulase-negative staphylococci from the BSAC Bacteraemia Surveillance Programme, 2013–2015. Eur J Clin Microbiol Infect Dis 37:1653–1659. https://doi.org/10.1007/s10096-018-3295-6.
9. Chan LC, Basuino L, Diep B, Hamilton S, Chatterjee SS, Chambers HF. 2015. Ceftobiprole- and ceftaroline-resistant methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 59:2960–2963. https://doi.org/10.1128/AAC.05004-14.
10. Greninger AL, Chatterjee SS, Chan LC, Hamilton SM, Chambers HF, Chiu CY. 2016. Whole-genome sequencing of methicillin-resistant *Staphylococcus aureus* resistant to fifth-generation cephalosporins reveals potential non-mecA mechanisms of resistance. PLoS One 11:e0149541. https://doi.org/10.1371/journal.pone.0149541.
11. Schaumburg F, Peters G, Alabi A, Becker K, Idelevich EA. 2016. Missense

mutations of PBP2a are associated with reduced susceptibility to ceftaroline and ceftobiprole in African MRSA. J Antimicrob Chemother 71:41–44. https://doi.org/10.1093/jac/dkv325.
12. Fishowitz J, Hermoso JA, Chang M, Mobashery S. 2014. Penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. IUBMB Life 66:572–577. https://doi.org/10.1002/iub.1289.
13. European Committee on Antimicrobial Susceptibility Testing. 2018. Breakpoint tables for interpretation of MICs and zone diameters. Version 8.0. European Committee on Antimicrobial Susceptibility TestingVaxjo, Sweden. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_8.0_Breakpoint_Tables.pdf.
14. Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, Bost DA, Riehman M, Naidich S, Kreiswirth BN. 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J Clin Microbiol 37:3556–3563.
15. Enright MC, Day NP, Davies CE, Peacock SJ, Spratt BG. 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol 38:1008–1015.
16. Mato R, Campanile F, Stefani S, Crisostomo MI, Santagati M, Sanches SI, de LH. 2004. Clonal types and multidrug resistance patterns of methicillin-resistant *Staphylococcus aureus* (MRSA) recovered in Italy during the 1990s. Microb Drug Resist 10:106–113. https://doi.org/10.1089/1076629041310109.
17. Brenciani A, Morroni G, Pollini S, Tiberi E, Mingoia M, Varaldo PE, Rossolini GM, Giovanetti E. 2016. Characterization of novel conjugative multiresistance plasmids carrying *cfr* from linezolid-resistant *Staphylococcus epidermidis* clinical isolates from Italy. J Antimicrob Chemother 71:307–313. https://doi.org/10.1093/jac/dkv341.
18. DeLeo FR, Otto M, Kreiswirth BN, Chambers HF. 2010. Community-associated methicillin-resistant *Staphylococcus aureus*. Lancet 375:1557–1568. https://doi.org/10.1016/S0140-6736(09)61999-1.
19. Tristan A, Ferry T, Durand G, Dauwalder O, Bes M, Lina G, Vandenesch F, Etienne J. 2007. Virulence determinants in community and hospital meticillin-resistant *Staphylococcus aureus*. J Hosp Infect 65:105–109. https://doi.org/10.1016/S0195-6701(07)60025-5.
20. Boyle-Vavra S, Ereshefsky B, Wang CC, Daum RS. 2005. Successful multiresistance community-associated methicillin-resistant *Staphylococcus aureus* lineage from Taipei, Taiwan, that carries either the novel staphylococcal chromosome cassette *mec* (SCC*mec*) type V or SCC*mec* type IV. J Clin Microbiol 43:4719–4730. https://doi.org/10.1128/JCM.43.9.4719-4730.2005.
21. Argudín AM, Dodémont M, Taguemount M, Roisin S, de Mendonça R, Deplano A, Nonhoff C, Denis O. 2017. *In vitro* activity of ceftaroline against clinical *Staphylococcus aureus* isolates collected during a national survey conducted in Belgian hospitals. J Antimicrob Chemother 72:56–59. https://doi.org/10.1093/jac/dkw380.
22. Becker K, van Alen S, Idelevich EA, Schleimer N, Seggewiss J, Mellman A, Kaspar U, Peters G. 2018. Plasmid-encoded transferable *mecB*-mediated methicillin resistance in *Staphylococcus aureus*. Emerg Infect Dis 24:242–248. https://doi.org/10.3201/eid2402.171074.
23. Banerjee R, Gretes M, Basuino L, Strynadka N, Chambers HF. 2008. *In vitro* selection and characterization of ceftobiprole-resistant methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 52:2089–2096. https://doi.org/10.1128/AAC.01403-07.
24. Banerjee R, Gretes M, Harlem C, Basuino L, Chambers HF. 2010. A *mecA*-negative strain of methicillin-resistant *Staphylococcus aureus* with high-level β-lactam resistance contains mutations in three genes. Antimicrob Agents Chemother 54:4900–4902. https://doi.org/10.1128/AAC.00594-10.