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ORIGINAL ARTICLE

# Evaluation of *in vitro* activity of ceftaroline on methicillin resistant *Staphylococcus aureus* blood isolates from Iran

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## ABSTRACT

**Background and Objectives:** Ceftaroline (CPT) is a novel cephalosporin with potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Despite its recent introduction, CPT resistance in MRSA has been described worldwide. We aimed in the current study to evaluate the *in vitro* activity of CPT against 91 clinical MRSA and 3 MSSA isolates.
**Materials and Methods:** Susceptibility of isolates to CPT was tested using E-test and disk diffusion (DD) method. The nucleotide sequence of the *mecA* gene and molecular types of isolates with reduced susceptibility to CPT were further studied to identify resistance conferring mutations in PBP2a and the genetic relatedness of the isolates respectively.
**Results:** Overall, 92.5% of isolates were found to be CPT susceptible (MICs≤1mg/l) and 7 MRSA isolates were characterized with MIC=2mg/l and categorized as susceptible dose dependent. Compared to E-test, DD revealed a categorical agreement rate of 93.6% and the obtained rates for minor, major /very major error were found to be 6.3% and 0% respectively. The MRSA isolates with increased CPT MICs (n=7), belonged to *spa* types t030 (n=6) and t13927 (n=1) and all carried N146K substitution in PBP2a allosteric domain, except for one isolate which harbored a wild-type PBP2a.
**Conclusion:** While resistance to CPT was not detected we found increased CPT MICs in 7.69% of MRSA isolates. Reduced susceptibility to CPT in the absence of *mecA* mutations is indicative of contribution of secondary chromosomal mutations in resistance development.

**Keywords:** Ceftaroline; Methicillin-resistant *Staphylococcus aureus*; Penicillin-binding protein 2a; Minimum inhibitory concentration; *mecA*

## INTRODUCTION

*Staphylococcus aureus* is one of the most common human pathogens isolated from both community-acquired (CA) and hospital-acquired (HA) infections. The ability to adopt and develop resistance to commonly used antibiotics renders *S. aureus* infections difficult to eradicate (1). Of particular concerns are

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infections arising from methicillin-resistant *S. aureus* (MRSA) which has acquired the ability to resist the action of most β-lactam antibiotics by expression of penicillin binding protein 2a (PBP2a), encoded by *mecA* (2, 3). The recent, alarming emergence of vancomycin-intermediate and -resistant (VISA & VRSA) MRSA strains has prompted increased need for new antibiotics to treat infections caused by these superbugs.

Ceftaroline (CPT), the active metabolite of the prodrug ceftaroline fosamil, is a novel last generation cephalosporin, with extended spectrum of activity against common Gram-negative and Gram-positive bacteria, notably MRSA. The anti-MRSA activity of CPT is explained by its distinct increased affinity for PBP2a, while maintaining high affinity for other essential *S. aureus* PBPs (3). It binds to the allosteric region of PBP2a causing a conformational change that facilitates binding of a second molecule of CPT to the active site, and blocking the enzyme activity (4-6). CPT has been found to retain activity against MRSA with reduced susceptibility to vancomycin, including heteroresistant VISA and daptomycin (7, 8). It has been approved by the Food and Drug Administration (FDA) for the treatment of Complicated skin and soft tissue infections (cSSTIs) and community-acquired pneumonia (CAP) (9, 10). Despite its recent approval, isolates with reduced susceptibility levels to CPT have been described worldwide (11-13). Resistance is mainly mediated by amino acid substitutions in PBP2a. While high level CPT resistance (MIC > 32 μg/ml) is linked to mutations in the transpeptidase active site domain (14), mutations in allosteric site of PBP2a or non-penicillin-binding domain (nPBD) have been found to confer low-level resistance to ceftaroline (MIC values of > 1-8 μg/ml) (4, 15). CLSI has recently (2019) re-evaluated ceftaroline breakpoints changing susceptible (S), intermediate (I), resistant (R) MIC breakpoints from ≤1, 2, ≥4 μg/ml to susceptible, susceptible dose-dependent (SDD), resistant MIC breakpoints of ≤1, 2-4, ≥8 μg/ml (16). On the other hand, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has established an MIC of ≤1 mg/l as the clinical breakpoint for CPT susceptibility.

There is limited reports regarding the activity of CPT on MRSA isolates from Iran. Therefore, in the current work we aimed to evaluate the *in vitro* activity of ceftaroline against MRSA isolates obtained from blood stream infections (BSIs). Also, the molecular types and *mecA* genes of isolates with reduced susceptibility to ceftaroline were further studied to identify the genetic relatedness of the isolates and PBP2a genetic alterations respectively.

## MATERIALS AND METHODS

**Bacterial isolates and antimicrobial susceptibility testing.** A total of 91 nonduplicated MRSA and three MSSA isolates obtained from separate inpatients with blood stream infections in two different provinces of Iran were included in this study (performed from September 2018 to August 2019). Identification of the isolates to species level was performed by conventional biochemical tests (17). Identification of MRSA isolates was performed by cefoxitin disks and detection of *mecA* gene using gene specific primers (MecA-F 5'-TGGCTCAGGTACTGCTATCCAC-3' and *mecA*-R 5'-AGTTCTGCAGTACCGGATTTGC-3') (18). Susceptibility of the isolates to CPT was tested using disk diffusion (DD) (disks containing 30 μg of CPT (Mast Co, Merseyside, UK)) and E-test MIC test strips (Liofilchem, Italy) containing concentration gradient range of CPT (0.016 to 256 mg/l) and interpreted according to the revised CLSI breakpoints (S≤1mg/l; SDD=2-4mg/l; R≥8mg/l for MIC method and S≥25 mm, SDD=20-24 mm, R≤19 for DD method). Here, we categorized SSD and R isolates as non-susceptible. As the confirmatory broth microdilution (BMD) method was not available in this study, performance characteristics of DD method was compared and categorized in relation to E-test method as the only available MIC method. Categorical agreement (CA), major errors (ME), very major errors (VME), and minor errors (MIE) were evaluated as follow: CA was defined as the number of tests with correct susceptibility categorization between two methods. A very major error was defined when isolates were categorized as susceptible by disk diffusion but resistant by E-test (false-susceptible results) and a ME was defined when isolates were resistant by disk diffusion but susceptible by E-test (false-resistance result). Minor errors (MIE) were defined as those in which either E-test or DD reported a result as intermediate (SDD) and the other method reported the result as resistant or susceptible (19).

Susceptibility of CPT non-susceptible isolates (SDD or R) to other classes of antibiotics (linezolid, ciprofloxacin, levofloxacin, gatifloxacin, tetracycline,

minocycline, chloramphenicol, sulfamethoxazole-trimethoprim (BBL Sensi-Disk™, Becton–Dickinson, Sparks, MD) and tigecycline (Mast Co, Merseyside, UK)) was determined by disk diffusion method (Kirby Bauer) according to the CLSI guidelines. Food and Drug Administration interpretative criteria were used to test tigecycline susceptibility by disk diffusion method (S≥19 mm).

**Identification of PBP2a mutations in MRSA isolates with reduced susceptibility to CPT.** Chromosomal DNA was extracted from CPT-nonsusceptible (CPT-NS) (SDD) isolates by the boiling method. The *mecA* gene was amplified using primers *mecA*-F1 (5'-AGTTGTAGTTGTCGGGTTTGG-3') and mecA-R1 (5'-CCGTTCTCATATAGCTCATCATAC-3'). The PCR products were sequenced and the nucleotides and deduced protein sequences were analyzed at the National Center for Biotechnology Information web site.

**Determination of molecular types of isolates by spa typing.** To investigate the genetic relatedness of CPT-NS isolates, molecular types of the isolates was determined using spa typing method. The x region of the *spa* gene was amplified by PCR with primers *spa*-1113f (5'-TAAAGACGATCCTTCGGTGAGC-3') and spa-1514r (5-CAGCAGTAGTGCCGTTTGCTT-3) (20). The sequences of PCR products were determined and analyzed by ChromasPro software. Isolates were assigned to particular *spa* types using the *spa* typing website (http://www.spaserver.ridom.de).

## RESULTS

The *in vitro* activity of CPT was tested on 94 *S. aureus* blood isolates (91 MRSA and 3 MSSA) obtained from patients in East Azerbaijan and Tehran provinces. Using the E-test method, 84 MRSA (92.3%) and all MSSA isolates were found to be CPT susceptible (MIC≤1mg/l). Only 7 MRSA isolates were characterized with MIC=2 mg/l and categorized as SDD. The CPT MIC distributions of the clinical isolates ranged from 0.1 mg/l to 2 mg/l (Table 1).

Compared to E-test, DD revealed a categorical agreement rate of 93.6% with no ME or VME. DD wrongly categorized 3 SDD isolates as susceptible and 3 susceptible isolates found by Etest as SDD (MIC=1mg/l) with diameter of inhibition zone being 24 mm (very close to susceptible breakpoint 25mm) which resulted in MIE error rate of 6.3%.

**Table 1.** Ceftaroline susceptibility testing results determined by E-test and disk diffusion methods

| Method | Number of isolates with MICs (mg/l) | | | | | | |
|---|---|---|---|---|---|---|---|
| **E-test** | **0.12** | **0.25** | **0.5** | **1** | **2** | **4** | **8≤** |
| MRSA | 14 | 15 | 25 | 30 | 7 | 0 | 0 |
| MSSA | 0 | 1 | 1 | 1 | 0 | 0 | 0 |
| Total | 14 | 16 | 26 | 31 | 7 | 0 | 0 |

| Disk diffusion | Number of isolates with specific inhibition zone (mm) | | | | | |
|---|---|---|---|---|---|---|
| | **29-31** | **26-28** | **25** | **23-24** | **20-22** | **19≥** |
| MRSA | 12 | 41 | 31 | 5 | 2 | 0 |
| MSSA | 1 | 1 | 1 | 0 | 0 | 0 |
| Total | 13 | 42 | 32 | 5 | 2 | 0 |

According to AST results, linezolid, tigecycline, minocycline and chloramphenicol were found to be the most active agents (resistance rate 0%) tested against CPT-nonsuscpetible MRSA isolates. Moreover, about 28.5% and 100% of isolates were resistant to sulfamethoxazole-trimethoprim and quinolones (CIP, LVX and GAT) respectively.

By using the *spa* typing method all 7 MRSA isolates with increased CPT MIC (2 mg/l) identified by Etest belonged to two different spa types including t030 which was the most frequent type being found in six isolates followed by t13927 (n=1). The nucleotide sequences of the *mecA* gene in CPT-NS MRSA isolates were studied to identify resistance conferring mutations. About 85.7% of isolates (n=6 out of 7) carried the previously reported N146K substitution in allosteric domain of PBP2a protein. However, one isolate belonged to spa type t030 lacked any genetic alteration in the *mecA* gene indicating that mutations in other loci may contributed to reduced CPT susceptibility in this isolate (Table 2).

## DISCUSSION

MRSA remains a major healthcare problem worldwide being responsible as difficulties in eradication of infections. CPT is a recently introduced cephalosporin with potent activity against MRSA. In the current study we tested the *in vitro* activity of CPT against a series of MRSA and MSSA isolates obtained from BSIs using E-test and DD methods.

**Table 2.** Characteristics of clinical MRSA strains with reduced susceptibility to ceftaroline

| Isolate | CPT MIC (mg/L) | CPT disk (mm)[a] | *spa* type | PBP2a | AST | | |
|---|---|---|---|---|---|---|---|
| | | | | | S | I | R |
| 256R | 2 | 22 | t030 | WT | LZD,TGC, MI, C | - | CIP, LVX, GAT, SXT |
| 257R | 2 | 25 | t030 | N146K | LZD,TGC, MI, C, SXT | - | CIP, LVX, GAT |
| 200R | 2 | 24 | t030 | N146K | LZD,TGC, MI, C, SXT | - | CIP, LVX, GAT |
| 178R | 2 | 27 | t030 | N146K | LZD,TGC, MI, C, SXT | - | CIP, LVX, GAT |
| 242R | 2 | 23 | t030 | N146K | LZD,TGC, C | MI | CIP, LVX, GAT, SXT |
| 239R | 2 | 25 | t13927 | N146K | LZD, TGC, MI, C, SXT | - | CIP, LVX, GAT |
| 253R | 2 | 22 | t030 | N146K | LZD,TGC, MI, C, SXT | - | CIP, LVX, GAT |

AST, Antimicrobial susceptibility testing; S, susceptible; I, intermediate; R, resistant;
LZD, linezolid; CIP, ciprofloxacin; LVX, levofloxacin; SXT, trimethoprim/sulphamethoxazole; C, chloramphenicol; GAT, gatifloxacin; TGC, tigecycline; MI, minocycline

[a] inhibition zone diameter

Agar diffusion methods (E-tests and DD) remain the most common procedures used in many clinical microbiology laboratories for testing susceptibility to CPT in conditions when an automated method is not available or performing a BMD test is impractical (21). A study from Australia found reasonably good MIC correlation between BMD and E-test with all CPT-NS MRSA isolates (with MIC=2 mg/l) being correctly detected by E-test (22). Canton et al. assessed the performance characteristics of E-test versus BMD for testing susceptibility of *S. ausreus* isolates to CPT and found a good concordance between two methods with EA and CA being >97% and >95% respectively. However E-test underestimated MICs relative to BMD, for some MRSA isolates with increased CPT MICs (23). On the other hand, CPT MICE strip results have been reported by other study to be 1 dilution higher than BMD results in 34% of tested MRSA bacteremia isolates (24).

Overall 92.5% of *S. aureus* isolates studied in this work (including 92.3% of MRSA isolates) were susceptible to CPT. Only 7 MRSA isolates were categorized as SDD according to revised CPT breakpoint issued by CLSI 2019. A recent study from Iran reported a CPT susceptibility rate of 97.3% among MRSA isolates as determined by disc diffusion method (25). A 12% ceftobiprole resistance rate (12/102 isolates) was observed among MRSA isolates from an Italian hospital collected in 2017–2018 time period (26). Evaluation of CPT susceptibility among 421 MRSA isolates from a hospital in Australia by E-test revealed a non-susceptibility rate (MIC>1mg/L) of 16.9% (27). Also the CPT resistance rate observed among MRSA isolated from CAP cases obtained from the Asia-Pacific region and South Africa was found to be 19.4% (28). We also studied the performance characteristics of DD method compared to E-test as DD has been recommended by EUCAST and CLSI and it is suitable for countries with limited resources to perform the ideal standardized test or a reliable commercial microdilution assay. An inhibition zone diameter of 22-31 mm was observed among the isolates with the majority being characterized with a diameter of 26-28 mm. DD revealed an acceptable CA rate (≥90%) compared to E-test. Among the three different types of errors a MIE of 6.3% was estimated for DD with the other two types of errors (VME and ME) being 0%. A more accurate error rate could be determined if CPT resistant isolates with MIC≥8 mg/l were present among the studied isolates.

The molecular typing of MRSA isolates with reduced CPT susceptibility by *spa* typing classified 7 isolates in to two different *spa* types including t030 (the most frequent type) and t13927. The CPT-NS MRSA isolates in a recent study from Iran were found to belong to *spa* types t030, t4864, and t969, respectively (25). The type t030 has been frequently found as the most common *spa* type among MRSA isolates in several studies from Iran (29-31). In a study from Belgium the 4 CPT-NS MRSA isolates (MIC=2mg/L) belonged to *spa* types t002, t032, t037 and t14057 (11). Moreover, CPT resistant MRSA isolates (n=12) in a study from Italy were found to belong to *spa* types t041, t18014, t1476, t5948 and t002 (26). Analyses of PBP2a sequence revealed single N146K substitution in 6 out of 7 MRSA isolates with

increased CPT MICs. This alteration is located within the allosteric domain of PBP2a and has been previously detected as single mutation or in combination with other substitutions in MRSA isolates with reduced CPT susceptibility (4, 6, 32-34). Other reported PBP2a amino acid substitutions identified in allosteric domain include E239K, E150K and G246E (6, 32). Binding of ceftaroline to this allosteric site causes a conformational change in the PBP2a, leading to the opening of the active site and binding of a second CPT to the opened active site and subsequent inhibiting of the target (35). It has been demonstrated that mutations located in the allosteric domain of PBP2a interfere with allosteric trigger by ceftaroline and results in increased MICs of CPT (35). One CPT-NS MRSA isolate lacked any amino acid substitutions in the *mecA* gene indicating that alterations in other genetic loci may contribute to increased CPT MIC in this isolate. Mutations in genes encoding PBP4 and GdpP signaling protein seem to be particularly important as they have been identified in some CPT-NS clinical MRSA or *in vitro* selected CPT or ceftobiprole resistant mutants (33, 36, 37).

## CONCLUSION

In summary we found increased CPT MICs in some of the studied MRSA isolates. Although CPT resistance (MIC≥8 mg/L) was not found in the current study, there is always a potential for these SDD isolates to become fully CPT resistant. While PBP2a allosteric site mutations were detected in most isolates with increased CPT MICs, one isolate carried a wild-type *mecA* indicating that secondary chromosomal mutations (such as those in PBP4 or GdpP) are likely to be involved in increased CPT MICs. The role of these newly described chromosomally mediated resistance mutations in MRSA needs further investigation. In general, prudent use of fifth-generation cephalosporins should be practiced and bacterial susceptibility should be carefully monitored during the course of treatment to prevent the emergence and dissemination of CPT resistant isolates.

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