In vitro activity of ceftaroline against methicillin-resistant *Staphylococcus aureus* isolates from blood and complicated skin and soft tissue infections and update on the EUCAST breakpoints

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Summary

Ceftaroline fosamil is a novel cephalosporin active against methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Streptococcus pneumoniae* (MDRSpn). The aim of the present study was the evaluation of the compound against MRSA isolates from blood and complicated skin and skin structure infections (CSSSIs), the comparison of the current CLSI and EUCAST MIC interpretation guidelines, the evaluation of the disk-diffusion method for susceptibility testing,

and the investigation of the underlying mechanisms in borderline and/or resistant isolates.
A total of 329 MRSA isolates were included in the study. Ceftaroline MIC range was 0.064 – 4 mg/L. MIC_{50} and MIC_{90} were 0.5 and 1 mg/L, respectively. Three isolates exhibited MIC = 4 mg/L, and were considered as resistant using the CLSI guidelines M100 28th Ed., whilst 15 more isolates exhibited MIC = 2 mg/L and were considered as intermediate. All these 18 isolates were categorized as resistant using the EUCAST guidelines v7.1, but using the updated EUCAST guidelines v8.0, the categorization fell in accordance with the CLSI ones. Disk diffusion (DD), according to EUCAST v7.1, resulted in very major errors at 0.3%, and major errors at 5.2% (among isolates exhibiting MIC = 1 mg/L); using the updated guidelines v8.0 the major errors rate decreased to 1.5%, but were replaced by minor errors at 3.0% rate. SCCmec typing revealed mainly the $N_{146}K$, $E_{150}K$ and $H_{351}N$ substitutions.
Ceftaroline has an excellent *in vitro* activity against MRSA isolates from blood and/or CSSSI. The updated EUCAST guidelines amended the discrepancies that were detected regarding the categorization of isolates exhibiting MIC = 2 mg/L and the DD major errors.

Key words
Ceftaroline, Staphylococcus aureus, Minimum Inhibitory Concentration, EUCAST, CLSI

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Introduction

Ceftaroline fosamil is the pro-drug of the active metabolite ceftaroline, a new, parenteral, broad-spectrum, bactericidal cephalosporin. Its spectrum of activity against mainly wild-type Gram-negative bacteria closely resembles that of cefotaxime and ceftriaxone;[1,2] nevertheless, its main advantage is its activity against resistant Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Streptococcus pneumoniae* (MDRSpn).

More specifically, anti-MRSA activity comes as a consequence of high binding affinity to penicillin-binding protein (PBP) 2a, whereas anti-MDRSpn activity comes as a consequence of high binding affinity mainly to PBP2x.[1] In that respect, the compound has been approved for the treatment of community-acquired bacterial pneumonia (CABP) and complicated skin and skin structure infections (CSSSIs); in addition, due to the combination of anti-Gram-positive and anti-Gram-negative activity, ceftaroline is considered to be an effective option for monotherapy.

Resistance to ceftaroline, among Gram-positive pathogens is scarce and only a limited number of resistant MRSA isolates have been reported.[3,4] Nevertheless, there are differences between CLSI[5] and EUCAST guidelines[6] regarding ceftaroline MIC interpretation. CLSI document M100 28th Ed.[5] specified breakpoints of S ≤ 1, intermediate = 2, and R ≥ 4 mg/L, whereas the respective EUCAST document v7.1, valid until 31/12/2017,[6] specified breakpoints of S ≤ 1 and R > 1 mg/L. In that respect, isolates exhibiting MIC=2 mg/L have been categorized as intermediate by CLSI and resistant by EUCAST. Recently, EUCAST has updated its guidelines and differentiated the ceftaroline bre-

akpoints and interpretation of isolates from blood and CSSSI cases from those from pneumonia cases.[7] These differences have never been investigated before comparatively and the number of isolates that produce discrepancies in the categorization process is not known.

The aim of the present study was the evaluation of the activity of ceftaroline against MRSA isolates from blood and CSSSIs, the comparison of the MIC interpretation results using the different international guidelines, the evaluation of the disk-diffusion method for susceptibility testing as compared to MIC determination, and finally the investigation of the underlying mechanisms in borderline and/or resistant isolates.

Materials and methods

A total of 329 MRSA strains, isolated from blood and CSSSI specimens of hospitalized patients in "Laikon" General Hospital, Athens, Greece, during 2012-2015 were included in the study. All isolates were one-per-patient (the first isolate only) and consecutively collected.

CSSSI cultures were performed on 5% sheep blood agar plates (Bioprepare, Gerakas, Greece) and incubated for 72 h at 37° C, 5% CO_2 conditions. Blood cultures were performed using the BacT/ALERT 3D automated instrument (bioMerieux, Marcy L' Etoile, France), followed by subculture of all positive bottles on 5% sheep blood agar plates, incubated for 72 h at 37° C, 5% CO_2 conditions. Genus and species identification was performed using standard microbiological methodology (Gram stain, colony morphology, positive catalase and coagulase tests) supplemented with the MicroScan autoSCAN-4 System (Leriva SA, 15127, Melissia, Greece).

For all antibiotics except ceftaroline (penicillin, oxacillin, cefoxitin, vancomycin, erythromycin, ciprofloxacin, moxifloxacin, gentamicin, linezolid and daptomycin) susceptibility testing was performed using the disk-diffusion method (DD) on Mueller-Hinton agar plates, incubated for 24h at 37° C, and interpreted according to EUCAST guidelines.[6,7] Minimum Inhibitory Concentrations (MIC) were determined using the microdilution method and the MicroScan autoSCAN-4 System (Leriva SA, 15127, Melissia, Greece), according to the manufacturer's instructions. Interpretation of the MIC results was comparatively performed using the EUCAST[6,7] and the CLSI guidelines.[5] Cefoxitin was tested for methicillin-resistance confirmation only.

Regarding ceftaroline, susceptibility was performed using the disk-diffusion method and 5 μg antibiotic-impregnated disks, on Mueller-Hinton agar plates, incubated for 24h at 37° C, and interpreted according to EUCAST guidelines.[6,7] Ceftaroline MIC was determined using the agar dilution method, on Mueller-Hinton agar plates supplemented with ceftaroline at concentrations of 0.016 to 256 mg/L, using a multiple inoculator (Mast Group Ltd, England). Interpretation of the MIC results was performed comparatively using the EUCAST [6,7] and the CLSI guidelines.[5]

S. aureus ATCC29213 was used as control strain throughout the study.

Isolates with MIC ≥ 2 mg/L were tested by mecA sequencing and SCCmec I-IV type identification, according to previously published protocols.[3]

Results

A total of 329 MRSA isolates were included in the study. Positive blood cultures were the source of 64 isolates, whereas the remaining 265 ones derived from CSSSI cultures. Methicillin resistance was confirmed for all isolates using the cefoxitin disk test.

Ceftaroline MIC range was 0.064 – 4 mg/L; MIC_{50} and MIC_{90} were 0.5 and 1 mg/L, respectively. A total of three isolates exhibited MIC = 4 mg/L, whereas 15 more isolates exhibited MIC = 2 mg/L. The limited number of non-susceptible isolates did not allow us to investigate for differences between isolates from blood and CSSSI cases. Representative agar dilution plates are shown in Figure 1.

Using the EUCAST v7.1 MIC guidelines[6] (S ≤ 1 mg/L, R > 1 mg/L), a total of 18 isolates (5.5%) were categorized as resistant (all those exhibiting MIC = 2 or 4 mg/L). Using the CLSI M100 28th Ed. MIC guidelines[5] (S ≤ 1 mg/L, intermediate = 2 mg/L, R ≥ 4 mg/L), three isolates (0.9%) were considered resistant (those exhibiting MIC = 4 mg/L), whereas 15 more isolates were considered intermediate (those exhibiting MIC = 2 mg/L). Using the updated EUCAST v8.0 guidelines[7] (S ≤ 1 mg/L, R > 2 mg/L) the categorization fell in accordance with the CLSI ones. Comparison of the guidelines is shown in Figures 2A, 2B and 2C.

Disk diffusion (DD) results, as compared to MIC determination, using the EUCAST v7.1 guidelines,[6] showed that DD very major errors (MIC resistant isolates exhibiting false DD susceptibility) were 0.3%, DD major errors (MIC susceptible isolates exhibiting false DD resistance) were 5.2%, whilst no DD minor errors were detected (MIC susceptible or resistant isolate exhibiting false DD intermediate susceptibility). All DD major errors were among isolates exhibiting MIC = 1 mg/L. The distribution graphs are shown in Figures 3A and 3B; the MIC distribution peak is on the susceptible

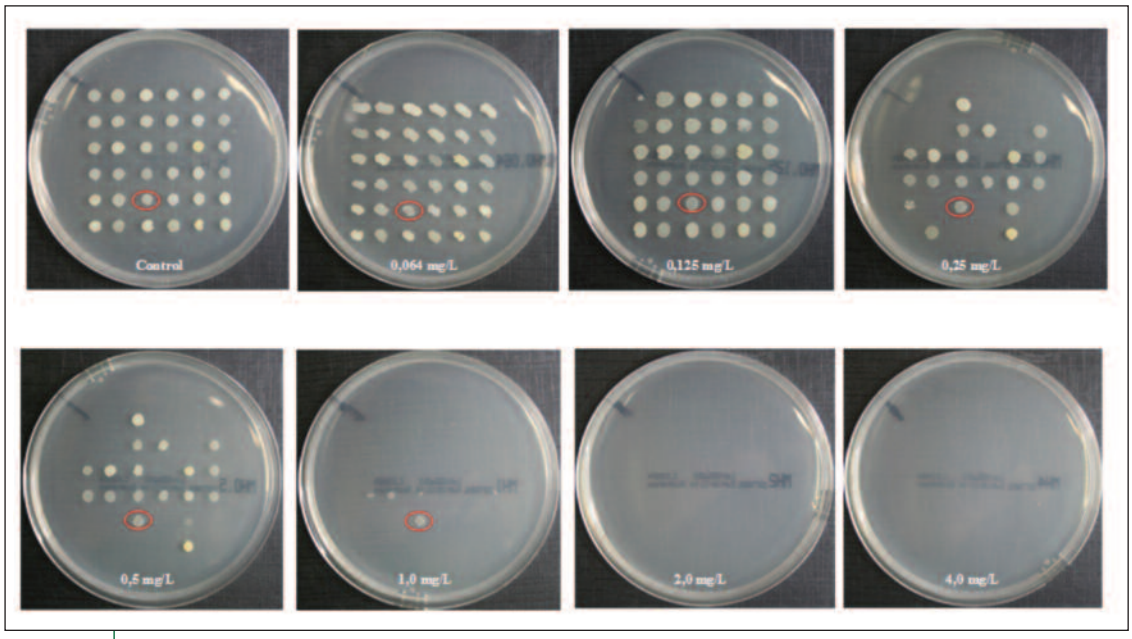
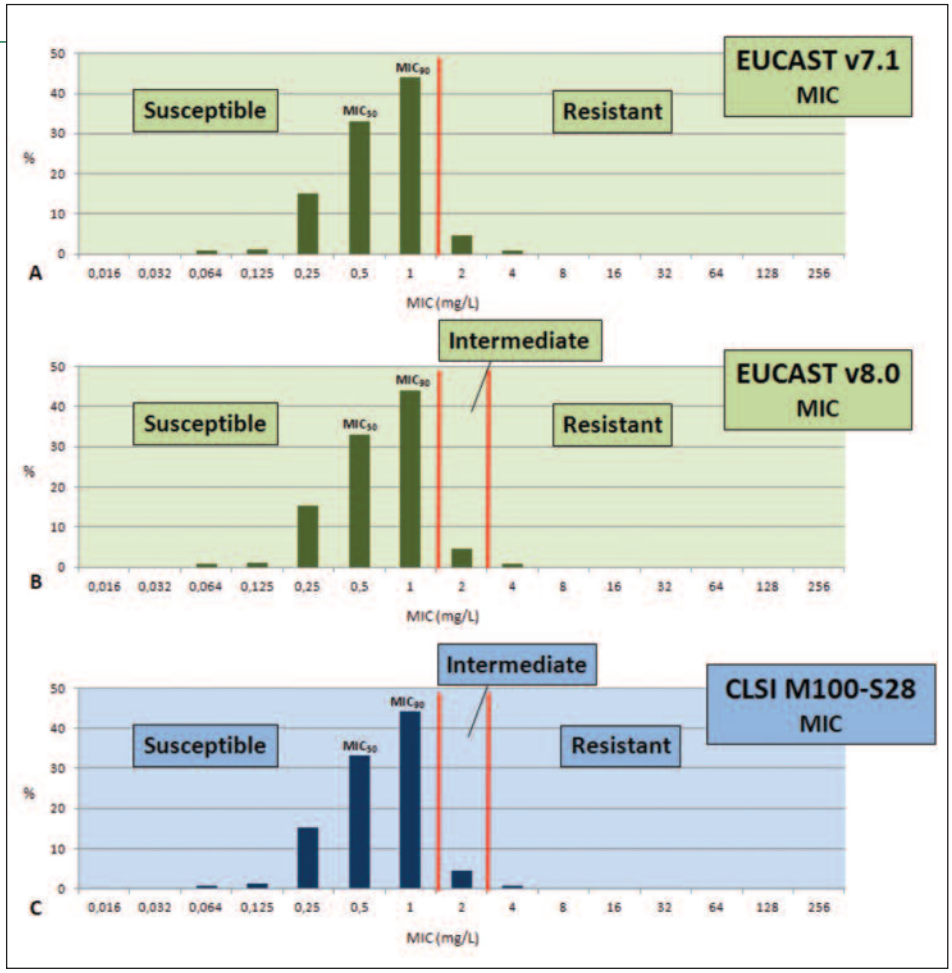


Figure 1 Representative agar dilution MIC plates. The isolate in line E, third column (E3, indicated in the red circle) exhibits a MIC = 2 mg/L.

Figure 2

Comparison of ceftarolin MIC interpretation according to EUCAST v7.1 (2A), EUCAST v8.0 (2B) and CLSI M100 28th Ed. (2C) guidelines. Red lines indicate the breakpoints.



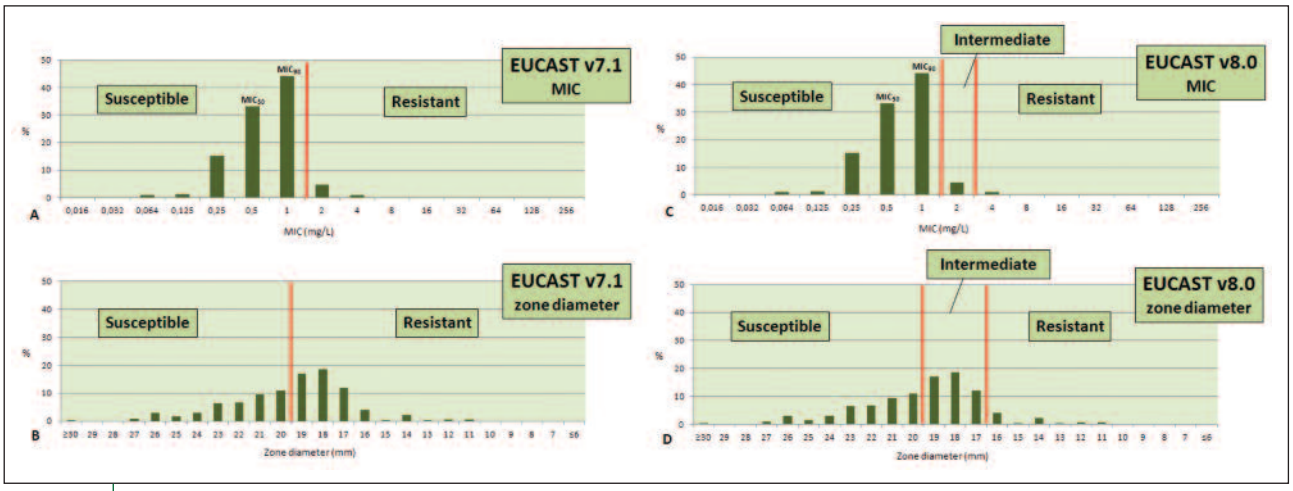


Figure 3 Comparison of MIC and disk diffusion distributions, and interpretation according to EUCAST v7.1 (3A, 3B) and v8.0 (3C and 3D) guidelines. Red lines indicate the breakpoints.

side of the graph, whereas the disk diameter distribution peak (17-19 mm) is on the resistant side of the graph, thus explaining the DD major errors observation. Use of the updated EUCAST v8.0 guidelines[7] resulted in decrease of the major errors rate to 1.5%, but with subsequent replacement by minor errors at 3.0%, as the distribution peak (17-19 mm) was moved to the intermediate range of the graph (Figures 3C and 3D).

SCCmec typing among the three isolates exhibiting MIC = 4 mg/L (Group A) and the 15 ones exhibiting MIC = 2 mg/L (Group B) revealed that all group A isolates harbored the type IIIA SCCmec, as well as the $N_{146}K$ and the $E_{150}K$ substitutions in the non-penicillin-binding domain and the $H_{351}N$ one in the transpeptidase domain. In contrast, group B isolates harbored types II, IIIA and IV SCCmec; six of the isolates harbored the $N_{146}K$ substitution, whilst five additional isolates harbored both $N_{146}K$ and $E_{150}K$ substitutions in the non-penicillin-binding domain.

Discussion

MRSA is a serious challenge, both in hospitals and the community. Vancomycin remains the major alternative for severe infections, although elevated vancomycin MICs and nephrotoxicity are major concerns.[8] Other antimicrobials being used are daptomycin and/or linezolid, both of which also may exhibit major side effects.

The new beta-lactam agent, ceftaroline fosamil, has demonstrated clinical success as an alternative to these agents and has been approved for severe MRSA CSSSIs.[9-11] Recommended dosing is 600 mg twice a day, although in severe infections administration every 8 hours may facilitate better results.[8-12]

Resistant isolates are scarce[4,8] and the relevant mechanisms are related to substitutions in PBP2a.[3,4] MICs of non-susceptible and/or resistant isolates are within the range of 2-8 mg/L and higher MICs are practically non-existing. Although isolates exhibiting MIC ≤ 1 mg/L are unanimously considered as susceptible, guidelines were controversial regarding isolates exhibiting MIC=2 mg/L, at least until 31/12/2017. EUCAST v7.1 (valid until 31/12/2017) and CLSI M100 28th Ed. documents categorized these isolates differently, a situation which has been questioned in the literature.

The present study indicated that ceftaroline has excellent *in vitro* activity against the collection of MRSA isolates from blood and CSSSI tested, and the MIC_{90} was within the susceptible range of all guidelines used, thus confirming previous results regarding the activity of ceftaroline against MRSA isolates from various sources.[8,10,11]

Only three isolates exhibited elevated MICs (4 mg/L, designated group A) and were all categorized clearly as resistant using all available guidelines. Nevertheless, discrepancies were noted regarding the 15 group B isolates (MIC = 2 mg/L), which were categorized as intermediated using CLSI M100 28th Ed.,[5] but resistant using EUCAST v7.1.[6]

When taking into account comparatively the MICs and the molecular typing results, a clear association was noted between all three group A isolates and known molecular mechanisms of resistance. In that respect, our observations confirmed previous studies[4]

which have described the molecular mechanisms of isolates exhibiting MIC ≥ 4 mg/L and categorized these isolates in the resistant side of the scatterplot.

Resistance to ceftaroline is conferred due to mutations at the *mec*A gene, thus disrupting the allosteric mechanism in various PBP2a sites near or in the transpeptidase domain.[10,11] The resistance is being built in steps, where the first-step is a change in the non-penicillin-binding domain (substitutions such as $N_{146}K$, or $E_{150}K$, among others), whilst the second step is a change in the penicillin-binding transpeptidase domain (usually substitution $H_{351}N$).[4] These changes have been proved to increase the ceftaroline MIC up to 8 mg/L.

Isolates exhibiting MIC = 2 mg/L have been described to harbor certain of these alterations, mainly the $N_{1546}K$ and/or the $E_{150}K$, usually in the non-penicillin-binding domain, although results are controversial on the actual presence, the number, the exact alterations and the combinations present.[4] In our study, six of the isolates were found to harbor the $N_{146}K$ substitution, whilst five additional isolates harbored both the $N_{146}K$ and the $E_{150}K$ ones. However, in the remaining four isolates no known alterations were detected, thus confirming previous observations about non-susceptible isolates that do not possess these alterations.[4]

Similar observations in the literature have led EUCAST to differentiate and update the breakpoints in the current guidelines,[7] valid from 1/1/2018. In that respect, for non-pneumonia *S. aureus* isolates (blood and CSSSI), the new EUCAST MIC breakpoints are S ≤ 1 and R > 2 mg/L, thus adding an intermediate zone for isolates exhibiting MIC = 2 mg/L, although EUCAST guidelines do not clearly specify intermediate zones for any species/antibacterial agent combination.[7] Regarding *S. aureus* isolates from pneumonia cases, the previous breakpoints are still valid, until further update.[6,7]

The DD method proved a reliable procedure, and using the EUCAST v7.1 guidelines (R < 20 and S ≥ 20 mm),[6] very major errors were extremely scarce and major errors were limited and solely among isolates having a borderline susceptibility of 1 mg/L. This was very clearly presented in Figure 3, where the peak of the MIC distribution curve was on 1 mg/L (susceptible), whilst the peak on the disk diameter distribution curve was on the 18-19 mm area (resistance), showing that isolates having MIC = 1 mg/L may result in DD results within the resistance zone (18-19 mm) of these guidelines. Nevertheless, the recent change in the EUCAST DD guidelines for blood and CSSSI isolates (R < 17 and S ≥ 20 mm) amended this situation also, and resulted in a substantial decrease of the major errors rate,[7] but with a subsequent increase of the minor errors, indicating that still the issue of DD misinterpretation of those isolates exhibiting MIC = 1 mg/L has not been entirely solved.

In conclusion, ceftaroline proved to be very active against our collection of MRSA isolates. The mechanism of resistance among non-susceptible isolates is substitutions in the *mec*A gene. The updated EUCAST guidelines amended the categorization discrepancies that were noted using the previous versions. DD is a reliable method and the number of errors (very major, major or minors) is limited.

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Περίληψη

In vitro δραστικότητα της κεφταρολίνης έναντι ανθεκτικών στην μεθικιλλίνη στελεχών *Staphylococcus aureus* από μικροβιαιμίες και επιπεπλεγμένες λοιμώξεις δέρματος και μαλακών μορίων και ενημέρωση σχετικά με τα νέα όρια ερμηνείας της EUCAST

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Η φοσαμιλική κεφταρολίνη είναι μια νέα κεφαλοσπορίνη δραστική έναντι στελεχών *Staphylococcus aureus* ανθεκτικών στην μεθικιλλίνη (MRSA) και πολυανθεκτικών στελεχών *Streptococcus pneumoniae* (MDRSpn). Ο στόχος της μελέτης είναι ο έλεγχος της δραστικότητας του σκευάσματος έναντι στελεχών MRSA από καλλιέργειες αίματος και επιπλεγμένων λοιμώξεων δέρματος και μαλακών μορίων (CSSSI), η σύγκριση των τρεχόντων οδηγιών ερμηνείας CLSI και EUCAST, η αξιολόγηση της μεθόδου διάχυσης του δίσκου για τον έλεγχο ευαισθησίας και η διερεύνηση των υποκείμενων μηχανισμών στα οριακά και / ή ανθεκτικά στελέχη.

Συνολικά 329 στελέχη MRSA ενσωματώθηκαν στην μελέτη. Το εύρος των τιμών της Ελάχιστης Ανασταλτικής Πυκνότητας (ΕΑΠ) της κεφταρολίνης ήταν 0,064-4 mg/L. Οι τιμές $ΕΠΑ_{50}$ και $ΕΑΠ_{90}$ ήταν 0,5 και 1 mg/L, αντίστοιχα. Τρία στελέχη είχαν ΕΑΠ = 4 mg/L και αξιολογήθηκαν ως ανθεκτικά, χρησιμοποιώντας τις κατευθυντήριες οδηγίες CLSI M100 28th Ed., ενώ 15 στελέχη είχαν ΕΑΠ = 2 mg/L και κατηγοριοποιήθηκαν ως ενδιάμεσα. Τα συγκεκριμένα 18 στελέχη αξιολογήθηκαν ως ανθεκτικά χρησιμοποιώντας της κατευθυντήριες οδηγίες EUCAST v7.1, όμως με την χρήση των ανανεωμένων οδηγιών EUCAST v8.0 η κατηγοριοποίηση συμφώνησε με εκείνη των οδηγιών της CLSI. Η μέθοδος διάχυσης δίσκων (ΔΔ), με την χρήση των οδηγιών EUCAST v7.1 παρουσίασε πολύ μεγάλα σφάλματα σε ποσοστό 0,3% και μεγάλα σφάλματα σε ποσοστό 5,2% (σε στελέχη με ΕΑΠ = 1 mg/L), όμως με τις αναθεωρημένες οδηγίες EUCAST v8.0 τα μεγάλα σφάλματα μειώθηκαν στο 1,5% και αντικαταστάθηκαν από μικρά σφάλματα σε ποσοστό 3,0%. Η τυποποίηση του γονιδιακού τόπου SCCmec αποκάλυψε κυρίως τις υποκαταστάσεις $N_{146}K$, $E_{150}K$ και $H_{351}N$.

Η κεφταρολίνη παρουσίασε εξαιρετική *in vitro* δραστικότητα έναντι στελεχών MRSA από αίμα ή/και CSSSI. Οι αναθεωρημένες κατευθυντήριες οδηγίες της EUCAST διόρθωσαν τις αναντιστοιχίες που είχαν παρατηρηθεί σε σχέση με την κατηγοριοποίηση στελεχών με ΕΑΠ = 2 mg/L, καθώς και τα μεγάλα σφάλματα που είχαν παρατηρηθεί στην μέθοδο ΔΔ.

Λέξεις κλειδιά

Κεφταρολίνη, Staphylococcus aureus, Ελάχιστη Ανασταλτική Πυκνότητα, EUCAST, CLSI.

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