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CONSEQUENCES OF THE INOCULUM EFFECT AGAINST B-LACTAMS IN METHICILLIN-SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* (MSSA) STRAINS

METİSİLİNE DUYARLI *STAPHYLOCOCCUS AUREUS* (MSSA) SUŞLARININ β-LAKTAMLARA KARŞI İNOKULÜM ETKİSİ GÖSTERMESİNİN SONUÇLARI

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Abstract

Objective: Some staphylococcal β-lactamases could destroy β-lactams other than penicillin, including cefazolin (Cz) and ampicillin-sulbactam (SAM), especially in the presence of higher inoculum, which is called the inoculum effect (InE). We investigated the incidence and clinical implications of InE against different β-lactams including in *S.aureus* (MSSA) strains isolated from bacteremic patients.

Material and Methods: Patients with MSSA bacteraemia were included. MSSA strains were tested with the disk diffusion and broth microdilution method at standard and high inoculum. The presence and type of β-lactamases were confirmed by PCR plus DNA sequencing. InE was defined as a 4-fold or greater increase in minimal inhibitor concentration (MIC) values at high inoculum. Patient data were obtained retrospectively.

Results: Among the 52 MSSA strains, 85% were Type A β-lactamase positive. Within these strains, 25%, 38.5%, and 3.8% showed InE against Cz, SAM, and ceftriaxone, respectively. The mortality rate in patients infected with MSSA strains showing SAMInE and treated with SAM was significantly higher than in those not treated with SAM (OR 7.8; 95% CI, 1.23-49.68, p=0.044).

Conclusion: SAM was the most affected β-lactam by type A β-lactamase of MSSA strains, followed by Cz, and this effect became more prominent with higher inoculum. SAM treatment of the patients infected with SAMInE strains was significantly associated with mortality.

Öz

Amaç: Bazı stafilokoksik β-laktamazlar, özellikle daha yüksek inokulum varlığında, sefazolin (Cz) ve ampisilin-sulbaktam (SAM) dahil olmak üzere penisilin dışındaki β-laktamları parçayalabilir ki buna inokulum etkisi (InE) denir. Bu çalışmada bakteriyemik hastalardan izole edilen *S.aureus*(MSSA) suşlarında farklı β-laktamlara karşı InE'nin insidansı ve klinik etkileri araştırılmıştır.

Gereç ve Yöntemler: Çalışmaya MSSA bakteriyemisi olan hastalar dahil edildi. MSSA suşları, standart ve yüksek inokulumda disk difüzyon ve sıvı mikrodilüsyon yöntemi ile test edildi. β-laktamazların varlığı ve tipi PCR artı DNA dizilemesi ile doğrulandı. InE, yüksek inokulumda minimal inhibtör konsantrasyon (MİK) değerlerinde 4 kat veya daha fazla artış olarak tanımlandı. Hasta verileri retrospektif olarak elde edildi.

Bulgular: Elli iki MSSA suşunun %85'i Tip A β-laktamaz pozitifti. Suşların %25'i, %38,5'i ve %3,8'i sırasıyla Cz, SAM ve seftriaksona karşı InE gösterdi. SAMInE gösteren MSSA suşlarıyla infekte olmuş ve SAM ile tedavi edilen hastalardaki ölüm oranı, SAM ile tedavi edilmeyenlere göre önemli ölçüde daha yüksekti (OR=7,8; 95% CI, 1,23-49,68, p=0,044).

Sonuç: MSSA suşlarının tip A β-laktamazı tarafından en çok etkilenen β-laktamın SAM olduğu, bunu Cz'nin takip ettiği ve bu etkinin daha yüksek inokulumla daha belirgin hale geldiği görülmüştür. SAMInE gösteren MSSA suşlarıyla infekte olmuş bakteriyemili hastaların SAM'la tedavisi ölüm oranını artırabilir.

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tients infected with the MSSA strains showing SAMInE may increase mortality.

Keywords Ampicillin-sulbactam · bacteraemia · cefazolin · inoculum effect · MSSA · *Staphylococcus aureus*

Anahtar Kelimeler Ampisilin-sulbaktam · bakteriyemi · sefazolin · inokulum etkisi · MSSA · *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is one of the most important causative agents of community and hospital-acquired bacteraemia and endocarditis worldwide. In recent years, there has been an increase in the incidence of community-acquired and hospital-acquired *S. aureus* bacteraemia (SAB) due to the ageing of the population, as well as increases in intravascular device usage and surgical procedures. SAB is associated with high morbidity, mortality, and healthcare costs (1).

Approximately 90% of methicillin-sensitive *S. aureus* (MSSA) strains are resistant to penicillin G (PG), which is related to the production of β-lactamase called penicillinase. Four types of staphylococcal β-lactamases exist, including type A, B, C, and D. Anti-staphylococcal penicillins (ASPs) such as nafcillin and oxacillin have been recommended as first-line antibiotics for the treatment of MSSA bacteraemia for years (2). However, after the publication of several recent studies showing that cefazolin (Cz) could be more effective but less nephrotoxic than ASPs, Cz became one of the first choice agents for the treatment of MSSA bacteraemia and other deep-seated MSSA infections, including bone and joint infections, deep-seated abscesses, osteomyelitis or pneumonia (3). Additionally, Cz and ampicillin-sulbactam (SAM) have been used as the first-choice agents for treating MSSA infections for years in countries where ASPs were unavailable, such as Türkiye, Argentina, and Japan.

The amount of the bacterial inoculum could decrease the in vitro effect of some β-lactam agents used to treat MSSA infections, which is called the "inoculum effect" (InE). The presence of InE and its clinical impact was most studied for Cz; while some studies have suggested that treatment failure may occur with Cz therapy of deep-seated MSSA infections caused by the strains showing Cz IE, some have reported no such effect. Although several studies reported that the activity of ASPs was not unaffected by the high inoculum of MSSA strains, other agents used to treat MSSA, including SAM, have not been adequately studied for this effect (2). We aimed to investigate the incidence, risk factors, simple definition methods, and clinical implication of InE against different β-lactams, including SAM and Cz, in MSSA strains isolated from patients with bacteraemia.

MATERIAL AND METHODS

All patients admitted to İstanbul University, İstanbul Faculty of Medicine between 2016 and 2018 and diagnosed with MSSA bacteraemia, with MSSA growth in blood culture and available stored strains, were included in the study. We recorded the clinical and laboratory information of each patient on the pre-prepared forms.

MSSA isolates were identified by classical methods. Disk diffusion susceptibility tests for penicillin G, cefoxitin, cefazolin, linezolid, clindamycin, erythromycin, trimethoprim-sulfamethoxazole, ciprofloxacin, fusidic acid, rifampicin, gentamicin, vancomycin, and teicoplanin were performed using the method defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) at standard (10^5 CFU/µl) and high (10^7 CFU/µl) inoculum and breakpoints of EUCAST were used for the interpretation of the results if available (4). Minimal inhibitor concentration (MIC) of Cz, SAM, cefuroxime, cefotaxime, and ceftriaxone for the MSSA strains were determined at standard and high inocula using the broth microdilution (BMD) method defined by the Clinical and Laboratory Standards Institute (CLSI) (5). After the MIC values of the control MSSA strain for SAM were not found within valid ranges using the microdilution method, the MIC values were re-determined using the SAM Etest®. A 4-fold or more significant increase in MIC values at high inoculum concentrations was defined as an InE (2), and if a susceptible strain at the standard inoculum became resistant at higher inoculum, it was described as "pronounced InE" (6).

The S. aureus ATCC 29213 strain, known to produce type A β-lactamase *(blaZ)*, and the *blaZ* gene-negative *S.aureus* ATCC 25923 strain were used as control strains.

The presence of β-lactamases in the isolates was qualitatively detected with the nitrocefin disc assay, PG disc diffusion method, PG edge test and then confirmed with PCR and Sanger sequencing (6).

The β-lactamase gene was identified using the primers F, 5'-CAA AGA TGA TAT AGT TGC TTA TTC-3′ and R, 5'-CAT ATG TTA TTG CTT GCA CCA C-3′ designed to amplify a 355-bp region within the *blaZ* gene structure. The amount of PCR products was confirmed by gel electrophoresis for all of the MSSA strains and then analysed by DNA sequencing (6).

DNA sequencing was performed using the Sanger method and the Applied Biosystem Abi3500 device. The BLAST network

service of the National Library of Medicine, National Centre for Biotechnology Information (NLM, NCBI) database was used to define the sequences. Classification of the β-lactamase of each strain relied on the amino acid content of the 128 and 216 residues encoded by *blaz*, which were threonine and serine, lysine and asparagine, threonine and asparagine, and alanine and serine, respectively, for bla type A, B, C, and D (6).

The study data was analysed using IBM SPSS Statistics for Windows, Version 21.0 (Statistical Package for the Social Sciences, IBM Corp., Armonk, NY, USA). For the analysis of continuous variables, the Student's t-test was used when the distribution was normal, and the Mann-Whitney U test was used when the distribution was abnormal; for the analysis of categorical variables, the χ2 test or Fisher's exact test was utilised. The risk was presented as the odds ratio (OR). A p-value <0.05 was considered statistically significant.

This study was conducted in line with the principles of the Declaration of Helsinki. Ethical approval was granted by the Clinical Research Ethics Committee of İstanbul University, İstanbul Faculty of Medicine (Date: 29.03.2019, No: 06).

RESULTS

A total of 52 patients with MSSA bacteraemia and 52 MSSA strains isolated from those patients were evaluated in the study. Forty-four of the 52 strains (84.6%) were β-lactamase positive by the nitrocefin disk, PG disk diffusion, and PG edge test methods, and the results were confirmed by the PCR method (Figure 1). Sequencing of the PCR products was suitable for 35 of 44 β-lactamase positive isolates and all of them were carrying type A β-lactamase.

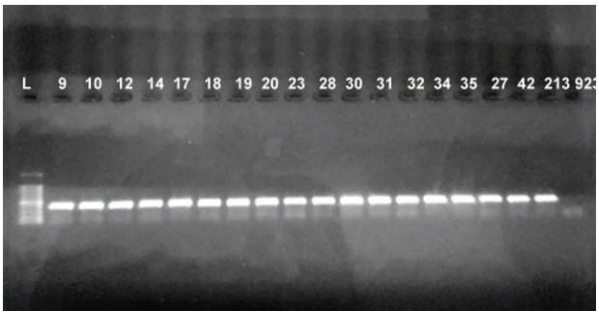

Figure 1. The image of the products obtained by amplifying the β-lactamase genes of *Staphylococcus aureus* strains using PCR in gel electrophoresis (PCR products of β-lactamase genes of strains numbered 9, 10, 12, 14, 17, 18, 19, 20, 23, 28, 30, 31, 32, 34, 35, 27, 42 and the positive control strain ATCC 29213 are present, with the β-lactamase-negative ATCC 25923 strain used as the negative control in the last row).

In disc diffusion susceptibility testing, 44 (85%), 8 (15%), 8 (15%), 6 (11.5%), 6 (11.5%), 5(9.6%), and 2 (3.8%) of the 52 MSSA strains were found to be resistant to PG, erythromycin, fusidic acid, clindamycin, rifampicin, ciprofloxacin, and trimethoprim-sulfamethoxazole, respectively, and all of the strains were susceptible to cefoxitin, Cz, gentamycin, and linezolid. In MIC testing, all strains were susceptible to SAM, cefazolin, cefuroxime, ceftriaxone, and cefotaxime. Only the cefoxitin geometric mean (GM) zone diameter was lower in the high inoculum than in the standard ones, but no InE were determined for cefoxitin, and the zone diameter of other antimicrobials including cefazolin did not show a significant difference between the high and standard inocula. In MIC testing, 13 (25%) and 20 (38.5%) of the 52 MSSA strains showed InE against Cz (three of 13 strains showed pronounced InE) and SAM (two of 20 strains showed pronounced InE), respectively, two (3.8%) strains showed InE against ceftriaxone, and none of the strains showed any InE against cefuroxime and cefotaxime. All of the GM MICs of the tested β-lactams were increased significantly with the high inoculum (p<0.001); SAM was the most affected one with a 2.94-fold increase in MIC, followed by Cz, cefuroxime ceftriaxone, and cefotaxime, with a 2.20, 1.27, 1.21 and 1.20 fold increase in MIC at high inoculum.

All of the β-lactamase negative strains were susceptible to PG, but all of the β-lactamase positive ones were resistant. The GM zone diameters of the 52 MSSA strains for PG and Cz were significantly lower for β-lactamase positive strains than β-lactamase negative strains, both at standard and high inoculum (<0.001). Among the β-lactams tested, only the MIC of SAM and Cz were significantly affected by the presence or absence of β-lactamases; GM MIC values of SAM against strains with β-lactamase were 23 times higher than those without, and this difference became even more pronounced at higher inoculum, reaching 61. GM MIC values of the Cz against the strains with β-lactamase were 1.66 and 3.86 times higher than those without, at standard and high inocula, respectively. MIC of cefuroxime, ceftriaxone, and cefotaxime was not affected by the presence of the β-lactamase (Table 1 and Figure 2).

While SAM InE was strongly related to the increase of only the MIC of SAM (p<0.001), CzInE was strongly related to the decrease of PG and Cz zone diameters (p<0.001 for both) and the increase of Cz MIC (p<0.001). The mean Cz zone diameter of the strains with CzInE was significantly lower than the zone diameters of the strains without CzInE, both at standard (27.94±3.09 vs. 23.85±1.99, p<0.001) and high (28.26±2.63 vs. 24.39±2.33, p<0.001) inoculum (Table 2); zone diameters of other tested antimicrobials, including cefuroxime, ceftriaxone, cefotaxime, vancomycin, teicoplanin, linezolid, clindamycin, erythromycin, trimethoprim-sulfamethoxazole, ciprofloxacin, fusidic acid, rifampicin, and gentamicin, did not show any decrease with either beta-lactamase positivity or SAMInE or CzInE.

Table 1. Comparison of the zone diameters and MIC values of β-Lactamase positive and negative MSSA strains at standard and high inoculum.

Antimicrobials	Total geometric mean (minimum-maximum) (MIC50-MIC90)		β-lactamase negative Geometric mean (minimum-maximum)	βlactamase positive Geometric mean (minimum-maximum)	p
Penicillin G, zone, mm					
Standard inoculum	15.44 (9-35)	p=0.430	33.35 (32-35)	13.42 (9-23)	**<0.001**
High inoculum	16.25 (0-35)		33.45 (31-35)	13.11 (0-25)	**<0.001**
Cefoxitin, zone ,mm					
Standard inoculum	27.85 (23-33)	**p=0.011**	29.07 (27.0-33.0)	27.64 (23.0-31.0)	0.147
High inoculum	26.70 (19-30)		27.72 (26.0-30.0)	26.53 (19.0-30.0)	0.133
Cefazolin, zone, mm					
Standard inoculum	26.72 (20-34)	p=0.252	32.10 (30-34)	25.84 (20-32)	**<0.001**
High inoculum	27.12 (18-35)		31.52 (26-35)	26.39 (18-32)	**<0.001**
Vancomycin, zone, mm					
Standard inoculum	15.34 (13-17)		14.86 (14.0-16.0)	15.44 (13.0-17.0)	
High inoculum	16.02 (14 –19)	p=0.001	15.48 (15.0-17.0)	16.12 (14.0-19.0)	
Teicoplanin, zone, mm					
Standard inoculum	14.53 (12-18)		14.35 (13.0-16.0)	14.57 (12-18)	
High inoculum	15.07 (13-17)	p=0.005	14.74 (14-16)	15.13 (13-17)	
Linezolid					
Standard inoculum	26.69 (21-32)		25.85 (23-31)	26.84 (21-32)	
High inoculum	26.75 (20-31)	p=0.836	26.65 (23-30)	26.77 (20-31)	
Fusidic acid, zone, mm					
Standard inoculum	25.57 (11-30)		25.38 (21-30)	25.61 (11-30)	
High inoculum	25.35 (10-31)	P=0.882	23.50 (12-28)	25.71 (10-31)	
Rifampin, zone, mm					
Standard inoculum	27.95 (23-31)		27.20 (25-30)	28.09 (23-31)	
High inoculum	27.80 (21-35)	P=0.809	27.10 (21-30)	27.93 (21-35)	
Co-trimoxazole, zone, mm					
Standard inoculum	25.09 (12-30)		24.97 (21-29)	25.12 (12-30)	
High inoculum	26.14 (10-33)	**p=0.049**	27.19 (25-31)	25.96 (10-33)	
Gentamycin, zone, mm					
Standard inoculum	22.91 (19-26)		22.53 (19-26)	22.97 (20-26)	
High inoculum	22.71 (19-26)	P=0.446	22.31 (19-24)	22.79 (19-26)	
Clindamycin, zone, mm					
Standard inoculum	25.03 (21-30)		24.43 (22-27)	25.15 (21-30)	
High inoculum	25.41 (21-30)	P=0.300	24.56 (22-27)	25.57 (21-30)	
Erythromycin, zone, mm					
Standard inoculum	22.27 (0-30)	P=1.000	18.5 (0-27)	22.96 (0-30)	
High inoculum	22.27 (0-31)		19.25 (0-27)	22.82 (0-31)	
Ciprofloxacin					
Standard inoculum	24.39 (0-30)		26.02 (22-30)	24.07 (0-30)	
High inoculum	23.92 (0-30)	P=0.245	26.07 (23-29)	23.52 (0-30)	
Ampicillin-sulbactam, MIC1, µg/ml					
Standard inoculum	0.36 (0.016-4) (0.5-1.35)	**p<0.001**	0.025 (0.016-0.032)	0.58 (0.064-4)	**0.017**
High inoculum	1.06 (0.023-12) (2- 6)		0.033 (0.023-0.125)	2.01 (0.25-12)	**<0.001**
Cefazolin, MIC, µg/ml					
Standard inoculum	0.787 (0.5-2) (1-1.7)		0.545 (0.5-1)	0.84 (0.5-2)	**<0.001**
High inoculum	1.73 (0.5-16) (1-8)	**p<0.001**	0.77 (0.5-1)	2 (1-16)	**<0.001**

Antimicrobials	Total geometric mean (minimum-maximum) (MIC50-MIC90)		β-lactamase negative Geometric mean (minimum-maximum)	βlactamase positive Geometric mean (minimum-maximum)	p
Cefuroxime, MIC, µg/ml					
Standard inoculum	0.79 (0.5-2) (1-1)		0.77 (0.5-2)	0.79 (0.5-2)	0.795
High inoculum	1 (0.5-2) (1-2)	**p<0.001**	1.19 (1-2)	0.97 (0.5-2)	0.172
Cefotaxime, MIC, µg/ml					
Standard inoculum	2.35 (1-4) (2-4)		2.18 (2-4)	2.38 (1-4)	0.328
High inoculum	2.83 (2-4) (3- 4)	**p<0.001**	3.36 (2-4)	2.74 (2-4)	0.132
Ceftriaxone, MIC, µg/ml					
Standard inoculum	3.02 (1-8) (4-4)	**p=0.001**	3.36 (2-8)	2.97 (1-8)	0.467
High inoculum	3.64 (2-16) (4-6.8)		4.36 (2-16)	3.53 (2-8)	0.329

MIC: Minimal inhibitor concentration

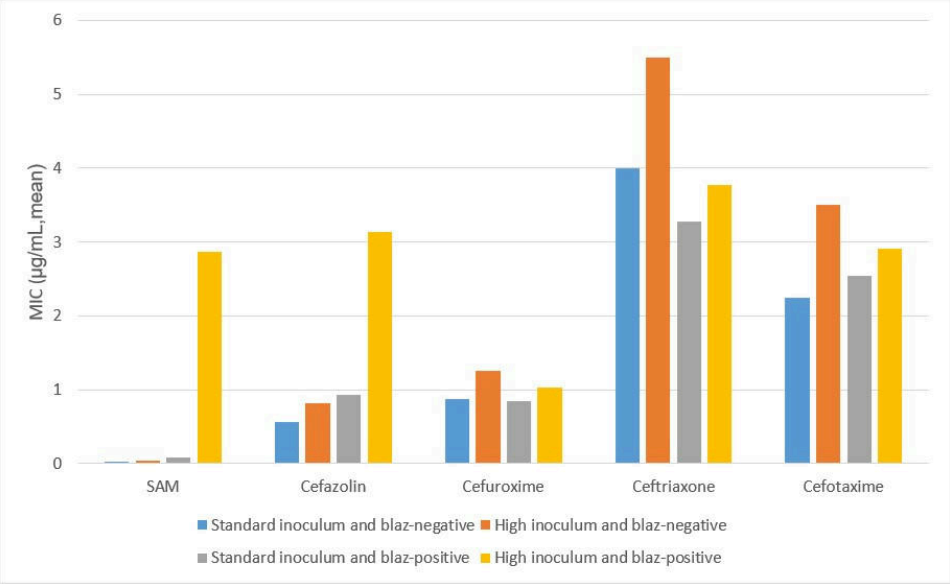

Figure 2. Mean minimal inhibitor concentration (MIC) values of ampicillin-sulbactam and cefazolin against β-lactamase positive and negative 52 strains of MSSA at standard and high inoculum.

Table 2. Effect of the SAMInE and CzInE on the antimicrobial susceptibilities of the 52 MSSA strains at standard and high inoculum.

		SAMInE			CzInE		
Tested Antimicrobials		**Negative**	**Positive**	**p**	**Negative**	**Positive**	**p**
PG zone diameter (mean±SD)	Standard inoculum	18.47±9.17	13.95±2.63	0.320	18.54±7.99	11.31±2.16	<0.001
	High inoculum	17.19±11.35	14.75±4.62	0.806	18.62±8.93	9.15±6.9	<0.001
Cefazolin zone diameter (mean±SD)	Standard inoculum	27.63±3.71	25.8±2.35	0.550	27.95±3.09	23.85±1.99	<0.001
	High inoculum	27.56±3.43	26.85±2.32	0.493	28.26±2.63	24.39±2.33	<0.001
Cefazolin zone diameter <28 mm, n (%)	Standard inoculum				22 (56)	13 (100)	0.005
	High inoculum				25 (64)	13 (100)	0.011
Ampicillin-sulbactam, MIC, (mean±SD)	Standard inoculum	0.67±0.77	0.64±0.45	0.569	0.59±0.49	0.86±1.01	0.565
	High inoculum	1.31±1.31	4.24±3.38	<0.001	2.30±2.86	2.85±2.58	0.187
Cefazolin MIC, (mean±SD)	Standard inoculum	0.91±0.48	0.83±0.37	0.723	0.79±0.31	1.12±0.65	0.178
	High inoculum	2.42±3.21	3.35±4.01	0.550	1.24±0.49	7.39±4.72	<0.001

SAMInE: Inoculum effect against ampicillin – sulbactam, CzInE: Inoculum effect against cefazolin, MIC: Minimal inhibitor concentration

Table 3. Effect of the SAMInE1 and CzInE2 on the antimicrobial susceptibilities of the 52 MSSA strains at standard and high inoculum.

Antimicrobials Affected by	Penicillin G (zone diameter)	Cefazolin (zone diameter)	Cefazolin (MIC)	Cefoxitin (zone diameter)	Others* (zone diameter)	SAM (MIC)	Cefuroxime (MIC)	Cephotaxime (MIC)	Ceftriaxone (MIC)
Inoculum**	Ø	Ø	↑↑	↓↓	Ø	↑↑↑	↑	↑	↑
Inoculum effect***			↑↑			↑↑↑	Ø	Ø	↑
β-lactamase	↓↓↓	↓↓↓	↑↑	Ø	Ø	↑↑↑	Ø	Ø	Ø
SAM InE	Ø	Ø	Ø	Ø	Ø	↑↑↑	Ø	Ø	Ø
Cz InE²	↓↓↓	↓↓↓	↑↑↑	Ø	Ø	Ø	Ø	Ø	Ø

SAM InE: Inoculum effect against ampicillin– sulbactam, CzInE: Inoculum effect against cefazolin, MIC: Minimal inhibitor concentration, *: Linezolid, clindamycin, erythromycin, trimethoprim- sulfamethoxazole, ciprofloxacin, fusidic acid, rifampicin, gentamicin, vancomycin and teicoplanin, **: Significant zone diameter decrease or MIC increase at the high inoculum, ***: A 4-fold or greater increase in MIC values at high inoculum concentrations

The effects of inoculum, β-lactamase positivity, SAmInE, and CzInE on the zone diameters or MIC of the tested antimicrobials against the MSSA strains are given in Table 3.

Compared with MIC testing, a Cz zone diameter of <28 mm was found to be 100% sensitive with a 100% negative predictive value in both standard and high inocula to define the CzInE, but the specificity and positive predictive values of that zone diameter were 44%-36%, and 37%-35.8% at standard and high inocula, respectively.

Gender, age, presence of comorbidities including diabetes mellitus, hypertension, chronic renal failure or immunosuppression, Charlson comorbidity index, having health-care or community-acquired infection, Pitt bacteraemia score, admission serum/blood test (WBC, Hb, platelet, AST, ALT, creatinine, albumin, CRP, PCT) results were not different between the patients infected with the strains showing or not showing SAMInE or CzInE (p>0.005). However, the rate of β-lactamase positivity (24/24 vs. 20/28, p=0.005) was significantly higher, and the rates of the presence of neutropenia (4/24 vs. 1/28, p=0.169) and mortality (6/24 vs. 2/28, p=0.123) were higher in the patients infected with the strains showing SAM InE or CzInE than in the patients infected with the strains not showing any InE.

The clinical and laboratory characteristics of the 52 patients with MSSA bacteraemia are given in Table 4. While 24 of bacteraemia were community-acquired (46.2%), 20 were hospital-acquired (38.5%), and 7 were healthcare-associated (13.5%). Catheter-related infections, endocarditis, bone-joint, and soft tissue infections and intra-abdominal infection were the source of bacteraemia in 20 (38.5%), nine (17.3%), eight (15.4%) and five (9.6%) of the patients, respectively.

Cz (23/52, 44.2%) was the most common definitive treatment for MSSA infection, followed by SAM (15/52, 28.5%). Vancomycin and combinations of vancomycin were de-escalated in 17 of 22 patients after the culture resulted as MSSA. The mean total duration of treatment was 22.5 days in those receiving Cz and 20.8 days in those receiving non-cefazolin treatment.

Mortality was observed in 8/52 patients (15.4%). Deceased patients had higher CCI (5.75 vs. 3.29, p=0.050) and Pitt score (3.25 vs. 1.21, p=0.078), lower serum albumin level (2.69 mg/dL vs. 3.26 mg/dL, p=0.99, more frequently infected with the strains showing InE against the used antibiotics for treatment (50% vs. 13.6%, OR=6; 95% CI, 1.17-30.73, p=0.041) and against SAM used for treatment (37.5% vs. 68%, OR=7.8; 95% CI, 1.23-49.68, p=0.044), causative MSSA strains of deceased patients showed higher Cz MIC value at higher inocula (5.68 µg/mL vs 2.25 µg/mL, p=0.054), CzIE (50% vs. 20%, p=0.096) and SAM InE (62.5% vs. 34%, p=0.235) than survived patients.

DISCUSSION

In this study, we found that the drugs whose activity was most affected by the inoculum of MSSA strains isolated from patients with bacteraemia were SAM (35% of the strains) and Cz (25% of the strains). Although the GM MIC of all the cephalosporins tested was significantly higher in the higher inoculum than in the standard inoculum (p<0.001), no InE was determined against cefuroxime or cefotaxime, and only limited (3.8% of the strains) InE was defined against ceftriaxone. The increase of GM MIC was also the highest for SAM with a 2.94 fold higher MIC at high inoculum, followed by Cz (2.20 times), cefuroxime (1.27 times), ceftriaxone (1.21 times), and cefotaxime (1.20 times). Similar to our findings, in a recent Korean study including 302 MSSA isolates of bacteraemia, InE was more frequent and prominent in β-lactam-β-lactamase inhibitor (BL/BLI) combinations (7). In an in-vitro study of 52 MSSA isolates from blood cultures, pronounced SAMInE (9.6%) was seen more frequently than a pronounced CzInE (5.8%), and no significant increase in MICs at a high inoculum of MSSA was observed with cefotaxime and ceftriaxone (8).

The rate of InE against Cz varies significantly between the studies. There has not been any report about the proportion of CzInE or SAMInE from Türkiye. The proportion of CzInE in MSSA strains was reported as a median of 14.4% (range 0-54.5%) and varied significantly from country to country, with the highest proportion being in South American countries (36.0%

Table 4. Clinical and laboratory findings of the total cohort and survived and deceased patients

Feature	Total cohort (n=52)	Deceased (n=8)	Survived (n=44)	*p-value*
Gender, female, n (%)	19	4 (50%)	15 (34%)	0.443
Age, average ±SD	58.4±18.3	64.87±21.12	57.22±17.8	0.282
Source of bacteraemia				
Community-acquired	22	3	19	0.705
Healthcare acquired	22	5	17	
Fever (°C), mean ±SD	38.1±0.9	37.63±1.08	38.18±0.88	0.117
Charlson comorbidity index, mean ±SD	3.94±2.61	5.75±2.81	3.59±2.46	0.050
Pitt bacteraemia score, mean ±SD	1.54±2.7	3.25±4.36	1.21±2.25	0.078
White blood cell l count (10^3/μL), mean ±SD	13x10^3/μL±8.9 x10^3/μL	9.8 x10^3/μL ±5.4 x10^3/μL	13.7 x10^3/μL±9.4 x10^3/μL	0.270
Blood haemoglobin value (gr/dL), mean ±SD	10.62±2.24	10.59±1.97	10.62±2.31	0.967
Blood platelet count (10^3/μl) , mean ±SD	202 910±108 349.7	141 214±78 856	212 953±10 984	0.105
Serum ALT level (U/L), mean ±SD	36.5±43.5	39.58±26.35	36.08±45.84	0.857
Serum creatinine value (mg/dL), mean ±SD	1.9±2.1	1.37±1.09	2.01±2.28	0.620
Serum procalcitonin value (ng/mL) mean ±SD	19.8±26.7	40.43±36.49	18.09±24.69	0.521
Serum CRP level (mg/L), mean ±SD	179±106	163.64±112	181.98±106.36	0.659
Serum albumin value (gr/dL), mean ±SD	2.9±2.1	2.69±0.45	3.26±0.74	0.099
Strain positive for *blaZ* gene, n (%)	44 (84.5%)	7 (87.5)	37 (84)	1000
Cefazolin MIC[1] value in the standard inoculum, μg/mL, mean ±SD	0.875±0.44	0.875±0.23	0.875±0.47	0.485
Cefazolin MIC[1] value in the high inoculum, μg/mL, mean ±SD	2.78 ±3.55	5.68±5.32	2.25±2.91	0.054
CzInE, n (%)	13 (25)	4 (50)	9 (20)	0.096
CzInE in patients treated with Cz, n (%)	4 (7.7)	1 (12.5)	3 (6.8)	0.514
SAM MIC, standard inoculum, μg/mL, mean ±SD	0.66±0.66	0.76±0.67	0.64±0.66	0.636
SAM MIC, high inoculum, μg/mL, mean ±SD	2.44±2.71	3.33±3.69	2.27±2.51	0.301
SAM InE, present, n (%)	20 (38.4)	5 (62.5)	15 (34)	0.235
SAM InE in patients treated with SAM, n (%)	6 (7.7)	3 (37.5)	3 (6.8)	0.044 (OR7.8, 95%CI;1.23-49.68)
Inoculum effect against the drug used for definite treatment, n (%)	10	4 (50)	6 (13.6)	0.041 (OR 6.0, 95%CI;1.17-30.73)
Intensive care unit admisision	7	6	1	<0.001

MIC: Minimal inhibitor concentration, CzInE: Inoculum effect against cefazolin, SAM: Ampicillin – sulbactam, SAMInE: Inoculum effect against ampicillin - sulbactam

to 54.5%), followed by Asian countries (5.8% to 21.8%), North American countries (0% to 18.7%) and European countries (2.5% to 11.0%) (9). Different proportions of InE among countries could be related to the definition of InE, prevalence and type of β-lactamase among MSSA strains, hydrolysing capacity of the prevalent MSSA β-lactamases in the region, β-lactam consumption rates and antimicrobial stewardship efforts in the community, presence of resistance-carrying genes on mobile genetic elements, and preventive infection control efforts to decrease the spread of resistance genes in the health care facilities (10). In some studies like ours, CzInE was defined as a 4-fold or more significant increase in MIC values at high inoculum concentrations and pronounced InE as a MIC of ≥16 μg/ml with the high-inoculum (2, 11, 12), while others defined it as a MIC of ≥16 μg/ml with the high-inoculum (6). In one study from South Korea using the same definitions as us, the proportion of CzInE and pronounced CzInE were reported to be 57.5% and 20%, respectively (12). In another study, the proportion of CzInE and pronounced CzInE were reported to be 20% and 4% CzInE, respectively, which are pretty similar to our rates of 25% and 5.7% (11). The definition of InE that we used is more sensitive than the latter definition. Assessment of the clinical implication of InE should include a more sensitive definition of InE to avoid the risk of falsely rejecting possible associations by using less specific metrics (13).

Some studies suggest that CzInE is related to either type A or type C β-lactamases of MSSA, and TZP InE is related to Type C β-lactamases (7, 14). In our study, all of the tested MSSA strains were found to carry type-A β-lactamase. While previous studies from various countries generally reported that all four enzyme types were present, in different proportions, type C

was seen in 94% of the MSSA strains with β-lactamase in Japan. Being a plasmid-mediated and antibiotic-inducible β-lactamase, spreading the same clones carrying the same enzyme is logical and possible, especially in a community with high antibiotic consumption, like Türkiye. Wide-spread dissemination of β-lactamase-associated resistance genes between the strains of bacteria has occurred several times in our country previously, such as the spread of *OXA-48* among *Klebsiella pneumoniae and the spread* of *CTX-M* among *E. coli* strains (15, 16).

Although all of the strains in our study were found to be carrying type A β-lactamase which has repeatedly been shown to be associated with a higher rate of CzInE, CzInE determined only 25% of our strains, and this finding suggests that type of β-lactamase is not the only factor affecting the rate of CzInE. Consistent with this, a recent study from Latin America, including 690 bloodstream MSSA isolates with whole-genome sequencing, revealed that the allotype rather than the type of β-lactamase could be a more accurate tool for identifying strains with a likelihood of exhibiting the CzInE. They reported that particular amino acid residues (E112A and G145E substitutions) were highly associated with allotypes that exhibited the CzInE (17). In addition, another study reported that single nucleotide polymorphisms (SNP) of the type A *blaZ* gene at codons 226 and 229 (Ser226Pro and Cys229Tyr) were closely associated with the CzInE (18). However, either those specific allotypes or those specific SNPs could also be determined in strains without the CzInE; it is clear that CzInE is a multifactorial phenomenon, and further studies analysing the mechanism of the CzIE are still needed.

Although the mechanism of the CzInE or SAM InE has not been defined completely, both were somehow related to the β-lactamases of MSSA. We found that while the GM MICs of cefuroxime, ceftriaxone, and cefotaxime were not affected by the presence or absence of β-lactamase, only the MICs of SAM and Cz were significantly affected by the presence or absence of β-lactamase;GM MIC values of SAM against strains with β-lactamase were 23 times higher than those without, and this difference became even more pronounced at higher inoculum, reaching 61. GM MIC values of the Cz against the strains with β-lactamase were 1.66 and 3.86 times higher than those without, at standard and high inoculum, respectively. Additionally, the GM zone diameter of the 52 MSSA strains for PG and cefazolin was significantly lower for β-lactamase positive strains than β-lactamase negative strains, both at standard and high inocula (<0.001). However, the way β-lactamases interact with InE seems different between SAMInE and Cz InE: While Cz InE is repeatedly reported to be related to either hyperproduction or better performance of type A and type C BlaZ on cefazolin (2), SAMInE probably resulted from the decreased inhibition of MSSA β-lactamase by sulbactam (7). In our study, we saw that while SAMInE strongly related with the increase of only the MIC of SAM (p<0.001), CzInE strongly related with the decrease of PG and Cz zone diameters (p<0.001 for both) and the increase of Cz MIC (p<0.001). The fact that SAMInE is specific to SAM and is not related to the lower susceptibility to other beta-lactams suggests that this effect is not due to excessive β-lactamase production of the MSSA strains, but to the insufficient inhibitory effect of sulbactam. If the presence of InE against one β-lactamase inhibitor was shown, then InE and the decreased activity of other β-lactamase inhibitors could be foreseen. TZP was shown to be affected by MSSA β-lactamases frequently (7). In an in vitro study, clavulanic acid and tazobactam were 93 and 11 times more active than sulbactam against the β-lactamases of MSSA strains (19).

The mean Cz zone diameter of the strains with CzInE was significantly lower than the zone diameters of the strains without CzInE, both in standard and high inoculum in our study (27.94±3.09 vs. 23.85±1.99, p<0.001). A Cz zone diameter of <28 mm was 100% sensitive for the definition of CzInE among type A β-lactamase carrying MSSA strains in our study. In a recent study from Australia, it was shown that type A and type C β-lactamases of MSSA could be defined by calculations using the zone diameters of cefazolin, cephalothin, and oxacillin with a sensitivity and specificity of 88.6% and 96.6%, respectively (13). These results show that both InE and β-lactamase type could be predicted using simple disk diffusion tests. The zone diameter of Cz could be used as a screening test to define the InE of Cz and the necessity of further testing.

We found that rate of β-lactamase positivity (26/26 vs. 18/26, p=0.004) of the infecting MSSA strains was significantly higher and the rates of the presence of neutropenia (4/26 vs. 1/26 p=0.350), admission to the ICU (6/26 vs. 1/26, p=0.99), and mortality (6/26 vs. 2/26, p=0.248) were higher in patients infected with the strains showing InE against SAM or Cz than in patients not. In another study, metastatic cancer, recent close contact with a chronically ill patient, and resistance to clindamycin and erythromycin among the causative MSSA strains were found to be risk factors for CzInE (7). As neutropenia develops frequently in patients with metastatic cancer, the incidence, underlying mechanism and clinical implication of CzInE among patients with cancer or neutropenia should be evaluated in further studies. We did not find any association between SAM or CzInE either with other comorbidities or test results of the patients or with the susceptibility or resistance of the strains to certain antibiotics.

It is unknown whether being less active against the β-lactamase of MSSA and having a more frequent InE among the

MSSA strains compared to Cz in SAM will make a difference in the treatment effectiveness between the two agents.

In our study, the mortality rate in patients infected with MSSA strains showing SAMInE and treated with SAM was higher than in those not treated with SAM (37.5% vs. 68%, OR=7.8; 95% CI, 1.23-49.68, p=0.044), and the causative MSSA strains of deceased patients showed higher SAM InE (62.5% vs. 34%, p=0.235) than the surviving patients. We could find only one study analysing the clinical impact of SAMInE. In that study including 302 cases with MSSA bacteraemia, the mortality of the SAM InE-positive (n=27) group who received empirical β-BL/BLIs was significantly higher than negative (n=23) (32.4% vs. 5.6%, p=0.04) ones and mortality rate of the SAM InE-positive (n=28) group who received definitive β-lactam/β-lactamase inhibitors for the treatment of MSSA bacteraemia was also higher than that of the negative (n=14) group, but this did not reach statistical significance (26.1% vs. 8.3%, p=0.38) (7).

There are also a couple of studies comparing the effectiveness of SAM or other BL/BLI combinations and other first-choice regimens including either Cz or ASPs: In a retrospective cohort study of 478 patients with MSSA bacteraemia, mortality was found to be similar between cloxacillin and cefazolin-treated patients, but it is nearly two times (OR=2.68, p=0.08) higher among patients treated with BL/BLI combinations including TZP, (n=32), ampicillin-clavulanate, (n=28) and SAM, (n=1) (20). In a retrospective cohort study of our group, including 127 MSSA bacteraemia cases, the mortality rate of patients treated with Cz (2/30, 6.6%) was lower than that in patients treated with SAM (9/47, 19%); however, it did not reach statistical significance (p=0.082) (21). In the study of Uda et al., another BL/BLI combination TZP for definitive therapy of MSSA bacteraemia was found to be associated with treatment failure for MSSA bacteraemia (OR=17, p=0.003) (22). In a USA study including more than 400 MSSA bacteraemia cases, while no difference in mortality was seen between ASP and cefazolin or fluoroquinolones, higher mortality was observed with TZP as compared with ASP/cefazolin (hazard ratio [HR]=0.10; 95% CI, 0.01-0.78), suggesting it may not be as effective as a monotherapy in MSSA bacteraemia (23).

Finally, in a recent retrospective observational study from Japan comparing the clinical efficacy of SAM (41 patients) versus Cz (30 patients) in patients with MSSA bacteraemia, mortality rates were found to be not different between the groups (24).

All of these data suggest that due to the lower effectiveness of sulbactam against β-lactamase of MSSA and SAMInE, SAM treatment of MSSA bacteraemia and other higher inoculum infections, could be less effective than other options, including Cz. SAM is a frequent replacement of the first choice antimicrobials for MSSA bacteraemia, including ASP and Cz, especially in the case of unavailability of those agents like here in Türkiye, Argentina and previously in Japan or if those agents could not be used due to the adverse effects. That's why additional studies are urgently needed on this subject, and the limited evidence along with our findings should create the hypothesis that BL/BLIs including SAM or TZP could have decreased activity against some β-lactamase of MSSA, which led to reduced activity of those agents during treatment. In our study, the mortality rate in patients infected with MSSA strains showing CzInE and treated with Cz was higher than in those not treated with Cz (12.5% vs. 6.8%, p=0.514); additionally, the mortality rate of the patients infected with the strains showing CzInE (4/13, 30%) was higher than the mortality rate of patients infected with the strains not showing (4/39, 10%, p=0.096). Still, the differences were not statistically significant in both of the findings and the numbers are insufficient to reach a definite conclusion. Cz is the most studied drug for the presence and clinical consequences of InE in MSSA strains caused by bacteraemia, but the results of the studies are conflicting. In a recent meta-analysis of 23 observational studies, CzInE was defined in 0%-55% of the cases. The mortality rate of serious infections caused by MSSA did not differ significantly between the strains with and without CIE. However, it was emphasised that the quality of the included studies was low (9). A well-designed study without limitations is urgently needed to answer the question about the clinical impact of CzInE.

As cefuroxime was found to be highly effective in-vitro against MSSA strains, with a similar GM MIC with Cz and was shown to be not affected by InE in our and some other studies, it could be evaluated as an alternative agent to SAM or cefazolin in the case of InE against those antimicrobials (25, 26). In a recent study of 268 patients with MSSA bacteraemia, empirically treated with a mean of three days of either ASP flucloxacillin or cefuroxime or ceftriaxone, duration of bacteraemia or SAB-related mortality did not show any difference between the groups (27). Therefore, it is essential to conduct comparative studies with Cz and cefuroxime in such MSSA infections. However, it should always be considered that as β-lactamases of MSSA are not constitutive but inducible with exposure to β-lactam antibiotics, close monitoring of the susceptibility of all used antibiotics along with rational antimicrobial usage efforts is of utmost importance (28).

Our study has some limitations, including a retrospective and observational design and a small number of patients for each group for some comparisons. However, our study contributes to the current limited knowledge about the incidence and clinical consequences of SAMInE in patients with MSSA bacteraemia. In addition, for the first time, we found a disk

diffusion test zone diameter breakpoint for the screening of CzInE.

CONCLUSSION

In conclusion, InE is more frequently encountered against SAM than Cz among MSSA strains caused bacteraemia, probably because of the decreased activity of the sulbactam against some of the type A β-lactamase of the MSSA strains. SAM treatment of the patients infected with the MSSA strains showing SAMInE may increase mortality. Additional studies that will provide stronger evidence are needed regarding the incidence and clinical consequences of InE among MSSA strains of deep-seated infections against β-lactam agents, including not only Cz but also BL/BLI. A cefazolin zone diameter of <28 mm could be used as a screening method to define the Cz InE.

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