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ORIGINAL ARTICLE

In-vitro activity of oxymino-cephalosporins with and without sulbactam against Class A Extended-spectrum β-lactamase producing *E.coli*

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ABSTRACT

Objectives: The primary aim of this study was to determine the activities of ceftazidime and cefepime combined to sulbactam against class A extended-spectrum β lactamases (ESBLs).

Materials and methods: Eight university hospitals participated to the study by submitting isolates those were recovered during a six-month period in 2010 from various clinical materials. Sulbactam was tested in two fixed concentrations of 4 mg/l and 8 mg/l. Isolates showing a fourfold or more decrease in the MIC of an oxyimino-cephalosporin with sulbactam were defined as ESBL producers. Isolates were screened for CTX-M group 1 extended-spectrum β lactamases by PCR.

Results: A total of 149 ESBL-positive *E.coli* were studied. Isolates were uniformly susceptible to carbapenems and highly resistant to ciprofloxacin. According to CLSI breakpoints, 28% (42/149) of isolates were susceptible to ceftazidime and 32% (47/149) to cefepime. With 4 mg/L and 8 mg/L sulbactam supplement, ceftazidime susceptibility rose to 69% (103/149) and 88% (131/149), while cefepime susceptibility rose to 86 % (128/149) and 95% (141/149), respectively. PCR screening revealed that 63% (94/149) of the isolates were positive for blaCTX-M and 38% (36/94) of these were on the O25b-ST131 clone.

Conclusion: Ceftazidime plus sulbactam and cefepime plus sulbactam showed remarkable activity against ESBL-positive *E.coli*. *J Microbiol Infect Dis 2011;1(3):87-92*

Key words: Extended-spectrum β-lactamase, Escherichia coli,ceftazidime, cefepim

A sınıfı genişlemiş spektrumlu beta-laktamaz üreten E.coli suşlarına karşı sulbaktam içeren ve içermeyen oksimino-sefalosporinlerin invitro aktivitesi

ÖZET

Amaç: Bu çalışmanın amacı seftazidim ve sefepim ile kombine sulbaktam'ın A sınıfı genişlemiş spektrumlu β laktamazlara (ESBL) karşı aktivitesini belirlemektir.

Gereç ve yöntem: Sekiz üniversite hastanesi 2010 yılı içerisinde altı aylık süre zarfında çeşitli klinik materyallerden izole edilmiş *E.coli* suşlarını göndererek çalışmaya katıldı. Sulbaktam 4 mg/L ve 8 mg/L şeklinde iki sabit konsantrasyonda test edildi. Sulbaktam eklenen oxyimino-sefalosporin MIC değerlerinde 4 ve daha fazla kat düşüş ESBL açısından kanıt sayıldı. İzolatlar PCR ile CTX-M grup 1 türü ESBL açısından tarandı.

Bulgular: Toplam 149 ESBL-pozitif *E.coli* suşu çalışıldı. İzolatlar tekdüze karbapenemlere duyarlı ve siprofloksasine yüksek derecede dirençli idi. CLSI referans değerine göre, izolatların % 28'i (42/149) seftazidime ve % 32'si (47/149) sefepime duyarlı idi. Test ortamına 4 mg/L ve 8 mg/L sulbaktam eklenmesiyle; seftazidim duyarlılığı, sırasıyla % 69 (103/149) ve % 88'e (131/149), sefepim duyarlılığı, sırasıyla % 86 ve (128/149) % 95'e (141/149) yükseldi. PCR taraması bu izolatların % 63'ünde (94/149) blaCTX-M ve % 38'inde (36/94) O25b-ST131 klonunun pozitif olduğunu ortaya çıkardı.

Sonuç: Seftazidim artı sulbaktam ve sefepim artı sulbaktam ESBL-pozitif *E.coli*'ye karşı dikkate değer bir aktivite gösterdi.

Anahtar kelimeler: Geniş spektrumlu beta-laktamaz, Escherichia coli, seftazidim, sefepim

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INTRODUCTION

Class A extended-spectrum β-lactamases (ESBLs) susceptible to β-lactamase inhibitors (BLi) are classified under group 2be of the functional classification scheme of Bush, Jacoby & Medeiros.[1] In the group 2be, TEM and SHV variants make the biggest cluster. CTX-M, VEB and PER families are other significant enzymes of this group.[2] ESBLs are disseminated worldwide among the members of the Enterobacteriaceae particularly in hospital settings due to the extensive pressure of antibiotics. The rate of ESBLs remained relatively low in the community setting until the recent pandemics of the CTX-M-like ESBL-producing *E.coli*.[3,4]

In life-threatening infections, administration of an effective antibiotic during the first few days, mostly before the microbial etiology is known, is the most important determinant of outcome.[5,6] *E.coli* is among the leading causes of such infections in both community and hospital settings. Therefore, the widespread emergence of ESBLs among *E.coli* establishes a significant pressure over the initial antibiotic choice of physicians in favor of a broad coverage with carbapenems. Over usage of carbapenems, on the other hand, causes the selection of carbapenem resistant Pseudomonas and Acinetobacter species in hospitals, which is another concern. An expanded spectrum, β-lactam antibiotic (BL) with BLi might replace carbapenems as the empirical antibiotic choice in selected situations.

In most countries, piperacillin with tazobactam is the single available expanded-spectrum BL plus BLi while in several countries cefoperazone with sulbactam is accessible as well. Recently, sulbactam also became available as a standalone drug in various countries to be used in combination with expanded-spectrum cephalosporins. The primary aim of this study is to test the performance of ceftazidime and cefepime with sulbactam against ESBL producing *E.coli* clinical isolates and to discuss the usability of sulbactam. The secondary aim is to demonstrate the prevalence of CTX-M group 1 enzymes and their association with O25b ST131 clone among the ESBL producers obtained from multiple centers.

MATERIALS AND METHODS

Bacterial isolates, identification, and MIC determination

Eight university hospitals located in different geographical parts of Turkey participated in the study by contributing clinical non-repetitive *E.coli* isolates possessing ESBL phenotypes to the study. Isolates were recovered during a six-month period in 2010 from various clinical materials.

The current researchers re-identified these isolates in the laboratory depending on colony characteristics, gram staining, oxidase reaction, sugar fermentation, and other biochemical characteristics. MICs were determined by the agar dilution technique using the Mueller–Hinton agar (Oxoid, Basingstoke, UK) as described by CLSI (formerly NCCLS).[7] Briefly, a bacterial inoculum of 10^4 cfu per spot was replicated on agar plates supplemented with doubling dilutions of antimicrobials. Endpoints were read after 18 h of incubation at 37^0C. *E.coli* ATCC 25922 was used as the control strain. Sulbactam was tested in two fixed concentrations of 4 mg/l and 8 mg/l. Isolates showing a fourfold or more decrease in the MIC of an oxyimino-cephalosporin with sulbactam were defined as ESBL producers.

Antimicrobial agents and their sources were as follows: ampicillin (Mustafa Nevzat); cefepime (Bristol-Myers Squibb); cefoperazone (Pfizer); ceftazidime (GlaxoSmithKline); ciprofloxacin (Bayer); ertapenem (Merck); doripenem (Janssen Pharmaceuticals); imipenem (Merck); meropenem (AstraZeneca); piperacillin/tazobactam (Pfizer) and sulbactam (Mustafa Nevzat).

PCR screening and Sequencing

PCR screenings were performed by inoculation of bacteria from agar plates to reaction tubes by the aid of a disposable sterile needle. This technique seems to be superior to other DNA preparation techniques in order to prevent false positive results due to contamination. Direct inoculation from an agar plate did not cause inhibition for *E.coli*, which was confirmed by including a 427 bp fragment of the trpA gene as an internal control.

To screen the CTX-M group 1 genes and to sequence analyze the products, two sets of primer pairs were used: 1. CTXoF 5' ATG GTT AAA AAA TCA CTG CGC-3' & CTXoR 5' TTA CAA ACC GTC GGT GAC GAT-3' to amplify the entire gene (876 bp) and 2. CTXinF-5'-AGT GAA AGC GAA CCG AAT CTG-3' and CTXinR 5' CGC CAA CGT GAG CAA TCA-3' to amplify a 181 bp inner hot region. Annealing temperatures used during the amplification reactions were 57 0C and 54 0C, respectively.

On the other hand, the primers O25pabBspe.F (5' TCCA GCA GGT GCT GGA TCG T-3') and O25pabBspe.R (5' GCG AAA TTT TTC GCC GTA CTG T-3') were used to amplify 347 bp fragment of the pabB allele specific to O25b-ST131 clone and primers trpA.F (5' GCT ACG AAT CTC TGT TTG CC-3') and trpA2.R (5' GCA ACG CGG CCT GGC GGA AG-3') were used to amplify 427 bp fragment of the trpA gene, as described elsewhere.[8]

Negative and positive controls were *E.coli* CAMB2 (CTX-M-15, phylogroup B2, O25b negative) and *E.coli* TN03 (CTX-M-15, phylogroup B2, O25b positive).[8] Sequencing was performed with purified amplicons of the full-length gene, using the dye terminator cycle sequencing method.[9]

RESULTS

A total of 149 *E.coli* were studied. The MIC 50 and MIC 90 values are shown in Table 1. Classically, MIC 50 and MIC 90 values for carbapenems remained below susceptible breakpoints,[7,10] whilst the MICs of ciprofloxacin were unacceptably high.

MIC 50 values of piperacillin/tazobactam, ceftazidime and cefepime were not promising. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints 21% (32/149), 7% (10/149) and 5% (8/149) of ESBL (+) ive *E.coli* were susceptible to piperacillin/tazobactam, ceftazidime and cefepime, respectively.

Sulbactam significantly enhanced the activity of ceftazidime and cefepime. The apparent shift in the MICs of ceftazidime and cefepime in comparison to piperacillin/tazobactam is presented in Table 2. Briefly, according to EUCAST breakpoints, the susceptibility to ceftazidime dramatically increased to 44 % (66/149) with 4 mg/l and to 70 % (104/149) with 8 mg/l of sulbactam. Similarly, the susceptibility to cefepime increased to 51% (76/149) and 68% (101/149) with 4 mg/l and 8 mg/l of the sulbactam supplement, respectively.

Table 1. MIC 50 and MIC 90 values of 149 ESBL-positive *E.coli* isolates (mg/dl)

Variable	MIC 50			MIC 90		
	Alone	+Sul[1]		Alone	+Sul	
		4 mg/l	8 mg/l		4 mg/l	8 mg/l
Ampicillin	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128	≥ 128
Ceftazidime	16	2	≤ 0.5	64	32	8
Cefepime	16	1	≤ 0.5	64	32	8
Cefoperazone	≥ 128	64	8	≥ 128	≥ 128	≥ 128
Piperacillin/Tazobactam[1]	16	-	-	64	-	-
Ertapenem	≤ 0.5	-	-	≤ 0.5	-	-
Imipenem	≤ 0.5	-	-	≤ 0.5	-	-
Doripenem	≤ 0.5	-	-	≤ 0.5	-	-
Ciprofloxacin	≥ 32	-	-	≥ 32	-	-

1: +Sul, plus sulbactam

Table 2. The distribution of isolates to MIC values

mg/l	CAZ [i,ii]	CAZ+ Sul (4)	CAZ+ Sul (8)	FEB	FEB+ Sul (4)	FEB+ Sul (8)	CEP	CEP+ Sul (4)	CEP+ Sul (8)	PIP+ TAZO
0.5		57	80	2	60	88	1	27	36	
1	10	9	24	6	16	13		5	11	
2	20	18	13	9	19	12		6	16	5
4	12	19	14	11	6	15		7	10	7
8	14	11	10	19	27	13	5	4	16	20
16	25	17	5	32	6	5	10	5	15	57
32	32	10	2	43	10	3	4	17	22	36
64	25	6	1	17	2		7	28	6	17
128	11	2		10	3		122	50	17	7

i. CAZ, Ceftazidime; CAZ+Sul (4), Ceftazidime+Sulbactam 4 mg/l; CAZ+Sul (8), Ceftazidime+Sulbactam 8 mg/l ; FEB, Cefepime; FEB+Sul (4), Cefepime+Sulbactam 4 mg/l; FEB+Sul (8), Cefepime+Sulbactam 8 mg/l ; CEP, Cefoperazone; CEP+Sul (4), Cefoperazone+Sulbactam 4 µg ml-1; CEP+Sul (8), Cefoperazone+Sulbactam 8 µg ml-1; PIP+TAZO, Piperacillin+Tazobactam (Tazobactam at ½ ratio)

ii. Isolates below breakpoints are indicated by bold number and shaded area. Dark shaded area is indicating EUCAST breakpoints

Clinical and Laboratory Standards Institute (CLSI) breakpoints of ceftazidime and cefepime are slightly different from the EUCAST ones: the ceftazidime breakpoint is ≤4 mg/l and cefepime is ≤ 8 mg/l whereas both are ≤ 1 mg/l according to EUCAST (7)(10). Susceptibility rates changed according to CLSI breakpoints. Briefly, 28% (42/149) of isolates were susceptible to ceftazidime and this was enhanced to 69% (103/149) and 88% (131/149) with 4 mg/l and 8 mg/l of the sulbactam supplement, respectively. The figure for cefepime susceptibility was similar; 32% (47/149) increased to 86 % (128/149) and 95% (141/149), respectively.

Unfortunately, neither EUCAST nor CLSI published revised breakpoints for cefoperazone. Therefore, researchers were not able to evaluate the performance of this antibiotic with confidence. However, it would not be wrong to say that the MIC shift for cefoperazone with sulbactam was not as good as that for ceftazidime or cefepime.

The PCR screening for CTX-M group 1 revealed that 63% (94/149) of the isolates were positive for these β-lactamases. The O25b-ST131 screening revealed that 37 % (55/149) of the isolates belonged to this particular clone. Of the 55 O25b-ST131-positive isolates, 36 were also positive for CTX-M group 1 enzymes. Sequencing was performed on 10 randomly selected ceftazidime/sulbactam or cefepime/sulbactam resistant isolates. Of these, five were CTX-M-1 and the rest were CTX-M-15-positive. Only one of the CTX-M-15-positive isolates was also O25b-ST131 PCR positive.

The MIC values between CTX-M-positive and CTX-M-negative isolates as well as between the positive and negative combinations of CTX-M and O25b-ST131 were compared but no significant difference was found.

DISCUSSION

This study documented that sulbactam significantly potentiates ceftazidime and cefepime against ESBL-positive *E.coli*. MICs of ceftazidime/sulbactam and cefepime/sulbactam were superior to the MICs of piperacillin/tazobactam and cefoperazone/sulbactam. The differences between the activities of these BL+BLi combinations largely depend on the stability of the BL component. Ceftazidime and cefepime are more stable than piperacillin and cefoperazone to the hydrolysis by β-lactamases. Previously, it has been shown that even TEM-1, a classical narrow spectrum enzyme, when overproduced, conferred resistance to piperacillin and piperacillin/tazobactam but not to ceftazidime.[11]

Piperacillin/tazobactam, and in various countries, cefoperazone/sulbactam are available in the market but ceftazidime or cefepime combined to a BLi are not manufactured. However, in some countries, now including Turkey, sulbactam is available as a standalone drug and licensed to use with an expanded-spectrum BL antibiotic.

In life-threatening infections, effective antibiotic treatments have to be initiated immediately, before the microbial etiology and the resistance pattern is known.[6] In infections of immunocompromised host including neutropenia with fever, empirical therapy is life saving and antibiotics have to be continued in spite of negative cultures and in spite of an unknown target. In routine medical practice, physicians hesitate to use ceftazidime or cefepime in the empirical regimen due to the ESBL concern. The consequence of this is the over usage of carbapenems.

The data from this study suggest that ceftazidime or cefepime plus sulbactam may be a reasonable alternative to carbapenems in the empirical regimen and that they are more active than piperacillin/tazobactam and cefoperazone/sulbactam. However, several points must be discussed before recommending sulbactam for use with ceftazidime or cefepime.

First, there is no consensus over the clinical breakpoints of ceftazidime and cefepime.12 If we accept the breakpoints recommended by CLSI, susceptibility rates will be 69% and 88% for ceftazidime and 86 % and 95 % for cefepime when supplemented with sulbactam 4 µg/ml and 8 mg/ml, respectively. Second, the sulbactam concentration used in the dilution for susceptibility testing must receive further investigation. Although 4 mg/L fixed concentration is suggested10, pharmacokinetic data imply that 8 mg/L is achievable in the bloodstream with even a 500 mg dose (20 mg/l peak).[13,14] In other words, the susceptibility testing of ceftazidime or cefepime with sulbactam must be optimized before these combinations are widely recommended.

Recent studies from Turkey document very high ESBL rates among *E.coli* such that 40% has been reported from a multi-center nosocomial survey 15 while 6% and 17% have been reported from uncomplicated and complicated community unset urinary tract infections, respectively.16 The latter study also reported that 90 % (46/51) of ESBLs among community isolates were CTX M 15. However, they did not study the clonality of these isolates. In the current study, researchers found that 63 % of ESBLs were from the CTX M group 1 and 57 % (36/63) of these were associated with the O25b-ST131 clone. This finding showed that the pandemic clone O25b-ST131 with CTX-M group 1 is already widespread in Turkey. However, this does not have any more significant consequence on antibiotic potency than other ESBLs do.

To investigate alternative treatment strategies to overcome the ESBL problem is of high interest.[17] This study showed that sulbactam significantly potentiates ceftazidime and cefepime against ESBL-positive *E.coli* and so deserves further investigation as an alternative strategy of empirical treatment in certain situations, including sepsis of urinary origin and febrile neutropenia where *E.coli* is a concern. Dissemination of the ST131 clone with CTX-M-type ESBLs does not affect the potency of these combinations.

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