ORIGINAL ARTICLE

Effect of inoculum size of *Enterobacteriaceae* producing SHV and CTX-M extended-spectrum ß-lactamases on the susceptibility to ß-lactam combinations with inhibitors and carbapenems

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ABSTRACT

Aim Many extended-spectrum β-lactamases (ESBL) producing isolates of *E. coli* and *K. pneumoniae* are susceptible in vitro to amoxycillin-clavulanate (AMC), ceftazidime-clavulanate (CAZ/cl), and piperacillin-tazobactam (TZP), but MICs increase substantially when higher inoculum is applied. The aim of this study was to determine the effect of inoculum size on the susceptibility of *E. coli* and *K. pneumoniae* isolates with well characterized ESBLs, to amoxycillin (AMX), AMC, ceftazidime (CAZ), CAZ/cl, piperacillin (PIP), TZP, imipenem (IMI) and meropenem (MEM).

Methods Minimum inhibitory concentrations (MIC) were determined by broth microdilution method using inocula that differed 100 fold in density.

Results Inoculum effect for CAZ/cl was detected in 52% of SHV-2 producing *K. pneumoniae* strains followed by AMC (43%) and TZP (38%). SHV-5 producing *K. pneumoniae* strains showed the most pronounced inoculum effect with CAZ/cl and AMC and to lesser extent with TZP. Inoculum effect was observed for AMC, CAZ/cl and TZP in 71% of SHV-12 producers. *E. coli* producing SHV-5 β-lactamase showed the most pronounced inoculum effect with AMC, followed by CAZ/cl and TZP. Strains producing CTX-M β-lactamases had a marked inoculum effect with CAZ/cl, AMC and TZP. Carbapenems did not show inoculum effect with any type of ESBLs.

Conclusion According to the results of this study, carbapenems remain the antibiotics of choice for the treatment of infections caused by ESBL-producing *Enterobacteriaceae*.

Key words: inoculum effect, extended spectrum β-lactamases, carbapenems, β-lactam/inhibitor combinations

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INTRODUCTION

Extended-spectrum β-lactamases are enzymes capable of hydrolyzing oxyimino-cephalosporins and aztreonam. They are produced by a variety of Gram-negative bacilli (1). A major problem with ESBLs is their capacity to cause therapeutic failures with cephalosporins and monobactams even when the causative agent appears susceptible in the laboratory tests (2-4). In response to this problem CLSI (Clinical and Laboratory Standard Institution, former NCCLS) recommends that laboratories should report ESBL producing isolates of K. pneumoniae and E. coli as resistant to penicillins, cephalosporins and monobactams regardless of the results of in vitro testing (5). There are also questions whether β-lactamase inhibitor combinations should be used for the therapy of infections caused by ESBL pathogens. Many ESBL producing isolates of E. coli and K. pneumoniae are susceptible in vitro to amoxycillin-clavulanate (AMC), ceftazidime-clavulanate (CAZ/cl and piperacillin-tazobactam (TZP), but MICs increases substantially when higher inoculum is applied (6-7). Efficacy has been reported in animal models but clinical failures were reported in patients. Previous studies have shown moderate inoculum effect of β-lactamase/inhibitor combinations against Enterobacteriaceae in general (6) but there are only few reports so far for ESBL positive enteric bacteria with well defined resistance mechanisms (7-9) .

The aim of this study was to determine the effect of inoculum size on the susceptibility of *E. coli* and *K. pneumoniae* isolates with well characterized ESBLs to amoxycillin (AMX), amoxycillin/clavulanate (AMC), ceftazidime (CAZ), ceftazidime/clavulante (CAZ/cl), piperacillin (PIP), piperacillin/tazobactam (TZP) and carbapenems in comparison with ESBL negative strains. Inoculum effect was determined according to the type of ESBL in order to determine if there are differences in its magnitude between different types of ESBLs.

MATERIAL AND METHODS

The experiments were performed on the set of *K. pneumoniae* and *E. coli* isolates with well defined resistance mehanisms: 52 *K. pneumoniae* strains producing SHV-5 β-lactamase, 21 *K. pneumoniae* with SHV-2 β-lactamase, seven *K. pneumonia* strains possessing SHV-12 β-lactamase, 41 *E. coli* strains producing SHV-5 β-lactamase (10-12) and fourteen *E. coli* strains positive for CTX-M β-lactamases (nine CTX-M-3 and five-CTX-M-15) (Bedenić B, unpublished data). Twenty six ESBL negative isolates of *K. pneumoniae* were used as the control group. The β-lactamases were characterized by isoelectric focusing, PCR and sequencing of bla_{ESBL} genes. Minimum inhibitory concentrations (MIC) of amoxycillin (AMX), amoxycillin/clavulanate (AMC), ceftazidime (CAZ), ceftazidime/clavulanic acid (CAZ/cl), piperacillin (PIP), piperacillin/tazobactam (TZP), imipenem (IMI) and meropenem (MEM) were determined by broth microdilution method using inocula that differed 100 fold in density according to CLSI (5). The inocula contained 10^5 CFU/ml and 10^7CFU/ml approximately in Mueller-Hinton broth. The MIC (minimum inhibitory concentration) was defined as the lowest antibiotic concentration that prevented the visible growth of bacteria after incubation at 37°C for 18 h. Inoculum effect was defined as at least eightfold increase in antibiotic MIC in the presence of high inoculum compared to standard inoculum size (7).

RESULTS

Inoculum effect for CAZ/cl was detected in 52% of SHV-2 producing *K. pneumoniae* strains followed by AMC (43%) and TZP (38%). Two strains showed inoculum effect with imipenem. SHV-5 producing *K. pneumoniae* strains showed the most pronounced inoculum effect with CAZ/cl (57%) and AMC (55%) and to lesser extent with TZP (44%). Two strains showed inoculum effect with meropenem (Table 1).

Table 1. Percentage of strains showing inoculum effect with ß-lactam/inhibitor combinations and carbapenems, according to the type of ESBL.

	Antibiotic*				
Type of β-lactamase	AMC	CAZ/cl	TZP	IMI	MEM
K. pneumoniae					
ESBL-negative	1/26 (3.8%)	0/26 (0%)	1/26 (3.8%)	0/26 (0%)	0/26 (0%)
SHV-2	9/21 (43%)	11/21 (52%)	8/21 (38%)	2/21 (9.5%)	0/21 (0%)
SHV-5	29/52 (55%)	30/52 (57%)	23/52 (44%)	0/52 (0 %)	2/52 (3.8 %)
SHV-12	5/7 (71%)	5/7 (71%)	5/7 (71%)	0/7 (0%)	0/7 (0%)
E. coli					
SHV-5	25/41 (61%)	21/41 (51%)	9/41 (22%)	0/41 (0%)	0/41 (0%)
CTX-M	8/14 (57%)	10/14 (71%)	7/14 (50)	0/14 (0%)	1/14 (7.1%)

*AMC, amoxycillin/clavulanate, CAZ/cl, ceftazidime/clavulanate; TZP, piperacillin/tazobactam; IMI, imipenem; MEM, meropenem;

Inoculum effect was observed for AMC, CAZ/cl and TZP in 71% of SHV-12 producers. None of the strains showed inoculum effect for the carbapenems (Table 1).

E. coli producing SHV-5 β-lactamase showed the most pronounced inoculum effect with AMC (61%) followed by CAZ/cl (51%) (Table 1). TZP had the least inoculum effect (22%). Carbapenems were not affected.

Strains producing CTX-M β-lactamases had a marked inoculum effect with CAZ/cl (71%), AMC (57%) and TZP (50%). One strain exhibited inoculum effect with meropenem (Table 1).

The concentration necessary to inhibit 90% of the SHV-2 producing *K. pneumoniae* rose from 128 to ≥1024 mg/l for AMC and TZP, from 256 to ≥1024 mg/L for CAZ, and from 4 to 32 for CAZ/cl when high inoculum was applied as

Table 2. MIC range, cumulative MIC values and percentage of resistant strains at standard and high inoculum testing, for *Klebsiella pneumoniae* strains producing SHV-ESBLs

	Standard inoculum				High inoculum			
Antibiotic	MIC range	MIC50	MIC90	%R	MIC range	MIC50	MIC90	%R
				***K. pneumoniae* SHV-2 (n=21)**				
amoxycillin	≥1024-≥1024	≥1024	≥1024	100	≥1024-≥1024	≥1024	≥1024	100
amoxycillin/clavulanate	1->1024	16	128	42.8	8->1024	128	≥1024	90.5
ceftazidime	4-512	32	256	76.2	16->1024	128	≥1024	95.2
ceftazidime/clavulanate	0.25-4	1	4	0	0.5-64	8	32	9.5
piperacillin	≥1024-≥1024	≥1024	≥1024	100	≥1024-≥1024	≥1024	≥1024	100
piperacillin/tazobactam	4-256	32	128	19	32-≥1024	128	≥1024	57
imipenem	≤0.016-1	0.12	1	0	0.06-4	0.25	4	0
meropenem	≤0.016-0.25	0.06	0.25	0	0.03-1	0.12	0.5	0
				***K. pneumoniae* SHV-5 (n=52)**				
amoxycillin	≥1024-≥1024	≥1024	≥1024	100	≥1024-≥1024	≥1024	≥1024	100
amoxycillin/clavulanate	1-≥1024	64	128	80.7	8->1024	128	≥1024	98
ceftazidime	1-≥1024	≥1024	≥1024	98	128->1024	≥1024	≥1024	100
ceftazidime/clavulanate	0.12-16	0.25	4	0	0.25-64	8	32	38.4
piperacillin	≥1024-≥1024	≥1024	≥1024	100	≥1024-≥1024	≥1024	≥1024	100
piperacillin/tazobactam	16-256	16	256	38.4	32-≥1024	128	≥1024	75
imipenem	0.06-1	0.25	0.5	0	0.06-4	0.25	1	0
meropenem	0.016-2	0.03	025	0.25	0.03-2	0.12	0.5	0
				***K. pneumoniae* SHV-12 (n=7)**				
amoxycillin	≥1024-≥1024	≥1024	≥1024	100	≥1024-≥1024	≥1024	≥1024	100
amoxycillin/clavulanate	32->1024	128	≥1024	100	256-≥1024	512	≥1024	100
ceftazidime	64-≥1024	128	≥1024	100	256-≥1024	512	≥1024	100
ceftazidime/clavulanate	0.25-4	1	4	0	1-32	8	32	14.1
piperacillin	≥1024-≥1024	≥1024	≥1024	100	≥1024-≥1024	≥1024	≥1024	100
piperacillin/tazobactam	32-128	128	128	57.1	128-≥1024	128	≥1024	100
imipenem	0.25-1	0.5	1	0	0.25-4	1	2	0
meropenem	0.06-0.25	0.12	0.25	0	0.12-2	0.12	1	0

Table 3. MIC range, cumulative MIC values and percentage of resistant strains at standard and high inoculum testing, for *Escherihia coli* strains producing SHV and CTX-M-ESBLs.

	Standard inoculum				High inoculum			
Antibiotic	MIC range	MIC50	MIC90	%R	MIC range	MIC50	MIC90	%R
				***E. coli* - SHV-5 (n=41)**				
amoxycillin	512≥1024	≥1024	≥1024	100	512≥1024	≥1024	≥1024	100
amoxycillin/clavulanate	4-64	8	32	14.6	16-128	>128	128	90
ceftazidime	16≥1024	256	≥1024	95.1	32->1024	≥1024	≥1024	100
ceftazidime/clavulanate	0.06-8	0.5	4	0	0.06-64	4	32	7.3
piperacillin	512≥1024	≥1024	≥1024	100	512≥1024	≥1024	≥1024	100
piperacillin/tazobactam	8-128	32	128	9.7	32-512	128	512	53.6
imipenem	0.06-0.25	0.12	0.5	0	0.06-2	0.25	2	0
meropenem	0.016	0.03	0.03	0	0.016-0.12	0.03	0.12	0
				***E. coli* - CTX-M (n=14)**				
amoxycillin	64-≥1024	≥1024	≥1024	100	≥1024-≥1024	≥1024	≥1024	100
amoxycillin/clavulanate	1-64	16	64	42.8	8-512	128	512	92.8
ceftazidime	0.25-32	4	32	35.1	2-512	32	512	57.1
ceftazidime/clavulanate	0.06-2	0.5	1	0	1-16	4	8	0
piperacillin	64-≥1024	≥1024	≥1024	100	≥1024-≥1024	≥1024	≥1024	100
piperacillin/tazobactam	2-128	15	128	42.8	16-≥1024	256	≥1024	64.2
imipenem	0.06-1	0.25	1	0	0.12-4	0.5	4	0
meropenem	0.03-0.25	0.12	0.25	0	0.06-2	0.25	1	0

compared to the standard. Meropenem and imipenem were not affected by inoculum size with MIC_{90} values of 0.25 and 1 mg/L respectively when standard inoculum was applied and MIC_{90} of 0.5 and 4 mg/L in high inoculum testing. With increased inoculum the percentage of SHV-2 producers resistant to AMC rose from 43 to 90%, to CAZ from 76% to 95% and from TZP from 19% to 57%. At the standard inoculum testing none of the SHV-2 producers were resistant to CAZ/cl whereas at high inoculum 9.5% of the strains became resistant (Table 2).

With SHV-5 producing *K. pneumoniae* the highest increase in MIC_{90} due to inoculum effect was observed for CAZ/cl (4 to 32 mg/L) and AMC (128 to >1024 mg/L) followed by TZP (256 to ≥1024 mg/L) whereas carbapenems showed only slight increase of the concentration necessary to inhibit 90% of the strains (0.5 to 1 mg/L for imipenem and 0.25 to 0.5 mg/L for meropenem). At the standard inoculum testing none of the SHV-5 producers was resistant to CAZ/cl while at high inoculum 38% of the strains showed resistance (Table 2). The percentage of resistant strains was also significantly increased due to inoculum effect for TZP (38% to 75%) and AMC (81 to 98%).

MIC_{90} for HV-12 producers at standard inoculum size was ≥1024 for AMX, for TZP 128 mg/L, for CAZ/cl 4 mg/l, for IMI 1 mg/L and for MEM 0.25 mg/L whereas at high inoculum size it reached ≥1024 mg/l for AMX, AMC, CAZ, PIP and TZP, 32 mg/l for CAZ/cl, 2 mg/L for IMI and 1 mg/L for MEM. At the standard inoculum testing all strains were resistant to AMX, AMC, PIP and CAZ, whereas 57% were resistant to TZP. No resistance to CAZ/cl, IMI and MEM was observed. At high inoculum percentage of strains resistant to TZP rose to 100 % and to CAZ/cl to 14.1% but the susceptibility IMI and MEM was maintained in spite of slightly higher MIC values for particular strains (Table 2).

The concentration necessary to inhibit 90% of the SHV-5 producing *E. coli* strains rose for two dilutions with increased inoculum for AMC (32 →128 mg/L), TZP (128→512 mg/L), imipenem (0.5→2 mg/L) and meropenem (0.03→0.12 mg/L) and for three dilutions in case of CAZ/cl (4→32 mg/L). When the inoculum was increased 100 fold, resistance increased from 14 to 90% for AMC, from 10 to 53% for TZP and from 0 to 7% for CAZ/c (Table 3).

The significant increase in the concentration that inhibited 90% of the CTX-M producers due to inoculum effect was obtained with AMC (64 →512 mg/L), CAZ/cl (1→8 mg/L) TZP (128 →≥1024 mg/L), where MICs of carbapenems did not have a marked increase in MIC_{90} at high inoculum testing (two dilutions). When the inoculum was increased 100 fold resistance of CTX-M positive *E. coli* strains was increased from 43 to 93% for AMC, from 35 to 57% for CAZ and from 43 to 64% for TZP. All CTX-M producers maintained susceptibility to CAZ/cl and carbapenems even with high inoculum testing (Table 3).

ESBL negative strains did not display inoculum effect with any antibiotic tested. MIC_{90} of AMX, AMC, CAZ, CAZ/cl, PIP, TZP, IMI, and MEM was 8, 2, 0.5, 0.25, 8, 4, 0.12 and 0.06 mg/L respectively in the presence of the standard inoculum while the values in the presence of high inoculum were 32, 8, 2, 1, 16, 8, 0.5 and 0.12 mg/L respectively (Table 4). All ESBL negative strains were susceptible to all tested antibiotics at the standard inoculum testing. When testing was performed with high inoculum 19% of the ESBL negative strains were resistant to AMX, but no resistance to any other antibiotic was observed (Table 4).

AMC and CAZ/cl were associated with inoculum effect against all type of ESBL producers: SHV-2, SHV-5, SHV-12 and CTX-M. TZP was less affected by the inoculum size then AMC, and CAZ/cl particularly with CTX-M producers. It was not possible to determine inoculum effect for AMX, PIP and CAZ alone because of the predominantly off- scale MIC values which exceeded 1024 mg/l even when tested with the standard inoculum size.

DISCUSSION

Clinicians rely on the results of in vitro susceptibility testing to choose appropriate antimicrobial agent for the therapy. Results of in vitro testing depend on many factors including inoculum effect (6). Inoculum effect was previously described for ceftazidime, cefotaxime, cefepime and other cephalosporins (13-15), but there are only few reports of inoculum effect with β-lactam/inhibitor combinations (6). Previous studies have shown small inoculum effect of β-lactamase/inhibitor combinations on Enterobacteriaceae in general (6), but in this research we studied the inoculum effect of these compounds in enteric bacteria with well defined resistance mechanisms. The studies on animal models have shown failures of ceftriaxone/sulbactam combination in experimental rabbit endocarditis due to the high density of *K. pneumoniae* producing TEM-3 β-lactamase (8) and *E. coli* producing SHV-2 β-lactamase in the cardiac vegetations (9). According to the results of this study, inoculum effect for all tested compounds was more pronounced for ESBL positive strains in comparison with ESBL negative. This is in concordance with previous reports which found the inoculum effect to be more significant if the antibiotic is susceptible to hydrolysis by a certain β-lactamase (7,13-14). It can lead to therapeutic failures if infections caused by ESBL producing microorganisms are treated with expanded-spectrum cephalosporins. Inoculum effect occurs when a bacterium produces enzyme capable of hydrolyzing an antibiotic (7). There are two explanations for the inoculum effect: antibiotic destruction by β-lactamases and filamentous transformations with continued growth (6). Susceptibility to AMC and CAZ/cl was more affected by inoculum size than TZP. There were slight differences observed in the magnitude of the inoculum effect with different types of ESBLs. The activity of TZP was mostly compromised in the presence of high density of SHV-5 producing *K. pneumoniae*. The fact that SHV-5 and SHV-12 producers showed the higher increase in the percentage of resistant strains for CAZ/cl in comparison with SHV-2 and CTX-M producers due to inoculum effect could be explained with higher hydrolysis rate of ceftazidime by SHV-5 and SHV-12 β-lactamase. Car-

Table 4. MIC range, cumulative MIC values and percentage of resistant strains at standard and high inoculum testing, for ESBL negative *Klebsiella pneumoniae* strains.

	ESBL negative *K. pneumoniae* (n=26)							
	Standard inoculum				High inoculum			
Antibiotic	MIC range	MIC50	MIC90	%R	MIC range	MIC50	MIC90	%R
amoxycillin	0.5-16	2	8	0	0.5-64	4	32	19.2
amoxycillin/clavulanate	0.5-4	2	2	0	1-16	2	8	0
ceftazidime	0.03-0.5	0.12	0.5	0	0.06-4	0.25	2	0
ceftazidime/clavulanate	0.03-0.5	0.12	0.25	0	0.06-1	0.25	1	0
piperacillin	0.5-8	4	8	0	1-32	4	16	0
piperacillin/tazobactam	0.5-4	2	4	0	1-16	2	8	0
imipenem	<0.016-025	0.06	0.12	0	0.03-0.5	0.12	0.5	0
meropenem	<0.016-0.06	<0.016	0.06	0	0.03-0.25	0.06	0.12	0

bapenems were the most stable to inoculum efect regardless of the type of ESBL. This observation is in concordance with previous reports (16-17). For that reason carbapenems which are stable in the presence of high inoculum should be antibiotics of choice for the treatment of infections caused by ESBL producing Enterobacteriaceae. β-lactamase/inhibitor combinations should be avoided in the therapy because of the inoculum effect and development of hyperproducing variants during treatment which are not sufficiently inhibited with therapeutic concentrations of clavulanic acid or sulbactam (18). AMC could be used for the treatment of urinary tract infections caused by ESBL producing Enterobacteriaceae due to its high concentrations in urine which overwhelm the inoculum effect and prevent development of hyperproducing mutants. Combinations of expanded-spectrum cephalosporins with β-lactamase inhibitors are not available at Croatian market but combination of cefoperazone with sulbactam is registered in France (18). The other important drawback of β-lactamase/inhibitor combinations is the selection of inhibitor resistant β-lactamases (18).

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REFERENCES

1. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev 2001; 14:933-51.
2. Paterson DL. Recommendation for treatment of severe infections caused by *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs). Clin Microbiol Infect 2000; 6:460-3.
3. Paterson DL, Ko WC, von Gottberg A, Casellas JM, Mulazimoglu L, Klugman KP, Bonomo RA, Rice L, McCormack J, Yu V. Outcome of cephalosporin treatment for serious infections due to apparently susceptible organisms producing extended-spectrum β-lactamases: implication for the clinical microbiology laboratory. J Clin Microbiol 2001; 39:2206-12.
4. Karas JA, Pillay DG, Muckart D, Sturm W. Treatment failure due to extended-spectrum β-lactamase. J Antimicrob Chemother 1996; 37:203-4.
5. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Eighteen Informational Supplement. CLSI document M100-S18. Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
6. Goldstein EJ, Citron DM, Cherubin CE. Comparison of the inoculum effects of members of the family *Enterobacteriaceae* on cefoxitin and other cephalosporins, β-lactamase inhibitor combinations, and the penicillin-derived component of these combinations. Antimicrob Agents Chemother 1991; 35:560-6.
7. Thomson KS, Smith-Molland E. Cefepime, piperacillin:tazobactam, and the inoculum effect in tests with extended-spectrum β-lactamase producing Enterobacteriaceae. Antimicrob Agents Chemother 2001; 45: 3548-54.
8. Caron F, Gutmann L, Bure A, Pangon B, Vallois JM, Pechinot A, Carbon C. Ceftriaxone-sulbactam combinations in rabbit endocarditis caused by a strain of *Klebsiella pneumoniae* producing extended-spectrum TEM-3 β-lactamase. Antimicrob Agents Chemother 1990; 34: 2070-4.
9. Fantin B, Pangon B, Potel G, Caron F, Vallee E, Vallois JM, Mohler J, Bure A, Philippon A, Carbon C. Activity of sulbactam in combinations with ceftriaxone in vitro and in experimental endocarditis caused by *Escherichia coli* producing SHV-2 likw β-lactamase. Antimicrob Agents Chemother 1990; 14. 581-6.

10. Bedenić B, Žagar Ž. Extended-spectrum β-lactamases in clinical isolates of *Klebsiella pneumoniae* from Zagreb, Croatia. J Chemother 1998;10:449-59.
11. Bedenić B, Randegger CC, Stobberingh E, Hachler H. Molecular epidemiology of extended-spectrum β-lactamases from *Klebsiella pneumoniae* strains isolated in Zagreb, Croatia. Eur J Clin Microbiol Infect Dis 2001; 20:505-8.
12. Bedenić B, Schmidt H, Herold S, Monaco M, Plečko V, Kalenić S, Skrlin J. Spread of *Klebsiella pneumoniae* producing SHV-5 β-lactamase in Dubrava University Hospital, Zagreb. J Chemother 2005; 17:367-75.
13. Queenan AM; Foleno B, Gownley C, Wira E, Bush K. Effects of inoculum and β-lactamase activity in AmpC and extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates tested by using NCCLS ESBL methodology. J Clin Microbiol 2004; 42:269-75.
14. Kang CI, Pai, H, Kim SB, Kim HB, Kim EC, Oh MD, Choe KW. Cefepime and the inoculum effect in tests with *Klebsiella pneumoniae* producing plasmid-mediated AmpC-type β-lactamase. J Antimicrob Chemother 2004; 54:1130-3.
15. Bedenić B, Beader N, Žagar Ž. Effect of inoculum size on the antibacterial activity of cefpirome and cefepime against *Klebsiella pneumoniae* strains producing SHV extended-spectrum β-lactamases. Clin Microbiol Infect 2001; 7: 626-35.
16. Wiseman LR, Wagstaff AJ, Brogden RN, Bryson HM: Meropenem: A Review of its antibacterial activity, pharmacokinetic properties and clinical efficacy. Drugs 1995; 50:73-101.
17. Betriu C, Salso S, Sanchez A, Culebras E, Gomez M, Rodrigez-Avial I, Picazo JJ. Comparative in vitro activity and the inoculum effect of ertapenem against Enterobacteriaceae resistant to extended-spectrum cephalosporins. Int J Antimicrob Agents 2006; 28:1-5.
18. Amyes SGB, Miles RS. Extended-spectrum β-lactamases: the role of inhibitors in the therapy 1998; 42:415-7.