

β -lactam resistance phenotype determination in *Escherichia coli* isolates from University Malaya Medical Centre

Jeanne Sze Lyn WONG, Zainal Abidin MOHD AZRI, *Geetha SUBRAMANIAM, BSc, PhD, **Siaw Eng HO, BSc, MMedSc, *Selvi PALASUBRAMANIAM, BSc, MMedSc and *Parasakthi NAVARATNAM, MBBS, FRCPath

*Department of Medical Microbiology, Faculty of Medicine, University of Malaya and **Faculty of Science, National University of Singapore

Abstract

β -lactamases have been identified as the major cause of antimicrobial resistance to β -lactam antibiotics in *Escherichia coli*. The activities of ampicillin-sulbactam and amoxicillin-clavulanate as well as a range of β -lactam antibiotics were studied with 87 clinical *E. coli* isolates from patients of the University Malaya Medical Center using the disc diffusion technique. Susceptible, intermediate and resistant categories were established based on the diameter of zones of inhibition set by the National Committee for Clinical Laboratory Standards (NCCLS). The isolates were then classified into 6 phenotypes according to the criteria stated in the methodology: S (susceptible to all β -lactams); TL (resistant to aminopenicillins; amoxicillin-clavulanate susceptible and susceptible or intermediate to ampicillin-sulbactam); TI (resistant to aminopenicillins and ampicillin-sulbactam; susceptible to amoxicillin-clavulanate); TH-IRT (resistant to aminopenicillins; intermediate or resistant to amoxicillin-clavulanate; resistant to ampicillin-sulbactam); ESBL (resistant to aminopenicillins and oxyimino cephalosporins; positive results with the double-disc diffusion test); and CP (resistant to aminopenicillins, β -lactam- β -lactamase inhibitor combinations, oxyimino cephalosporins and cephamycins). Results showed that the TL phenotype was the commonest (40.2% of the isolates) followed by S (31%), TH-IRT (16.1%), ESBL and CP (3.4% each) and TI (2.3%). One isolate showed both ESBL and CP phenotypes while two isolates were classified as inconclusive. Representatives from each phenotype were further analysed for the presence of β -lactamases which revealed a predominance of TEM and SHV enzyme producers. PCR-SSCP analysis of the SHV gene from all the ESBL and CP isolates revealed the predominance of SHV 5-type enzyme which was concurrent with our previous studies.

Key Words: *E. coli*, β -lactamase, resistant phenotypes

INTRODUCTION

Escherichia coli isolates that exist as normal flora in man and animals, and if unexposed to antibiotic pressure remain virtually susceptible to all antibiotics. However, selective pressure exerted by the continuous exposure to antimicrobial agents could lead to the development of antibiotic resistance. The most commonly used antibiotics worldwide in the treatment of infections are the β -lactams, due to their efficacy, broad-spectrum activity and low toxicity. Not surprisingly, the most common mechanism of resistance in Gram negative bacilli, including *E. coli*, is the production of β -lactamases, which hydrolyze these antibiotics¹. The widespread and often indiscriminate use of β -lactams has led to the emergence of bacteria

that hyperproduce β -lactamases or variants of these enzymes. This has undermined the value of ampicillin and 3rd generation cephalosporins used against many Gram-negative bacteria. Derivatives of these β -lactamases which include TEM-1, TEM-2 and SHV enzymes have led to the emergence of extended spectrum β -lactamases (ESBLs) which cause resistance to most penicillins, cephalosporins and aztreonam². ESBLs are encoded for by genes which are generally located on plasmids, and are therefore, easily transmissible.

Combinations of β -lactam- β -lactamase inhibitors have proven successful in treating infections caused by ESBL-producing organisms³. However, there are increasing number of reports on resistance to β -lactam- β -

lactamase inhibitor combinations⁴. In *E. coli*, this resistance has been reported to be due mainly to the hyperproduction of TEM-1 β -lactamase, usually encoded by small, multicopy plasmids⁵. However, susceptibility to these combinations could be the result of other mechanisms of resistance which includes the production of inhibitor-resistant TEMs (IRTs), and the hyperproduction of chromosomally-mediated AmpC⁶.

There is a need to closely monitor the incidence of antibiotic resistance in clinical isolates in order to determine the trend of resistance and thereby to help confine the spread of ESBL-encoded plasmids. In this study, we analysed the antimicrobial susceptibility of 87 *E. coli* isolates from patients in the UMMC, and subsequently characterized the β -lactamases produced by the β -lactam-resistant strains in order to study the pattern of β -lactam resistance phenotypes in *E. coli* and the probable mechanisms of resistance.

MATERIALS AND METHODS

Bacterial isolates

A total of 87 *E. coli* isolates were obtained from clinical samples (blood, pus and urine) over a period of 4 months from March to June, 2001. These were consecutive, non-duplicate isolates from patients in the University Malaya Medical Center (UMMC). The identification of the *E. coli* isolates were confirmed using the conventional bacteriological methods⁷.

Antimicrobial Susceptibility Testing

Susceptibility profiles were obtained using the standard disc diffusion method (National Committee for Clinical Laboratory Standards 2003 (NCCLS)⁸. Antibiotic discs used in this study were amoxicillin-clavulanate (30 μ g), ampicillin-sulbactam (20 μ g), aztreonam (30 μ g), meropenem (10 μ g), cefepime (30 μ g), ceftazidime (30 μ g), cefotaxime (30 μ g), and ceftioxin (30 μ g). All discs were obtained from Becton Dickinson, USA, with the exception of the ceftioxin and aztreonam discs that were obtained from OXOID, England.

After overnight incubation, the diameter of the inhibition zones for each disc was measured and susceptible, intermediate and resistant categories were established based on the standard criteria designed by the NCCLS. The *E. coli* isolates were then classified into 6 different phenotypes according to the following criteria⁹: (i) S phenotype isolates were susceptible to all

β -lactams tested; (ii) TL phenotype isolates were resistant to aminopenicillins, susceptible to amoxicillin-clavulanate and susceptible or intermediate to ampicillin-sulbactam; (iii) TI phenotype isolates were resistant to aminopenicillins, susceptible to amoxicillin-clavulanate, and resistant to ampicillin-sulbactam; (iv) TH-IRT phenotype isolates were resistant to aminopenicillins, intermediate or resistant to amoxicillin-clavulanate, and resistant to ampicillin-sulbactam; (v) ESBL phenotypes were resistant to aminopenicillins and oxyimino cephalosporins and gives a positive result in synergy testing between ceftazidime and clavulanate in the double disc synergy test (DDST)¹⁰; (vi) CP phenotypes are resistant to aminopenicillins, β -lactam- β -lactamase inhibitor combination, oxyimino cephalosporins, and cephamycins.

Isoelectric focusing

Crude β -lactamase extracts, prepared according to the method of Neuwirth *et al.*¹¹, were subjected to analytical isoelectric focusing on ampholine polyacrylamide gels with broad pH range (3-9) (Pharmacia Biotech, Sweden) using a PhastSystem (Pharmacia Biotech, Sweden). Visualization of β -lactamases was done by staining with nitrocefin, a chromogenic cephalosporin (OXOID, UK).

PCR Amplification

PCR amplification was carried out on 17 selected isolates with known pI values and were β -lactamase producers. DNA template preparation and subsequent PCR amplification of the SHV gene was carried out based on the method of M'Zali *et al.*¹². A pair of primers 5'-TCAGCGAAAAACACCTTG-3' and 5'-TCCCGCAGATAAATCACCA-3' were used to amplify the 475-bp sequence of the *bla*_{SHV} gene¹³ while the 971-bp fragment of the TEM gene was amplified using primers 5'-TCGGGGAAATGTGCG-3' and 5'-TGCTTAATCAGTGAGGCACC-3'. All primers were synthesized by Genemed Biotechnologies Inc., USA.

Polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) analysis

PCR-SSCP was carried out using the method of M'Zali *et al.*¹². Briefly, the 475-bp SHV amplicons were digested with *Pst*I, denatured by mixing with a formamide dye, prior to heating at 95°C for 5 mins. The denatured products were

β-LACTAM RESISTANCE PHENOTYPES IN ESCHERICHIA COLI

separated on a 12% denaturing polyacrylamide gel.

RESULTS

The 87 *E. coli* isolates were classified resistant, intermediate, or sensitive to β-lactams as well as β-lactam-β-lactamase inhibitor combinations, and were subsequently grouped into 6 phenotypes based on their antibiotic profiles obtained from the disc diffusion method (Table 1). *E. coli* isolates of the TL phenotype which encompassed

resistance to aminopenicillins only, had the highest percentage of isolates (41.4%) followed by the S phenotype (31%) which were sensitive to all antibiotics. Most of these isolates were from in-patients in UMMC (24 and 17 respectively). There was a fairly high percentage of *E. coli* isolates with the TH-IRT phenotype. However, there were only 3 *E. coli* isolates with the ESBL and CP phenotypes and were from in-patients with the exception of 1 isolate that had been isolated from an out-patient.

TABLE 1: Number and percentage of *E. coli* isolates for each phenotype

Phenotype	Number of isolates (%)	Number of patients (%)	
		In-patient	Out-patient
S	27 (31.0)	17 (29.3)	10 (34.5)
TL	35 (40.2)	24 (41.4)	11 (38.0)
TI	2 (2.3)	2 (3.5)	-
TH-IRT	14 (16.1)	7 (12.0)	7 (24.1)
ESBL	3 (3.4)	2 (3.4)	1 (3.4)
CP	3 (3.4)	3 (5.2)	-
ESBL and CP	1 (1.1)	1 (1.7)	-
Inconclusive	2 (2.3)	2 (3.5)	-
Total	87 (100)	58 (100%)	29 (100%)

TABLE 2: Characterization of β-lactamases via IEF, PCR and PCR-SSCP

Phenotype	No. of strain	pI range	Subtype	Confirmed via PCR		PCR-SSCP
				TEM	SHV	
S	2	-	-	ND	ND	ND
	1	7.8	SHV	-	+	
TL	5	5.1-6.0	TEM	+	-	ND ND ND
	3	5.4-8.2	TEM,SHV	+	-	
	1	8.2	SHV	-	+	
TI	2	5.1-6.0	TEM	+	-	
TH-IRT	2	5.1-8.2	TEM,SHV	+	+	ND
ESBL	1	5.1	TEM	+	-	SHV-5 SHV-5
	1	5.1-8.2	TEM,SHV	+	+	
	1	8.2	SHV	-	+	
CP	1	6.5	TEM	+	-	SHV-5
	1	6.5,8.2	TEM,SHV	+	+	

ND – not done

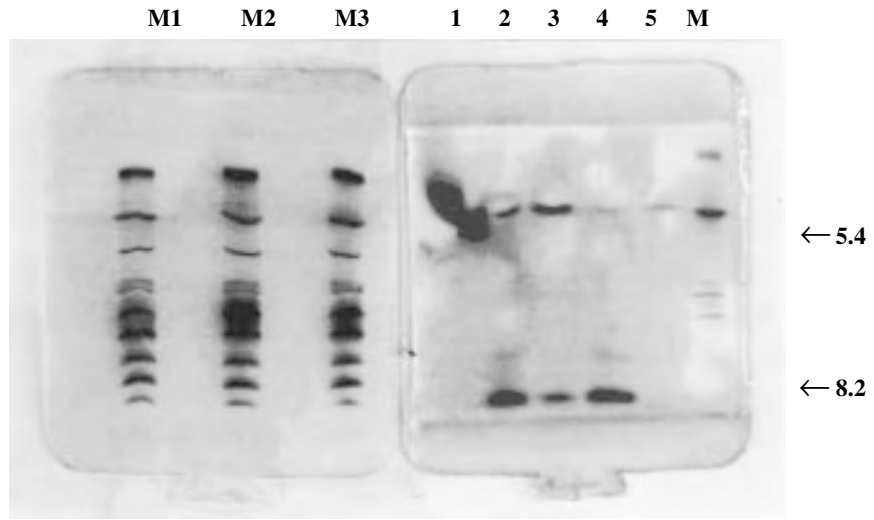


FIG. 1: Isoelectric focusing of β -lactamases and deduction of pI values using pI Markers (M1, M2 M3 and M - IEF markers stained with Coomassie blue and nitrocefin respectively; Lanes 1 – 5 - beta-lactamases extracted from *E. coli* isolates and stained with nitrocefin).

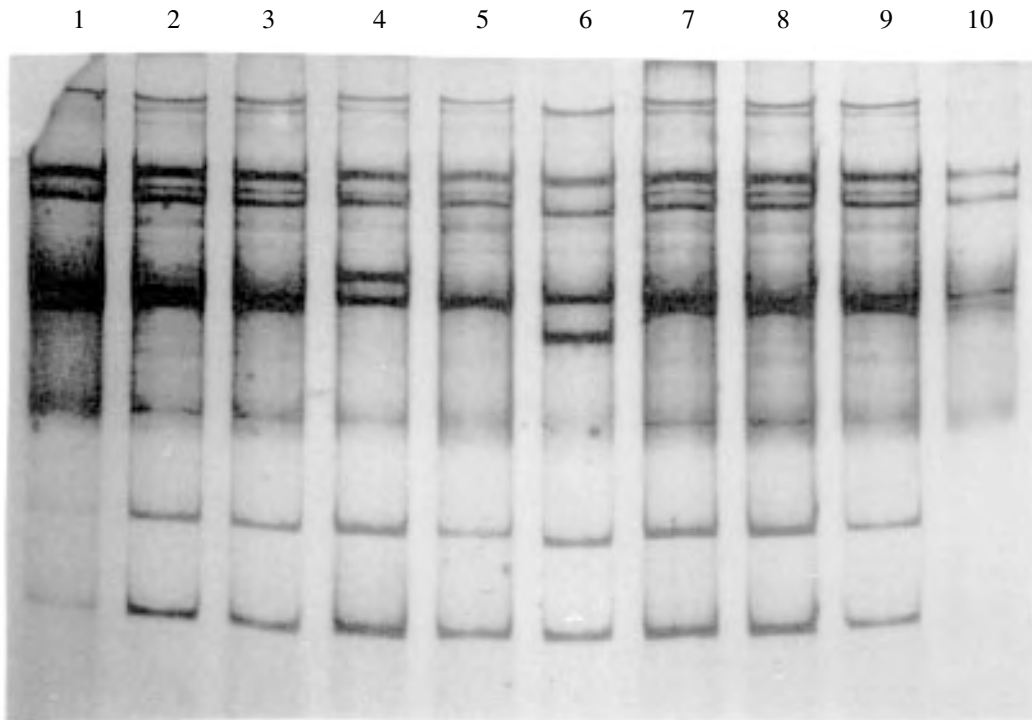


FIG. 2: PCR-SSCP of amplified SHV gene products from *E. coli*. (Lanes 1,2,7,8,9,10 – isolates; Lane 3 – SHV-5 control; Lane 4 – SHV-4 control; Lane 5 – SHV-2 control; Lane 6 – SHV-1 control)

Isoelectric focusing was carried out on 21 selected isolates which were representatives of all 6 phenotype groups (Table 2). The β -lactamases detected were predominantly TEM enzymes with a pI range of 5.4 to 6.8, and were found in 76% of the isolates tested (Figure 1). SHV enzymes were detected in 35% of the isolates. Only one of the three isolates tested from the S phenotype produced a β -lactamase. However, this could also be due to the low level of production of the TEM-1 enzyme within this phenotypic group which did not confer resistance. The isolates that produced the TEM or SHV enzymes were further analysed using PCR to confirm the presence of these genes (Table 2, Fig. 1). Isolates that harboured the SHV-type gene were further analysed using PCR-SSCP in order to determine the identity of the SHV subtype (Fig. 2). Data indicated that the SHV genes from the ESBL and CP isolates were SHV 5 type β -lactamase, which has been shown to be predominant in *K. pneumoniae* and *E. coli* isolates in UMMC (Table 2, Figure 2)¹⁴.

DISCUSSION

Antibiotic resistance is a growing threat worldwide due to the increasing incidence and spread of this resistance which has been further intensified by emergence of resistance to new antibiotics and spread of these resistant organisms among patients in both hospital as well as community settings. This problem might be further aggravated by the introduction of antibiotics in animal feed, thereby creating a reservoir for the development of resistant strains in livestock and subsequent transmission to man via food consumption.

As in other members of the family Enterobacteriaceae, resistance to β -lactam antibiotics in *E. coli* is mainly due to the production of β -lactamases, the most common being the TEM-1 enzyme and SHV enzymes^{1,9}. However, other mechanisms affecting the activity of β -lactams which include AmpC hyperproduction, porin-deficient mutants, could also contribute to the resistance in *E. coli* to the β -lactams.

The commonest phenotype among isolates from both in-patients and out-patients was TL, S and TH-IRT in order of frequency. However, among in-patients, a wider phenotype distribution was seen which included TI, CP and ESBL phenotypes (Table 1). Overall, there was a higher number of pathogens isolated from in-patients than from out-patients. These findings are

supported by a study carried out in 8 hospitals in America which showed that there was a significantly higher percentage of resistant isolates from in-patients than from out-patients¹⁵. This could be attributed to the overall increase in the number of immunocompromised patients coupled with factors such as an increase in antimicrobial usage in hospitals and indwelling devices such as catheters and intravascular lines. These factors contribute to the rise in antimicrobial-resistant pathogens causing nosocomial infections in hospitals, particularly in intensive care units (ICUs)^{15,16}.

The TL and S phenotypes were the predominant phenotypes in *E. coli* seen in this study, with TL phenotypes having the highest percentage of isolates (40.2%) followed by S phenotypes (31.0%). The high TL phenotype percentage could be due to the widespread prescription of ampicillin in Malaysia. Low level production of plasmid-mediated TEM-1 enzymes could be mainly responsible for the pattern of resistance seen in TL phenotypes of *E. coli* in this study. These isolates are resistant to ampicillin and susceptible or intermediately susceptible to β -lactamase inhibitor combinations. This is concurrent with previous reports on β -lactam resistance in *E. coli* due to the production of TEM-1^{5,17}.

The percentage of isolates with the TH-IRT phenotype was the 3rd highest (16.1%). The resistance to the β -lactam- β -lactamase inhibitor combinations of ampicillin-sulbactam and amoxicillin-clavulanate have been attributed to various mechanisms, including hyperproduction of TEM-1 and the presence of an inhibitor resistant TEM (IRT)¹⁸. The high percentage of TEM β -lactamases in these isolates (74%) coupled with the administration of β -lactam- β -lactamase inhibitor combination suggests that the predominant mechanisms of β -lactamase resistance in *E. coli* isolates in UMMC are the hyperproduction of TEM 1 and, to a lesser degree, the presence of IRTs. The high level of TEM 1 β -lactamase production has been shown to correlate with resistance to β -lactam- β -lactamase inhibitor combinations^{18,19}.

ESBL phenotypes however, could attribute the resistance to the extensive usage of expanded spectrum β -lactams to other mechanisms. Although the percentage of ESBL-producing *E. coli* were relatively low (3.4%), the prevalence of this phenotype should be monitored closely because of its ability to hydrolyze a wide range of β -lactams and to transmit this resistance both within the species and among other Gram-

negatives as well. The presence of the SHV-5 type enzyme in the ESBL phenotype could be responsible for resistance to the extended-spectrum β -lactams. The predominance of SHV-5 in *E. coli* is concurrent with previous studies in UMMC whereby we found a predominance of SHV-5 type β -lactamase in clinical isolates of ESBL-producing *K. pneumoniae* and *E. coli*¹⁴. Widespread use of expanded-spectrum cephalosporins for treatment of severe infections could be responsible for the emergence of this phenotype.

CP phenotypes were also uncommon (3.4%) in the strains studied. This phenotype is associated with the hyperproduction of AmpC and/or TEM-1 enzymes in permeability modified isolates and were resistant to virtually all the β -lactams tested. Inadequate testing of AmpC hyperproduction in the laboratories has been reported⁶. However, PCR analyses revealed only 1 isolate harbouring the AmpC gene, therefore, AmpC hyperproduction does not seem to be a major mechanism of resistance in *E. coli* in this study. Isolates with ESBL and CP phenotypes were still susceptible to the carbapenems, meropenem and imipenem. Hence, these antibiotics still remain as effective therapeutics against the growing number of multiresistant *E. coli* isolates in UMMC. However, there is a need to implement surveillance of microbial pathogens in nosocomial infections over periods of time as this can provide valuable information that could aid in the decision of physicians as to the most suitable antimicrobial therapy to administer to patients.

As in other members of the family Enterobacteriaceae, resistance to β -lactam antibiotics in *E. coli* is mainly due to the production of β -lactamases, the most common being the TEM-1 enzyme and SHV enzymes^{1,9}. However, other mechanisms affecting the activity of β -lactams which include AmpC hyperproduction, porin-deficient mutants, could also contribute to the resistance in *E. coli* to the β -lactams.

ACKNOWLEDGMENTS

We are grateful to Dr. Peter Hawkey for providing the SHV controls. This work was funded by the Ministry of Science, Technology and the Environment, Malaysia IRPA Grants 06-02-03-0759 and 06-02-03-0017.

REFERENCES

1. Livermore DL. Extended-spectrum β -lactamases in Resistance: Evolution and Epidemiology. In Proceedings of the Symposium of Antibiotic Resistance: the challenge of the New Millenium, Birmingham,UK,1999.
2. Heritage J, M'Zali FH, Gascoyne-Binzi DM, Hawkey PM. Evolution and spread of SHV extended-spectrum beta-lactamases in gram-negative bacteria. J Antimicrob Chemother 1999; 44: 309-18.
3. Maddux M. Effects of β -lactamase-mediated antimicrobial resistance: the role of β -lactamase inhibitors. Pharmacotherapy 1991;11(Suppl 2): 40-50.
4. Martinez JL, Cercenado E, Rodriguez-Creixems M, Vicente-Perez, Delgado-Iribaren A, Baquero F. Resistance to beta-lactam/clavulanate. Lancet 1987; ii:1473.
5. Wu PJ, Shannon K, Phillips I. Mechanisms of hyperproduction of TEM-1 β -lactamase by clinical isolates of *E. coli*. J Antimicrob Chemother 1995;36:927-39.
6. Thompson KS. Controversies about extended-spectrum and AmpC β -lactamases. Emerging Infect Dis 2001; 7:333-5.
7. Cowan ST. Manual for the identification of Medical Bacteria. Second edition. Cambridge University Press. 1974; Appendix C: 166-180.
8. National Committee for Clinical Laboratory Standards 2003. Performance Standards for Antimicrobial Disk Susceptibility Tests. Approved Standard M2-A6. NCCLS Document. Wayne,Pa.
9. Oliver A., P'erez-V'azquez M, Martinez-Ferrer M., Baquero F, Rafael LD, Canton R. Ampicillin-Sulbactam and amoxicillin-clavulanate susceptibility testing of *E. coli* isolates with different β -lactam resistance phenotypes. Antimicrob Agents Chemother 1999; 43:862-867.
10. Jarlier V, Nicolas MH, Fournier G and Philippon A. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactams in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Rev Infect Dis 1988; 10: 867-878.
11. Neuwirth C, Siebor E, Lopez J, Pechinot A and Kazmierczak A. Outbreak of a TEM-24 producing *Enterobacter aerogenes* in an intensive care unit and dissemination of the extended-spectrum beta-lactamase to other members of the family Enterobacteriaceae. J Clin Microbiol 1996; 34:76-9.
12. M'Zali FH, Heritage J, Gascoyne-Binzi DM, Denton M, Todd NJ, Hawkey PM. Transcontinental importation into the UK of *Escherichia coli* expressing a plasmid-mediated AmpC-type β -lactamase exposed during an outbreak of SHV-5 extended-spectrum β -lactamase in a Leeds hospital. J Antimicrob Chemother 1997; 40: 823-831.
13. Billot-Klein D, Guttmann L, Collatz E. Nucleotide sequence of the SHV-5 β -lactamase gene of *Klebsiella pneumoniae* plasmid. Antimicrob Agents Chemother 1990; 34:2439-41.

14. Palasubramaniam S. Characterization of extended-spectrum β-lactamases from multi-resistant strains of *Klebsiella pneumoniae*. 1998. Dissertation submitted in fulfillment of Masters in Medical Sciences Degree in University of Malaya.
15. Archibald L, Phillips L, Monnet D *et al*. Antimicrobial resistance in isolates from inpatients and outpatients from the United States : increasing importance of the intensive care unit. *Clin Infect Dis* 1997; 24:211-5.
16. Valles J, Leon C Alvarez-Lerma *et al*. Nosocomial bacteremia in critically ill patients: a multicenter study evaluating epidemiology and prognosis. *Clin Infect Dis* 1997;24:387-95.
17. Marre R, Aleksic S. Beta-lactamase types and beta-lactam resistance of *Escherichia coli* strains with chromosomally mediated ampicillin resistance. *Eur J Clin Microbiol Infect Dis* 1990; 9:44-6
18. Seetulsingh PS, Hall LM, Livermore DM. Activity of clavulanate combinations against TEM-1 β-lactamase producing *Escherichia coli* isolates obtained in 1982 and 1989. *J Antimicrob Chemother* 1991; 24:749-59.
19. Vanjak D, Muller-Serieys C, Picard B, Bergogne-Berezin E, Lambert-Zechovsky N. Activity of β-lactamase inhibitor combinations on *E. coli* isolates exhibiting various patterns of resistance to β-lactam antibiotics. *Eur J Clin Microbiol Infect Dis* 1995; 14:927-8.