

## Original Article

### Detection of CTX-M- $\beta$ Lactamases in Isolated *Klebsiella pneumoniae*

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#### ABSTRACT

**Background and Objective:** Organisms producing CTX-M  $\beta$ -lactamases are emerging as a source of resistance to oxyiminocephalosporins such as ceftriaxone and ceftazidime. However, the laboratory detection of these strains is not well defined. In this study, phenotypic assay for screening of extended-spectrum  $\beta$ -lactamases producing strains and molecular assay for the identification of CTX-M  $\beta$ -lactamases genes was developed and used to investigate the prevalence of these enzymes among clinical isolates of *Klebsiella pneumoniae* in three general hospitals of Tehran, Iran.

**Materials and Methods:** Phenotypic detection was used for screening of isolates by agar dilution method. A decrease of  $\geq 3$  doubling dilution in an MIC for either ceftriaxone or ceftazidime tested in combination with 4 mg/l clavulanic acid (prepared from Glasco Smith company) versus its MIC when tested alone, confirmed an ESBL-producing organism. The PCR assay consisted of four primer sets.

**Results:** In initial screening test, 117 (69%) from 168 clinical isolates were positive and 51 isolates (31%) were negative. From the positive isolates, 96 isolates were positive in phenotypic confirmatory test. Using molecular assay, 117 strains potentially producing extended-spectrum- $\beta$ -lactamases were examined for the presence of CTX-M enzymes. 88 strains (75.2%) were positive for bla<sub>ctx-m</sub> group I genes, 1 strain (0.85%) was positive for bla<sub>ctx-m</sub> group III genes, and 2 strains (1.7%) were positive for bla<sub>ctx-m</sub> group IV.

**Conclusion:** The prevalence of extended-spectrum  $\beta$ -lactamases (ESBLs) are increasing significantly in hospitals of Tehran. In other side, we found that the CTX-M I group had the most prevalence than other CTX-M groups.

**Keywords:** Beta Lactamases, *Klebsiella pneumoniae*, Antibacterial Drug Resistance

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## Introduction

Resistance to the extended-spectrum cephalosporins can occur in *Escherichia coli* and *Klebsiella* species via the production of extended-spectrum  $\beta$ -lactamases (ESBLs) that are capable of hydrolyzing the oxyimino cephalosporins and monobactams (1-3). Recently, a family of ESBLs which preferentially hydrolyze cefotaxime (CTX), the CTX-M- $\beta$ -lactamases, have been recognized and reported in the literature with increasing frequency (4). The CTX-M-type enzymes are group of molecular class A ESBLs that exhibit an overall preference for cefotaxime (CTX, hence the CTX-M name) and ceftriaxone. Nearly 40 variants of the CTX-M-type enzymes have been identified and registered to date (5-9).

According to a review and new data within Embank, CTX-M  $\beta$ -lactamases can be divided into five groups based on their amino acid sequence identities (4-8). Group I includes CTX-M -1, -3, -10 to -12, -15 (UOE-1), -22, -23, -28, -29 and -30. Group II includes CTX-M -2, -4 to -7, -20 and TOHO-1. Group III includes CTX-M -8. Group IV includes CTX-M -9, -13, -14, -16 to -19, -21, -27 and TOHO-2. Finally, group V includes CTX-M-25 and -26. The members of this group exhibit >94% amino acid identity among groups (4).

The  $\text{bla}_{\text{CTX-M}}$  genes are often carried on transferable plasmid (4,10). This resistance mechanism is wide spread through the world, with reports of clinical isolates producing these  $\beta$ -lactamases from Europe, Africa, Asia, South America, and most recently North America (11-15).

The aim of this work was to recognize the epidemiology and prevalence of ESBLs strains (particular CTX-M enzymes) in general hospitals of Tehran, capital of Iran.

## Materials and Methods

### Standards and clinical isolates:

One hundred and sixty eight clinical isolates of *K. pneumoniae* were collected during September 2006 to February 2007 from 3 general hospitals of Tehran,

Iran. All the strains were identified and stored at  $-70^{\circ}\text{C}$ . The quality control strains used for this study were *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 2913, and *K. pneumoniae* ATCC 700603.

### Susceptibility testing:

#### Initial screening test:

MICs of ceftriaxone and ceftazidime (prepared from Glaxo Smith Company, UK) were determined by agar dilution method according to NCCLS (CLSI) recommendations (16, 17). The wild-type MIC distribution of these Gram-negative species suggests that with MICs above 1 mg/l for any of the ESBL screening agents are abnormal and therefore likely to possess an acquired resistance mechanism.

#### Phenotypic confirmatory test:

Phenotypic confirmation was done by agar dilution method. A decrease of  $\geq 3$  doubling dilution in an MIC for either ceftriaxone or ceftazidime tested in combination with 4 mg/l clavulanic acid (prepared from Glaxo Smith Company) versus its MIC when tested alone, confirmed an ESBL-producing organism (16,17).

#### PCR detection of $\text{bla}_{\text{CTX-M}}$ genes:

DNA template preparation and PCR amplification for CTX-M  $\beta$ -lactamase genes were carried out on a thermal cycler 9600 instrument (Techne Company). The primers, and size of the expected are listed in Table 1 (primers were prepared from Metabion company). Magnesium chloride concentration was 1.5 mM for all PCRs. The following reaction parameters for CTX-M group I, II, III, were used: Initial denaturation at  $94^{\circ}\text{C}$  for 3min; denaturation at  $94^{\circ}\text{C}$  for 30s, annealing at  $55^{\circ}\text{C}$  for 30s, and elongation at  $72^{\circ}\text{C}$  for 60s, repeated for 25 cycles, final extension at  $72^{\circ}\text{C}$  for 7min, and then hold the reaction at  $4^{\circ}\text{C}$ .

**Table1:** Primers used in PCRs

Targets	Primer sequence	Prout size(bp)	Annealing temp(°c)	Nucleoted position (pb)	Genbank accession no.
<b>CTX-M group I</b>	CTX-M1-F3 GAC GAT GTC ATC GGC TGA GC CTX-M1-R2 AGC CGC CGA CGC TAA TAC A	499	55	416-435	X <sub>92506</sub>
<b>CTX-M group II</b>	TOHO1-2F GCG ACC AGG TTA ACT ACAATC C TOHO1-1R CGG TAG TAT TGC CCT TAA GCC	351	55	313-334 663-643	X <sub>92507</sub>
<b>CTX-M group III</b>	CTX-M825F CGC TTT GCC ATG TGC AGC ACC CTX-M825R GCT CAG TAC GAT CGA GCC	307	55	475-495 481-764	AF18921
<b>CTX-M group IV</b>	CTX-M914F GCT GGA GAA AAG CAG CGG AG CTX-M914R GTA AGC TGA CGC AAC GTC TC	474	62	1857-1876 2330-2311	AF252622

The following reaction parameters for CTX-M group IV were used: initial denaturation at 94°C for 3min ; denaturation at 94°C for 30s, annealing at 62°C for 30s, and elongation at 72°C for 60s, repeated for 25 cycles, final extension at 72°C for 7min, and then hold reaction at 4°C.

## Results

Number of isolation of *K. pneumoniae* from wards of ICU (n= 34), emergency (n= 25), urology (n= 23)

and infants (n= 21), were more than other wards. The most isolates of *K. pneumoniae* were from urine (n= 82), respiratory tract (n= 21) and blood (n= 16) specimens.

From 168 isolates of *K. pneumoniae*, 117 isolates were resistant to ceftriaxone and ceftazidime (MIC >1mg/l) in initial screening test. These isolates marked as P<sup>+</sup> and potentially had ESBLs enzymes (screen positive isolates). From 117 P<sup>+</sup> strains, 96 isolates were recognized by phenotypic confirmation test (Table 2).

**Table 2:** Frequency of ESBLs producing isolates by phenotypic assay

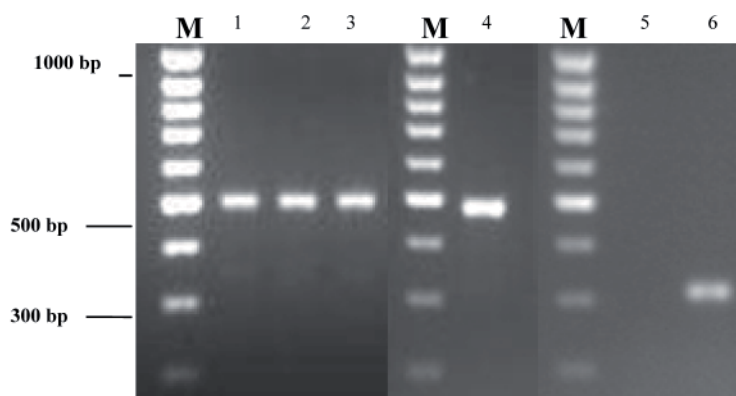
Tests	Positive N (%)	Negative N (%)	Total N (%)
<b>Initial screening</b>	117(69)	51(31)	168 (100)
<b>Confermation of screening test</b>	96(82)	21(18)	117 (100)

All clinical isolates resistant to ceftriaxone or ceftazidime in initial screening test, were tested for the presence of genes coding for the CTX-M family of ESBLs with specific primers. Of the 117 *K. pneumoniae* strains, 88 strains (75.2%) were positive for bla<sub>CTX-M</sub> genes from the CTX-M group I, and 2

strains (1.7%) were positive for bla<sub>CTX-M</sub> genes from the CTX-M group IV. We had not positive strains for CTX-M group II. One strain has both genes of CTX-M group I and CTX-M group III (Table 3, Fig. 1).

**Table 3:** Frequency of ESBLs producing isolates by PCR (117 cases)

Group of CTX-M	Positive N (%)	Negative N (%)
CTX-M group I	88 (75.2)	29 (24.8)
CTX-M group II	0.0 (0.0)	117 (100)
CTX-M group III	1(0.85)	116 (99.15)
CTX-M group IV	2(1.7)	115 (98.3)



**Fig. 1:** PCR for detection of bla<sub>CTX-M</sub> genes. M: Molecular weight marker; 1-3: CTX-M group I; 4: CTX-M group IV; 5: Negative isolate of CTX-M group III; 6: CTX-M group III

## Discussion

There has been a dramatic increase in number of organisms reported in the literature producing CTX-M-β-lactamases (5). This class of β-lactamases has been recognized world wide as an important mechanism of resistance to oxyimino cephalosporins used by Gram-negative pathogens (5). In most cases, organisms producing these enzymes display higher levels of resistance to CTX and ceftriaxone than CAZ (5). However, differentiation between organisms producing some CTX-M β-lactamases from organisms producing other types of ESBLs can be difficult. The difficulty is due to overlapping phenotypes resulting in interference from other β-lactamases produced by the organism capable of hydrolyzing CAZ (12, 16, 18). Therefore, susceptibility testing which relies on identifying organisms that are resistant to CTX and/or ceftriaxone but susceptible to CAZ is not a reliable approach (14). PCR amplification and sequencing

of bla<sub>CTX-M</sub> genes have been used to characterize organisms producing CTX-M β-lactamases (8, 19-21). Data generated using several control strains known to produce specific CTX-M β-lactamases validated the specificity and sensitivity of these primer sets. Even though this PCR assay involves the use of four sets of primers, a single DNA fragment is amplified for each CTX-M group. Therefore, interpretation of results is simple and can be adapted in reference laboratories for screening multiple isolates for the presence of group-specific CTX-M β-lactamases genes. The four-primer pair PCR-based detection system was used to screen 117 ESBL-producing *K. pneumoniae* strains for the presence of genes encoding CTX-M β-lactamases recovered from the three hospitals in Tehran during September 2006 to February 2007. The majority of ESBL-producing (75.2%) isolated in the three hospitals in Iran carried a CTX-M β-lactamases gene. The limitation of unknown mutations, which might

occur in the primer target region or the evolution of gene products, has not been identified at the genetic level. Therefore, any negative PCR result must be evaluated. 24.8% of 117 ESBL-producing strains in this report were negative by PCR for bla<sub>CTX-M</sub>. These data could indicate that the ESBLs phenotype was due to production of ESBLs other than the CTX-Ms. However, the negative PCR results in this report do not negate the possibility that modified bla<sub>CTX-Ms</sub> were present in these isolates. Due to the increased complexity of  $\beta$ -lactam resistance in Gram-negative organisms, the key to effective surveillance is the use of both phenotypic and genotypic analyses in concert.

Only 8% of microbiology laboratories from rural hospitals in the United States routinely screen for ESBL-producing organisms (22). Since the majority of patients infected with a strain of ESBL-producing *E. coli* or *Klebsiella* spp. identified in other study originated from the community (23), it is conceivable to predict that ESBL-producing organisms are present in the community in Iran but are not being reported. Outbreaks due to dissemination of ESBL-producing strains of *K. pneumoniae* vary geographically (10, 24). In many parts of the world, 10-40% of *K. pneumoniae* isolates expressed ESBLs. The highest rate of ESBLs has been reported in Latin America (45.4%), followed by the Western Pacific (24.6%), Europe (22.6%), the United States (7.6%), and Canada (4.9%) (10, 24).

Before this study, it was shown that 44.5% of isolated strains were ESBL-producing (18) in Iran, but in this study the prevalence of ESBL-producing strains were more than 60%.

### Conclusion

We showed that the prevalence of ESBLs is increasing significantly. Moreover, we found that between different groups of CTX-M the prevalence of CTX-M I group was more than other groups in Iran's hospitals.

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### References

1. Baraniak A, Fiett J, Sulikowska A, Hryniewicz W, Gniadkowski M. Countrywide spread of CTX-M-3 extended-spectrum beta-lactamase-producing microorganisms of the family Enterobacteriaceae in Poland. *Antimicrob Agents Chemother* 2002;46(1):151-9.
2. Ensor VM, Shahid M, Evans JT, Hawkey PM. Occurrence, prevalence and genetic environment of CTX-M beta-lactamases in Enterobacteriaceae from Indian hospitals. *J Antimicrob Chemother* 2006;58(6):1260-3.
3. Maltezou HC. Metallo-beta-lactamases in Gram-negative bacteria: introducing the era of pan-resistance? *Int J Antimicrob Agents* 2009;33(5):405-7.
4. Bonnet R. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother* 2004;48(1):1-14.
5. Bradford PA. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev* 2001;14(4):933-51, table.
6. Brenwald NP, Jevons G, Andrews JM, Xiong JH, Hawkey PM, Wise R. An outbreak of a CTX-M-type beta-lactamase-producing *Klebsiella pneumoniae*: the importance of using cefpodoxime to detect extended-spectrum beta-lactamases. *J Antimicrob Chemother* 2003;51(1):195-6.
7. Bush K. The impact of beta-lactamases on the development of novel antimicrobial agents. *Curr Opin Investig Drugs* 2002;3(9):1284-90.
8. Pitout JD, Nordmann P, Laupland KB, Poirel L. Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) in the community. *J Antimicrob Chemother* 2005;56(1):52-9.
9. Walther-Rasmussen J, Hoiby N. Cefotaximases (CTX-M-ases), an expanding family of extended-spectrum beta-lactamases. *Can J Microbiol* 2004;50(3):137-65.
10. Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ. CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. *Int J Antimicrob Agents* 2000;14(2):137-42.
11. Bonnet R, Sampaio JL, Labia R, De CC, Sirot D, Chanal C, *et al.* A novel CTX-M beta-lactamase (CTX-M-8) in cefotaxime-resistant Enterobacteriaceae isolated in



Brazil. Antimicrob Agents Chemother 2000;44(7):1936-42.

12. Moland ES, Black JA, Hossain A, Hanson ND, Thomson KS, Pottumarthy S. Discovery of CTX-M-like extended-spectrum beta-lactamases in *Escherichia coli* isolates from five US States. Antimicrob Agents Chemother 2003;47(7):2382-3.

13. Dong F, Xu XW, Song WQ, Lu P, Yu SJ, Yang YH, *et al.* Characterization of multidrug-resistant and metallo-beta-lactamase-producing *Pseudomonas aeruginosa* isolates from a pediatric clinic in China. Chin Med J (Engl ) 2008;121(17):1611-6.

14. Ryoo NH, Lee K, Lim JB, Lee YH, Bae IK, Jeong SH. Outbreak by meropenem-resistant *Pseudomonas aeruginosa* producing IMP-6 metallo-beta-lactamase in a Korean hospital. Diagn Microbiol Infect Dis 2009;63(1):115-7.

15. Pena A, Donato AM, Alves AF, Leitao R, Cardoso OM. Detection of *Pseudomonas aeruginosa* producing metallo-beta-lactamase VIM-2 in a central hospital from Portugal. Eur J Clin Microbiol Infect Dis 2008;27(12):1269-71.

16. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing- Tenth informational supplement. M100-S10 ed. Wayne, PA:2000.

17. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard-6th ed. M7-A6 ed. Wayne, Pa: 2003.

18. Feizabadi MM, Etemadi G, Yadegarinia D, Rahmati M, Shabanpoor S, Bokaei S. Antibiotic-resistance patterns and frequency of extended-spectrum beta-lactamase-producing isolates of *Klebsiella pneumoniae* in Tehran.

Med Sci Monit 2006;12(11):BR362-5.

19. Pitout JD, Hanson ND, Church DL, Laupland KB. Population-based laboratory surveillance for *Escherichia coli*-producing extended-spectrum beta-lactamases: importance of community isolates with blaCTX-M genes. Clin Infect Dis 2004;38(12):1736-41.

20. Poirel L, Naas T, Le T, I, Karim A, Bingen E, Nordmann P. CTX-M-type extended-spectrum beta-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob Agents Chemother 2001;45(12):3355-61.

21. Woodford N, Zhang J, Kaufmann ME, Yarde S, Tomas MM, Faris C, *et al.* Detection of *Pseudomonas aeruginosa* isolates producing VEB-type extended-spectrum beta-lactamases in the United Kingdom. J Antimicrob Chemother 2008;62(6):1265-8.

22. Prodinger WM, Fille M, Bauernfeind A, Stemplinger I, Amann S, Pfausler B, *et al.* Molecular epidemiology of *Klebsiella pneumoniae* producing SHV-5 beta-lactamase: parallel outbreaks due to multiple plasmid transfer. J Clin Microbiol 1996;34(3):564-8.

23. Shah AA, Hasan F, Ahmed S, Hameed A. Characteristics, epidemiology and clinical importance of emerging strains of Gram-negative bacilli producing extended-spectrum beta-lactamases. Res Microbiol 2004;155(6):409-21.

24. Stevenson KB, Samore M, Barbera J, Moore JW, Hannah E, Houck P, *et al.* Detection of antimicrobial resistance by small rural hospital microbiology laboratories: comparison of survey responses with current NCCLS laboratory standards. Diagn Microbiol Infect Dis 2003;47(1):303-11.