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*Full Length Research Paper*

# CTX-M-15 type extended spectrum β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae*: A developing problem in infected outpatients and hospitalised patients in Istanbul, Turkey

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**Extented-spectrum β-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* were shown to be a significant cause of both community-acquired and hospital-acquired infections worldwide. The aim of this study was to compare the epidemiological feature of CTX-M, TEM and SHV producing pathogenic *E. coli* and *K. pneumoniae* strains in outpatients and hospitalised patients. Antimicrobial susceptibilities of 551 *E. coli* and 62 *K. pneumoniae* strains isolated as pathogenic bacteria were determined by disc diffusion method and ESBLs were characterised by isoelectric focusing and PCR. ESBL production was found in 17.4% of the *E. coli* and 33.9% of the *K. pneumoniae* strains. CTX-M type ESBL production was determined in 94.8% of the *E. coli* and 81% of the *K. pneumoniae* strains and their sequence analysis revealed the presence of CTX-M-15. Our ESBL producing strains had an excellent susceptibility to imipenem, meropenem and ertapenem. According to the usage of molecular methods, the epidemiologic character of CTX-M type ESBL producing *E. coli* and *K. pneumoniae* was found to be related particularly to the presence of CTX-M-15 in outpatients and hospitalised patients. Prudent usage of extented-spectrum cephalosporins is inevitable to reduce the propagation of multidrug resistant ESBL-producing organisms and additionnaly to succeed in the treatment.**

**Key words:** Extented-spectrum β-lactamase, CTX-M, resistance, *Escherichia coli*, *Klebsiella pneumoniae*.

## INTRODUCTION

The first plasmid-encoded β-lactamase that was able to destroy extended-spectum β-lactam antibiotics was described in Germany in 1983. It was related to the production of a variant of the SHV-1 enzyme, a broad spectrum penicillinase found in *Klebsiella pneumoniae* (Jacoby, 2006). This report was followed by the description of TEM-1 and TEM-2 enzymes with hydrolytic properties similar to SHV-1 derivatives. CTX-M enzymes were reported in Germany and Argentina in 1989 and since the increase of these strains have been reported worldwide and they have become the most prevalent ESBLs (Canton and Coque, 2006). These enzymes have not been observed only in the nosocomial environment but also in the community setting (Valverde et al., 2004; Romero et al., 2005).

Detection of CTX-M by molecular methods in ESBL-producing bacteria and their pattern of antimicrobial

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**Abbreviations: OP,** Outpatients; **HP,** hospitalized patients; **ESBL,** extented-spectrum β-lactamase; **CLSI,** Clinical and Laboratory Standards Institute; **MIC,** minimum inhibitory concentrations; **PCR,** polymerase chain reaction; **IEF,** isoelectric focusing; **RAPD,** randomly amplified polymorphic DNA (RAPD) analyses.

**Table 1.** Distribution of ESBL producing *E. coli* and *K. pneumoniae* clinical isolates in HP and OP according their source of isolation.

| Source of isolation | *E. coli* n:96 | | *K. pneumoniae* n:21 | |
|---|---|---|---|---|
| | HP | OP | HP | OP |
| Urine | 5 | 23 | - | 4 |
| Blood | 10 | - | 3 | - |
| Respiratory secretions | 8 | 4 | 5 | - |
| Skin and soft tissues | 29 | 17 | 9 | - |

resistance can provide useful information about its epidemiology and aid in rational antimicrobial therapy (Jain and Mondal, 2008). Data on the prevalence and distrbution of CTX-M enzymes are limited in Turkey (Gulamber et al., 2012; Gur et al., 2008; Gonullu et al., 2008). The aim of this study was to determine the current prevalence of CTX-M, TEM and SHV enzymes among clinical isolates of *Escherichia coli* and *K. pneumoniae* isolated as pathogenic bacteria from different samples of outpatients (OP) and hospitalized patients (HP) to obtain valuable informations about their epidemiology.

## MATERIALS AND METHODS

### Patients population

Our patient population includes outpatients and hospitalised patients living in different cities of Turkey who registered at Cerrahpasa Medical Faculty which is a University Hospital of Istanbul University. 27 of the outpatients that registered with a urinary tract infection, 4 with a chronic obstructive pulmonary disease (COPD) and 17 with skin and soft tissue infection (11 with diabetic foot ulcer, 2 with axillary abscesses and 4 with gluteal abscesses). Additionnally, a skin and soft tissue infection was detected in 38, a respiratory tract infection in 13, septisemia in 13 and urinary tract infection was detected in 5 of the 69 hospitalised patients.

### Bacterial isolates

In this study, clinical isolates of *E. coli* and *K. pneumoniae were* collected between the years 2008 and 2011 from clinical samples of OP and HP in Cerrahpaşa Medical Faculty in Istanbul, Turkey and these isolates were screened for the production of extented-spectrum β-lactamase (ESBL). The ESBLs producing isolates were stored in a stock solution (Microbank; Pro-Lab Diasnostics, Ontorio, Canada) at -80°C until the following analyses.

### Antimicrobial susceptibility testing and screening for extended-spectrum β-lactamases

Antimicrobial susceptibilities of *E. coli* and *K. pneumoniae* strains for 14 antibiotics were determined by disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidlines. All the *E. coli* and *K. pneumoniae* strains were screened for ESBL production by double-disc method and the results were interpreted according to the CLSI guidlines. *K. pneumoniae* ATCC 700603 strain (positive control) and *E. coli* ATCC 25922 strain (negative control) were used for quality control.

### Polymerase chain reaction (PCR)

PCR amplification and gene sequencing were performed for 117 clinical isolates, determinated as ESBL producer by phenotypic methods. Specific primers were used to detect and identify $bla_{TEM}$, $bla_{SHV}$ and $bla_{CTX-M}$ genes as described previously (Mendonca et al., 2006). Primer used for PCR amplification are shown in Table 2.

### Isoelectric focusing (IEF)

Crude extracts of isolates were prepared by sonication and analytic IEF was performed using a Model 111 Mini IEF Cell (Bio-Rad, Hercules, CA, USA) described previously (Bauernfeind et al.,1999). Enzymes were detected by nitrocefin (0.1 nM) overlay (Calbiochem US and Canada) and isoelectric points (pI) were estimated relative to the known enzymes TEM-1 (pI 5.4), TEM-8 (pI 5.9), SHV-3 (pI 7), CMY-1 (pI 8) and CMY-2 (pI 9) used in the experiment.

### Randomly amplified polymorphic DNA (RAPD) analyses

RAPD analyses were performed using an ERIC 2 primer (AAGTAAGTGACTGGGGTGAGC) (Davin-Regli et al.,1996).

### Statistical analysis

Statistical analyses were performed with Stat Wiew-J software (version 5.0; SAS Institut Inc, Cary, NC). The chi-square test or Fisher's exact test was used to evaluate the statistical significance of the differences. P values of <0.05 were considered statistically significant.

## RESULTS

The incidence of ESBLs producing *E. coli* and *K. pneumoniae* clinical isolates determined by IEF and PCR were found to be 17.4% (96/551) and 33.9% (21/62), respectively. From 96 ESBLs producing *E. coli* isolates, 44 were isolated from OP and 52 from HP, and from 21 ESBL producing *K. pneumoniae* isolates, 17 were isolated from HP and 4 from OP. The distribution of ESBL produsing *E. coli* and *K. pneumoniae* clinical isolates, according their source of isolation, is shown in Table 1. The most common site from which we isolated ESBL producing *E. coli* and *K. pneumoniae* isolates were skin and soft tissue samples (88 and 53%, respectively) of HP and urine samples (63 and 100%, respectively) of OP. In skin and soft tissue samples of HP, ESBL producing *E.*

**Table 2.** Primer sequence and thermal cycling conditions used for PCR amplification.

| Gene | Primer sequence (5′-3′) | Thermal cycling conditions |
|---|---|---|
| $bla_{TEM}$ | AGAGTATGAGTATTCAACATT<br>ATCTCAGCGATCTGTCTAT | 94°C 3 min → 44 x [94°C 3 min, 50°C 1 min, 72°C 2.5 min] → 72°C 15 min |
| $bla_{SHV}$ | AGATCCACTATCGCCAGCA<br>TCATTCAGTTCGGTTTCCCA | 94°C 3 min → 44 x [94°C 3 min, 50°C 1 min, 72°C 2.5 min] → 72°C 15 min |
| $bla_{CTX-M}$ | ATCTGACGCTGGGTAAAGC<br>ATATCGTTGGTGGTGCCATA | 94°C 3 min → 44 x [94°C 3 min, 50°C 1 min, 72°C 2.5 min] → 72°C 15 min |
| $bla_{CTX-M\ group\ 1}$ | CCCATGGTTAAAAAATCACTG<br>CCGTTTCCGCTATTACAAAC | 94°C 3 min → 44 x [94°C 3 min, 50°C 1 min, 72°C 2.5 min] → 72°C 15 min |
| $bla_{CTX-M\ group\ 2}$ | ATGATGACTCAGAGCATTCGC<br>TCGCTCCATTTATTGCATCA | 94°C 3 min → 44 x [94°C 3 min, 50°C 1 min, 72°C 2.5 min] → 72°C 15 min |
| $bla_{CTX-M\ group\ 8}$ | ATGTTAATGACGACAGCCTGTG<br>CCGGTTTTATCCCCGACA | 94°C 3 min → 44 x [94°C 3 min, 50°C 1 min, 72°C 2.5 min] → 72°C 15 min |
| $bla_{CTX-M\ group\ 9}$ | ATGGTGACAAAGAGAGTGCA<br>CCCTTCGGCGATGATTCTC | 94°C 3 min → 44 x [94°C 3 min, 50°C 1 min, 72°C 2.5 min] → 72°C 15 min |
| $bla_{CTX-M-15}$ | CACACGTGGAATTTAGGGACTGCCGTCT<br>AAGGCGATAAACA | 94°C 3 min → 44 x [94°C 3 min, 50°C 1 min, 72°C 2.5 min] → 72°C 15 min |

*coli* isolates were found to be significantly higher than ESBL producing *K. pneumoniae* isolates (P<0.05). Additionnally, the number of ESBL producing *E. coli* isolates were found to be higher than ESBL producing *K. Pneumoniae* isolates in urine and skin and soft tissue samples of OP but a statistically significant difference was not determined. (P > 0.05) (Table 1). Comparison in terms of patient population, a statistically significant difference was not detected between the distribution rate of ESBLs producing *E. coli* and *K. pneumoniae* strains that we isolated from OP and HP (*E. coli*, p = 0.147 and *K. Pneumoniae*, p = 0.445)

Imipenem, meropenem and ertapenem were found to be the most active antimicrobial agents against all the ESBLs producing isolates. The amoxicillin + clavulanat, cefepime, ceftasidime, levofloxacin and amikacin susceptibility rates of *K. pneumoniae* strains, isolated from outpatients, were found to be statistically higher than the susceptibility rates of *K. pneumoniae* strains isolated from hospitalised patients. (p<0.05) .

CTX-M, TEM and SHV type ESBLs were detected by IEF and PCR screening in all ESBL producing isolates. In 64.6% of the *E. coli* and in 57% of the *K. pneumoniae* strains, we determined only the production of CTX-M. The presence of CTX-M together with TEM and SHV enzymes was found in 30.2% of the *E. coli* and in 23.8% of the *K. pneumoniae* strains.

The distribution rate of *bla* genes in ESBLs producing *E. coli* isolates was found as 94.8% $bla_{CTX-M}$, 25% $bla_{TEM}$ and 15.6% $bla_{SHV}$ (Table 5). In *K. pneumoniae*, this rates of *bla* gene was found as 81% $bla_{CTX-M}$, 38% $bla_{TEM}$ and 62% $bla_{SHV}$. Comparison in terms of patient population, a statistically significant difference in the distribution of $bla_{CTX-M}$, $bla_{TEM}$ and $bla_{SHV}$ genes was not found between *E. coli* and *K. pneumoniae* strains isolated from hospitalises patients and outpatients.

In this study, we did not detect any strain containing the $bla_{CTX-M-2}$, $bla_{CTX-M-8}$ and $bla_{CTX-M-9}$ enzymes, CTX-M-1 type beta lactamase was detected as CTX-M-15 subgroup in all the ESBL producing strains (Tables 3 and 4; Figure 1).

According to the bands (200, 250, 275, 300, 320, 400, 450, 550, 600, 700, 875, 900, 1100, 1300 and 1400 bp) detected by RAPD-PCR, it was found that, two *E. coli* strains isolated from blood samples of two different patients, hospitalized in the same intensive care unit, have the same bands with a clonal similarity.

## DISCUSSION

The prevalence of CTX-M type β-lactamases increased substantially since 1992 (Canica et al., 1997) and the distribution of CTX-M ESBLs were different in geographic areas. Nowadays, it is worth noting that an endemic situation is dominant in most European countries and in Asia and South America (Canton and Coque, 2006). Some of the CTX-M enzymes such as CTX-M9 and CTX-M14 are widely present in specific countries, especially in the countries surrounding the Mediterranean sea and in the United Kingdom (Mendonca et al., 2007; Livermore et al., 2007; Novais et al., 2006; Hernandez et al., 2005). CTX-M1 is present in Italy and CTX-M2 in most South American countries, Japan and Israel (Bonnet, 2004; Brigante et al., 2005; Chmelnitsky et al, 2005), whereas other CTX-M such as CTX-M 15 have been detected worldwide (Bonnet, 2004; Lartigue et al, 2005). Data on the prevalence and distrubution of CTX-M enzymes are limited in Turkey (Gulamber et al., 2012; Nazik et al., 2011; Celik et al., 2010; Gur et al., 2008; Gonullu et al., 2008) and there is no data comparing CTX-M, TEM and

**Table 3.** The antimicrobial susceptibility rates of ESBL producing *E. coli* and *K. pneumoniae* clinical isolates according to their source of isolation (HP and OP).

| Antimicrobial agent | *E. coli* (%) | | *K. pneumoniae* (%) | |
|---|---|---|---|---|
| | HP(n:52) | OP(n:44) | HP(n:17) | OP(n:4) |
| Amoxicillin | 0 | 0 | 0 | 0 |
| Amoxicillin + clavulanate | 30 | 20 | 2 | 25 |
| Cefepime | 36 | 44 | 10 | 75 |
| Ceftazidime | 8 | 14 | 5 | 25 |
| Cefotaxime | 1 | 3 | 0 | 0 |
| Ceftriaxone | 7 | 10 | 0 | 0 |
| Aztreonam | 3 | 9 | 0 | 0 |
| Ciprofloxacine | 25 | 26 | 12 | 25 |
| Levofloxacin | 30 | 33 | 6 | 50 |
| Imipenem | 100 | 100 | 100 | 100 |
| Meropenem | 100 | 100 | 100 | 100 |
| Ertapenem | 98 | 100 | 94 | 100 |
| Amikacin | 84 | 89 | 76 | 100 |
| TMP/SMX[c] | 30 | 39 | 32 | 50 |

HP: Hospitalized patient, OP: outpatient, TMP/SMX: Trimethoprim/Sulfamethoxazole.

**Table 4.** Distribution of TEM, SHV and CTX-M types ESBL among *E. coli* and *K. pneumoniae* strains.

| Specie | CTX-M | TEM | SHV | CTX-M +TEM | CTX-M +SHV | TEM +SHV | CTX-M + TEM + SHV | TOTAL |
|---|---|---|---|---|---|---|---|---|
| *E. coli* | 62 | 3 | 1 | 16 | 9 | 1 | 4 | 96 |
| *K.pneumoniae* | 12 | 1 | 1 | 3 | 1 | 2 | 1 | 21 |
| Total | 74 | 4 | 2 | 19 | 10 | 3 | 5 | 117 |

**Table 5.** Distribution of *bla* genes in ESBL producing *E. coli* and *K. pneumoniae* strains isolated from hospitalized patients and outpatients.

| Bacteria | Patient | Beta lactamase genes, n (%) | | |
|---|---|---|---|---|
| | | $bla_{CTX-M}$ | $bla_{TEM}$ | $bla_{SHV}$ |
| ***E. coli*** | HP (n:52) | 50 (96) | 15 (28.8) | 8 (15.4) |
| | OP (n:44) | 41 (93) | 9 (20.5) | 7 (15.9) |
| | P values | 0.699 | 0.602 | 0.657 |
| ***K. pneumoniae*** | HP (n:17) | 14 (82,4) | 7 (41.2) | 11 (64.7) |
| | OP (n:4) | 3 (75) | 1 (25) | 2 (50) |
| | P values | 0.477 | 0.489 | 0.501 |

SHV type ESBLs in hospitalized and outpatients. In this study, the rate of CTX-M type ESBL producing *E. coli* and *K. pneumoniae*, when compared with the results reported from different cities in our country, was found to be quite high as 94.8 and 81%, respectively. There is no enough data reported from Turkey that we can use to speculate the factors influencing the high rate of ESBL producing *E. coli* and *K. pneumoniae* strains. Due to inappropriate usage of cephalosporins, the fecal carriage seems to be one of the causes of this high rate but there is no recent data on the fecal carriage rate of ESBL producing bacteria reported from Turkey. According to the patient's informations, we can estimate that, usage of cephalosporins prior admission, longer hospital stay and usage of invasive device can be some of the factors influencing this high rate in HP and repeated usage of cephalosporins prior admission, food chain, trade and human migration can be one of the factors influencing this high rate in OP. Among these ESBLs, CTX-M-15 was determined as the most prevalent type. Such predominance of the CTXM-15 enzyme was also reported in different studies from Turkey and from other European countries (Canton

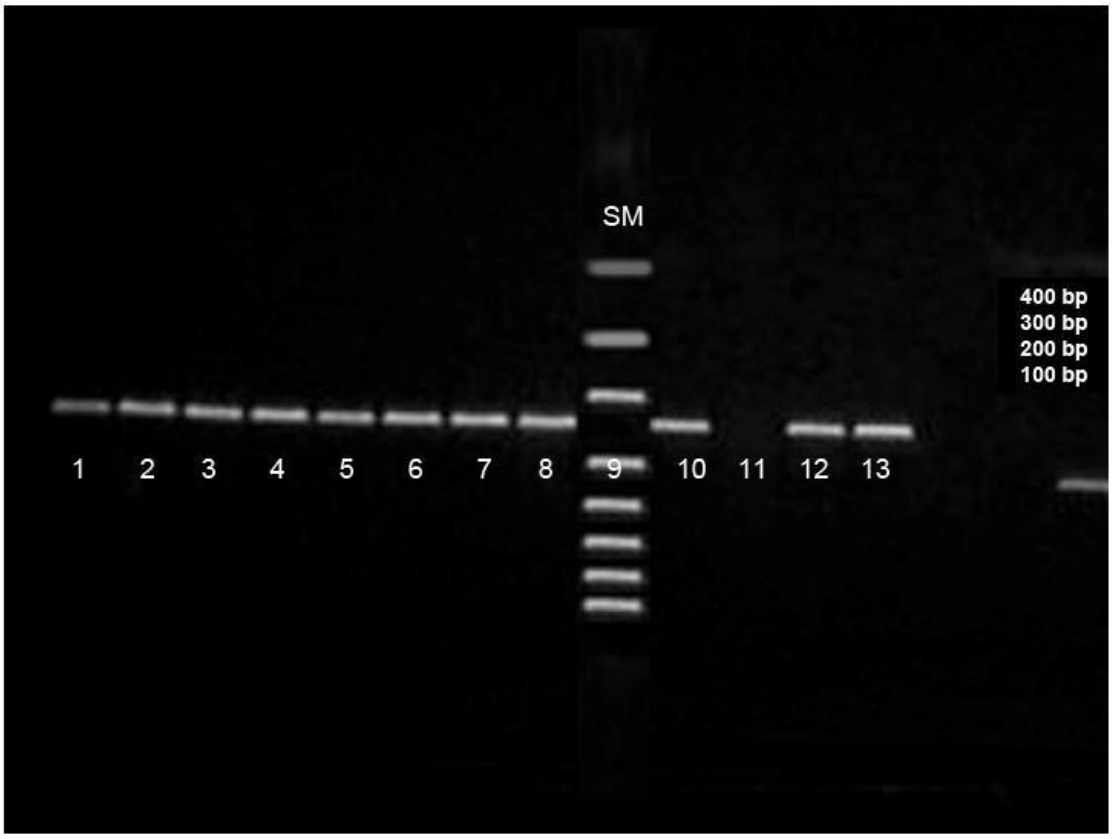


**Figure 1.** Agarose gel showing CTX-M products of *E. coli* and *K. pneumoniae* strains after PCR amplification. Lanes 1 to 8 and lanes 12, 13: CTX-M products of *E. coli* and *K.pneumoniae* strains; Lane 10: positive control; Lane 11: negative control; SM: molecular weight size markers (sizes are shown on the right in base pairs).

and Coque, 2006; Romero et al., 2005; Gulamber et al., 2012; Gur et al., 2008; Gonullu et al., 2008; Mendonca et al., 2007; Nazik et al., 2011; Livermore et al., 2007; Bonnet, 2004; Celik et al., 2010). In this study, this predominance was found to be similar in the strains obtained from hospitalised patients and outpatients. TEM- and SHV-beta-lactamases were firstly described especially in *K. pneumoniae* isolates causing nosocomial outbreaks. Years ago, hospitalisation was a serious risk to get infections due to ESBL producing *K. pneumoniae* but today, first CTX-M then TEM and SHV-beta-lactamases are mostly determined from *E. coli* strains isolated from community-acquired infections. The wide presence of CTX-M 15, especially in *E. coli* strains isolated from community patients was also reported from UK (Woodford et al., 2004). Similar to our results, all these isolates were found to be susceptible to carbapenems. Dissemination of specific clones and epidemic plasmids in community and nosocomial settings has been the main reason for the increase in ESBLs belonging to the SHV, TEM and CTX-M families in Europe. Because of excessive usage of antibiotics in human and animals, hospital crossinfection, food chain, trade and human migration are accepted as factors influencing the dissemination of ESBLs outside hospitals; hospitalisation could be a risk factor only to a small number of people who are influenced by these factors (Coque et al., 2008).

In a study in Iran, *E. coli* and *K. pneumoniae* were found as the most common bacteria among *Enterobacteriaceae* isolates and the prevalence of $bla_{SHV}$ was reported as 15%. This rate was found to be similar with the rate of $bla_{SHV}$ of the ESBL producing *E. coli* isolates in our study, but when compared with the rate of $bla_{SHV}$ that was determined as 62% in our ESBL producing *K. Pneumoniae* isolates, this rate was evaluated as very low. In the same study, the rate of $bla_{TEM}$ reported as 65.5% was two fold high than our rate of $bla_{TEM}$ that was determined as 25% in the *E. coli* and as 38% in the *K. pneumoniae* isolates in our study. Interestingly, $bla_{CTX-M}$ was not detected in their ESBL-producing strains (Moosavian et al., 2012).

Co-expression of CTX-M enzymes and ESBLs from TEM and SHV enzymes families are common (Romero et al., 2005). In our study, the co-expression of CTX-M together with TEM and SHV enzymes was found in 30.2% of the *E. coli* and in 23.8% of the *K. pneumoniae* strains in our study. Our ESBL producing strains presented the highest antimicrobial susceptibility rates for imipenem, meropenem and ertapenem.

When compared with the strains isolated from hospita-

lized patients, the higher ceftasidime, levofloxacin and amikacin susceptibility rates of *K. pneumoniae* strains isolated from outpatients can be due to the wide usage of these antibiotics generally in hospitalised patients.

## Conclusion

It was concluded that in Cerrahpasa Medical Faculty, the rate of CTXM-15 production was high (94.8 and 81%) in *E. coli* and *K. pneumoniae* strains isolated from hospital and community acquired infections. The usage of carbapenems will still be suitable for a successful treatment of infections by these bacteria. After carbapenems, aminoglycosides can be considered as the second most effective agent. If the usage of quinolons or other antibiotics was requested, the prescription have to be due to the result of their antimicrobial susceptibility tests.

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