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Full Length Research Paper

Characterization of CTX-M type extended spectrum β-lactamases of *Escherichia coli* isolated from urinary tract infections in Southern Turkey: The first report of CTX-M-14 producing *Escherichia coli* from Turkey

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The aim of this study was to investigate the frequency of CTX-M type extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* strains which have become emerging etiologic agents of urinary tract infections (UTIs). A total of 310 *E. coli* isolates recovered from UTIs were screened for ESBL production by VITEK2 system and the double-disk synergy tests. In addition, ESBL positive isolates were selected for typing of bla_{CTX-M} genes by PCR screening followed by DNA sequencing analysis permitting the differentiation of the CTX-M subtypes. A pulsed-field gel electrophoresis (PFGE) assay was also performed to check for a possible clonal relation among these isolates. ESBL positivity was detected in 77 (24.8%) of isolates. Subsequently, 46 (14.8%) of ESBL positive *E. coli* isolates were shown to be carrying bla_{CTX-M} genes of which 30 (9.6%) were found to be CTX-M-15 producers, while the remaining 16 (5.1%) were CTX-M-14 producers. No major clonal relationship among these bla_{CTX-M} producers was found. We demonstrated the presence of CTX-M-14 producer *E. coli* strains for the first time in Turkey and provided epidemiologic data suggesting the evidence for the ongoing dissemination of CTX-M type ESBLs.

Key words: CTX-M-14, extended-spectrum beta-lactamase (ESBL), *Escherichia coli*, pulsed-field gel electrophoresis (PFGE).

INTRODUCTION

The early extended-spectrum beta-lactamases (ESBLs) arose as a result of a number of amino acid substitutions from the common plasmid-mediated TEM and SHV-1 beta lactamases. In the early 1990s, a new class A type of ESBL was characterised in the first reports on the CTX-M-1 (MEN-1) enzyme (Barthélémy et al., 1992; Bauernfeind et al., 1992). The first CTX-M β-lactamase was characterised in *Escherichia coli* strains isolated from German and Italian patients (Barthélémy et al., 1992; Bauernfeind et al., 1996). The rate of dissemination of CTX-M β-lactamases produced by gram-negative bacteria such as *E. coli* and *Klebsiella pneumoniae* has been reported increasingly with an increasing resistance worldwide (Bradford, 2001; Bonnet, 2004; Kimura et al., 2004; Lartigue et al., 2005; Lartigue et al., 2007a; Pitout et al., 2007; Ogbolua et al., 2011).

Plasmid-mediated CTX-M type ESBLs of which there are over 80 different variants described so far can be divided into five groups acoording to their amino acid identities as follows: (i) CTX-M-1 group, (ii) CTX-M-2 group, (iii) CTX-M-8 group, (iv) CTX-M-9 group and (v) CTX-M-25 group. Currently, among CTX-M-type ESBLs, CTX-M-15 is the most widely distributed in *E. coli*, and CTX-M-14, CTX-M-3 and CTX-M-2 are the other widespread enzymes (Bonnet, 2004).

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E. coli strains carrying *bla*$_{CTX-M}$ determinants are assigned for nosocomial and community-associated outbreaks which can affect narrow to wide areas. This emergence and dissemination of these enzymes involve plasmid or strain epidemics, but they can involve mobile elements, like ISEcp1, while strains producing CTX-M enzymes seem to be implicated also in nosocomial infections (Woodford et al., 2004).

Limited data revealed that CTX-Ms are already disseminated throughout Turkey (Yumuk et al., 2008; Celik et al., 2010). Therefore, in this study, we focused on the following: (i) characterizing the CTX-M-type ESBLs of *E. coli* isolated from outpatients with UTIs, (ii) assessing the antibiotic resistance rates of *E. coli* isolates and (iii) discussing the mechanism of enzyme transmission among *bla*$_{CTX-M}$ producers in Adana, Turkey.

MATERIALS AND METHODS

Bacterial isolates

A total of 310 isolates, recovered from patients who registered at Çukurova University Hospital with symptoms resembling those of community acquired UTIs were screened for ESBLs over a period of 9-months (February to October) in 2010. Community-onset infections were recognised in individuals who were either (i) outpatients or (ii) hospitalized patients whose first positive cultures were obtained within 48 h of admission to the hospital.

Identification and the minimal inhibitory concentration values of the selected *E. coli* isolates were obtained by the VITEK2 system (bio-Mérieux, Marcyl'Etoile, France) followed by biochemical testing and a double-disk synergy test performed to confirm the VITEK2. The results were interpreted according to the 2011 guidelines of the Clinical and Laboratory Standards Institute.

Amplification and sequencing of bla$_{CTX-M}$ encoding genes

Plasmid DNA of study isolates was extracted using a QIAGEN plasmid mini kit according to the manufacturer's instructions (QIAGEN Inc. Valencia, CA). DNA extracts were screened for the presence of *bla*$_{CTX-M}$ genes with consensus PCR primers matching the conserved sequences at positions 205 to 227 and positions 748 to 727 with respect to the CTX-M translational starting point to amplify a 544-bp fragment from all known *bla*$_{CTX-M}$ genes using a method desribed previously (Edelstein et al., 2003).

Sequence analysis was performed with the same primers on both strands of the PCR products after purification process using the High Pure PCR Product Purification Kit (Roche Diagnostics, Basel, Switzerland). Sequence analysis was done using the dye terminator cycle sequencing method and an ABI Prism BigDye Terminator kit (Applied Biosystems, Foster City, CA, USA). The assay was carried out according to the standard protocol. Data were collected on an ABI 3100 automated fluorescence sequencer (Applied Biosystems). The types of *bla*$_{CTX-M}$ genes were identified by comparing the sequences of the database of G. Jacoby and K. Bush (http://www.lahey.org/Studies/) and the sequences in GenBank (http://www.ncbi.nlm.nih.gov/BLAST).

Pulsed-field gel electrophoresis (PFGE)

All CTX-M-producing *E. coli* were investigated by PFGE following the extraction of genomic DNA and digestion with XbaI by using the standardised protocol, as described previously (Durmaz et al., 2009). The PFGE analysis was performed on a CHEF-DR II apparatus (Bio-Rad Laboratories, La Jolla, CA). Gel images were exported to Gelcompar II software (version 3.0; Applied Maths, Sint-Martens-Latem, Belgium) for analysis. Comparisons were made by using the band-based Dice coefficient, which is a binary coefficient that measures similarity based on common and different bands. Dendrograms were generated by using the unweighted pair group method by arithmetic averaging method with 1% position tolerance. DNA relatedness was calculated on the basis of the Dice coefficient, and isolates were considered to be genetically related if the Dice coefficient correlation was 80% or greater, which corresponds to the "possibly related (four- to six-band difference)" criteria of Tenover et al. (1995).

Statistical analysis

Fisher's exact t-test was used to determine the significant differences in resistance using SPSS version 16 for Windows (SPSS, Chicago, IL, USA). A P value of < 0.05 was considered statistically significant.

RESULTS

A total of 310 *E. coli* samples of community acquired UTIs were included in the study. Generally, CTX-M-producing isolates revealed high antimicrobial resistance rates, except for amikacin and carbapenems (Table 1). No significant differences of antimicrobial resistance rates were found between CTX-M-14 and CTX-M-15 producing *E. coli* isolates for most of the antimicrobial agents tested. Notably, CTX-M-15-producing *E. coli* isolates demonstrated a higher resistance rate of ceftazidime than CTX-M-14-producing isolates (83.3 vs. 50%; p = 0.036). The amikacin resistance rate was also higher in CTX M-14-producing *E. coli* isolates than in CTX-M-15-producing isolates (56.3 vs. 33.3%; p = 0.060). Only one CTX-M-15-producing *E. coli* isolate was found resistant to imipenem (MIC, >32 µg/ml).

ESBL positivity was found in 72 of the 310 (23.2%) isolates. CTX-M type ESBLs were detected in 46 of the 72 (63.9%) isolates by PCR screening. The primers used for screening were specific to all the known *bla*$_{CTX-M}$ genes and sequence analysis of the fragments amplified by these primers further suggested that the *bla*$_{CTX-M}$ genes belonged to 2 different phylogenetic subgroup. Subsequently, sequence analysis among the *bla*$_{CTX-M}$ producers revealed that 30 of the 46 (65.2%) *E. coli* isolates were CTX-M-15 producer, but above all 16 of the 46 (34.8%) ESBL producer *E. coli* strains were found to be CTX-M-14 producer (Table 2).

The clonal relationship among these 46 *bla*$_{CTX-M}$ producing isolates was established based on PFGE of XbaI-digested genomic DNA (Figure 1). Thirty-seven distinct pulsotypes of which six are two-membered and one is four-membered clusters were obtained. PFGE profiles of imipenem resistant CTX-M producer strain (E1) and the other strain (E2) which showed intermediate resistance to carbapanems (MIC = 2 µg/ml) were totally

Table 1. Antimicrobial resistances of CTX-M-producing *E. coli* isolates.

Antimicrobial agent	Subtotal (n=46)			CTX-M-14 (n=16)			CTX-M-15 (n=30)			
	S[a]	I[b]	R[c]	S[a]	I[b]	R[c]	S[a]	I[b]	R[c]	P[d]
AMP[e]	- (0)	5 (10.9)	41 (89.1)	- (0)	3 (18.7)	13 (81.3)	- (0)	2 (6.7)	28 (93.3)	0.324
CAZ[f]	13 (28.3)	- (0)	33 (71.7)	8 (50)	- (0)	8 (50)	5 (16.7)	- (0)	25 (83.3)	0.036
CTX[g]	- (0)	- (0)	46 (100)	- (0)	- (0)	16 (100)	- (0)	- (0)	30 (100)	0.999
ATM[h]	11 (23.9)	- (0)	35 (76.1)	6 (37.5)	- (0)	10 (62.5)	5 (16.7)	- (0)	25 (83.3)	0.153
AMK[i]	43 (93.5)	3 (6.5)	- (0)	4 (25)	3 (18.7)	9 (56.3)	18 (60)	2 (0.7)	10 (33.3)	0.060
GEN[j]	18 (39.1)	- (0)	28 (60.9)	4 (25)	- (0)	12 (75)	14 (48.7)	- (0)	16 (53.3)	0.209
CIP[k]	20 (43.5)	2 (4.3)	24 (52.2)	7 (43.7)	- (0)	9 (56.3)	13 (43.3)	2 (0.7)	15 (50)	0.782
IPM[l]	44 (95.6)	1 (2.2)	1 (2.2)	16 (100)	- (0)	- (0)	28 (93.3)	1 (3.3)	1 (3.3)	0.999
MEM[m]	45 (97.8)	1 (2.2)	- (0)	16 (100)	- (0)	- (0)	29 (96.7)	1 (3.3)	- (0)	0.999
SXT[n]	9 (19.6)	5 (10.9)	32 (69.6)	3 (18.7)	2 (12.5)	11 (68.8)	6 (20)	3 (10)	21 (70)	1.0
TZP[o]	4 (8.7)	13 (28.3)	29 (63)	1 (6.2)	4 (25)	11 (68.8)	3 (10)	9 (30)	18 (60)	0.896

[a]: Number of susceptible isolates; [b]: number of isolates with intermediate resistance; [c]: number of resistance isolates; [d]: p values between CTX-M-14 and CTX-M-15 producer isolates; [e]: ampicillin; [f]: ceftazidime; [g]: cefotaxime; [h]: aztreonam; [i]: amikacin; [j]: gentamicin; [k]: ciprofloxacin; [l]: ımipenem; [m]: meropenem; [n]: co-trimoxazole; [o]: piperacillin-tazobactam.

Table 2. Chart which summarizes the results of characterization study.

Results	ESBL rate	CTX-M Rate	CTX-M-15 rate	CTX-M-14 rate
Positive	77	46	30	16
Negative	310	31	16	30
Percentage	24.8%	14.8%	9.6%	5.1%

indifferent suggesting that they are clonally of the same origin.

DISCUSSION

E. coli producing CTX-M enzymes have emerged worldwide as important causes of community-onset UTIs, as well as various nosocomial infections; and this is of great concern as we are facing the CTX-M pandemic (Canton and Coque, 2006). Although, we do not know the exact prevalence of these enzymes in Turkey, current studies focusing on these strains which produce CTX-Ms suggest that CTX-M-producers have already began to disseminate throughout the Turkey (Celik et al., 2010). Yumuk et al. (2008) reported that CTX-M is common among the ESBL producing *E. coli* isolates obtained from community acquired UTIs in Izmir, Turkey. Consequently, the successful spread of *E. coli* producing CTX-Ms is described by two mechanisms as follows: (i) the spread of an epidemic clone (such as ST131) and (ii) the horizontal transfer of plasmids or such mobility elements that carry bla_{CTX-M} alleles, furthermore there is a clonal dissemination of bla_{CTX-M} positive *E. coli* isolates throughout clinical isolates but studies focusing on community-onset infections revealed that diffusion of CTX-M genes among isolates may be associated with the spread of mobile elements possibly carrying other resistance determinants rather than a clonal

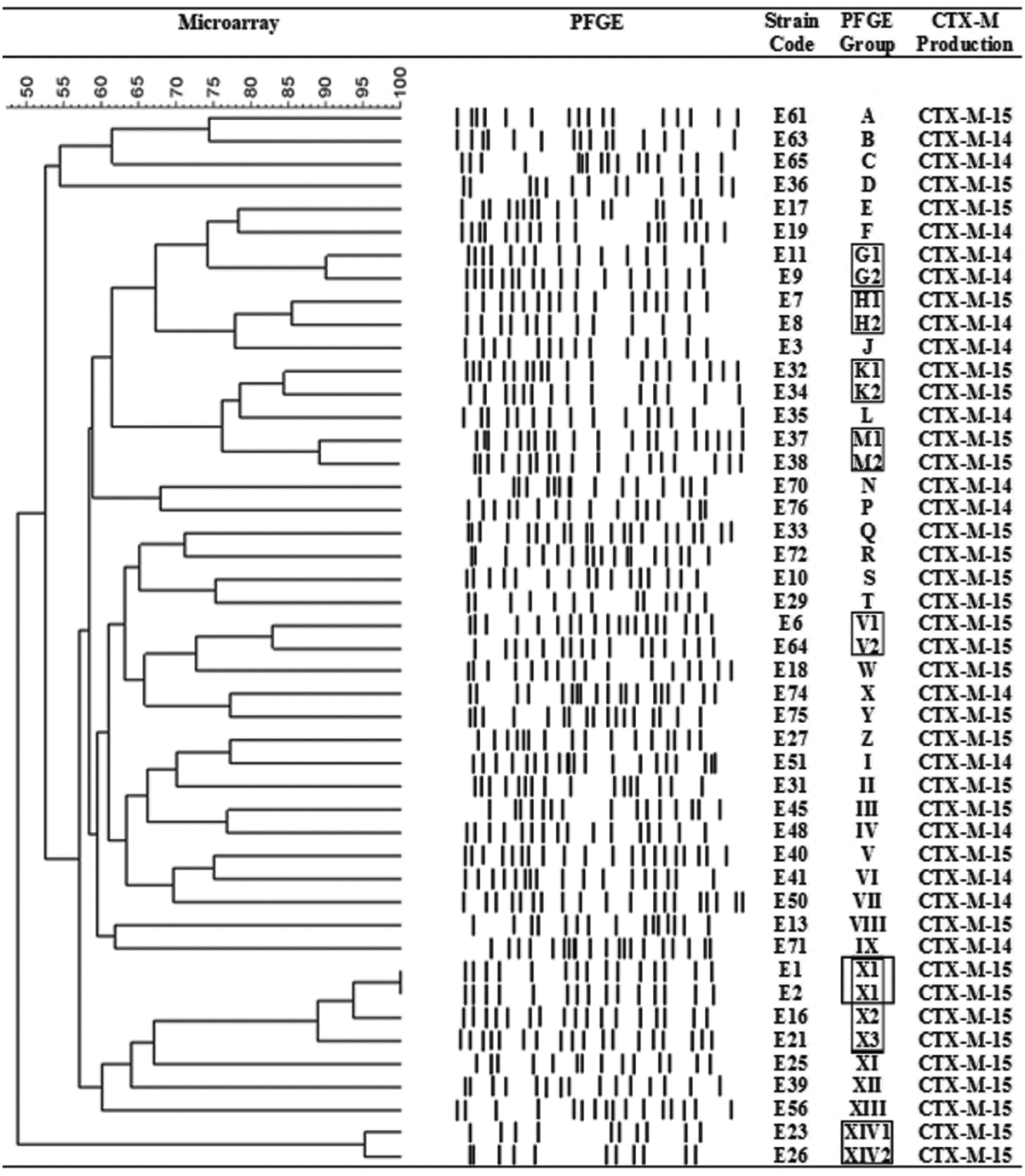

Figure 1. Dendrogram based on PFGE typing showing genetic relatedness among CTX-M-producing *E. coli* isolates.

dissemination (Woodford et al., 2004; Pitout et al., 2005; Ho et al., 2007; Coque et al., 2008; Peirano and Pitout, 2010; Peirano et al., 2010). In our study, we identified bla_{CTX-M} genes on plasmids with the help of a plasmid extraction protocol and isolates which harbor plasmids carrying bla_{CTX-M} genes did not share identical macrodigestion patterns by PFGE. This finding promotes the idea of dissemination by mobility elements with requirements of further examinations of mobility mechanisms.

In this study, CTX-M-15-producing isolates were found to be more resistant, as compared CTX-M-14-producing *E. coli* isolates with regards to ceftazidime and carbapenems as expected. Two isolates that developed or were developing resistance against carbapenems were CTX-M-15-producers and they exhibited same antimicrobial resistance profiles; they were resistant simultaneously to ampicillin, ceftazidime, cefotaxime, aztreonam, gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole and piperacillin-tazobactam, and both were susceptible to amikacin. Despite carbapenem resistance has been reported only rarely for *E. coli*, carbapenems are sometimes the only effective agents for the treatment of severe infection caused by multiresistant *E. coli*. Thus, our study result shows that with one carbapenem resistant strain and one strain with intermediate resistance must be of great concern. Carbapenem resistance is thought to be resulting from the production of chromosomal and plasmid-mediated cephalosporinases combined with decreased drug permeability through the outer membrane or from carbapenem-hydrolysing enzymes (Poirel et al., 2004; Bratu et al., 2007; Lartigue et al., 2007b; Liu et al., 2008).

This study highlights the serious emergence and dissemination of CTX-M-15 producer *E. coli* strains, and also it is the first report demonstrating the presence of CTX-M-14 producer *E. coli* strains isolated in Turkey. In terms of dissemination, this study indicates the role of mobility elements in addition to clonal spread which is reported as the primer way in the literature (Peirano et al., 2010; Peirano and Pitout 2010). Furthermore, identification of two isolates developing resistance to carbapenems is a worrying output.

Finally, serious diffusion of CTX-M producing *E. coli* isolates especially in outpatients in Turkey should be considered in selection of proper antibiotic therapy as this is a key factor relating to the control of ESBL producing organisms where antibiotics are available without prescription in developing countries like Turkey. Further studies are required to elucidate the phenotype/genotype interactions in the antimicrobial resistance in addition to mechanism of dissemination of *E. coli*.

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