CTX-M-15 Carried On Incn-Type Plasmids in *Klebsiella pneumoniae*

Klebsiella pneumoniae'da Incn Tipi Plazmidler ile Taşınan CTX-M-15

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ABSTRACT Objective: The aim of this study was to characterize the CTX-M genes in clinical isolates of *Klebsiella pneumoniae*, particularly, the plasmid types that carry them. **Material and Methods:** Antimicrobial susceptibilities were determined by the agar dilution and E-test. Beta-lactamase production as analyzed by phenotypic tests [E-test MBL and extended spectrum β-lactamase (ESBL), isoelectric focusing, and bioassay] and molecular methods [polymerase chain reaction (PCR) detection of ESBL-encoding genes and IS elements, DNA sequencing and analysis of *bla*CTX-M and *ISEcP1* PCR amplicons, typing by randomly amplified polymorphic DNA (RAPD) analysis and plasmid isolation, transformation, rep-typing and IncN plasmid confirmation]. **Results:** Thirty four (14.8%) out of 230 clinical isolates of *K. pneumoniae* were found as ESBL producers. The isolates produced one to five different β-lactamases, according to the isoelectric focusing results. The prevalence of the CTX-M-type ESBLs was found as 35% and sequencing proved all as CTX-M-15. RAPD analysis showed no clonal relation between the strains. Previous studies have shown that the *bla*CTX-M-15 gene was carried on FII plasmids. In 10 strains in this study, the CTX-M-15 gene was on 95 kb-larger plasmids typing to IncN. In two isolates the *bla*CTX-M-15 was carried on an approximately 60 kb plasmid and possessed an Inc/rep type of FII. **Conclusion:** This is the first report of IncN carrying *bla*CTX-M-15 and confirms the rapid emergence of CTX-M-15 enzymes among *K. pneumoniae* in Istanbul. Through this study, it was aimed to underline the risk of spread of IncN type plasmids, among gram-negative bacteria in Turkey, as shown previously in Greece.

Key Words: *Klebsiella pneumoniae;* beta-lactamase CTX-M-15

ÖZET Amaç: Bu çalışmada çeşitli klinik örneklerden izole edilen *Klebsiella pneumoniae* suşlarında CTX-M genlerinin genetik içeriği ve özellikle bu genleri taşıyan plazmidler açısından karakterize edilmesi amaçlanmıştır. **Gereç ve Yöntemler:** Suşların antimikrobiyal duyarlılıkları agar dilüsyon ve E-test yöntemleriyle araştırılmıştır. Beta-laktamaz üretimi fenotipik testler [E-test metalo-beta-laktamaz, genişlemiş spektrumlu beta-laktamaz (GSBL), izoelektrik odaklama ve biyoassay] ve moleküler yöntemler [polimeraz zincir reaksiyonu (PCR) ile GSBL kodlayan genler ve IS elementlerinin saptanması, *bla*CTX-M ve *ISEcP1* PCR amplikonlarının analizi ve DNA dizi analizi, "randomly amplified polymorphic DNA (RAPD)" ve plazmid analizi, transformasyon, rep tiplemesi ve IncN plazmidlerinin doğrulanması] kullanılarak analiz edilmiştir. **Bulgular:** İki yüz otuz *K. pneumoniae* klinik izolatında 34 (%14.8) suşun GSBL oluşturduğu saptanmıştır. İzoelektrik odaklama sonuçlarına göre bu izolatların 1-5 arasında farklı beta-laktamaz tipi oluşturduğu görülmüş, CTX-M tipi GSBL prevalansı %35 olarak bulunmuş ve dizi analizi yöntemiyle bunların CTX-M-15 olduğu doğrulanmıştır. RAPD analizi ile suşlar arasında klonal ilişki saptanmamıştır. *bla*CTX-M-15 geninin genellikle FII plazmidinde taşındığı bilinmekle birlikte, bu çalışmadaki 10 suşta *bla*CTX-M-15 geninin 95 kb'lik IncN tipinde büyük bir plazmid üzerinde olduğu saptanmıştır. İki suşta ise *bla*CTX-M-15 geni yaklaşık 60 kb büyüklüğünde bir plazmid üzerinde taşınmaktadır ve plazmid FII Inc/rep tipindedir. **Sonuç:** Bu çalışma İstanbul'da *K. pneumoniae* suşlarında CTX-M-15 tipi enzimlerin hızla ortaya çıktığını doğrulayan ve *bla*CTX-M-15 geninin *K.pneumoniae* suşlarında IncN tipi plazmidler üzerinde taşındığını gösteren ilk bildirimdir. VIM-4 metalo beta-laktamaz genini de taşıdığı Yunanistan'daki bir çalışmada gösterilmiş olan IncN plazmidlerinin, Türkiye'de gram-negatif bakteriler arasında yayılma riskine bu çalışma ile dikkat çekilmek istenmiştir.

Anahtar Kelimeler: *Klebsiella pneumoniae*; CTX-M-15

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More than 150 genetically distinct TEM-types and about 90 SHV-type extended spectrum β-lactamases (ESBL) have been characterized up to date and other types of ESBLs have been documented (www.lahey.org/studies). CTX-M-15 type β-lactamase is an emerging enzyme among Enterobacteriaceae.[1-3] Plasmid mediated CTX-M enzymes have been detected widely in a variety of species of enteric gram-negative bacilli.[4-6] CTX-M ESBLs have become dominant, with a much greater penetration into *Escherichia coli*, and with many infections in complicated patients community in the, usually with an underlying disease, recent antibiotic use, or healthcare contact.[7]

According to a recent review and new data in GenBank, CTX-M β-lactamases can be divided into five groups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25) based on their amino acid sequence identities (http://www.lahey.org/studies/webt.stm).[5,8,9] Cluster 1 is globally dominant and *bla*CTX-M-15, a member of this cluster, has been reported throughout Europe, South America, Central Asia, South East Asia and Africa.[10-17] CTX-M enzymes are more active against cefotaxime and ceftriaxone than ceftazidime, but point mutations can increase the activity against ceftazidime. Thus, CTX-M-15 and 32 differ from CTX-M-3 and -1, respectively, solely by Asp-240Gly substitutions, however they are 100-fold more active against ceftazidime.[7,18]

Recent studies have shown that Inc/rep type FI and FII are the predominant carriers of these ESBL genes in *E. coli.*[19] Comparatively, few reports have associated *Klebsiella* with CTX-M-15, and to date there is no information on the plasmid types and whether they are the same as those found in *E. coli.*[20] The earliest *K. pneumoniae* isolates that were reported to carry *bla*CTX-M-15 are those that originated in India prior to 2000, and were associated with the IS element IS*EcP1*.[9] Hitherto, *K. pneumoniae* isolates carrying *bla*CTXM-15 have been reported mainly from countries in India, Southern/Eastern Europe and North Africa including Algeria, Tunisia, Lebanon, Italy, Portugal, Hungary, Bulgaria and Turkey.[21-29]

We characterized the CTX-M genes with respect to their genetic context and, in particular, the plasmid types that carry them.

MATERIAL AND METHODS

STRAINS

Between 2002-2004, 230 consecutive non-duplicate clinical isolates of *K. pneumoniae* were evaluated for the presence of ESBLs by using a double disk synergy test (DDST) with ceftazidime, ceftazidime/clavulanic acid and cefotaxime and cefotaxime/clavulanic asid. Thirty four (14.8%) strains were found as ESBL producers and were included in this study. The majority of the isolates were from urine specimens (31/34) from outpatients (24/34), while the rest were from hospitalized ones treated in eight different medical and surgical wards.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibilities were determined by the agar dilution test, and the results were evaluated according to the breakpoints and recommendations of the Clinical and Laboratory Standards Institute (CLSI).[30] Cefotaxime, ceftriaxone, ceftriaxone/clavulanic acid, piperacillin, ceftazidime, cefepime, aztreonam, amikacin, cefoxitin, imipenem, ampicillin and ciprofloxacin were tested. For β-lactam/ β-lactamase-inhibitor combinations, the constant concentrations of clavulanic acid and tazobactam were 4 and 2 μg/mL respectively. *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control purposes in the susceptibility testing.

ISOELECTRIC FOCUSING (IEF)

Analytical isoelectric focusing (IEF) was performed according to the method of Matthew et al[31] followed by overlaying the gel to visualize the β-lactamases,[4] using a mini IEF cell system (Bio-Rad Laboratories, Hercules, CA,USA). pIs of the β-lactamases were estimated from pIs of the previously known enzymes (TEM-1: 5.4, TEM-8: 5.8, SHV-3: 7, CMY-1: 8, and CMY-2: 9) and commercial pI markers (Bio-Rad Laboratories, Hercules, CA, USA).

BIOASSAY

After IEF, the cefotaxime hydrolyzing activities of particular β-lactamases were detected by the bioassay approach, as described by Bauernfeind et al.[4] Gels were stained with nitrocefin (Calbiochem, Darmstadt, Germany) to identify the β-lactamase bands. The polyacrylamide gel was covered with a cefotaxime (1 mg/L) containing 0.75% tryptic soy agar (TSA) overlay, to be tested for inactivation. After two hours of incubation at 35°C, a second overlay with a cefotaxime-susceptible indicator strain was applied. Following overnight incubation, growth of the indicator strain on the β-lactamase band identified the hydrolytic activity of that enzyme against cefotaxime.

CONJUGATION

In CTX-M positive isolates, plasmid transfer was performed in triptic soy broth (Oxoid, Basingtone, UK) by mixing 1 mL portions of each 18-24 hours tryptic soy broth (Oxoid, Basingtone, UK) cultures of the donor and the recipient (*E. coli* K12:W3110 RifRLac-) strains. Transconjugants were selected on MacConkey agar containing cefotaxime 2 mg/L and rifampicin 128 mg/L.

PCR DETECTION OF ESBL-ENCODING GENES AND IS ELEMENTS

Total DNA preparations from clinical isolates were used as templates in specific PCR reactions. For the $bla_{\text{CTX-M-1}}$ group (which is known to include CTX-M-1, 3, 10, 11, 12, 15, 22 and UOE-1 subgroups), the primers used are listed in Table 1.

PCR amplicons for linking IS*Ecp1* with *bla*CTX-M were obtained by anchoring one primer to the 3' end of *bla*CTX-M (*bla*CTX-M reverse and the other to the 5' end of IS*Ecp1*) (Table 1). Oligonucleotides designed to detect IS*CR* elements were based on the consensus sequence as reported by Toleman et al, 2006 (Table 1).[32]

Cycling conditions for amplification were: 5 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at 67°C, and 45 s at 72°C and finally 10 min at 72°C for the *bla*CTX-M. PCR was carried out in a 50 μL volume with 100 μM 10X PCR buffer, 50 pmol of each primer, 10 mM deoxynucleoside triphosphates, 2.5 mM MgCl2, and 2.5U Taq DNA polymerase. The PCR products were separated in 1% agarose gels [1.5% for Typing by randomly amplified polymorfic DNA analysis (RAPD) PCR] stained with ethidium bromide and visualized under UV light. ΦX174 replicative-form DNA *Hae*III fragments were used to assess the PCR product size. Strains encoding CTX-M-group-1 were used as positive controls for PCR amplification. The negative control strain was *E. coli* ATCC 25922.

DNA SEQUENCING AND ANALYSIS OF *bla*CTX-M AND IS*EcP1* PCR AMPLICONS

Sequencing was carried out on both strands by the dideoxy-chain termination method with a Perkin Elmer Biosystems 377 DNA sequencer. Sequence analysis was performed using the Lasergene DNASTAR software package. Sequence alignments were done using the Clustal W program and the PAM 250 matrix.

TYPING BY RANDOMLY AMPLIFIED POLYMORPHIC DNA ANALYSIS

RAPD analysis were performed using an ERIC2 primer.[33] Cycling conditions for amplification were: 3 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 40°C, and 2 min at 72°C and finally 5 min at 72°C.

PLASMID ISOLATION, TRANSFORMATION, REP-TYPING AND INcN PLASMID CONFIRMATION

The *bla*CTX-M-positive *K. pneumoniae* isolates had their plasmids fully characterized to determine the genetic context of the β-lactamase gene. Bacterial plasmids were isolated by the alkaline lysis method.[34] Essentially, an overnight 10 mL culture was centrifuged (12.000 g) and suspended in water (250 μL) before 200 μL of lysis solution (0.2M NaOH, 1% SDS) was added. After lysis, 125 μL of neutralising solution (0.3 M potassium acetate, 1 mM EDTA) was added. After precipitation, the suspension was centrifuged (12.000 g) and washed twice with 500 μL of a 50/50 (V/V) phenol/chloroform solution. The DNA was precipitated from the solution by adding 0.7 volume of isoamyl-alcohol. The DNA/RNA pellet was washed twice in 1 mL of 70% alcohol before it dried. The DNA was dissol-

TABLE 1: List of primers.

Primer name (s)	Sequence of primer(s)	Gene (s)	Reference
ERIC-2	5' AAGTAAGTGACTGGGGTGAGCG 3'	- (RAPD)	33
CTX-M-1 grp F	F: 5' CGCTTTGCGATGTGCAG 3'	CTX-M-1 group	15
CTX-M-1 grp R	R: 5' TAGAATTAATAACCGTCGGT 3'		
CTX-M-15rev	5' CACTTTGTCGTCTAAGGCG 3'	CTX-M-15 – ISEcp1	This study
ISEcp1F	5' AATACTACCTTGGCTTTCTGA 3'		
CRF	5' CACGCCACTGCTGTAAC 3'	ISCR elements	This study
MOV1	5' GGTATAGGAGTTCAACCGCC 3'		
HI1 FW	5′-GGAGCGATGGATTACTTCAGTAC-3′	parA-parB	32
HI1 RV	5′-TGCCGTTTCACCTCGTGAGTA-3′		
HI2 FW	5′-TTTCTCCTGAGTCACCTGTTAACAC-3′	iterons	32
HI2 RV	5′-GGCTCACTACCGTTGTCATCCT-3′		
I1 FW	5′-CGAAAGCCGGACGGCAGAA-3′	RNAI	32
I1 RV	5′-TCGTCGTTCCGCCAAGTTCGT-3′		
X FW	5′-AACCTTAGAGGCTATTTAAGTTGCTGAT-3′	ori γ	32
X RV	5′-TGAGAGTCAATTTTTATCTCATGTTTTAGC-3′		
L/M FW	5′-GGATGAAAACTATCAGCATCTGAAG-3′	repA,B,C	32
L/M RV	5′-CTGCAGGGGCGATTCTTTAGG-3′		
N FW	5′-GTCTAACGAGCTTACCGAAG-3′	repA	32
N RV	5′-GTTTCAACTCTGCCAAGTTC-3′		
FIA FW	5′-CCATGCTGGTTCTAGAGAAGGTG-3′	iterons	32
FIA RV	5′-GTATATCCTTACTGGCTTCCGCAG-3′		
FIB FW	5′-GGAGTTCTGACACACGATTTTCTG-3′	repA	32
FIB RV	5′-CTCCCGTCGCTTCAGGGCATT-3′		
W FW	5′-CCTAAGAACAACAAAGCCCCCG-3′	repA	32
W RV	5′-GGTGCGCGGCATAGAACCGT-3′		
Y FW	5′-AATTCAAACAACACTGTGCAGCCTG-3′	repA	32
Y RV	5′-GCGAGAATGGACGATTACAAAACTTT-3′		
P FW	5′-CTATGGCCCTGCAAACGCGCCAGAAA-3′	iterons	32
P RV	5′-TCACGCGCCAGGGCGCAGCC-3′		
FIC FW	5′-GTGAACTGGCAGATGAGGAAGG-3′	repA2	32
FIC RV	5′-TTCTCCTCGTCGCCAAACTAGAT-3′		
A/C FW	5′-GAGAACCAAAGACAAAGACCTGGA-3′	repA	32
A/C RV	5′-ACGACAAACCTGAATTGCCTCCTT-3′		
T FW	5′-TTGGCCTGTTTGTGCCTAAACCAT-3′	repA	32
T RV	5′-CGTTGATTACACTTAGCTTTGGAC-3′		
FII_S FW	5′-CTGTCGTAAGCTGATGGC-3′	repA	32
FII_S RV	5′-CTCTGCCACAAACTTCAGC-3′		
F_{repB} FW	5′-TGATCGTTTAAGGAATTTTG-3′	RNAI/repA	32
F_{repB} RV	5′-GAAGATCAGTCACACCATCC-3′		
K/B FW	5′-GCGGTCCGGAAAGCCAGAAAAC-3′	RNAI	32
K RV	5′-TCTTTCACGAGCCCGCCAAA-3′		
B/O RV	5′-TCTGCGTTCCGCCAAGTTCGA-3′	RNAI	32
TRA F	5'-CGATTACGTCAATGGTGAGC- 3'	TraD	This study
TRA R	5'-CTGCTTCCTCCGCTGTTGC-3'		
STB F	5'-CACTTCAGTTGATGTTGCCG-3'	StrB	This study
STB R	5'-CTCTTTATCAATAATGCCGG-3'		
ARD F	5'-CCATAATAGGCATCTCTAAACAG-3'	ArdA	This study
ARD R	5'-CATAAATACAACTGCGGAAG-3'		
RES F	5'-CGCGCAATGCCTTCAGACAGT-3'	ResA	This study
RES R	5'-CTGTCTGAAGGCATTGCGCG-3'		

ved in 30 μL with 0.1 Unit of RNAse. Isolated plasmids were used to transform *E. coli* TOPO cells (Invitrogen, Paisley, UK) via electroporation, using previously described conditions.[35] Transformants were isolated using cefotaxime (20 mg/L) and checked by PCR for carriage of the CTX-M type 1 gene. Plasmids were restricted using *Eco*R1 and their size was assessed,[36] and they were typed by PCR according to the method described by Carattoli et al[36,37] Primers used for plasmid typing are listed in Table 1. Initially, multiplex PCR was undertaken using the conditions described above and then refined with single PCR to obtain clear amplicons for sequencing.

Plasmid identification was verified by a PCR technique using primers based on the published IncN plasmid R46 (GenBank Accession number AY046276). Housekeeping genes chosen were *tra*D, *sta*B, *ard*A and *res*A (GenBank Accession number AY046276) and primers used are listed in Table 1. PCR conditions were as described above and amplicons were verified by sequencing.

RESULTS

ANTIMICROBIAL SUSCEPTIBILITIES

The MIC_{90} was 0.25 mg/L for imipenem; > 512 mg/L for ampicillin, piperacillin, piperacillin-tazobactam and ceftazidime; 512 mg/L for aztreonam; 128 mg/L for cefotaxime, ceftriaxone and ciprofloxacin; 64 mg/L for amikacin; 32 mg/L for cefepime and cefoxitin; 1 mg/L for cefotaxime-clavulanate and 8 mg/L for ceftriaxone-clavulanate and ceftazidime-clavulanate showed a CTX-M positive strains high degree of diversity of the levels of resistance to cefotaxime, as illustrated by the broad range of MICs (1- > 512 mg/L). Ten isolates showed resistance to cefoxitin, but all isolates were sensitive to imipenem (Table 2).

ISOELECTRIC FOCUSING (IEF)

Ten isolates produced only one β-lactamase; while 10 isolates produced two, 10 isolates three, three isolates four, and two isolates five different β-lactamases. pI values, at which β-lactamase bands were detected, ranged from 5.2 to 8.4 (Table 3).

TABLE 2: Cumulative distribution of MICs for ESBL producing *K. pneumoniae* isolates.

	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	>512	MIC range	MIC90	S%	I%	R%
Ampicillin																			34	> 512		-	-	100
Piperacilin															2		1	7	24	64-> 512	> 512	-	5.9	94.1
Piperacilin-tazobactam										2	7	1	3		2	2	1		16	2-> 512	> 512	38.2	5.9	55.9
Cefotaxime									1	2	2	2	1	10	8	2	4	1	1	1-> 512	128	20.6	32.4	47
Cefotaxime-clavulanate					7	7	12	2	2	1	2	1								0.06-8	1			
Ceftriaxone								3		2		2	11	4	5	3	1	3		0.5-512	128	20.6	44.1	35.3
Ceftriaxone-clavulanate				3	1	5	16	2	4	2	1									0.03-4	1			
Ceftazidime												3	3	1	7	4	4	5	7	8-> 512	> 512	8.8	8.8	82.4
Ceftazidime-clavulanate							1	2	9	6	6	4	2		1	3				0.25-128	8			
Aztreonam																	10	24		256-512	512	-	-	100
Cefepime							1		1	1	1	1	17	9		1	1	1		0.25-512	32	14.7	50	35.3
Cefoxitin										10	5	5	4	5	2	3				2-128	32	58.8	11.8	29.4
Imipenem	1				5	16	12													0.004-0.25	0.25	100	-	-
Ciprofloxacin			1	14	3	1	1	1	1	1	1	1	1		1	3	3		1	0.015-> 512	128	64.7	2.9	32.4
Amikacin								2	9	5	3		3	8	3	1				0.5-128	64	65	-	35

S: Susceptible, I: Intermediate, R: Resistant.

TABLE 3: Results for ESBL producing *K. pneumoniae* isolates.

Isolate no	MIC (≒g/mL) CTX	CAZ	FOX			pI		blaCTX-M	RAPD	Inc/rep typing (# plasmids)
1	32 (I)	8(S)	4 (S)	5.4		7.4	8.4	+	A	FII, N (2)
2	64 (R)	>512(R)	16 (I)			7.4	8.4	-	B	-
3	64 (R)	512(R)	8 (S)			7.4	8	-	C	-
4	>512(R)	512(R)	32(R)		6.5	7.2		-	D	-
						7.6				
5	32(I)	>512(R)	16 (I)			7.4	8	-	E	-
6	32(I)	8(S)	2 (S)	5.4		7.2	8.4	+	F	Y-FI, N (2)
7	4(S)	64(R)	2 (S)				8.2	-	G	-
8	32(I)	256(R)	4 (S)				8.2	-	H	-
9	4(S)	64(R)	2 (S)	5.4		7.2		-	İ	-
10	8(S)	64(R)	2 (S)	5.6		7.4	8.2	-	İ	-
11	256(R)	64(R)	32(R)				8.4	-	J	-
12	64(R)	8(S)	2 (S)				8.4	+	K	N
13	16(I)	64(R)	2 (S)			7		-	L	-
14	32(I)	32(R)	2 (S)	5.4		7.4	8.4	+	M	N
15	256(R)	512(R)	4 (S)	5.2		7.2	8.4	+	N	FII
16	64(R)	>512(R)	128(R)	5.2			8.4	-	O	-
17	64(R)	>512(R)	128(R)	5.2 5.4	6.6	7	8.4	-	O	-
18	64(R)	64(R)	4 (S)	5.4			8.4	+	P	N
19	64(R)	>512(R)	64 (R)	5.2	6.6	7.6	8	-	O	-
20	32(I)	>512(R)	2 (S)				8.2	-	P	-
21	32(I)	512(R)	128(R)	5.2		7.8	8	-	O	-
22	8(S)	256(R)	8 (S)			7.4		-	R	-
23	32(I)	16(I)	4 (S)				8.2	-	M	-
24	128(R)	16(I)	16 (I)			7	8.4	+	M	N
25	2(S)	16(I)	2 (S)			7.4	8.4	+	A	N
26	256(R)	>512(R)	32 (R)			7.4	8	-	O	L/M
27	1(S)	64(R)	8 (S)	5.4	6.8	7	8.4	-	İ	-
28	32(I)	256(R)	8 (S)	5.2 5.4	6.8		8.4	-	P	-
29	2(S)	128(R)	2 (S)	5.2		7.4	8.4	+	R	N
30	64(R)	512(R)	8 (S)	5.4		7.4	8.4	-	M	-
31	128(R)	128(R)	16 (I)	5.4	6.8	7	8.4	+	N	N
						7.4				
32	256(R)	128(R)	32 (R)				8.4	-	N	P
33	32(I)	128(R)	32 (R)	5.4			8.4	+	S	FII
34	512(R)	256(R)	64 (R)	5.4		7	8.4	+	T	N

CTX: Cefotaxime; CAZ: Ceftazidime; FOX: Cefoxitin; R: Resistant; I: Intermediate; S: Susceptible.

BIOASSAY

All of the 12 *K. pneumoniae* strains were confirmed to possess cefotaxime hydrolysing activity in the subsequent bioassays. pIs at which cefotaxime was hydrolized were 8.4.

Detection of *bla*CTX-M genes and association with *ISEcP1*.

The prevalence of the CTX-M-type ESBLs was as high as 35% (12 of 34) in this study (Table 3). Three of the isolates (strain no: 11, 20 and 32 with pIs of 8.4, 8.2) were negative for CTX-M genes.

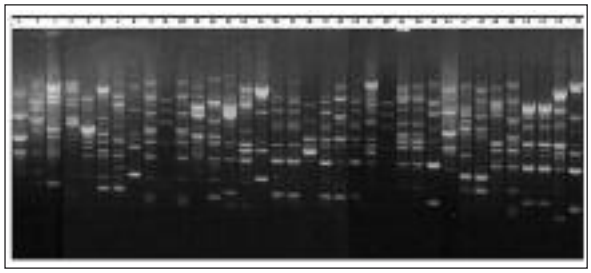

FIGURE 1: RAPD profiles of *K. pneumoniae* isolates (n= 34).
Lane M: OX174 DNA *Hae*III size marker.

All *bla*CTX-M amplicons were shown to be *bla*CTX-M-15. Determination of the genetic context of *bla*CTX-M-15 was undertaken using primers based on IS*EcP1* sequences.[22,36] Interestingly 3 of the 12 isolates (strain no: 14, 31 and 34) were negative for IS*EcP1* despite possessing *bla*CTX-M-15.

RAPD-PCR

Most strains were found as clonally unrelated (Table 3). Isolates 1 and 6 carried FII and N; and Y-FI and N (RAPD types A and F), respectively (Figure 1).

Resistance Transfer Experiments and Characterization of Plasmids Carrying *bla*CTX-M Genes

Plasmid transfer to the recipient strain (*E. coli* K12:W3110 RifRLac) was successful in nine out of the 12 CTX-M-15 producing isolates. High conjugation efficiency of about 10^{-3}, 10^{-4} recombinants per donor cell were observed and the transconjugants expressed a high-level resistance to cefotaxime.

Plasmid analysis indicated that in two cases, strains possessed more than one plasmid. The presence of *bla*CTX-M-15 genes in cefotaxime resistant transconjugates was confirmed by PCR and sequencing. Once confirmed, plasmid was isolated from each of the nine transconjugates for characterization. The three strains where transfer was not demonstrated had their plasmids extracted and were used to transform *E. coli* (K12:W3110 RifRLac) via electroporation. All 12 plasmids carrying *bla*CTX-M-15 was subject to multiplex PCR to determine the plasmid Inc/rep type followed by simplex PCR as previously described.[12,36,37] Amplicons were isolated, purified and sequenced to verify the multiplex/simplex data. In two isolates (15 and 33), the *bla*CTX-M-15 was carried on an approximately 60 kb plasmid and possessed an Inc/rep type of FII (Table 3). However, each of these isolates gave different RAPD profiles. The remaining, 10/12 carried the *bla*CTX-M-15 gene on larger plasmids (95 kb) typing to IncN, again exhibited markedly distinct RAPD profiles (Table 3), and were not clonal apart from two groups having two members (A and M). As these data clearly indicate the possible dissemination of an IncN-type plasmid, plasmids from the transconjugates were subjected to a PCR analysis examining the IncN housekeeping genes. The plasmids from all transconjugates carrying *bla*CTX-M-15 also carried intact *traD*, *staB*, *ardA* and *resA* genes similar to the R46 plasmid backbone indicating no internal arrangements in this part of the plasmid (GenBank Accession number AY046276).

DISCUSSION

In this study which included primarily the urinary isolates of *K. pneumoniae* from outpatients, the overall prevalence rate of ESBL production was 14.7% (34 of 230 isolates). ESBL-producing bacteria have been reported from Turkey, but enzyme-specific prevalence studies are still few.[25,38-40] In the present study, analytical IEF of crude extracts of the 34 isolates revealed heterogeneous patterns with multiple β-lactamase bands in some isolates.

CTX-M has been recognized recently in Turkish strains. Until now, CTX-M-2 has been isolated from *K. pneumoniae*; CTX-M-3 from *E. coli*, *Salmonella typhimurium*, *Shigella sonnei* and *Morganella morganii*; and CTX-M-15 from *K. pneumoniae* and *E.coli*.[38-42] The only prevalence study on CTX-Ms is a multi-center work which revealed that CTX-M enzymes, particularly CTX-M-3, were disseminated in Enterobacteriaceae in Turkey. Enzyme production was detected in 76.5% of 34 *E. coli*, 82.6% of 23 *K. pneumoniae* and half of eight *Enterobacter* spp. isolates. In the present study, 35% of the *K. pneumoniae* strains produced CTX-M, while in another recent study again from our hospital, the frequency of the enzyme in *E. coli* was

found as high as 86.8%.[42] These studies and results of the present study infer that CTX-M-type ESBLs are spreading in Turkey.

In this study, all *bla*CTX-M genes were *bla*CTX-M-15 as determined by sequencing. The widespread dissemination of the CTX-M genes, particularly *bla*CTX-M-15, is thought to be due to clonal spread and/or the IS element, IS*EcP1*, that is ubiquitously associated with it. Interestingly three of the *bla*CTX-M-15 positive *K. pneumoniae* isolates were negative for IS*EcP1*, suggesting that an unusual mobile genetic element was associated with its mobility. As IS*CR* elements have been associated with *bla*CTX-M genes,[42] these mobile elements were investigated as a possible source of carriage of *bla*CTX-M-15 but none were found (data not shown).

The RAPD typing on the *bla*CTX-M-15 positive isolates showed that they were phylogenetically unrelated and apart from the two groups, RAPD type A and M. This would suggest that the increase in *bla*CTX-M-15 during this period was likely due to a common plasmid being disseminated throughout *K. pneumoniae* isolates. We typed all strains possessing *bla*CTX-M-15 and found that only 2 isolates possessed multiple plasmids: isolates 1 and 6 carried FII and N, and Y-FI and N, respectively. Transconjugates were created, ensuring expression of the *bla*CTX-M-15 genotype, and also typed. All plasmids carried by the *bla*CTX-M-15 positive transconjugates were IncN type, except 15 and 33 that possessed IncFII type plasmids. The plasmids were confirmed as typical R46-like IncN rep plasmids as confirmed by the presence of the housekeeping genes *traD*, *staB*, *ardA* and *resA*. It is known that *bla*CTX-M-15 is carried on FII plasmids and is commonly found in *E. coli,* as shown previously in Turkey and France.[19,42] This is the first report of *bla*CTX-M-15 that is associated with IncN type plasmids and this finding is in contrast with that of a previous study from Spain which showed that *bla*CTX-M-15 in *K. pneumoniae* was carried on FII plasmids.[16] Studies using *bla*CTX-M-15 positive *E. coli* isolates from France, Tunisia, Bangui, Pakistan, Central America and UK showed that *bla*CTX-M-15 was carried frequently on IncFII plasmids[12,43-46] or, in few *E. coli* and *Salmonella* producers, it was associated with IncI1 plasmids.[47] Intriguingly, a recent report on plasmid typing of strains conferring resistance to carbapenems showed that *bla*VIM-4 in *K. pneumoniae* from Greece was also carried on an IncN type plasmid. In a study on a nonbiased population of Enterobacteriaceae demonstrated that plasmids belonging to the IncFII group were prevalent in *E. coli* (58%) and infrequent in *K. pneumoniae* (5%).[42] Thus, *K. pneumoniae* isolates have a propensity for IncN type plasmids and *E. coli* for FII. The results of this study and that of Loli *et al*[48] which used strains from South East Europe (Greece and Turkey) might indicate the dominance of IncN plasmids in this area.

In conclusion, this study confirms the emergence of *bla*CTX-M-15 and its spread in a major city hospital in Istanbul. RAPD and plasmid typing indicates that IncN plasmids are disseminating the *bla*CTX-M-15 through *K. pneumoniae* populations.

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