

Full Length Research Paper

Phenotypic and molecular characterization of SHV, TEM, CTX- M and extended-spectrum - lactamase produced by *Escherichia coli*, *Acinobacter baumannii* and *Klebsiella* isolates in a Turkish hospital

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A total of 94 clinical isolates were collected from Gazi University Hospital, Turkey. Presence of ESBL positivity was detected using the double disk synergy test (DDST). ESBL isolates were further typed for the *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M} and *bla*_{OXA} using designed primers. ESBLs were found in 65 (69.14%) isolates using DDST. Plasmid DNAs of potentially ESBL positive strains were isolated. About 7.69% of the ESBL positive isolates did not harbour plasmid DNA. According to the PCR technique, only 2 additional isolates were found to be ESBL producers. *bla*_{TEM} was the commonest genotype (73.43%), followed by *bla*_{SHV} (21.87%) and *bla*_{CTX-M} (17.18%), either alone or in combination. ESBL positive strains of *Klebsiella pneumoniae*, *Escherichia coli*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are increasingly found in hospital isolates. Because these strains become resistant to available antibiotics and they can pass the gene to other clinical strains, the quick detection of these strains in clinical laboratories is very important.

Key words: ESBL, double disk synergy test, plasmid, PCR.

INTRODUCTION

Extended-spectrum -lactamases (ESBLs) were first described in the 1980s and they have been detected in *Klebsiella* species, and later in *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia marcescens* and other gram- negative bacilli (Kiratin et al., 2008; Cheng et al., 2008; Morris et al., 2003). ESBLs are also able to hydrolyze 3 and 4 generation cephalosporins and monobactams. ESBL producing strains are inhibited by -lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) (Bradford, 2001; Pitout et al., 2007; Giraud-Morin and Fosse, 2003). ESBLs are a group of enzymes encoded by genes described predominantly on plasmid that are common among Enterobacteriaceae (Poole, 2004). ESBL are an increasingly important cause of transferable multidrug resistance in gram-negative bac-

teria throughout the world. These bacteria have spread rapidly and have become a serious threat to human health worldwide (Giraud-Morin and Fosse, 2003; Poole, 2004; Gupta, 2007).

ESBLs are undergoing continuous mutation, causing the development of new enzymes showing expanded substrate profiles. At present, there are more than 300 different ESBL variants, and these have been clustered into nine different structural and evolutionary families based on amino acid sequence. TEM and sulphhydryl variable SHV were the major types. However, CTX-M type is more common in some countries (Paterson et al., 2003). Determination of TEM and SHV genes by molecular techniques in ESBL producing bacteria and their pattern of antimicrobial resistance can supply useful data about their epidemiology and risk factors associated with these infections (Jain and Mondal, 2008). The aim of this study was to isolate and identify the types of extended-spectrum beta- lactamases (ESBL) produced by *E. coli*, *A. baumannii* and *Klebsiella* spp. (*K. pneumoniae*, *K. oxy-*

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Table 1. Oligonucleotide primers used for detection of beta-lactamase genes.

Primers	(°C)	Nucleotide Sequences (5'-3')	References (GenBank no)	Expected amplicon size (bp)
SHV-F	60	CGCCTGTGTATTATCTCCCT	EF125011	293
SHV-R	62	CGAGTAGTCCACCAGATCCT		
TEM-F	60	TTTCGTGTCGCCCTTATTCC	AB282997	403
TEM-R	62	ATCGTTGTCAGAAGTAAGTTGG		
CTX-M-F	60	CGCTGTTGTTAGGAAGTGTG	DQ303459	569
CTX-M-R	62	GGCTGGGTGAAGTAAGTGAC		
OXA-F	64	ATGGCGACTACTGGATAGATGG	L07945	701
OXA-R	62	AGTCTTGGTCTTGGTTGTGAG		

toca, *K. terrigena* and *K. ornithinolytica*) isolated from various specimens (urine, blood, sputum, wound, abscesses, catheter, peritoneum and cerebrospinal fluid) of patients hospitalized in different units of Gazi University Medical Faculty Hospital, Turkey.

MATERIALS AND METHODS

Bacterial isolates and detection of ESBL

Ninety-three clinical isolates of *E. coli*, *A. baumannii*, *K. pneumoniae*, *K. oxytoca*, *K. terrigena* and *K. ornithinolytica* from various clinical specimens (urine, blood, sputum, wound, abscesses, catheter, peritoneum and cerebrospinal fluid) were obtained in 2006 - 2007 and identified with API ID 32E (Bio Mérieux, Marcy l'Etoile, France). *E. coli*, *A. baumannii* and *Klebsiella* isolates were screened for ESBL production by DDST using ceftazidime (30 g), cefotaxime (30 g), ceftriaxone (30 g) and cefpodoxime (10 g) antibiotic on Mueller–Hinton agar as recommended by the Clinical and Laboratory Standards Institute (CLSI guideline 2009). ESBL production was confirmed by disk potentiation test using ceftazidime (30 g) and cefotaxime (30 g) antibiotic disks with and without clavulanic acid (10 g) and by DDST (Koneman et al., 1997; Jarlier et al., 1988; Philippon et al., 1989).

Plasmid and chromosomal DNA analysis

Plasmid DNA was isolated from clinical isolates using the alkaline lysis method (Stürenburg and Mack, 2003). All clinical isolates were grown for 12 h on nutrient agar plates. A loopful of cells from a single colony was transferred to 0.1 ml of H₂O, and the mixture was boiled for 10 min to lyse the cells. The resulting cell lysate was centrifuged briefly (10 s at 10,000 rpm), and 15 µl of the supernatant was used as the DNA sample for the PCR reaction (Sambrook and Russel, 2001).

Detection of ESBL types by PCR

Primer design

The specific oligonucleotide primers were designed according to TEM, SHV, CTX-M-type and OXA -lactamase DNA sequences deposited in the GenBank nucleotide sequence database with the accession numbers AB282997, EF125011, DQ303459 and L07945 (Table 1). Multiple nucleotide sequence alignments were performed with the CLC Workbench program. Coding sequences were detect-

ed with the Artemis program and primers were designed with the Perl Primer program.

Amplification of the ESBL genes

ESBL producing isolates were amplified using *bla*_{TEM/SHVCTX-M}, *OXA* specific primers listed in Table 1. Amplification reactions were carried out under the following conditions: initial denaturation at 94°C for 3 m, followed by 35 cycle of denaturation at 94°C for 45 s, annealing at 60°C for TEM, SHV and CTX-M and at 62°C for OXA for 30 s and extension at 72°C for 1 min, and a final extension at 72°C for 3 min. A molecular marker (Fermentase SM0241 effective size range: 80 to 1000 kb) was used to assess PCR product size.

RESULTS AND DISCUSSION

During an 8-month period, a total of 94 clinical specimens from Gazi University Hospital were identified, 50 as *E. coli*, followed by *A. baumannii* (n = 19), *K. pneumoniae* (n = 17), *K. oxytoca* (n = 4), *K. terrigena* (n = 2) and *K. ornithinolytica* (n = 2). Sixty-five of the 94 isolates were confirmed as potentially ESBL producers using ceftazidime/clavulanate and cefotaxime/clavulanate disks (Table 2). Occurrence of ESBL in isolates was as follows: 42 (84%) of 50 *E. coli*, 1 (5.26%) of 19 *A. baumannii*, 15 (88.23%) of 17 *K. pneumoniae*, 4 (100%) of 4 *K. oxytoca*, 2 (100%) of 2 *K. terrigena*, and 1 (50%) of 2 *K. ornithinolytica* (Table 2). Of the 65 isolates, 48 (73.84%) were from urine, 5 (7.69%) from blood, 4 (6.15%) from sputum, 1 (1.53%) from a wound, 1 (1.53%) from an abscess, 2 (3.07%) from catheters, 2 (3.07%) from peritoneums and 1 (1.53%) from cerebrospinal fluid (CSF).

The plasmid DNAs were isolated from 58 isolates (90.76%) of potentially ESBL positive *E. coli*, *A. baumannii*, *Klebsiella* spp., potentially ESBL positive *E. coli*, *A. baumannii* and *Klebsiella* species that harboured plasmid varying from 1 to 10 (Figure 1 a, b and c). Five of the ESBL positive *E. coli* and 2 of the *K. pneumoniae* isolates had no plasmid DNA. All ESBL-producing isolates were screened by PCR using *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M} and *bla*_{OXA} specific primers (Figure 2). Some of the TEM, SHV, and CTX-M PCR products were subsequently se-

Table 2. According to clinical material, distribution of ESBL positive isolates using disk diffusion (P*: Peritoneum, CSF*: cerebrospinal fluid).

Positive for ESBL by DDST / Source	Urine	Blood	Sputum	Wound	P*	Catheter	CSF*	Abscess	Total
<i>E. coli</i>	34	2	2	1	-	2	-	1	42
<i>K. pneumoniae</i>	8	2	2	-	2	-	1	-	15
<i>K. oxytoca</i>	4	-	-	-	-	-	-	-	4
<i>K. terrigena</i>	1	1	-	-	-	-	-	-	2
<i>K. ornithinolytica</i>	1	-	-	-	-	-	-	-	1
<i>A. baumannii</i>	-	-	1	-	-	-	-	-	1
Total	48	5	5	1	2	2	1	1	65

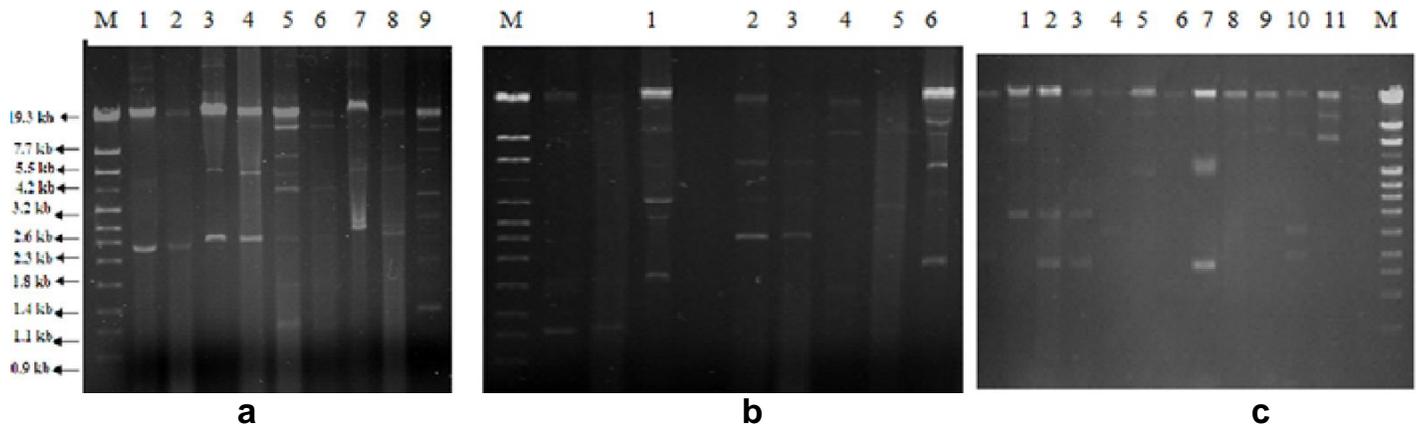


Figure 1. Plasmid profiles of some *Klebsiella* (a), *E. coli* (b) and *A. baumannii* (c) isolates. M. -pUC mix.

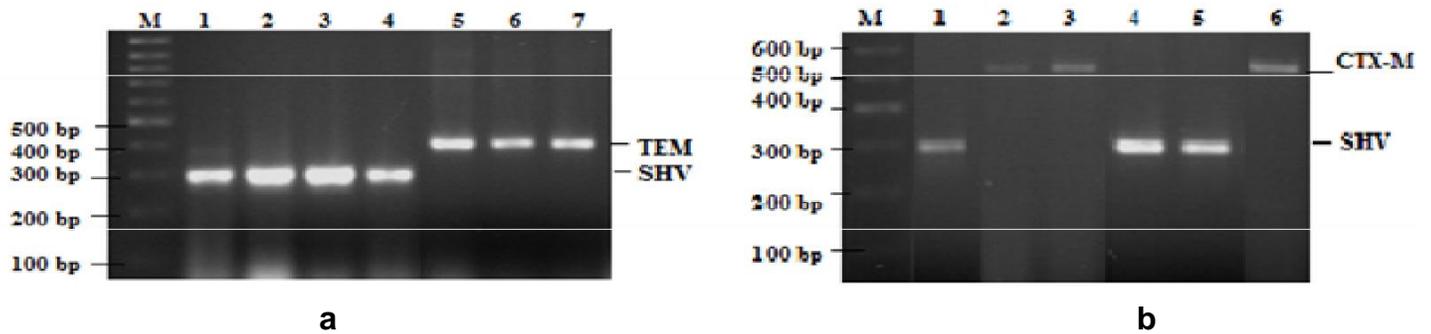


Figure 2. PCR products of *bla_{SHV}* (a: lines 1-4; b: lines 1, 4, 5), *bla_{TEM}* (a: Lines 5-7) and *bla_{CTX-M}* (b: Lines 2, 3, 6). M: 1000 bp DNA ladder.

sequenced and compared with DNA GenBank sequences using BLAST search. ESBLs were found in 65 isolates (68.14%): 44 *E. coli* and 21 *Klebsiella* isolates. Some of the isolates harboured two or more ESBL genes. However, the *A. baumannii* isolate had none of the ESBL genes.

TEM type ESBLs were found in 72.72% of *E. coli*, 73.33% of *K. pneumoniae*, 25% of *K. oxytoca* and 100% of *K. terrigena* and *K. ornithinolytica* isolates (Table 3). None of the isolates had *bla_{OXA}* genes. Eighteen (19.2%)

out of 65 isolates carried more than one type of - lactamase genes, with nine isolates harbouring *bla_{TEM}* and *bla_{SHV}* genes and nine isolates harbouring *bla_{TEM}* and *bla_{CTX-M}* genes. No isolates with *bla_{TEM}*, *bla_{SHV}*, and *bla_{CTX-M}* genes together were detected. *bla_{TEM}* genes were the most common ESBLs detected in *E. coli* (72.72%).

In recent years, the problem of gradually increasing resistance to antibiotics has threatened the entire world. Production of beta-lactamase, which hydrolyses and inactivates beta-lactam antibiotics, has been one of the

Table 3. Distribution of ESBL positive isolates including *bla*TEM, *bla*SHV and *bla*CTX-M genes by PCR. Total ESBL positive isolates number 64.

ESBL positive isolates / ESBL types	TEM	SHV	CTX-M
<i>E. coli</i> (n = 44)	32	4	10
<i>K. pneumoniae</i> (n = 15)	11	8	1
<i>K. terrigena</i> (n = 2)	2	-	-
<i>K. ornithinolytica</i> (n = 1)	1	1	-
<i>K. oxytoca</i> (n = 2)	1	1	-

most important resistance mechanisms of many bacterial species, mainly in the family Enterobacteriace (Akcam et al., 2004). Resistance to extended-spectrum -lactams among gram-negative pathogens is increasingly associated with ESBLs (Kimura et al., 2007). ESBL positive enterobacterial species are becoming widespread throughout the world (Timko, 2004).

In Turkey, several studies have revealed that the distribution of ESBLs has been observed in different rates. Bülüç et al. (2003) found that ESBL frequencies were 48% for *K. pneumoniae*, 40% for *K. oxytoca* and 14% for *E. coli* isolates. However, Delialioğlu et al. (2005) stated that ESBL frequencies of *K. pneumoniae*, *K. oxytoca* and *E. coli* isolates were 29.7, 4.2 and 18.3%, respectively. The prevalence of confirmed ESBL- positive isolates in the USA, Europe, Latin America, the Middle East and Asia/Pacific was 3, 5, 10 and 13% for *E. coli* and 17, 7, 11, 14, 20 and 18% for *Klebsiella* spp. (Paterson et al., 2005). ESBL production in *Acinetobacter* strains in India was 28% (Sinha et al., 2007).

In this study, ESBL positive isolates were detected by DDST and PCR. Sixty- five isolates were positive in the disk diffusion test and 67 isolates were positive using PCR. The fact that only two additional isolates were ESBL positive proves that the disk diffusion test is quite accurate. One of the positive isolates with DDST had no ESBL with PCR; these isolates may have different ESBL types than the ESBL tested in this study. TEM type ESBLs were found in 72.72% of *E. coli*, 73.33% of *K. pneumoniae*, 25% of *K. oxytoca* and 100% of *K. terrigena* and *K. ornithinolytica* isolates. ESBLs are mostly encoded by large plasmids (up to 100 kb or even more) that are transferable from strain to strain and between bacterial species (Jarlier et al., 1988). However, in different studies, different ESBL plasmid sizes have been detected; one study in South Africa proved that plasmid sizes of 25 isolates of *K. pneumoniae* varied from 5 to 186 kb in size (Essack et al., 2001). In another study, 48 *E. coli* isolates were investigated and it was found that the size of plasmid DNA varied from 95 to 120 kb for CTX-M type ESBL and from 60 to 160 kb for SHV type ESBL (Velasco et al., 2007). In our study, we found that ESBL positive *E. coli*, *A. baumannii* and *Klebsiella* species harboured plasmid DNA from 1 to 10 of size 1 kb to 19 kb. The other finding was that ESBL positive isolates showed plasmid DNA more often than ESBL

negative isolates. This may be considered evidence that most ESBL isolates depended on plasmids. These finding support the result of relation between the number of plasmids harbored by an isolate resistance to antibiotic. However, there were seven ESBL positive isolates with no plasmid, showing that some of the ESBL genes were coded potentially by chromosomal DNA.

TEM and SHV beta lactamases are mainly found in *E. coli* and *Klebsiella pneumoniae*, but can occur in other members of the family Enterobacteriaceae and in nonenteric organisms, such as *Acinetobacter* species (Turner, 2005). In Turkey, SHV-2 (*K. pneumoniae*, *E. coli*), SHV-5 (*K. pneumoniae*, *Enterobacter aerogenes* and *Enterobacter cloacae*), SHV-12 (*K. Pneumoniae* and *E. coli*), OXA-16 (*Pseudomonas aeruginosa*), PER-1 (*Acinetobacter baumannii* and *P. aeruginosa*), CTX-M-2, CTX-M-15 and TEM-1 (*K. pneumoniae*) type ESBLs were reported (Gupta, 2007; Velasco et al., 2007).

In summary, from a total of 94 isolates, we found 50% (n = 47), 14.89% (n = 14) and 11.70% (n = 11) ESBL rates for TEM, SHV and CTX-M type beta lactamases, respectively. There were no strains harboring OXA type beta lactamase. TEM and CTX-M type ESBL were observed in 72.72 and 22.72% of *E. coli* isolates, respectively. SHV type ESBL was frequently found in *K. pneumoniae* (53.3%) isolates. ESBL positive strains of *K. pneumoniae*, *E. coli*, *A. baumannii* and *P. aeruginosa* are increasingly found in hospital isolates. These strains are usually multi-drug resistant. Because these strains become resistant to available antibiotics and they can pass the gene to other clinical strains, the quick detection of these strains in microbiology laboratories is very important. Molecular typing would determine which types of ESBL are present in each isolate. Molecular detection and identification of beta lactamases would be essential for a reliable epidemiological investigation of antimicrobial resistance. Antimicrobial therapy has played an important role in the treatment of human bacterial infections, but the drug resistance that has emerged in the treatment of bacterial infections due to ESBL enzymes degrades all beta lactam antibiotics and thus bacteria become multi-drug resistant (Gupta, 2007). These enzymes can be chromosomal or plasmid mediated. The gene code for the enzymes may be carried on integrons. Integrons help in the dissemination of antimicrobial drug resistance in health care settings (Gupta, 2007). Therefore, ESBL pro-

producing organisms should be identified quickly so that appropriate antibiotic usage and infection control measures can be implemented.

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