Jpn. J. Infect. Dis., 58, 162-167, 2005

Original Article

Molecular Characterization of TEM- and SHV-Derived Extended-Spectrum Beta-Lactamases in Hospital-Based *Enterobacteriaceae* in Turkey

Hüseyin Taşlı* and İ. Hakkı Bahar

Department of Microbiology and Clinical Microbiology, Faculty of Medicine, Dokuz Eylul University, Izmir, Turkey

(Received September 15, 2004. Accepted March 3, 2005)

SUMMARY: TEM- and SHV-derived extended-spectrum β-lactamases (ESBLs) producing *Enterobacteriaceae* have been reported from throughout the world, but there has been limited data for the molecular characterization of these enzymes in Turkey. The aim of this study was to investigate and to type the TEM- and SHV-derived ESBLs in 63 ESBL-producing clinical isolates of *Enterobacteriaceae*, and it included further analysis; transfer experiments, isoelectric focusing, PCR, PCR-restriction fragment length polymorphism, and DNA sequencing. According to PCR results the transconjugant strains included 52.7% TEM, 74.3% SHV, and 32.4% of both the TEM and SHV genes. Using PCR/*Nhe*I restriction analysis, 45 of the 46 ESBL detected in transconjugants were determined to be SHV-derived. DNA sequencing was performed for the identification of TEM- and SHV-derived ESBLs for 18 selected transconjugants. SHV-2, SHV-5, and SHV-12 were detected in five, seven, and five samples, respectively. This is the first description of SHV-12 in Turkey.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs), such as the plasmid-mediated class A TEM- and SHV-derived enzymes, have developed by stepwise mutations in their structural genes, resulting in either single or multiple amino acid changes in the encoded enzymes (1-4). These enzymes are often the cause for resistance to newer cephalosporins and monobactams in the members of the family of *Enterobacteriaceae*. ESBLs have been widely found worldwide, including in Turkey (5,6), and they are emerging as a serious problem. Today, over 150 different ESBLs have been described. Most ESBLs are derivatives of TEM or SHV enzymes. There are now >90 TEM-derived β-lactamases and >25 SHV-derived enzymes (5). TEM- and SHV-derived ESBLs are most often found in *Escherichia coli* and *Klebsiella pneumoniae* (7,8). The phenotypic tests only presumptively identify the presence of an ESBL. Although isoelectric focusing (IEF) has become traditional for characterization of β-lactamase, this approach can not distinguish among several SHV-ESBLs having the same isoelectric point (5). Recently, several molecular methods that will aid in the detection and differentiation of ESBLs without sequencing have been suggested (9-13). Nucleotide sequencing remains the standard for determination of the specific β-lactamase gene present in a strain (14). In Turkey, several studies have shown that ESBL was found in high ratios in clinical isolates in *Enterobacteriaceae* (6,15-17). Unfortunately, published information on the molecular characterization of these enzymes in Turkey remains limited. The present investigation was undertaken to asses the types of ESBLs belonging to TEM- and SHV-families in the ESBL-producing clinical isolates of *Enterobacteriaceae*.

*Corresponding author: Mailing address: Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Ege University, 35100-Bornova, Izmir, Turkey. Tel: +90-232-3884000/1359, Fax: +90-232-3885258, E-mail: taslih@pharm.ege.edu.tr

MATERIALS AND METHODS

A total of 166 consecutive nonduplicate ampicillin-resistant clinical isolates of *Enterobacteriaceae* were collected at the Dokuz Eylul University Hospital, identified by conventional bacteriological methods and the Vitek ID system (BioMerieux Vitek Inc., Hazelwood, Mo., USA), and stored at –70°C until analysis. The MICs of amoxicillin, amoxicillin-clavulanic acid, cefoxitin, aztreonam, ceftazidime, ceftriaxone, cefotaxime, and imipenem were determined by the microdilution method according to the guidelines of the NCCLS (18). *E. coli* ATCC 25922 was used as the reference strain for antimicrobial susceptibility testing. These isolates were screened for ESBL production according to NCCLS criteria (18). ESBL production was confirmed using the double disk synergy test (19), the modified double disk synergy test (20), and the NCCLS confirmatory test (18) as previously described. Conjugational transfer of β-lactam resistance was performed by the plate mating method (21), with the clinical isolates as donors and *E. coli* K-12 SA362 (Str^R) as the recipient. Transconjugants were selected on Mueller Hinton Agar supplemented with 1,000 μg of streptomycin and 10 μg of ampicillin or 2 μg of ceftazidime per mL. Transconjugants were assessed for their β-lactam susceptibilities and ESBL production. The crude sonic extracts containing β-lactamases were examined using a PhastSystem apparatus (Pharmacia, Uppsala, Sweden) and precast polyacrylamide gels with a pH range of 3 to 9 (PhastGel IEF 3-9; Pharmacia). β-lactamase bands were visualized by staining with nitrocefin (500 μg/ml) (Oxoid, Hampshire, UK). Isoelectric points (pIs) were obtained by comparison with those for β-lactamases (TEM-1, SHV-1) with known pIs and protein markers (IEF-MIX 3.6-3.9; Sigma, Steinheim, Germany). Plasmid DNA extracted from transconjugant cells was used as a template in specific PCRs for detection of the bla_{TEM} and bla_{SHV} genes. Plasmid DNA was purified from bacterial cells by the alkaline lysis method (22,23). A pair of primers (TEM/F: 5′-GAA GAC GAA AGG

GCC TCG TG-3′ and TEM/R: 5′-GGT CTG ACA GTT ACC AAT GC-3′) was used for the amplification of a 1074-bp fragment that covers the entire bla_{TEM} genes. A pair of primers (SHV/F: 5′-CGC CGG GTT ATT CTT ATT TGT CGC-3′ and SHV/R: 5′-TCT TTC CGA TGC CGC CGC CAG TCA-3′) was used for the amplification of a 1016-bp fragment that covers the entire bla_{SHV} gene (24). *E. coli* transconjugant strains harboring plasmids incorporating bla_{TEM-1} or bla_{SHV-2} were used as positive controls, and the *E. coli* K-12 recipient strain was used as a negative control. A single reaction mixture contained 1 μL of plasmid DNA extract, 20 pmol of each primer, 100 μM (each) dNTPs, 1.25 U of *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania) and buffer with 1.5 mM $MgCl_2$ supplied by the manufacturer of the enzyme in a total volume of 50 μl. A Perkin-Elmer 9600 apparatus (Perkin-Elmer, Norwalk, Conn., USA) was used, and the reactions were run under the following conditions: 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and, finally, 10 min at 72°C for the bla_{TEM} amplification and 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 68°C, and 50 s at 72°C, and, finally, 10 min at 72°C for the bla_{SHV} amplification. The resulting PCR products were run in 1.5% agarose gels. SHV gene amplicons were subjected to the *Nhe*I restriction enzyme digestion suggested by Nuesch-Inderbinen et al. (referred to as the PCR/*Nhe*I test and employing PCR-RFLP) (24). Following PCR, the amplified DNA is digested with restriction enzyme *Nhe*I, which detects a single mutation Gly[238]Ser known to distinguish the majority of the early SHV-derived ESBLs from SHV-1. The ESBL-positive strains were grouped according to the patterns of IEF and antimicrobial susceptibility, and one or two samples from each group were selected to decrease cost. DNA sequencing was performed for the identification of the TEM- and SHV-derived ESBLs for selected samples. TEM and SHV PCR products were purified with a PCR Purification Kit (MBI Fermentas) and were subjected to direct sequencing of both strands performed by the Macrogen Company (Seoul, Korea) using primers used in PCR reactions on a full automatic ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, Calif., USA).

RESULTS

The presence of ESBLs in clinical isolates and transconjugants, and the results of PCR and PCR/*Nhe*I for transconjugants are shown in Table 1. We found ESBLs in 38% of clinical isolates by both the double disk method and NCCLS confirmatory test. Although the phenotypic confirmation tests failed to detect ESBL in two strains of *Enterobacter cloacae* because of the high-level resistance, these strains were shown to produce ESBL in their transconjugants. In the conjugation experiments, 44.6% of the isolates were detected to transmit their β-lactam resistance (amoxicillin, oxymino cephalosporins or aztreonam) to the recipient *E. coli* K-12 strain. The percentage of ESBL transmission was 73%; therefore, there was a difference in the numbers of ESBLs of clinical isolates and transconjugants. The cefoxitin resistance of clinical isolates was not transferred to the transconjugant strains. All the clinical isolates and transconjugants were resistant to amoxicillin (MIC, ≥256 μg/ml) and were susceptible to imipenem (MIC, 4≤ μg/ml). Using PCR, it was found that 52.7% of the transconjugants included TEM, 74.3% included SHV, and 32.4% included both the TEM and SHV genes. PCR products of the expected size of about 1074 and 1016 bp, covering the entire coding sequence of the bla_{TEM} and bla_{SHV} gene, respectively, were detected. By *Nhe*I restriction analysis of SHV-PCR products, 45 of the 46 ESBLs detected at transconjugants were determined to be SHV-derived. The G-to-A nucleotide change that gives the glycine-to-serine substitution at position 238 was confirmed by the nucleotide sequencing in selected amplimers. The 18 transconjugant strains (nine including both TEM and SHV, nine only SHV) shown in Table 2 were selected from the groups described in the Materials and Methods. *Citrobacter freundii* was not included in the analysis, because it does not produce an ESBL. The sequencing of SHV genes revealed that five strains contained SHV-2, seven strains contained SHV-5, five strains contained SHV-12, and one strain carried SHV-1, a non-extended-spectrum SHV. In addition, the sequencing of the TEM genes revealed that they encoded the TEM-1 β-lactamase. TEM-1 was found along with SHV-2 in four, with SHV-5 in three, and with SHV-12 in two strains (Table 2). The deduced nucleotide sequences of the SHV genes were compared with the sequence of bla_{SHV-1}, and the amino acid sequences were compared with the amino acid sequence of SHV-1 β-lactamase. The SHV-ESBLs were characterized by point mutations in the SHV-1 precursor (GenBank X98098 SHV-1 strain) according to the Ambler nomenclature (Table 3). According to IEF, the crude sonic extracts containing β-lactamases from the *E. coli* transconjugant strains contained 1 to 4 different bands. As shown

Table 1. The presence of ESBLs in clinical isolates and transconjugants and the results of PCR and PCR/*Nhe*I test for transconjugant strains

Species	Clinical isolates		Transconjugants				
							PCR/*Nhe*I test[2)]
	n[1)]	No. (%) of ESBL-positive	*n*[2)]	No. (%) of TEM-PCR-positive	No. (%) of SHV-PCR-positive	No. of ESBL-positive	No. of SHV-ESBL-positive
Klebsiella pneumoniae	91	52 (57.1)	45	14 (31.1)	42 (93.3)	35	34
Escherichia coli	53	9 (17)	24	21 (87.5)	8 (33.3)	7	7
Enterobacter cloacae	5[3)]	1 (20)	3	2 (66.7)	3 (100)	3	3
Klebsiella oxytoca	5	0 (0)	0	0 (0)	0 (0)	0	0
Serratia marcescens	4	0 (0)	0	0 (0)	0 (0)	0	0
Enterobacter aerogenes	3	1 (33.3)	1	1 (100)	1 (100)	1	1
Morganella morganii	3	0 (0)	0	0 (0)	0 (0)	0	0
Citrobacter freundii	2	0 (0)	1	1 (100)	1 (100)	0	0
TOTAL	166	63 (38)	74	39 (52.7)	55 (74.3)	46	45

[1)]: number of the isolates; [2)]: number of the transconjugants; [3)]: ESBL could not be detected in 2 strains of *E. cloacae* due to high-level resistance.

Table 2. Properties of clinical isolates and transconjugants

Strain		MICs (μg/mL)																ESBL		PCR[1)]		*Nhe*I[1)]	β-lactamase[1)]	
		AMX		AMC		FOX		ATM		CAZ		CRO		CTX		IMP								
No.	Sp.	CI	TR	CI	TR	CI	TR	CI	TR	CI	TR	CI	TR	CI	TR	CI	TR	CI	TR	TEM	SHV	RES	pIs	Types
DE2	kp	>256	>256	16	2	128	4	>128	128	128	16	64	4	128	2	4	1	+	+	–	+	+	8.7, 9.1	SHV-5
DE4	kp	>256	>256	4	4	8	4	128	128	128	128	16	16	8	8	4	2	+	+	–	+	+	7.6, 8.0, 8.7	SHV-5
DE5	kp	>256	>256	4	2	64	2	>128	1	128	2	64	2	64	4	4	1	+	+	–	+	+	7.6	SHV-2
DE7	kp	>256	>256	4	2	4	2	>128	32	>128	16	128	4	128	4	1	0.5	+	+	–	+	+	8.0	SHV-5
DE8	kp	>256	>256	8	2	4	4	64	32	64	32	32	8	32	8	2	1	+	+	–	+	+	8.1	SHV-12
DE35	kp	>256	>256	8	8	16	2	>128	32	>128	16	64	32	32	32	0.5	0.5	+	+	+	+	+	5.4, 5.8, 6.9-7.0, 8.2-8.3	SHV-12 + TEM-1
DE40	kp	>256	>256	4	2	128	2	>128	64	64	16	64	2	32	2	1	1	+	+	–	+	+	8.1, 8.5, 9.1	SHV-12
DE41	kp	>256	>256	4	1	4	2	128	16	64	16	64	2	64	2	2	1	+	+	–	+	+	8.7, 9.1	SHV-12
DE43	kp	>256	>256	8	8	8	8	>128	>128	>128	>128	>128	128	128	64	2	1	+	+	+	+	+	5.4, 8.0, 8.7	SHV-5 + TEM-1
DE9446	kp	>256	>256	32	4	4	4	1	<0.25	16	<0.25	8	<0.25	8	0.25	4	1	+	–	–	+	–	7.6	SHV-1
DE17720	kp	>256	>256	16	8	2	2	>128	>128	>128	>128	>128	>128	>128	>128	1	1	+	+	+	+	+	5.4, 6.6, 7.6	SHV-2 + TEM-1
DE20658	kp	>256	>256	16	8	32	2	32	<0.25	32	<0.25	>128	0.5	>128	0.5	1	1	+	+	+	+	+	5.4, 9.1	SHV-2 + TEM-1
DE21072	kp	>256	>256	8	8	2	2	0.5	<0.25	1	<0.25	4	2	2	2	1	1	+	+	+	+	+	5.4, 7.6, 8.8	SHV-2 + TEM-1
DE33	ecoli	>256	>256	4	4	8	2	128	32	128	16	32	32	32	32	0.5	0.5	+	+	+	+	+	5.4, 5.8, 6.9-7.0, 8.2-8.3	SHV-12 + TEM-1
DE16792	ecoli	>256	>256	4	4	8	4	>128	16	4	2	>128	>128	>128	>128	2	1	+	+	+	+	+	7.6, 8.0, 8.7	SHV-2 + TEM-1
DE21195	ea	>256	>256	32	8	>128	8	128	32	>128	>128	16	8	64	32	2	1	+	+	+	+	+	5.4, 6.6, 7.4, 8.7	SHV-5 + TEM-1
DE10962	ec	>256	>256	64	16	128	1	32	<0.25	128	<0.25	128	<0.25	128	<0.25	4	1	+	+	+	+	+	5.4-5.7, 8.2-8.5	SHV-5 + TEM-1
DE8154	ec	>256	>256	8	4	4	4	>128	4	>128	4	128	<0.25	128	<0.25	2	1	+	+	–	+	+	8.5, 9.1	SHV-5

[1)]: Transconjugants data.
CI, clinical isolates; TR, transconjugant; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; FOX, cefoxitin; ATM, aztreonam; CAZ, ceftazidime; CRO, ceftriaxone; CTX, cefotaxime; IMP, imipenem; kp, *K. pneumoniae*; ecoli, *E. coli*; ec, *E. cloacae*; ea, *E. aerogenes*.

Table 3. The amino acid substitutions in SHV-ESBL derivates compared with SHV-1 at selected positions

Species	Strains	SHV-type β-lactamases	Amino acid (Codon) at positions[1)]		
			35	238	240
		Consensus SHV-1[2)]	Leu (CTA)	Gly (GGC)	Glu (GAA)
K. pneumoniae	DE9446	SHV-1	Leu (CTA)	Gly (GGC)	Glu (GAG)
K. pneumoniae	DE5, DE17720, DE20658, DE21072	SHV-2	Leu (CTA)	Ser (AGC)	Glu (GAG)
E. coli	DE16792	SHV-2	Leu (CTA)	Ser (AGC)	Glu (GAG)
K. pneumoniae	DE2, DE4, DE7, DE43	SHV-5	Leu (CTA)	Ser (AGC)	Lys (AAG)
E. cloacae	DE8154,DE10962	SHV-5	Leu (CTA)	Ser (AGC)	Lys (AAG)
E. aerogenes	DE21195	SHV-5	Leu (CTA)	Ser (AGC)	Lys (AAG)
E. coli	DE33	SHV-12	Gln (CAA)	Ser (AGC)	Lys (AAG)
K. pneumoniae	DE8, DE35, DE40, DE41	SHV-12	Gln (CAA)	Ser (AGC)	Lys (AAG)

[1)]: Numbering is according to Ambler nomenclature. [2)] GebBank X98098 SHV-1 strain.

in Table 2, the SHV-2, SHV-5, and SHV-12-producing strains contained bands suggestin the SHV-2 (pI 7.6), SHV-5 (pI 8.2 ± 0.2), and SHV-12 (pI 8.2 ± 0.1) enzymes, except strains DE2, DE41, DE8154, DE20658, and DE21195. All strains, except strains DE5, DE7, and DE8, also contained additional band(s), indicating production of non SHV-derived β-lactamase(s). The strains DE33, DE35, DE43, DE10962, DE17720, DE20658, DE21072, and DE21195 produced β-lactamases with pIs of approximately 5.4 characterized as TEM-1.

DISCUSSION

The increased prevalence of *Enterobacteriaceae* producing ESBLs creates a great need for laboratory testing methods that accurately identify the presence of these enzymes in clinical isolates (5,7,25). Several studies revealed that ESBLs are widespread in our country; with the percentage of ESBL production being reported as 0 - 27% and 33 - 86% in clinical *E. coli* and *Klebsiella* isolates, respectively (6,15-17,26). In this study, the prevalence of ESBL producers among *E. coli* strains was 17% and that among *K. pneumoniae* strains was 57.1% (Table 1). A number of different testing methods have been suggested since ESBLs were first described (18,19,27, 28). These tests are based on the phenotype and identify only the presence of an ESBL. In the early days of studying ESBLs, determination of pI was usually sufficient to identify the ESBL that was present. More recently, this approach has failed to distinguish among several ESBLs having the same isoelectric point (5). In this study, SHV-2, SHV-5, or SHV-12-producing strains contained bands suggesting several SHV-ESBLs. The strains DE2, DE41, DE20658, and DE21195 did not contain bands suggesting SHV-2, SHV-5, or SHV-12. We thought that SHV-2, SHV-5, or SHV-12 was not expressed at detected level phenotypically in these strains. All strains, except strains DE5, DE7, and DE8, also contained additional band(s), indicating production of non SHV-derived β-lactamase(s) (Table 2). The MICs of the strain DE21072 to ceftazidime (1 μg/mL) and to cefotaxime (2 μg/mL) were low (Table 2). The reason for this could be the low level of ESBL activity. Although most ESBLs confer resistance to one or more of the oxymino-β-lactams, the β-lactamase does not always increase the MICs (7). Therefore, NCCLS has developed ESBL screening tests. According to the NCCLS broth microdilution screening test, when the MIC of one of five ESBL antibiotics is ≥2 μg/mL, it should be considered as suspicious for the presence of an ESBL (18). Recently, molecular methods have been shown to be absolutely necessary to determine the specific ESBL type (9-13). In Turkey, except for a few cases, these enzymes were not characterized at the molecular level (29-36). The ESBLs reported from Turkey are OXA-10-derived, PER-1, SHV-2, SHV-5, CTX-M-2, CTX-M-3, and CTX-M-15. In this study, we examined the molecular types of TEM- and SHV-derived ESBL variants produced by clinical *Enterobacteriaceae* isolates. As far as we know, the typing of TEM- and SHV-derived ESBL using DNA sequence analysis was applied for the first time in Turkey in this study. A number of different molecular tests have been proposed for the detection and identification of TEM and SHV derivatives. The simplest of these is the PCR/*Nhe*I test suggested by Nuesch-Inderbinen et al. (24), which allows for the detection of a single mutation, $Gly^{238}Ser$, known to distinguish the majority of the early SHV-derived ESBLs from SHV-1. This method cannot determine which SHV-derived ESBL is present. In our study, using the PCR/*Nhe*I method, 45 of the 46 ESBLs detected at transconjugants were determined to be SHV-derived. Our study also demonstrated that the PCR/*Nhe*I test allows rapid and sensitive detection of the single mutation $Gly^{238}Ser$. We identified the strains carrying $bla_{SHV\text{-}ESBL}$ genes by PCR/*Nhe*I test and confirmed the results by nucleotide sequencing. The specific SHV-ESBL variants were detected by DNA sequencing.

SHV-2, SHV-5 and SHV-12 ESBLs had been reported from several countries in the world (37-43). SHV-2 has been widely found in Turkey. SHV-5 produced by *K. pneumoniae* isolates from Turkey was first reported by Paterson et al. (33). In this study, SHV-5 was found in clinical *K. pneumoniae*, *Enterobacter aerogenes*, and *E. cloacae* strains, and SHV-12 was found in clinical *K. pneumoniae* and *E. coli* isolates. There are no other published reports of the discovery of SHV-12 in Turkey.

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