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Original Contribution

IDENTIFICATION OF SHV-2, TEM-3, TEM-15, CTX-M-3, CTX-M-15 AND PER-1 EXTENDED-SPECTRUM BETA-LACTAMASES IN CHROMOSOMAL AMPC-PRODUCING ENTEROBACTERIACEAE ISOLATES FROM CANCER PATIENTS IN BULGARIA

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ABSTRACT

PURPUSE: To identify and characterize extended-spectrum beta-lactamases (ESBLs) present in the chromosomal AmpC-producing enterobacterial isolates. METHODS: Screening for genes encoding ESBLs (bla_{PER-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB-1} and bla_{GES-1}) was carried out by PCR amplification with specific primers in 37 non-duplicate, clinically relevant ESBL-producing isolates including 19 *Citrobacter freundii*, 9 *Enterobacter cloacae*, 5 *Serratia marcescens*, 2 *Enterobacter aerogenes*, 1 *Morganella morganii* and 1 *Providencia rettgeri.* For isolates with PCR-positive results, sequencing was performed. Susceptibility to antimicrobials was determined by standard disk diffusion or Etest procedures. RESULTS: The 37 chromosomal AmpC-producing *Enterobacteriaceae* were found to coproduce the following ESBLs: 14 (37.8%) CTX-M-3, nine (24.3%) TEM-3, eight (21.6%) SHV-2, four (10.8%) CTX-M-15, one (2.7%) TEM-15 and one (2.7%) PER-1. Sixteen isolates (43.2%) also carried bla_{TEM-1}, and one of them carried bla_{SHV-1} as well. In vitro, all isolates were susceptible to imipenem. Susceptibility to other drugs was as follows: 78 % for ciprofloxacin, 43% for amikacin and 32 % for gentamicin. Associated resistance to amikacin and ciprofloxacin was observed most frequently among CTX-M-positive isolates. CONCLUSIONS: The most prevalent ESBLs were CTX-M enzymes (CTX-M-3 and CTX-M-15) followed by TEM-3 and SHV-2. This is the first report of TEM-15 and PER-1-producing *Enterobacteriaceae* in Bulgaria.

Key words: *Citrobacter freundii*, *Enterobacter*, *Serratia marcescens*, *Morganella morganii*, *Providencia rettgeri*, beta-lactamase

INTRODUCTION

The resistance of *Enterobacter* spp., *Citrobacter freundii*, *Serratia* spp., *Morganella morganii* and *Providencia* spp. to beta-lactam antibiotics is most frequently mediated by hyperproduction of chromosomal AmpC beta-lactamase, caused either by induction or, more likely, by selection of derepressed mutants (1). In the last decade, the production of plasmid-mediated extended-spectrum beta-lactamases (ESBLs) has been recognized as an additional important emerging mechanism of resistance among members of the family *Enterobacteriaceae*, including chromosomal AmpC-producing species (2). Although less common than AmpC hyperproduction, ESBLs among these species are a problem of great concern due to the potential transmission of resistance to other bacterial species and because ESBLs are usually encoded by plasmids that also harbour genes for resistance to non-beta-lactam antibiotics such as aminoglycosides (3, 4).

The aim of this study was to identify and characterize ESBLs present in the chromosomal AmpC-producing enterobacterial isolates from a Bulgarian hospital.

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MATERIALS AND METHODS

Thirty-seven non-duplicate, clinically relevant ESBL-producing enterobacterial isolates obtained at a 242-bed oncology hospital in Sofia were studied. These included 19 *Citrobacter freundii*, 9 *Enterobacter cloacae*, 5 *Serratia marcescens*, 2 *Enterobacter aerogenes*, and 1 each of *Morganella morganii* and *Providencia rettgeri*. The isolates were collected between September 1996 and December 2005. Species identification was done by standard biochemical tests and confirmed with an automated identification system (Vitek AMS; bioMerieux Vitek Systems Inc., Hazelwood, MO).

Susceptibility to antimicrobials was determined by disc diffusion, following the Clinical and Laboratory Standards Institute (CLSI) recommendations (5). Suggestive evidence of ESBL production was defined as synergy between amoxicillin/clavulanate and at least one of the following antibiotics: cefotaxime, ceftazidime, aztreonam or cefepime. MICs of the beta-lactam antibiotics cefotaxime, ceftazidime, cefepime, and imipenem were determined by Etest (AB Biodisk, Solna, Sweden). Susceptibility interpretations were defined according to CLSI-2007 breakpoints (6). *E. coli* ATCC 25922 was used as an antibiotic-susceptible control.

PCR screening for genes encoding ESBLs was performed on boiled cell lysate of clinical isolates with the Expand High Fidelity PCR system (Roche Diagnostics, Penzberg, Germany). The $bla_{\text{PER-1}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{VEB-1}}$ and $bla_{\text{GES-1}}$ genes were amplified using specific primers and reaction conditions, as previously described (7, 8). Amplification of DNA was performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). Amplicons were purified with a QIAquick PCR purification kit (QIAGEN, K.K., Tokyo, Japan) and sequenced with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequence analyses and comparison with known sequences were performed with the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Using PCR and sequence analysis, ESBL-encoding genes were identified in all 37 isolates expressing ESBL-producing phenotype. ESBLs belonged to CTX-M (48.6%, 18/37), TEM (27%, 10/37), SHV (21.6%, 8/37) and PER (2.7%, 1/37) families and corresponded to 6 distinct types: CTX-M-3 (37.8%, 14/37), CTX-M-15 (10.8%, 4/37), TEM-3 (24.3%, 9/37), TEM-15 (2.7%, 1/37), SHV-2 (21.6%, 8/37) and PER-1(2.7%, 1/37). As shown on **Table 1**, TEM-type ESBLs were particularly present in *E. aerogenes* (TEM-15) and *C. freundii* (TEM-3) isolates recovered between 1996 and 2002. The only SHV-type ESBL identified during this work (SHV-2) was distributed in *C. freundii* and *E. cloacae* isolates obtained from 1997 through 2005. In contrast, CTX-M-type ESBLs were widely disseminated among *E. aerogenes* (CTX-M-15), *C. freundii* (CTX-M-3 and CTX-M-15), *S. marcescens* (CTX-M-3), *E. cloacae* (CTX-M-3) and *M. morganii* (CTX-M-15) isolates recovered from 2002 to 2005. Finally, PER-1 ESBL was found only in *P. rettgeri* strain isolated in 1997, which also had TEM-1 penicillinase. Of interest is that CTX-M-type genes were frequently associated (72%, 13/18) with TEM-1 determinant, whereas the association between SHV-2 and TEM-1 determinants was rarely observed (25%, 2/8).

AmpC-producing *Enterobacteriaceae* isolates carrying TEM and CTX-M-type ESBLs were mostly non-susceptible (intermediated or resistant) to tobramycin (100% versus 94.4%, respectively), kanamycin (100% versus 77.8%), gentamicin (100% versus 72.2%), sulfonamides (100% versus 77.8%), netilmicin (100% versus 66.7%) and amikacin (90% versus 61.1%). In addition, TEM-positive isolates showed non-susceptibility patterns to chloramphenicol (100%), whereas CTX-M-positive isolates showed non-susceptibility patterns to trimethoprim (83.3%) and tetracycline (77.8%). Non-susceptibility to ciprofloxacin was lower than that observed for other antimicrobials among both TEM and CTX-M-positive isolates (20% versus 33.3%, respectively). Regarding SHV-positive isolates, resistance rates to non-beta-lactams were significantly lower than that observed among TEM and CTX-M-positive isolates, and varied from 0% for ciprofloxacin to 37.5% for sulfonamides. In contrast, PER-1-producing *P. rettgeri* strain was coresistant to all non-beta-lactams tested. Among all ESBL-producers 78.4% were resistant to tobramycin, 70.3% to sulfonamides, 70.3% to kanamycin, 67.6% to gentamicin, 64.9% to netilmicin, 56.7% to amikacin, 48.6% t

Table 1. *Characteristics of ESBL-producing Enterobacter spp., Citrobacter freundii, Serratia marcescens, Morganella morganii and Providencia rettgeri clinical isolates*

Species (no. of isolates)	Year of isolation	Genotypic evaluation				MICs of -lactams (mg/L)[a]				Other resistances[b]
		CTX-M	SHV	TEM	PER	CTX	CAZ	FEP	IPM	
E. aerogenes (1)	1966			TEM-15		4	8	1	0.5	Cm Gm Km Nt Su Tc Tm
C. freundii (2)	1997		SHV-2			8	16	1	0.25	Su
P. rettgeri (1)	1997			TEM-1	PER-1	8	64	8	2	Ak Cm Cp Gm Km Nt Nx Su Tc Tm Tp
E. cloacae (1)	1997		SHV-2			4	2	0.5	0.25	-
E. cloacae (1)	1998		SHV-2	TEM-1		16	16	2	0.5	Cm Gm Km Nt Su Tc Tm
C. freundii (1)	1998		SHV-2			8	8	1	0.25	-
C. freundii (4)	1998-1999			TEM-3		16-32	32-64	1-2	0.25	Ak Cm Gm Km Nt Su Tm
C. freundii (2)	2002			TEM-3		>256	>256	48-64	1	Ak Cm Cp Gm Km Nt Nx Su Tc Tm Tp
C. freundii (3)	2002			TEM-3		48-64	32-64	3-4	0.25	Ak Cm Gm Km Nt Nx Su Tm
E. aerogenes (1)	2002	CTX-M-15				64	12	8	0.19	Gm Km Tc Tm
C. freundii (3)	2003-2004	CTX-M-3		TEM-1		16-32	2-4	3	0.25	Ak Cp Gm Km Nt Nx Su Tc Tm Tp
S. marcescens (2)	2004	CTX-M-3		TEM-1		>256	12	64	0.5	Ak Gm Km Nt Su Tc Tm Tp
S. marcescens (2)	2004-2005	CTX-M-3				128	4	24	0.5	Nx Tc Tm Tp
E. cloacae (1)	2005	CTX-M-3		TEM-1		24	2	3	0.5	Ak Gm Km Nt Su Tm Tp
C. freundii (1)	2005	CTX-M-15		TEM-1		>256	64	12	0.5	Cm Cp Gm Km Nt Nx Su Tc Tm Tp
C. freundii (1)	2005	CTX-M-3		TEM-1		24	12	3	0.38	Ak Gm Km Nt Nx Su Tc Tm Tp
E. cloacae (2)	2005		SHV-2			16	2	0.5	0.25	-
E. cloacae (1)	2005		SHV-2	TEM-1		16	16	2	0.5	Ak Gm Km Nt Tm
C. freundii (1)	2005	CTX-M-3		TEM-1		24	2	3	0.38	Ak Gm Km Nt Nx Su Tm Tp
E. cloacae (2)	2005	CTX-M-3		TEM-1		64	6	16	0.5	Ak Gm Km Nt Nx Su Tc Tm Tp
E. cloacae (1)	2005	CTX-M-3		TEM-1		32	4	16	0.5	Gm Nx Su Tm Tp
C. freundii (1)	2005	CTX-M-15				>256	32	16	0.5	Gm Km Nx Tc Tm
M. morganii (1)	2005	CTX-M-15				64	2	4	1	Ak Cm Cp Gm Km Nt Nx Su Tc Tm Tp
S. marcescens (1)	2005	CTX-M-3	SHV-1	TEM-1		>256	4	64	0.5	Ak Cm Cp Gm Km Nt Nx Su Tc Tm Tp

[a] *CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem.*

[b] *Resistance abbreviations: Ak, amikacin; Cm, chloramphenicol; Cp, ciprofloxacin; Gm, gentamicin; Km, kanamycin; Nt, netilmicin; Nx, nalidixic acid; Su, sulfonamides; Tc, tetracycline; Tm, tobramycin, Tp, trimethoprim*

tetracycline, 45.9% to trimethoprim, 37.8 % to chloramphenicol and 21.6% to ciprofloxacin. Associated resistance to amikacin and ciprofloxacin was observed 27.7% of CTX-M-producers, in 20% of TEM-producers and in PER-1-producing *P. rettgeri* strain. All ESBL-producing isolates were susceptible to imipenem with MICs ranging from 0.19 to 1 mg/L (**Table 1**).

DISCUSSION

ESBLs among the isolates of *Enterobacter* spp., *C. freundii* and *S. marcescens* have been described from several countries worldwide and have become increasingly more prevalent (3, 9-13). Initially, the ESBLs among these species were typical TEM or SHV enzymes (1, 3, 14), but enzymes of the CTX-M class have been described more recently (9-11, 13). In Bulgaria, TEM-3, SHV-2, CTX-3 and CTX-M-15 ESBLs have been reported among isolates of the family *Enterobacteriaceae* (15, 16). In this study, CTX-M enzymes such as CTX-M-3 and CTX-M-15 were commonly identified among the isolates of *Enterobacter* spp. *C. freundii*, *S. marcescens* and *M. morganii*, suggesting dissemination of the enzymes among these species. Other enzymes such as TEM-15 and PER-1 found in *E. aerogenes* and *P. rettgeri*, respectively, were first identified in Bulgaria.

Consistent with the notion that ESBL determinants are often associated with other resistance traits (4), TEM-, CTX-M- and PER-type determinants were associated in over 60% of cases with both aminoglycoside and sulfonamide resistance. Since ESBL producers express their beta-lactamase genes from plasmids, these findings suggest that genes coding for ESBLs and genes coding for resistance to these antibiotics may reside within the same plasmids and therefore be spread together. This means that resistance to different kinds of drugs may be co-selected by the use of either one and each of these antimicrobials could be a selective pressure for spreading of such isolates.

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