HVM BIOFLUX
Human & Veterinary Medicine
International Journal of the Bioflux Society

The genetic characteristics of extended-spectrum β-lactamases (ESBLs) among *Escherichia coli* and *Klebsiella pneumoniae* isolates from urine specimens

[1]Mirela Petraşcu, [2]Mirela Flonta, and [2]Ariana M. Almaş

[1]"Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca, Cluj, România;
[2]Microbiology Laboratory, Infectious Diseases Clinical Hospital, Cluj-Napoca, Cluj, România;
Corresponding author: M. Petraşcu, mpetrascu@hotmail.com

Abstract. Background & objectives: Extended spectrum β-lactamases (ESBLs) have emerged as a major threat worldwide with limited treatment options. The genotypes of ESBL producing strains largely remain unknown in Romania; hence the present study was aimed to determine the genetic characteristics of ESBLs in *Escherichia coli* and *Klebsiella pneumoniae* isolates from urine specimens. Material and methods: Total 152 consecutive non-duplicate clinical isolates of *E. coli* (n=70) and *K. pneumoniae* (n=82) collected between January 2007 and April 2010 were isolated from urine samples in the Laboratory of Infectious Diseases Hospital Cluj-Napoca. The isolates were examined phenotypically for ESBL production. ESBL strains were further typed for the *bla* $_{TEM/SHV/CTX-M/OXA}$ genes by PCR using specific primers. Results: The TEM gene was detected in 77.63% (118 of 152) of the isolates followed by CTX-M (110 of 152 [73.36%]), OXA (101 of 152 [66.44%]), and SHV (93 of 152 [61.18%]). From 70 *E. coli* isolates, 50 (71.42%) were positive for CTX-M followed by OXA type (47; 67.14%), TEM (40; 57.14%) and SHV (23; 32.86%). From 82 *K. pneumoniae* isolates, 78 (95.12%) were positive for TEM followed by SHV type (71; 86.58%), CTX-M (60; 73.17%) and OXA (53; 64.63%). Conclusions: The results of this study provide insights into the genetic characteristics of ESBLs among *E. coli* and *K. pneumoniae* isolates from urine specimens. The ESBL-positive *E. coli* isolates investigated here encoded mainly CTX-Ms (71.42%), followed by OXA type (67.14%), TEM-type (57.14%), and SHV-type (32.86%) β-lactamases. The ESBL-positive *K. pneumoniae* isolates investigated here encoded mainly TEM type (95.12%) followed by SHV type (86.58%), CTX-M (73.17%) and OXA (64.63%).
Keywords: *Escherichia coli, Klebsiella pneumoniae*, ESBL, CTX-M.

Rezumat. Obiectiv: Transmiterea β-lactamazelor cu spectru extins (BLSE) între speciile bacteriene şi răspândirea genelor ce determină rezistenţa la acţiunea antibioticelor constituie la ora actuală o mare problemă din cauza opţiunilor limitate în tratamentul infecţiilor cu enterobacterii. Genotipul BLSE in România a rămas in mare masură necunoscut; prin acest studiu ne-am propus sa determinăm caracteristicile genetice ale BLSE la tulpini de *Escherichia coli* si *Klebsiella pneumoniae* izolate din eşantioane de urină. Material si metodă: 152 de tulpini de *E. coli* (n=70) si *K. pneumoniae* (n=82) colectate între ianuarie 2007 si aprilie 2010 au fost izolate din eşantioane de urină în laboratorul de microbiologie al Spitalului de Boli Infecţioase, Cluj-Napoca. Tulpinile au fost analizate fenotipic pentru identificarea prezenţei BLSE şi au fost analizate prin amplificare PCR pentru detectarea si identificarea genelor BLSE. Rezultate: Gena TEM a fost identificată la 77,63% (118 tulpini din 152), fiind urmată de CTX-M (110 tulpini din 152 [73,36%]), OXA (101 tulpini din 152 [66,44%]), şi SHV (93 tulpini din 152 [61,18%]). Dintre cele 70 tulpini de *E. coli,* 50 (71,42%) au fost pozitive pentru CTX-M fiind urmate de OXA (47; 67,14%), TEM (40; 57,14%) şi SHV (23; 32,86%). Dintre cele 82 tulpini de *K. pneumoniae*, 78 (95,12%) au fost pozitive pentru TEM fiind urmate de SHV (71; 86,58%), CTX-M (60; 73,17%) şi OXA (53; 64,63%). Concluzii : Acest studiu a permis identificarea genelor BLSE pentru tulpini de *E. Coli* si *K. pneumoniae* izolate din infecţii urinare. Pentru tulpinile de *E. coli* investigate genetic rezultatele sunt: CTX-M (71,42%), urmate de OXA (67,14%), TEM (57,14%), şi SHV (32,86%) β-lactamaze. Pentru tulpinile de *K. pneumoniae* investigate genetic rezultatele sunt : TEM (95,12%) urmate de SHV (86,58%), CTX-M (73,17%) şi OXA (64,63%).
Cuvinte cheie: *Escherichia coli, Klebsiella pneumoniae*, ESBL, CTX-M.

Introduction and History. Emergence of resistance to β-lactam antibiotics began even before the first β-lactam, penicillin, was developed. The first β-lactamase was identified in *Escherichia coli* prior to the release of penicillin for use in medical practice (Abraham & Chain 1940). In 1983, the first outbreak involving extended spectrum beta-lactamase (ESBL) producing organisms was reported in Germany (Paterson & Bonomo 2005).

These ESBL-producing pathogens are now recognized globally as major causes of nosocomial and community-acquired infections (Knothe et al 1983). ESBLs arise because of mutations in the TEM-1, TEM-2, or SHV-1 genes, commonly found in the *Enterobacteriaceae* family (Bradford 2001). While these enzymes are found predominantly in *Klebsiella* species and *Escherichia coli*, they have also been described in other genera of *Enterobacteriaceae* including *Citrobacter*, *Serratia*, *Proteus*, *Salmonella*, and *Enterobacter* (Bradford 2001; Paterson & Bonomo 2005). They are capable of hydrolyzing penicillins, broad-spectrum cephalosporins and monobactams, but they do not affect the cephamycins or carbapenems and their activity is inhibited by clavulanic acid (Paterson & Bonomo 2005). Most ESBLs have evolved by genetic mutation from native β-lactamases, particularly TEM-1, TEM-2, and SHV-1. These parent enzymes are commonly found in gram-negative bacteria, particularly in *Enterobacteriaceae* (Bradford 2001).

Until the 2000s, most of the ESBLs were structurally related to the narrow-spectrum TEM- and SHV-type β-lactamases, with one to several amino acid substitutions surrounding their active site (Bradford 2001). The genetic mutations that give rise to ESBLs, broaden the parental resistance pattern to a phenotype that includes resistance to broad-spectrum cephalosporins (e.g., cefotaxime [CTX] and ceftazidime [CAZ]) and monobactams (e.g., aztreonam) as it was shown by Jacoby & Carreras (1990). Furthermore, in the late 1990s, a novel type of ESBLs, the CTX-M enzymes, emerged worldwide, mostly from *Escherichia coli* (Bradford 2001).

The OXA-type enzymes are another growing family of ESBLs and are unique among the ESBLs because they are most often found in *Pseudomonas aeruginosa* rather than in members of the *Enterobacteriaceae* (Bradford 2001; Pfaller & Segreti 2006). Because ESBL-producing strains are resistant to a wide variety of clinically used antimicrobials, their proliferation poses a serious global health concern that has complicated treatment strategies for a growing number of hospitalized patients (Pfaller & Segreti 2006). Because ESBL-producing strains often arise in focal outbreaks, their prevalence can vary greatly from one site to another and even over time for a given site (Pfaller & Segreti 2006). As a result, regional and local surveillance are crucial for clinical decision making and infection control. The prevalence of bacterial isolates expressing the ESBL phenotype varies across different geographical regions with low rates of 3-8% reported in Sweden, Japan and Singapore compared to much higher prevalence rates documented in studies from Portugal (34%), Italy (37%), New York (44%), Latin American countries (30-60%) and Turkey (58%) (Paterson & Bonomo 2005). The present study was carried out to explore the occurrence of ESBLs in *E. coli* and *K. pneumoniae* strains isolated from urine specimens by phenotypic methods. Commonly prevalent molecular genotypes of ESBL (*bla* $_{TEM/SHV/CTX-M/OXA}$) were detected by PCR.

Material and Methods. Bacterial isolates, specimens: a total of 152 consecutive non-duplicate isolates of *E. coli* (n=70) and *K. pneumoniae* (n=82) from urine specimens were studied prospectively for ESBL production between January 2007 and April 2010 in the Microbiology Laboratory of Infectious Diseases Clinical Hospital in Cluj-Napoca.

Isolates were identified as followed: *E. coli* isolates were identified on chromagar media (Oxoid) and K. pneumoniae on Vitek 2 Compact system (BioMerieux). Phenotipic methods for detection of ESBL: all 152 *E. coli* and *K. pneumoniae* isolates were screened for ESBL detection using the double disc diffusion method based on the Clinical Standard Laboratory Institute (CLSI) criteria.

The CLSI recommends that screening and confirmatory tests should be routinely undertaken for *K. pneumoniae, K. oxytoca, E. coli* and *Proteus mirabilis*. The screening tests are based on a reduction in susceptibility zone diameter for one or more of five antimicrobial agents (cefpodoxime, aztreonam, ceftriaxone, cefotaxime and ceftazidime). Confirmatory tests are based on the increased susceptibility to antimicrobial agents tested in combination with clavulanic acid *versus* susceptibility when tested alone. The test inoculum (0.5 McFarland turbidity) is spread onto Mueller-Hinton agar using a sterile cotton swab. A disc of Augmentin (20 µg amoxycillin + 10 µg clavulanic acid) is placed on the surface of Mueller-Hinton agar; then discs of ceftazidim-CAZ (30 µg), cefotaxim-CTX (30 µg) and alternative cefpodoxime (30 µg) are placed around/on the opposite sides of Augmentin disc. The plate is incubated at 37°C for 16-24 hours. Distances between the discs are required to be suitably adjusted for each strain in order to accurately detect the synergy. The organisms are considered to be producing ESBL when the zone of inhibition around any third-generation cephalosporins discs showed a clear-cut increase towards the Augmentin disc. Synergy can be discreet, atypical or absent, leading to wrong interpretation (false susceptibility). In the absence of synergy producing ESBLs will suspected in case of any reduction in the diameter of inhibition around a third-generation cephalosporins; CTX (cefotaxime) 27mm, CAZ (ceftazidime) 22 mm, CRO (cefuroxime) 25 mm or monobactam: Aztreonam 27 mm. The antibiotics discs were provided by Bio-rad. PCR amplification for detection of β-lactamase genes: all isolates were screened for *bla* $_{TEM/SHV/CTX-M/OXA}$ by a multiplex PCR assay using universal primers (Table 1).

All analyses by PCR amplification for detection of β-lactamase genes were typed in the Bacteriology Clinic Laboratory of Sahlgrenska University Hospital, Gothenburg, Sweden.

Table1

Primers used for detection of different β-lactamase genes in the multiplex PCR

Amplicon	*Primer sequence (5′ to 3′)*	*Size*	*References*
*bla*SHV	CTT TAT CGG CCC TCA CTC AA	237	Fang et al 2004
	AGG TGC TCA TCA TGG GAA AG		
*bla*TEM	CGC CGC ATA CAC TAT TCT CAG AAT GA	445	Monstein et al 2007
	ACG CTC ACC GGC TCC AGA TTT AT		
*bla*CTX-M	ATG TGC AGY ACC AGT AAR GTK ATG GC	593	Boyd et al 2004
	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		
*bla*OXA	ACA CAA TAC ATA TCA ACT TCG C	813	Ouellette et al 1987
	AGT GTG TTT AGA ATG GTG ATC		

Bacterial DNA extraction was performed in a MagNA Pure LC System (Roche DiagnosticsGmbH, Mannheim, Germany) by using a MagNA Pure LC DNA isolation kit I. PCR amplification reactions were performed in a volume of 25 µl containing 12.5 µl of 2x PCR Master Mix (Roblokon, Germany), 0.2 µM concentrations of each primer, and 2 µl of DNA template. We used a Termocycler AmpGene PCR System 2100 from Applied Biosystems. The cycling parameters were as follows: an initial denaturation at 95°C for 5 min; followed by 35 cycles of 94°C for 1 min., 68°C for 1 min, and 72°C, 1 min; and with a final extension at 72°C for 10 min. The amplified PCR products were subjected to electrophoresis at a 2 %

agarose gel in 1x TAE buffer. Strains with known β-lactamase types were included as references.

Results and Discussion. Detection and spread of β-lactamase genes: the dissemination of the isolates each year is illustrated in Fig. 1.

Among the 152 ESBL isolates recovered from January 2007 to April 2010, 65 (42.76%) were isolated during 2009 (see Fig. 1).

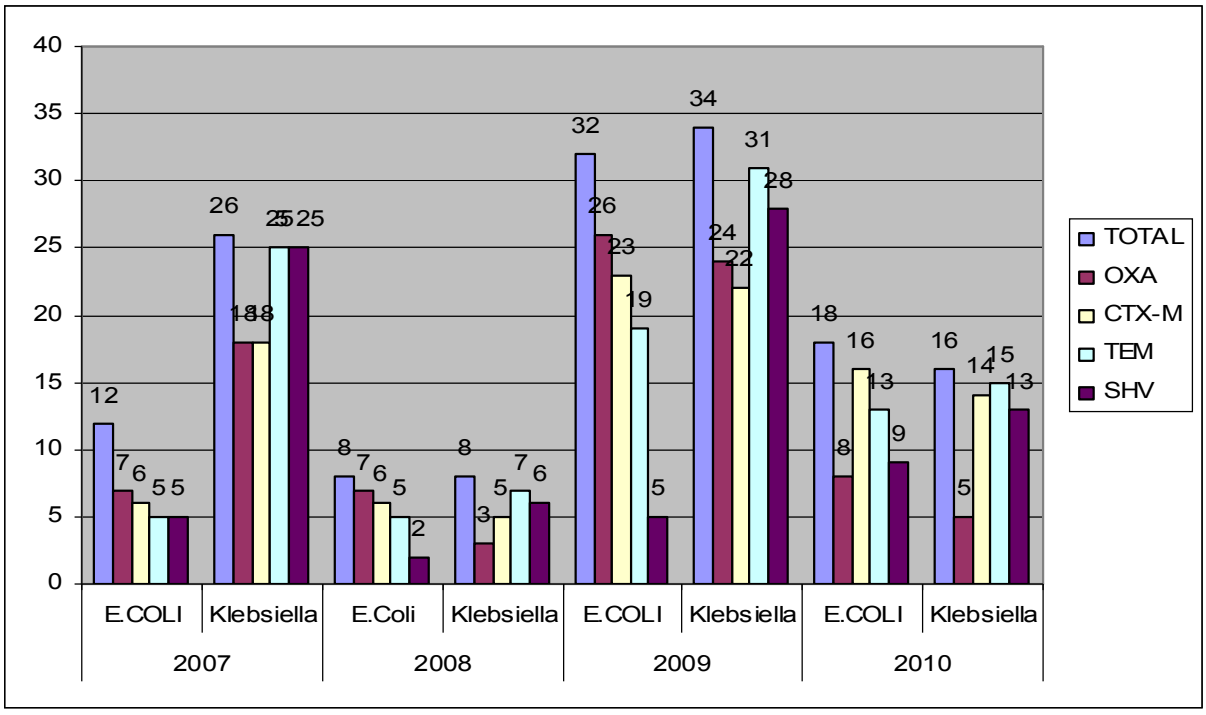

Figure 1. Distribution of different β-lactamase genes among ESBL-positive *E. coli* and *K. pneumoniae* isolates.

Lane 1 2 3 4 5 6

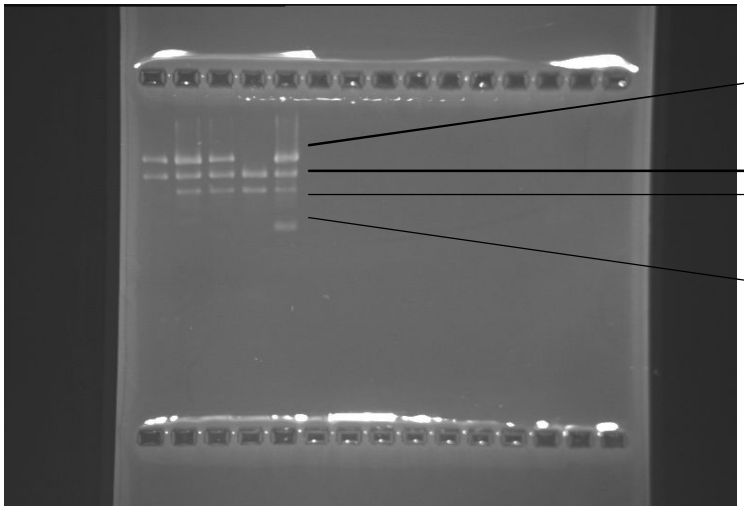

Fig. 2. Multiplex PCR assay for *bla* $_{OXA}$, *bla* $_{CTX-M}$, *bla* $_{TEM}$, *bla* $_{SHV}$. Lane 5, positive control, lane 6, negative control; lanes 1 to 4, clinical isolates of ESBL-positive *E.coli* and *K. pneumoniae.*

The multiplex PCR was discriminatory to genes encoding SHV, TEM, CTX-M, and OXA enzymes (Fig. 2). The TEM gene was detected in 77.63% (118 of 152) of the isolates followed by CTX-M (110 of 152 [73.36%]), OXA (101 of 152 [66.44%]), and SHV (93 of 152 [61.18%]) (Table 2).

Table 2

β-Lactamase genes profile

Year	*Bacterial isolate*	*No. of isolates*	*TEM*	*CTX-M*	*OXA*	*SHV*
2007	*E.coli*	12	5	8	7	5
	K. pneumoniae	25	25	18	18	25
2008	*E.coli*	8	5	6	7	2
	K. pneumoniae	8	7	5	3	6
2009	*E. coli*	32	17	20	25	7
	K. pneumoniae	33	31	23	27	27
2010	*E.coli*	18	13	16	8	9
	K. pneumoniae	16	15	14	5	13
Total		152	118	110	101	93

From 70 *E. coli* isolates, 50 (71.42%) were positive for CTX-M followed by OXA type (47; 67.14%), TEM (40; 57.14%) and SHV (23; 32.86%) (Fig. 3).

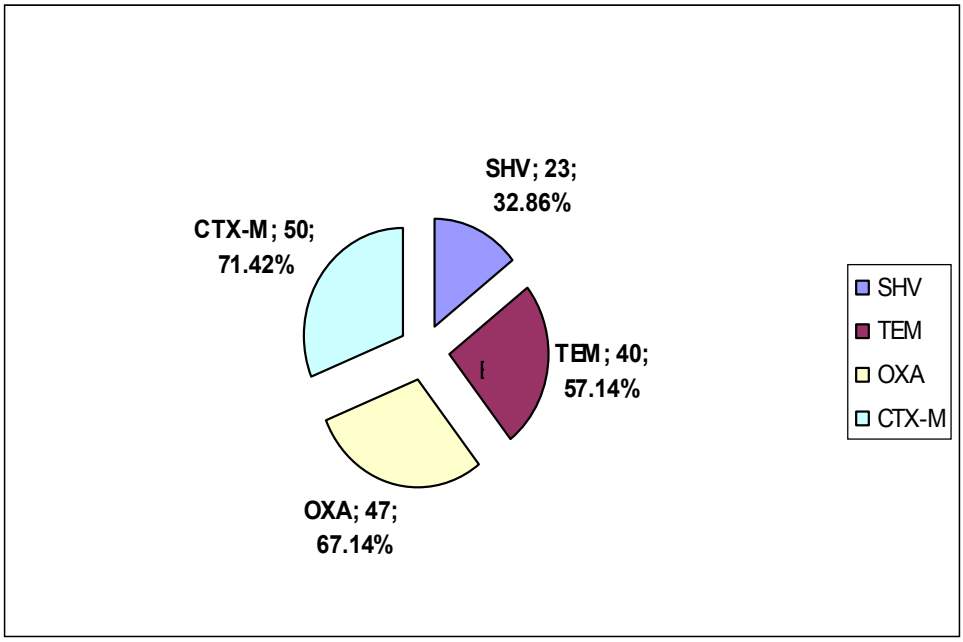

Figure 3. β-Lactamase genes profile for *E. coli* isolates.

The *bla* $_{CTX-M}$ was the most common and was present either alone or in combination with other ESBL-type(s).

The CTX-M type enzymes family of plasmid-mediated ESBLs preferentially hydrolyzes cefotaxime. They have mainly been found in strains of *Salmonella enterica* serovar Typhimurium and *E. coli*, but have also been described in other species of *Enterobacteriaceae* (Bradford 2001). Strains expressing CTX-M-type β-lactamases have been isolated from many parts of the world, but have most often been associated with focal outbreaks in Eastern Europe, South America, and Japan (Bradford 2001). There have been a

few reports of these enzymes in isolates from patients in Western Europe, mostly in isolates from immigrants from the outbreak areas (Tzouvelekis et al 1986). However, Sabete et al (2000) reported that 23 strains of *E. coli* and *Salmonella* isolated in Spain expressed the CTXM-9 β-lactamase, suggesting that there may be an endemic focus of this enzyme in western Europe as well. Several institutions in the areas where outbreaks have occurred reported that the CTX-M type enzyme is the most frequently isolated ESBL among clinical isolates in their laboratories (Sabete et al 2000).

E. coli isolates were positive for *bla* $_{OXA}$ in 67.14% cases (Fig. 3). The OXA-type enzymes are another growing family of ESBLs. The OXA-type β-lactamases confer resistance to ampicillin and cephalothin and are characterized by their high hydrolytic activity against oxacillin and cloxacillin and the fact that they are poorly inhibited by clavulanic acid (Bush et al 1995). While most ESBLs have been found in *E. coli, K. pneumoniae*, and other *Enterobacteriaceae*, the OXA-type ESBLs have been found mainly in *P. aeruginosa* (Bradford 2001). The OXA-type ESBLs provide weak resistance to oxyiminocephalosporins when cloned into *E. coli*, but provide fairly high-level resistance in *P. aeruginosa* transconjugants (Hall et al 1993).

E. coli isolates were positive for *bla* $_{TEM}$ in 57.14% cases (Fig. 3). Most ESBLs are derivatives of TEM or SHV enzymes as it was shown by Bush et al (1995) and Jacoby & Han 1996. There are now more than 90 TEM-type β-lactamases and more than 25 SHV-type enzymes. TEM- and SHV-type ESBLs are most often found in *E. coli* and *K. pneumoniae*; however, they have also been found in *Proteus* spp., *Providencia* spp., and other genera of *Enterobacteriaceae* (Bradford 2001).

From 82 *K. pneumoniae* isolates, 78 (95.12%) were positive for TEM followed by SHV type (71; 86.58%), CTX-M (60; 73.17%) and OXA (53; 64.63%) (Fig. 4).

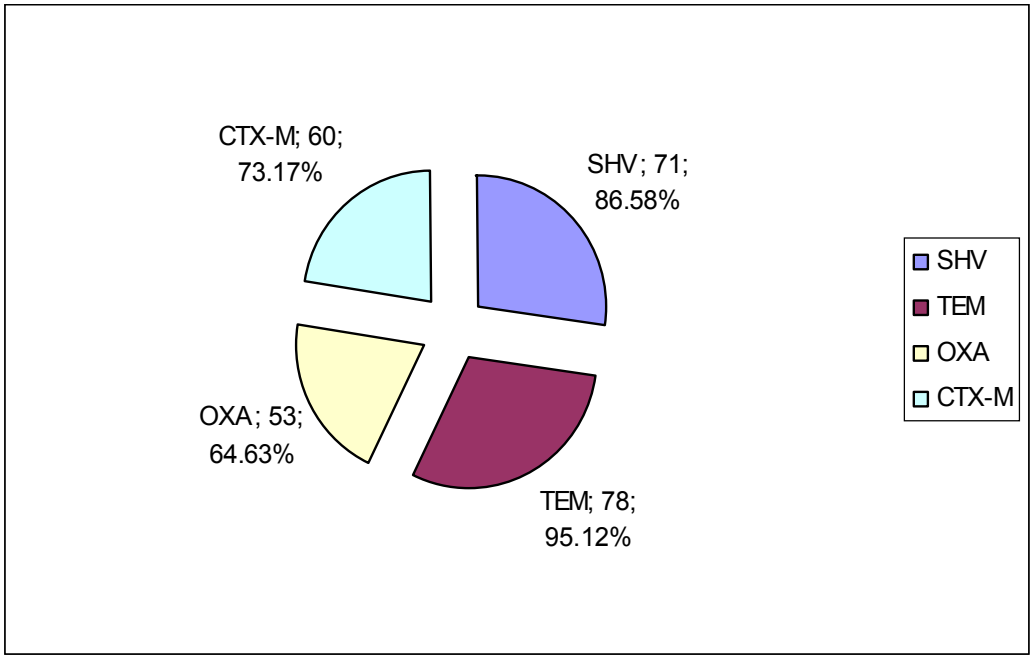

Figure 4. β-Lactamase genes profile for *K. pneumoniae* isolates

The *bla* $_{TEM}$ and the *bla* $_{SHV}$ were the most common for *K. pneumoniae* isolates (95.12% and 86.58%). The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs (Jacoby 2003). SHV refers to *s*ulfhydryl *v*ariable (Paterson & Bonomo

2005). In 1983, a *Klebsiella ozaenae* isolate from Germany possessed a β-lactamase which efficiently hydrolyzed cefotaxime, and to a lesser extent ceftazidime (Knothe et al 1983).

SHV-type ESBLs have been detected in a wide range of *Enterobacteriaceae.* Outbreaks of SHV-producing *Pseudomonas aeruginosa* and *Acinetobacter spp*. have now been reported (Knothe et al 1983; Poirel et al 2004). Although TEM-type β-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found in other species of gram-negative bacteria with increasing frequency (Paterson & Bonomo 2005). TEM-1 is the most commonly encountered β-lactamase in gram-negative bacteria. Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 (Bradford 2000).

TEM-type ESBLs have been reported in genera of *Enterobacteriaceae* such as *Enterobacter aerogenes, Morganella morganii, Proteus mirabilis, Proteus rettgeri*, and *Salmonella* spp. (Paterson & Bonomo 2005). Furthermore, TEM-type ESBLs have been found in non-*Enterobacteriaceae* gram-negative bacteria. The TEM-42 β-lactamase was found in a strain of *P.aeruginosa* (Paterson & Bonomo 2005).

Conclusions. The results of this study provide insights into the genetic characteristics of ESBLs among *E. coli* and *K. pneumoniae* isolates from urine specimens.

Extended-spectrum β-lactamases confer resistance to third- and fourth-generation cephalosporins and monobactams, in addition to the earlier generation cephalosporins.

ESBLs are capable of hydrolyzing penicillins, broad-spectrum cephalosporins and monobactams, but they do not affect the cephamycins or carbapenems and their activity is inhibited by clavulanic acid. ESBLs are most common in *Klebsiella pneumoniae* and *Escherichia coli,* but do occur in other Enterobacteriaceae and in *Pseudomonas aeruginosa*.

Enterobacteriaceae have become one of the most important causes of nosocomial and community acquired infections. As a result, regional and local surveillance are crucial for clinical decision making and infection control.

Acknowledgements. We are grateful to Prof. Dr. Edward R. B. Moore for all support and for offering us the possibility to work in his laboratory and with his group. Many thanks to Nahid Karami for instructions and advices.

References

Abraham E. P., Chain E., 1940 An enzyme from bacteria able to destroy penicillin. Nature **146**:837.

Boyd D. A., Tyler S., Christianson S., McGeer A., Muller M. P., Willey B. M., et al, 2004 Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extendedspectrum beta-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. Antimicrob Agents Chemother **48**:3758–3764.

Bradford P. A., 2001 Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev **14**:933-951.

Bush K., Jacoby G. A., and Medeiros A. A., 1995 A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother **39**:1211–1233.

Fang H., Lundberg C., Olsson-Liljequist B., Hedin G., Lindback E., Rosenberg A., Struwe J., 2004 Molecular epidemiological analysis of *Escherichia coli* isolates producing extended-spectrum beta-lactamases for identification of nosocomial outbreaks in Stockholm, Sweden. J Clin Microbiol **42**:5917–5920.

Jacoby G. A., 1997 Extended-spectrum beta-lactamases and other enzymes providing resistance to oxyimino-beta-lactams. Infect Dis Clin N Am **11:**875–887.

Jacoby G., Han P., Tran J., 1997 Comparative in vitro activities of carbapenem L-749,345 and other antimicrobials against multiresistant gram-negative clinical pathogens. Antimicrob Agents Chemother **41**:1830–1831.

Knothe H., Shah P., Krcmery V., Antal M., Mitsuhashi S., 1983 Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. Infection **11**:315-317.

Monstein H. J., Östholm-Balkhed Å., Nilsson M. V., Nilsson M., K. Dornbusch, and Nilsson L. E., 2007 Multiplex PCR amplification assay for the detection of *bla*SHV, *bla*TEM, and *bla*CTX-M genes in *Enterobacteriaceae.* APMIS **115**:1400–1408.

Ouellette M., Bissonnette L., and Roy P. H., 1987 Precise insertion of antibiotic resistance determinants into Tn*21*-like transposons: nucleotide sequence of the OXA-1 beta-lactamase gene. Proc Natl Acad Sci USA **84**:7378–7382.

Paterson D. L., Bonomo R. A., 2005 Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev **18**:657-686.

Pfaller M. A., Segreti J., 2006 Overview of the epidemiological profile and laboratory detection of extended-spectrum beta-lactamases. Clin Infect Dis **42**(Suppl.4):S153–S163.

Poirel L., Lebessi E., Castro M., Fevre C., Foustoukou M., Nordmann P., 2004 Nosocomial outbreak of extended-spectrum beta-lactamase SHV-5-producing isolates of *Pseudomonas aeruginosa* in Athens, Greece. Antimicrob Agents Chemother **48**:2277–2279.

Sabaté M., Tarragó R., Navarro F., Miró M., Vergés C., Barbé J., Prats G., 2000 Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing β-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob Agents Chemother **44**:1970–1973.

Tzouvelekis L. S., Gazouli M., Markogiannakis A., Paraskaki E., Legakis N. J., Tzelepi E., 1998 Emergence of resistance to third-generation cephalosporins amongst *Salmonella typhimurium* isolates in Greece: report of the first three cases. J Antimicrob Chemother **42**:273–275.

Received: 18 June 2011. Accepted: 08 July 2011. Published online: 08 July 2011.
Authors:
Mirela Petraşcu, "Iuliu Hatieganu" University of Medicine and Pharmacy, Department of Microbiology, Pasteur Street, no. 6, 400023, Cluj-Napoca, Cluj, Romania, EU, e-mail: mpetrascu@hotmail.com
Mirela Flonta, Infectious Diseases Clinical Hospital, Iuliu Moldovan Street, no. 23, 400348, Cluj-Napoca, Cluj, Romania, EU, e-mail: labinfecluj@yahoo.com
Ariana Mihaela Almaş, Infectious Diseases Clinical Hospital, Iuliu Moldovan Street, no. 23, 400348, Cluj-Napoca, Cluj, Romania, EU, e-mail: ari295@yahoo.com
How to cite this article:
Petraşcu M., Flonta M., Almaş A. M., 2011 The genetic characteristics of extended-spectrum β-lactamases (ESBLs) among *Escherichia coli* and *Klebsiella pneumoniae* isolates from urine specimens. HVM Bioflux **3**(2):58-65.