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Genotype-phenotype correlation in multiresistant *Escherichia coli* and *Klebsiella pneumoniae* strains isolated in Western Romania

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Abstract. – OBJECTIVE: Bacterial multidrug-resistance (MDR) to antimicrobials has become an important public health issue all over the world and it involves both hospital and community-acquired strains.

MATERIALS AND METHODS: A number of 75 Escherichia coli and 77 *Klebsiella pneumoniae (K.)* strains identified in biological samples collected from community (CA) and hospital-acquired (HA) infections were found to be resistant to the third generation cephalosporins. Of these, 93 MDR strains were subjected to microarray analysis to detect the expression of 31 antimicrobial resistance genes.

RESULTS: We found that all HA extended-spectrum β-lactamase (ESBL) producing *E. coli* strains had at least one resistance gene to third generation cephalosporins, while in 54% of all CA strains genetic substrates justifying their antibiotic resistance were identified. Almost 81% of HA-ESBL (Extended-Spectrum β Lactamase) *K. pneumoniae* strains had at least one resistance gene to third generation cephalosporins, while in only 6% of the CA strains a similar genotype was identified. In the HA group, the blaCTX-M-15 genotype proved to be most frequent in multidrug-resistant *E. coli* strains and second most frequent (after *ampC*) in *K. pneumoniae,* while in the CA group, this genotype was the fourth most frequent in ESBL *E. coli* (after *ampC*, *sul1*, *tet(R)*).

CONCLUSIONS: Overall, in 67% of all ESBL producing Enterobacteriaceae strains a genetic substrate justifying the resistance to beta-lactam antibiotics was identified; most of the remaining 33.33% strains were CA with a predominance of *K. pneumoniae*, in which a different antibiotic resistance genetic substrate (outside the detection limit of the kit used in this study) might have been involved.

Key Words:
Bacterial multi-resistance, Microarray, *E. coli*, *K. pneumoniae*.

Introduction

Bacterial multidrug-resistance (MDR) to antimicrobials has become an important public health issue all over the world and it involves both hospital (HA) and community-acquired (CA) strains.

According to the European Union reports[1], the most common MDR bacteria are *Escherichia (E.) coli* (11.9% of the invasive strains resistant to 2 classes, 7.1% to 3 classes and 3.9% to 4 classes of antibiotics, respectively) and *Klebsiella (K.) pneumoniae* (7.3% of the invasive strains resistant to 2 classes and 22.3% to 3 classes of antibiotics, respectively). The resistance to third generation cephalosporins ranges between 2-3% (in Sweden and Norway) and 30% (for *E. coli* in Cyprus and Slovakia) and even 81% (data reported on *K. pneumoniae* in Bulgaria). Similarly, the resistance to a combination of third generation cephalosporins, aminoglycosides and fluoroquinolones was increased in HA-*E. coli* and *K. pneumoniae* strains (3% and 20%, respectively), as compared to CA infection values (2.1% and 5%, respectively). The same study indicates that in Romania, 22% of *E. coli* strains are resistant to third generation cephalosporins (10.9% are resistant to the combination of the three antibiotic classes), while 44% of *K. pneumoniae* strains are resistant to third generation cephalosporins (with 30% exhibiting multiple resistances).

It is, therefore, highly important to properly screen and evaluate bacterial response to antibiotics prior to the onset of antibiotherapy as a first measure aiming to avoid/reduce the phenomenon of bacterial MDR.

Classical antibiotic sensitivity testing methods (broth dilution, disk diffusion, E-test) are rather

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laborious and time consuming (≥ 48h); the automated systems are fast and precise, but the costs related to their acquisition, installation and maintenance might represent a problem, especially in developing countries. On the other hand, the genotyping tests (by PCR and/or hybridisation methods) are precise, relatively cheap and much faster (up to 24 hours when performed on bacterial cultures, down to a couple of hours when performed directly in the biological product)[2]. Furthermore, these tests may provide important epidemiological data regarding the spatial/temporal distribution of the resistance genes in different pools, populations and environments[3]. DNA microarray is a method allowing the concomitant scan of multiple genes and has been described for typing resistance genes in enterobacteria, but it has not become a commonly used diagnostic method due to prohibitive costs of reagents, machines and lack of qualified personnel[4-6].

The aim of the present study was the molecular diagnosis of MDR by microarray technology in both HA- and CA-ESBL (Extended-Spectrum *b* Lactamase) producing *E. coli* and *K. pneumoniae* strains in Western Romania. This topic is of utmost importance in the context of the major epidemiological changes of the last decade: the increasing prevalence of CTX-M β-lactamase producing strains (as compared to TEM or SHV, which were predominant in Europe at the end of 90's), including CA infections, most probably caused by the ongoing inflow from hospital to community[7].

Materials and Methods

Bacterial Strains Collection and Microbiological Method

A prospective study was performed between February 2009 and October 2010, with the aim to investigate bacterial resistance of both *E. coli* and *K. pneumoniae* from CA (samples provided by S.C. Bioclinica S.A, Timisoara, Romania) and HA infections (samples provided by the ICUs of Timisoara County Emergency Clinical Hospital, Timisoara Institute of Cardiovascular Diseases, "Louis Turcanu" Pediatrics Emergency Clinical Hospital Timisoara, and the Municipal Obstetrics and Gynecology Clinical Hospital). The ICU of the County Hospital provides healthcare assistance for both medical and surgical cases; the other 3 ICUs deal predominantly with surgical patients.

Only the strains identified after at least 48 h of hospitalization were included as HA infections (all strains identified upon admission were discarded); in both HA and CA infections, we only included the first clinically relevant strain in order to avoid duplication and phenotypic changes induced by antibiotic selection pressure. No age, gender, infection site or prior antibiotic use exclusion criteria were applied.

E. coli and *K. pneumoniae* strains identified in bronchial aspirates, blood, urine samples and wound secretions and found to be resistant to third generation cephalosporins (as assessed by the Kirby-Bauer disc diffusion method) were subjected to further identification and phenotyping. Identification was done using the VITEK 2 Compact (BioMerieux®, Marcy l'Etoile, France) automated system with VITEK 2 GN cards. The sensitivity of bacterial strains was analyzed by the microdilution method (AST cards) and interpreted by the VITEK 2 Compact System according to the minimum inhibitory concentration breakpoints set by the National Committee on Clinical Laboratory and Standards Institute Inc. (CLSI M100-S16, 2006). Detection of ESBL strains was performed with the VITEK-ESBL test (AST-GN27 card), which includes cephotaxime, ceftazidime, cefepime with/without clavulanic acid. The following quality control strains were used: *E. coli* ATCC 3521, *E. coli* ATCC 25922, *K. pneumoniae* ATCC BAA 1705 and *K. pneumoniae* BAA 1706.

MDR was defined as acquired resistance to at least one agent in three or more antimicrobial categories[8].

A total of 4583 HA and 36236 CA biological samples were screened and assessed for MDR. We isolated 2452 non-repetitive Gram negative bacterial strains in hospital samples and 3521 in samples collected from ambulatory patients. Among these, 75 *E. coli* strains and 77 *K. pneumoniae* were identified as ESBL producing. In ninety-three (61.18%) of these strains MDR was confirmed and analysed by microarray (Table I).

Genomic DNA Isolation and Labeling

DNA was extracted from fresh (24 h from inoculation) colonies, grown on sheep blood agar medium. The total bacterial DNA was extracted from half a loopful of bacterial cells suspended in 200 ml phosphate buffered saline (PBS) using High Pure PCR Template Preparation Kit (Roche Applied Science, Basel, Switzerland, cat. no. 1179682800). The quality and concentration of

Table I. Distribution of ESBL strains tested by microarray according to the species and type of sample from which they are isolated.

Environment	Species	Total of isolates		Tested by microarray	
		N	%	N	%
Hospital	*E. coli*	49	32.24	30	32.26
	K. pneumoniae	45	29.60	21	22.58
Community	*E. coli*	26	17.10	26	27.96
	K. pneumoniae	32	21.05	16	17.20
Total	152	100	93	100	

DNA was determined with the NanoDrop ND1000 spectrophotometer. DNA was labelled with Alexa Fluor 3/5 by a randomly primed polymerization reaction and purified using BioPrime Total Genomic Labeling System (Invitrogen, Carlsbad, CA, USA – cat. no. 18097-011) according to the manufacturer's directions.

Oligonucleotide Design and Microarray Construction

Oligonucleotide probes were designed according to Frye[9] and represented 31 genes of the following classes of antimicrobials: *b*-lactam antibiotics, sulfanilamides, tetracyclines and macrolides. The kit was designed to determine resistance genes not only for *E. coli* and *K. pneumoniae*, but also for other gram negative species. The oligonucleotides were manufactured and spotted in triplicate by Arrayit Corporation (Sunnyvale, CA, USA) in an 18 well subarray format on standard glass slide (25 × 76 × 0.96 mm).

Hybridisation, Scanning and Analysis

Dye-labelled DNA was dried, re-suspended in HybIt 2 hybridisation buffer (Arrayit Corporation, cat. no. HHS2) and applied to a specific well subarray prepared according to the manufacturer's directions. Hybridization was performed in 3 h at 42°C. Protocols suggested by the manufacturer were used for post-hybridisation washing procedures. Microarrays were scanned with SpotLight CCD Scanner (Arrayit Corporation, Sunnyvale, CA, USA). Images were analysed using GenePix Pro 7 (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analysis

The 3.2.2.version of the EPI-INFO program was used for statistical analysis. Percent values were compared by contingency tables, using the chi-squared test and Fisher correction. The study was approved by the Ethical Committee of the "Victor Babes" University of Medicine and Pharmacy Timisoara (No.10/11.10.2008), and by the partner hospitals involved in the project. Before being included in the study, informed consent was obtained from patients or their relatives.

Results

The 93 MDR-ESBL strains were preponderantly isolated from urine samples (CA strains) and from urine samples, broncho-alveolar lavage liquid, wound secretions or other biological samples (HA strains from ICUs) (Figure 1).

MDR was confirmed in all the 93 EBSL strains by the microdilution method.

According to phenotype analysis, the average number of antimicrobial classes in which resistance to at least one agent was found was 4.2 for HA- ESBL *E.coli* strains (minimum 3, maximum 6) and 4.14 for HA-ESBL *Klebsiella pneumoniae* strains (minimum 3, maximum 5). For the strains isolated from ambulatory patients (CA infections), the respective average numbers were four in ESBL producing *E. coli* strains (minimum 3, maximum 6), and five in ESBL producing *K. pneumoniae* strains (minimum 3, maximum 6).

Among the HA *E. coli* strains tested by microarray, the average number of identified resistance genes was also four, with a minimum of one and a maximum of eight resistance genes. The CA-*E. coli* strains tested by microarray presented an average number of two resistance genes (minimum 0, maximum 10).

Among the HA-*K. pneumoniae* strains subjected to microarray analysis, the average number of resistance genes was three, with a minimum of zero, and a maximum of nine, while in the community *K. pneumoniae* group, the average number of resistance genes was basically zero.

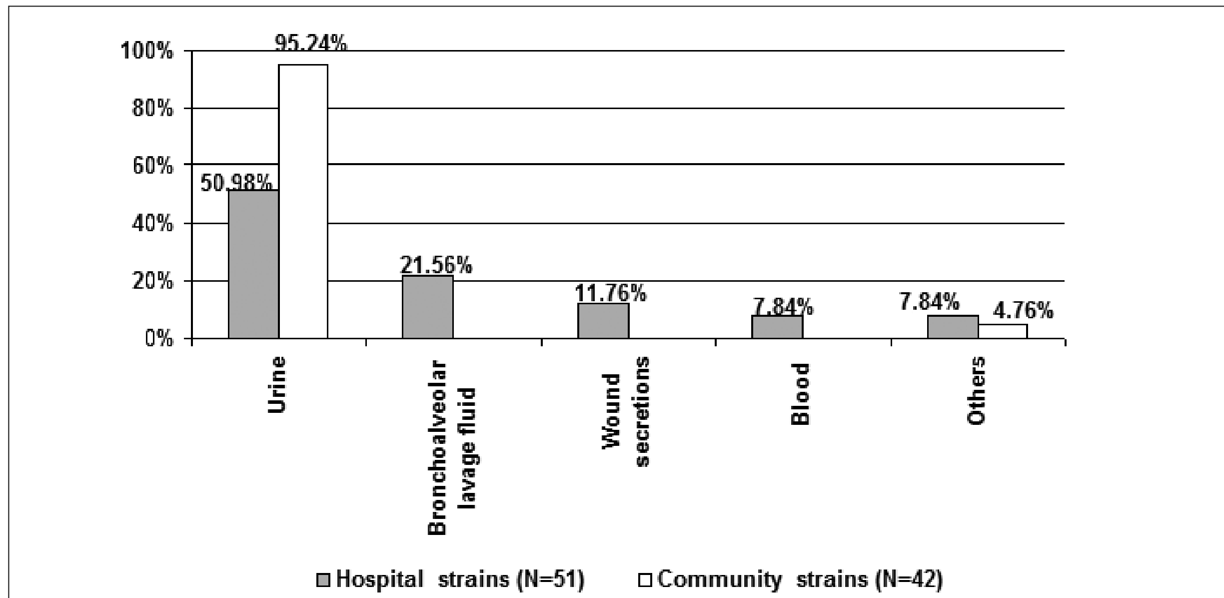

Figure 1. Distribution of MDR *E. coli* and *K. pneumoniae* isolates by sample origin.

Most of the HA-*E. coli* strains belonged to the CTX-M-15 β-lactamase (76.66%) followed by AmpC or TEM; one third of strains tested positive for the *tet(R)* gene. In the CA- *E. coli* group, nearly half were AmpC positive (46.15%), followed by positivity for sulphamethoxazole (*sul1* 30.76%) and tetracycline (*tet(R)*, 26.92%) resistance genes. In contrast to the HA-*E. coli* group, CTX-M -ESBL positive strains represented only 23.07% of the *E. coli* tested strains (Table II).

Among *K. pneumoniae* strains isolated from ICUs, AmpC positive bacteria were most frequent (61.90%), followed by *bla*CTX-M-15 (47.61%), *tet(R)* (38.9%) and *sul1* (33.33%) (Table III).

Table II. The genetic substrate identified in *E. coli* ESBL strains.

Genes detected	*E. coli strains*						p
	Hospital (N=30)			Community (N = 26)			
	n	%	95% CI	n	%	95% CI	
*amp*C	22	73.33	54.1- 87.7	12	46.15	26.6-66.6	**0.037**
*amp*R	0	0	/	0	0	/	/
*bla*CTX-M-15	23	76.66	57.5-90.1	6	23.07	9.0-43.6	**< 0.001**
*bla*TEM	22	73.33	54.1- 87.7	4	15.38	4.4-34.9	**< 0.001**
*bla*OXY-K1	7	23.33	9.9-42.3	4	15.38	4.4-34.9	0.455
tet(R)	10	33.33	17.3-52.8	7	26.92	11.6-47.8	0.602
tet(A)	7	23.33	9.9-42.3	2	7.69	0.9-25.1	0.153
sul1	5	16.66	5.6-34.7	8	30.76	14.3-51.8	0.212
sul2	6	20.00	7.7-38.6	5	19.23	6.6-39.4	0.942
mphA	7	23.33	9.9-42.3	5	19.23	6.6-39.4	0.709
*bla*CMY-2	3	10.00	2.1-26.5	3	11.53	2.4-30.2	1
blaOXA -9	0	0	/	0	0	/	/
*bla*OXA -27	0	0	/	0	0	/	/
*bla*SHV-37	0	0	/	0	0	/	/
*bla*OXA-2b	0	0	/	0	0	/	/
*bla*PSE-1	0	0	/	0	0	/	/
ereA	0	0	/	0	0	/	/
ereA2	0	0	/	0	0	/	/

Table III. The genetic substrate identified in *K. pneumoniae* ESBL strains.

Genes detected	*K. pneumoniae strains*						p
	Hospital (N = 21)			Community (N = 16)			
	n	%	95% CI	n	%	95% CI	
*amp*C	13	61.90	38.4-81.9	0	0	/	**< 0.001**
*amp*R	1	4.76	0.1-23.8	0	0	/	1
*bla*CTX-M-15	10	47.61	25.7-70.2	1	6.25	0.2-30.2	**0.010**
*bla*TEM	6	28.57	11.3-52.2	1	6.25	0.2-30.2	0.113
*bla*OXY-K1	5	23.80	8.2-47.2	1	6.25	0.2-30.2	0.206
tet(R)	8	38.09	18.1-61.6	0	0	/	**0.005**
tet(A)	3	14.28	3.0-36.3	0	0	/	0.243
sul1	7	33.33	14.6-57.0	1	6.25	0.2-30.2	0.103
sul2	4	19.04	5.4-41.9	1	6.25	0.2-30.2	0.364
mphA	0	0	/	0	0	/	/
blaCMY-2	0	0	/	0	0	/	/
*bla*OXA -9	3	14.28	3.0-36.3	0	0	/	0.243
*bla*OXA -27	1	4.76	0.1-23.8	0	0	/	1
*bla*SHV-37	2	9.25	1.2-30.4	0	0	/	0.495
*bla*OXA-2b	1	4.76	0.1-23.8	0	0	/	1
*bla*PSE-1	1	4.76	0.1-23.8	0	0	/	1
ereA	1	4.76	0.1-23.8	0	0	/	1
ereA2	2	9.25	1.2-30.4	0	0	/	0.495

Discussion

This is for the first time when a microarray analysis was correlated with the phenotypes of ESBL producing *E. coli* and *K. pneumonia*e strains circulating in the Western part of Romania.

We found that all HA-ESBL producing *E. coli* strains have at least one resistance gene to third generation cephalosporins, while in 54% of all CA strains we identified genetic substrates justifying their antibiotic resistance. On the other hand, 81% of HA-ESBL *K. pneumoniae* strains have at least one resistance gene to third generation cephalosporins while only 6% of the CA strains show a similar genotype. Overall, 67% of all ESBL producing *Enterobacteriaceae* strains presented a genetic substrate justifying the resistance to beta-lactam antibiotics; most of the remaining 33.33% strains were isolated from CA *K. pneumoniae* infections in which the genotype was not identifiable with the kit used in the present study.

However, the MDR phenotype of ambulatory strains isolated exclusively from urine samples, raises questions upon their community acquired character, a nosocomial inflow towards the community being also possible. More data on the admission history of ambulatory patients would be needed to answer this question.

Of the 27 strains testing positive for *tet(R)* or/and *tet(A)* resistance genes, 25 (92.59%) were tetracycline resistant and 2 strains (7.4%) were considered non-susceptible. We did not identify any silent genes (*tet* positive strains sensitive to tetracycline), although there are international studies which demonstrated this possibility for the *tet(A)* gene[11]. Only 4% of the *sul1, sul2* sau *sul1* + *sul2* resistance genes identified in the ESBL *Enterobacteriaceae* strains were silent.

In the HA group, the *bla*CTX-M-15 genotype proves to be the most frequently encountered in multidrug-resistant *E. coli* strains and second most frequent (after *amp*C) in *K. pneumoniae* strains, while in the CA group, this genotype comes fourth in ESBL *E. coli* (after *ampC, sul1, tet(R)*). There are significant differences between the HA and CA – ESBL producing *E. coli* regarding the frequencies of the *ampC*, *bla*CTX-M-15 and *bla*TEM genotypes. Similarly, we found significant differences between HA and CA strains regarding the distribution of *ampC*, *bla*CTX-M-15 and *tet(R)* genotypes in *K. pneumoniae* ESBL strains.

CTX-M-15 was identified in 2004 in Romania as well as in Bulgaria, Turkey and Poland[10,11]. The frequency of the *E. coli* CTX-M HA strains revealed by our study is similar to the data reported by two previous studies focussed on the population of North-Eastern Romania, which both reported a high incidence of the CTX-M genotype in both *E. coli* and *K. pneumoniae*[12,13].

The situation seems to be similar in Central and Eastern Europe. In Hungary, one of Romania's neighbouring countries, out of 281 *K. pneumoniae* ESBL strains collected in 2005 from 41 medical centres, 202 were CTX-M type, 97% being ciprofloxacin resistant CTX-M-15[14]. In Croatia, 162 strains of third generation cephalosporins resistant *K. pneumoniae*, collected in 2007 and analysed by PCR multiplex assay were group 1 CTX-M *b*-lactamase positive and the sequencing process revealed the *bla*CTX-M-15 gene[15]. Furthermore, in Turkey, a 2011 study found 80% of the ESBL *E. coli* isolated from urine samples to be quinolone resistant, 92% of these expressing CTX-M-15[16].

The high prevalence of CTX-M genotype might explain the emergence of non-responsiveness to other classes of antibiotics. Several studies have shown that the *bla*CTX-M genes are usually found on large plasmids, which are often carriers of other genes of resistance to aminoglycosides, fluoroquinolones, chloramphenicol, tetracyclines[17]. It is actually not uncommon to find plasmids with multiple resistance genes (aminoglycozides, chloramphenicol, quinolones, sulphonamides, tetracyclines, trimethoprime and other *b*-lactam antibiotics) in *E. coli* and *K. pneumoniae*, bacteria which usually have a weak expression of the chromosomal *bla ampC* genes[18]. Microarray technology has allowed to automatically detect AmpC *b*-lactamase when phenotyping cannot distinguish between the plasmidic and chromosomal AmpC enzymes underestimating this resistance mechanism.

Given that *E. coli* strains may represent true reservoirs of macrolide resistance genes, *ereA*, *ereB* (esterase encoding) and *mphA* (phosphotransferase encoding), we included them in our model. A 2009 study showed that *mphA* gene is one of the most frequently detected macrolide resistance gene in *E. coli* strains from five countries on four continents. This gene was especially identified in ESBL strains and also in amoxicillin and cotrimoxazole co-resistant strains[19]. In the present study, only approximately 20% of the *E. coli* strains were positive for *mphA*.

Conclusions

Determination of bacterial resistance phenotypes is essential not only for correct antimicrobial therapy but also for patient isolation and quality control procedures. Consequently, rapid and more accurate laboratory methods are required to accomplish these goals. This first microarray experimental model proposed and used in the present study revealed the diversity of the molecular substrate of ESBL producing *E. coli* and *K. pneumoniae* strains. It proved very useful for the concomitant analysis of a large number of strains and also for monitoring the circulation of HA-MDR bacteria, when financial resources and personnel qualifications allowed it. The kit must be completed with other resistance genes identified in our region, such as those encoding for resistance to aminoglycosides and fluoroquinolones as well as genes for other beta-lactam antibiotics, given the significant percent of Gram negative strains phenotypically associated with the above.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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