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Beta-lactam and quinolone resistance markers in uropathogenic strains isolated from renal transplant recipients

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Abstract

Our objectives were to investigate the extended-spectrum beta-lactamases (ESBLs) and carbapenemases (CR) genetic determinants and to assess the association between ESBL production and quinolone resistance in bacterial strains isolated from renal transplant recipients with urinary tract infections.

***Material and methods**: A number of 30 isolates were recovered from urine specimens of patients with renal transplant from October 2015 to March 2016. The isolates were analyzed for ESBL production using double disc synergy test and for CR production by the Hodge test. Phenotypically confirmed isolates were screened by PCR for the identification of ESBL, CR and fluoroquinolone resistance genes.*

***Results**: The 30 clinical bacterial strains isolated from urinary tract infections in renal transplant recipients were identified as Klebsiella pneumoniae (17), Pseudomonas aeruginosa (7), Morganella morganii (2), Escherichia coli (2), Edwardsiella tarda (1) and Enterobacter cloacae (1). Out of them, 22 isolates were ESBL producers and 20 multi-drug resistant (MDR) (i.e., 13 K. pneumoniae and 7 P. aeruginosa strains). More than half of the ESBL clinical strains (14/22, 63.63%) revealed at least one ESBL gene, the most frequent being bla_{CTX-M} type (18/22, 81.81%), either alone (4/22, 18.18%) or in combination with another ESBL gene (17/22, 77.27%), followed by bla_{TEM} (13/22, 59.09%). The bla_{OXA}-48 was present in 10 isolates (33.33%). The most frequent association of ESBLs and CR genes (5/14, 35.71%) was revealed by bla_{CTX-M}- bla_{TEM} - bla_{OXA}-48, encountered particularly among K. pneumoniae isolates (4/17, 23.52%). The qnrB gene was identified in five strains, i.e. one P. aeruginosa ESBL isolate (expressing the bla_{CTX-M} gene) and four K. pneumoniae ESBL isolates (harboring the bla_{CTX-M} - bla_{TEM} genes combination).*

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***Conclusions**: The uropathogenic strains isolated from renal transplant recipients exhibited high rates of MDR and beta-lactam resistance. The selective pressure exerted by quinolones could enable uropathogenic bacteria to acquire resistance to this class of antibiotics.*

***Keywords**: renal transplant recipients, urinary tract infections ESBL producers, bla genes, quinolone resistance*

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Introduction

Renal transplant recipients (RTRs) represent one of the categories of patients exhibiting the highest risk to develop UTIs, that could be one of the causes for allograft dysfunction. Additionally, the widespread use of antibiotics employed for prophylaxis and treatment of transplant recipients is leading to an increasing resistance to antibiotics among uropathogenic isolates [1, 2]. Different studies reported that infections caused by beta-lactam resistant-*K. pneumoniae* in kidney transplant recipients were associated with higher mortality [3], recurrent UTIs being also reported [4,5]. Therefore, management strategies employing corrrect use of antibiotics and early diagnosis are essential for successful allograft and patient outcome. Our objective was to investigate the extended-spectrum beta-lactamases (ESBLs) and carbapenemases (CR) and to assess the association between beta-lactamases production and quinolone resistance in bacterial strains isolated from RTRs with urinary tract infections.

Materials and Methods

Phenotypic characterization of bacterial isolates

The isolates were obtained from urine specimens of RTR submitted to the Bacteriology Department of Fundeni Hospital, Bucharest, Romania, between September 2015 and March 2016. The urine cultures were performed in the Bacteriology Department of Fundeni Hospital, Bucharest, Romania. Microbial identification was performed using the Phoenix automated system (Becton Dickinson Company). The Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines were employed for antibiotic susceptibility testing. ESBL production was determined by double-disk synergy test, while carbapenemase production was screened using the modified Hodge test [6]. A total of 30 bacterial isolates from urine samples of RTRs exhibiting increased antimicrobial resistance to beta-lactam antibiotics were selected.

Molecular analysis of ESBL-positive strains

ESBL producers were submitted to molecular characterization of the ESBL-encoding genes. Whole-cell DNAs were extracted using the alkaline extraction method. Simplex and multiplex PCR amplifications were performed for bla_{TEM} [7], bla_{SHV} [8], bla_{CTX-M} [9], bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{OXA-48} [10] and bla_{PER} [11], using a reaction mix of 20µL (PCR Master Mix 2x, Thermo Scientific), containing 1 µl of bacterial DNA. The ESBL-producing clinical isolates exhibiting a fluoroquinolone resistance phenotype were further investigated for identification of fluoroquinolone resistance genetic determinants (*gyrA*, *gyrB*, *parC*, *parE*, *qnrA*, *qnrB* and *qnrS*) [12, 13]. The amplification reactions were carried out in *PCR thermal Corbett* thermocycler under the following conditions: initial denaturation at 95°C for 10 min, followed by 36 cycles of denaturation (94°C for 30 sec), annealing (52°C for 40 sec), extension (72° for 50 sec) and a final extension step (72°C for 5 min). DNA fragments were analyzed by electrophoresis in a 1% agarose gel, migrated at 100 V for 1 h in 1× TAE (40 mmol/L Tris–HCl [pH 8.3], 2 mmol/L acetate, 1 mmol/L EDTA) containing 0.05 mg/L ethidium bromide [10].

Results

Antimicrobial susceptibility profiles

Among the 30 clinical bacterial strains isolated from urine samples of RTRs, 17 *Klebsiella pneumoniae,* 7 *Pseudomonas aeruginosa*, 2 *Morganella morganii*, 2 *Escherichia coli,* 1 *Edwardsiella tarda* and 1 *Enterobacter cloacae* were identified. Antimicrobial susceptibility test results showed that 20 isolates were MDR (all *P. aeruginosa* strains and 13 strains of *K. pneumoniae*), exhibiting resistance to cephalosporins (third [ceftazidime] and fourth generation [cefepime], carbapenems (imipenem and meropenem), and quinolones (ciprofloxacin, levofloxacin) and 22 were ESBL-producers. Of these, 14 were *K. pneumoniae*, four *P. aeruginosa,* two *M. morganii*, one *E. coli* and one *E. tarda*. Furthermore, all ESBL producers were resistant to trimetoprim-sulphamethoxazole and gentamicin (*Figure 1*). A total of 13 *K. pneumoniae* isolates were carbapenemase producers, exhibiting also resistance to cephalosporins (third and fourth generation), quinolones (ofloxacin, levofloxacin, ciprofloxacin), monobactams (aztreonam), trimetoprim-sulphamethoxazole and gentamicin. A higher susceptibility rate was observed only in case of amikacin (*Table 1 and 2*).

Table 1. *In vitro* antimicrobial susceptibility of clinical strains isolated from urine samples of renal transplant recipients.

Antimicrobial agent	No. isolates tested	No. susceptible isolates
Amoxicillin/ clavulanate	23	0
Cefotaxime	23	0
Ceftazidime	30	0
Cefepime	30	0
Piperacillin/Tazobactam	30	10
Ertapenem	23	3
Meropenem	30	16
Imipenem	30	18
Aztreonam	30	0
Ciprofloxacin	30	0
Norfloxacin	30	4
Levofloxacin	30	0
Amikacin	30	30
Gentamicin	30	8
Trimethoprim/Sulphamethoxazole (TMP/SXT)	23	0
Tigecycline	23	3

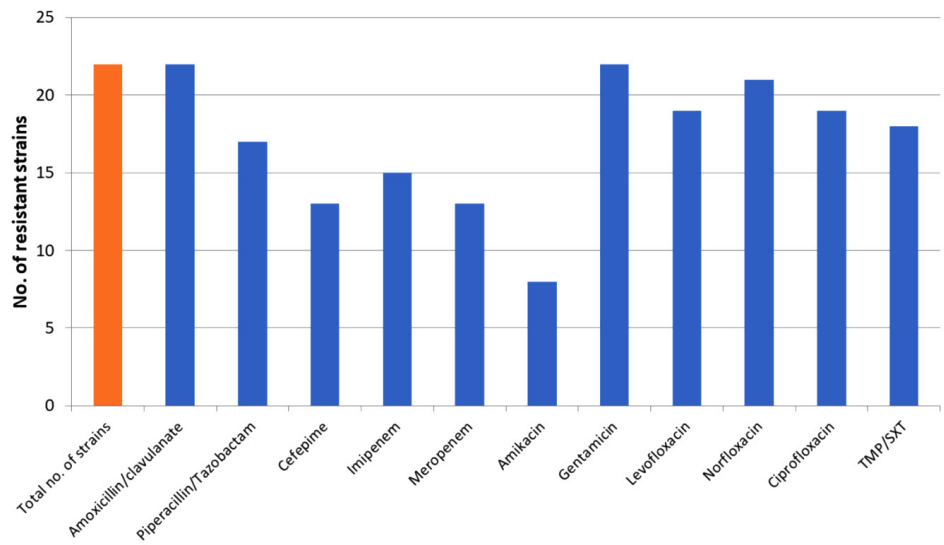

Figure 1. Antimicrobial resistance profiles for the 22 ESBL-producing urinary isolates.

Table 2. Antimicrobial susceptibility (%) of ESBL-producing isolates isolated from urine samples of renal transplant recipients

Antimicrobials	Total (30)				*K. pneumoniae* spp. (17)				*M. morganii* (2)		*E. coli* (2)		*E. tarda* (1)		*E. cloacae (1)*		*P. aeruginosa* (7)	
	ESBL (10)		MDR (20)		ESBL (4)		MDR (13)		ESBL (2)		ESBL (2)		ESBL (1)		ESBL (1)		MDR (7)	
	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S
Amoxicillin/ clavulanate	10	0	13	0	4	0	13	0	2	0	2	0	1	0	1	0	nt	nt
Cefotaxime	10	0	20	0	4	0	13	0	2	0	2	0	1	0	1	0	nt	nt
Ceftazidime	10	0	20	0	4	0	13	0	2	0	2	0	1	0	1	0	7	0
Cefepime	4	0	20	0	-	-	13	0	1	0	2	0	1	0	nt	nt	7	0
Pip/Tazo	6	4	18	2	2	2	13	0	2	0	1	1	0	1	1	0	5	2
Ertapenem	2	7	19	1	2	2	12	1	0	1	0	2	0	1	0	1	nt	nt
Meropenem	1	8	20	0	1	2	13	0	0	2	0	2	0	1	0	1	7	0
Imipenem	1	9	20	0	0	4	12	1	1	1	0	2	0	1	0	1	7	0
Aztreonam	nt	nt	9	nt	nt	nt	9	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt
Ciprofloxacin	6	0	20	0	2	0	13	0	nt	nt	2	0	1	0	1	0	7	0
Norfloxacin	8	2	20	0	3	1	13	0	1	1	2		1	0	1	0	7	0
Levofloxacin	6	2	20	0	2	0	13	0	0	2	2	0	1	0	1	0	7	0
Amikacin	0	9	5	13	0	3	0	13	0	2	0	2	0	1	0	1	7	0
Gentamicin	5	4	20	0	3	1	13	0	0	1	1	1	0	1	1	0	7	0
TMP/SXT	9	0	13	0	4	0	13	0	1	0	2	0	1	0	1	0	nt	nt
Tigecycline	0	0	2	11	nt	nt	2	11	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt

Legend: nt – not tested

Screeening of ESBL, CR and quinolone resistance genes

CTX-M enzymes were the majority of the revealed ESBLs (18/22), alone (4/22) or in combination with another ESBL gene (17/22) followed by TEM (13/22). The OXA-48 carbapenemase was present in 10 isolates. More than half of the ESBL clinical strains (14/22,) revealed at least one ESBL gene. Among the *K. pneumoniae* isolates, $bla_{\text{CTX-M}}$ - bla_{TEM} (6/17, 35.29%) was the most frequent association, followed by the association between 2 ESBLs and one CRG (carbapenem resistance gene) [$bla_{\text{CTX-M-}}$ $bla_{\text{TEM -}}$ $bla_{\text{OXA-48}}$ (4/17, 23.52%)] and by one ESBL and one CRG [$bla_{\text{CTX-M-}}$ $bla_{\text{OXA-48}}$ (2/17, 11.76%)]. A low number of the studied strains expressed only one β-lactam resistance gene, i.e.: $bla_{\text{CTX-M}}$ (13.33%), bla_{TEM} (6.66%) and $bla_{\text{OXA-48}}$ (6.66%) (*Table 3).*

Among the quinolone resistant clinical strains the PMQR *qnr*B gene was identified in one *P. aeruginosa* isolate also harboring $bla_{\text{CTX-M}}$ and in four *K. pneumoniae* ESBL-positive isolates (harboring the $bla_{\text{CTX-M-}}$ bla_{TEM} gene combination) (*Table 3*).

Discussion

RTRs are reported to exhibit the highest incidence of UTIs [14,15]. Although third generation cephalosporins [16] and trimethoprim-sulfamethoxazole have been shown to prevent the occurence of UTI in the post-transplantation period [17], however, chemoprophylaxis has led to the selection of resistance, including the emergence of ESBL producing isolates [18].

K. pneumoniae and *E. coli* isolates are according to ECDC (*European Centre for Disease and Control*) the most frequent etiological agents of UTIs. The latest data are indicating that Romania is one of the UE countries with

Table 3. Distribution of ESBLs, CRGs and QNRBs among clinical strains isolated from urine samples of renal transplant recipients

Species	Number of isolates	Type of ESBL and CR (number of isolates)				QRDRs (number of isolates)
		CTX-M	TEM	OXA-48	ESBLs and Carbapenemases (different associations)	QNRB
K. pneumoniae	17	1		1	CTX-M – TEM **(6)** CTX-M – OXA-48 **(2)** CTX-M – TEM – OXA-48 **(4)**	4
M. morganii	2	1	1			
E. coli	2	1				
E. tarda	1	1				
Enterobacter cloacae	1					
P. aeruginosa	7		1	1	CTX-M **(1)** CTX-M – TEM **(1)**	1
Number of strains (%)	30	4 (13.33%)	2 (6.66%)	2 (6.66%)	14 (47.66%)	5 16.66

the highest resistance rates of *K. pneumoniae* to third generation cephalosporins (65-83%), fluoroquinolones (55-77%), aminoglycosides (48-60%), carbapenems (20-30%) and MDR (45-56%) (https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/antimicrobial-resistance-europe-2015.pdf). In a recent Romanian study performed in an infectious disease hospital, the resistance rates to the third generation cephalosporins (cefotaxime) were of 39% for *Klebsiella* spp. and 12% for *E. coli*. A non-interventional, retrospective study performed in "Prof. Dr. Matei Bals" National Institute of Infectious Diseases in Bucharest, revealed lower resistance rates of *K. pneumoniae* blood isolates to fluoroquinolones, aminoglycosides, third generation cephalosporins, carbapenems, and MDR, as compared to those reported by our country to EARS-Net [19].

The present study was carried out in a healthcare setting for kidney transplant in Bucharest, Romania. Over a six-month period (September 2015 to March 2016) a total of 30 clinical isolates from urine samples of RTR exhibiting increased antimicrobial resistance were selected. Phenotypic and molecular characterization of the clinical isolates revealed that 22 (73.33%) were ESBL producers, i.e.: *K. pneumoniae* (14), *P. aeruginosa* (4), *M. morganii* (2), *E. coli* (1) and *E. tarda* (1). The findings of our study are in accordance with several other studies that are describing an elevated frequency of ESBL infections among RTRs that received prophylactic antibiotic treatment [20, 21, 4, 22, 23]. We found that all of them exhibited resistance to third generation cephalosporins, that it is reported to be linked to the emergence of MDR isolates [24]. All ESBL producers were also resistant to gentamicin and trimethoprim-sulphamethoxazole, as reported by other authors [25]. Trimethoprim-sulphamethoxazole resistance is most frequently encoded by plasmids, including some coexpressing ESBLs. The increased proportion of ESBL simultaneous with resistance to other classes of antibiotics, constitutes a major therapeutic problem for infections produced by resistant *K. pneumoniae* or *E.coli* isolates, relying on carbapenems, as last therepeutic option.

However, this could contribute to the emergence of carbapenem resistant bacteria, as also confirmed by our study in which a very high number of *K. pneumoniae* isolates displayed carbapenem resistance (13). In 2014, Romania reported 81 carbapenemase resistant *K. pneumoniae* isolates (31.5%), the number decreasing in 2015 to 67 isolates.

Our molecular assays showed that the majority of the ESBL-producing strains harbored a $bla_{\text{CTX-M}}$ gene alone, but also in association with another ESBL or CRG, followed by bla_{TEM}. Approximatively half of the clinical strains (14/22%) expressed more than one beta-lactam resistance gene, the most frequent being the association $bla_{\text{CTX-M-}}$ $bla_{\text{TEM -}}$ $bla_{\text{OXA-48}}$, particularly among *K. pneumoniae* strains. These findings are in accordance with previous investigations performed in Romania. The first confirmed cases of OXA-48 - NDM-1 producing *Enterobacteriaceae*, mostly *K. pneumoniae*, were detected in 2012 [26]. A more recent study carried out on clinical strains isolated from patients hospitalized in ICUs in southern Romania revealed a high prevalence and a wide diversity of ESBL genes ($bla_{\text{CTX-Mlike}}$, bla_{TEMlike} and bla_{SHVlike} in *Enterobacteriaceae* isolates and bla_{GESlike}, bla_{SHVlike} and bla_{VEBlike} in nonfermenting rods (*P. aeruginosa* and *Acinetobacter baumannii*) [27]. Also, the CRGs $bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-181}}$ in *Enterobacteriaceae* strains isolated in northern Romania, $bla_{\text{OXA-23}}$ in *A. baumannii* in west Romania, and $bla_{\text{VIM-2}}$ in *P.aeruginosa* in north-east Romania have been reported [28].

Another pathogen frequently involved in the etiology of UTIs in RTRs is MDR *P. aeruginosa* [29]. Our results indicated that 23.33% (7/30) of *P. aeruginosa* exhibited a MDR phenotype and of these, 28.57% (2/7) were carrying ESBL genes. In *P. aeruginosa* isolates, the ESBLs are still less frequently detected; the most common are OXA derivatives (class D β-lactamases), PER enzymes [30] and VEB enzymes (class A β-lactamases) [31]. The ESBL coding genes combination $bla_{\text{CTX-M}}$-bla_{TEM} was also detected in the analysed *P. aeruginosa* isolates. These findings are suggesting the possible exchange of resistance determinants between *Enterobacteriaceae* and non-*Enterobacteriaceae* isolates.

Significantly elevated trends with regard to the national percentages of fluoroquinolone resistant isolates were reported by Romania for the period 2012-2015 (www.ecdc.europa.eu). In our study all investigated strains were resistant to fluoroquinolone. These antimicrobials are employed in the treatment of infections caused by uropathogens resistant to most β-lactams, particularly the extended-spectrum cephalosporins and, more recently, the carbapenems. However, an increasing percentage of *Klebsiella* sp. strains developed resistance to fluoroquinolones [19]. In another study, the phenotypic analysis of 61 *P. aeruginosa* isolates from a laboratory hospital from north-east Romania revealed a resistance level of 17.69%.

The high incidence of ESBLs isolates with combined resistance to fluoroquinolones has become an important concern nowadays [32]. EARS-Net reported in 2015 that among the *K. pneumoniae* resistant isolates, combined resistance to fluoroquinolones, third-generation cephalosporins and aminoglycosides was the most prevalent phenotype (https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/antimicrobial-resistance-europe-2015.pdf). In Turkish hospitals, a dramatic increase of resistance rates to quinolones among uropathogenic *E.coli*, from 30.4% in 2003, 41.3% in 2007, to 59.4% in 2012 in RTRs was reported [33]. We found that all bacterial strains with ESBL phenotype exhibited also a fluoroquinolone-resistance phenotype.

The coexpression of the *qnr* gene and efflux pumps on plasmids carrying ESBLs may encourage the development of *gyrA*- and *parC*-mediated fluoroquinolone resistance and emergence

of highly resistant strains [34]. In our study the PMQR *qnr*B gene, was identified in five (16.16%) clinical strains, i.e.: one *P. aeruginosa* ESBL isolate (expressing the bla_{CTX-M} gene) and four *K. pneumoniae* ESBL isolates (harboring the association between 2 ESBLs genes (bla_{CTX-M} - bla_{TEM}). Co-resistance to fluoroquinolones, which is more commonly due to chromosomal mutations in genes coding for DNA gyrase rather than plasmids carrying genes for low-level resistance, is more likely to have been acquired independently of a plasmid-borne ESBL gene. One possible explanation for the significant co-resistance among ESBL-producing isolates is the presence of multiple plasmid-borne resistance loci [35]. Until now the efflux-based resistance mechanisms has never been documented in ESBL-bearing plasmids. Lagacé-Wiens reported that *qnr*-mediated quinolone resistance remains extremely rare in ESBL-bearing plasmids and has not yet been reported in Canadian *E. coli* isolates. Molecular characterization of a collection of Romanian isolates of ciprofloxacin-resistant *E. coli* isolates from human extraintestinal specimens showed that they were carrying double mutations in the QRDR of *gyr*A. A high percentage of them (69%) possessed $bla_{CTX-like}$ genes, and with one exception, all were ESBL producers [36].

Considering the recent studies involving monitoring, antimicrobial resistance rates have been shown to significantly increase, threatening the available treatment options for common infections [37-39]. In case of RTRs, prolonged hospitalization, broad-spectrum antimicrobial treatment, the use of urinaty catheters and the renal replacement therapy are all contributing to the occurence of MDR bacteria.

To our knowledge this report represents the first study regarding the antibiotic resistance markers in uropathogenic strains isolated from RTRs in Romania. In our study, very high rates of resistance have been observed in the bacteria isolated RTRs, more than 70% of the bacteria carring a β-lactamase genes that virtually could reach any Gram-negative bacterium and become a major threat in the future. Of major importance is the detection of a high number of *K. pneumoniae* isolates resistant to carbapenems (76.47%), the last-treatment option for severe community and hospital acquired infections. Moreover, all ESBL urinary isolates were fluoroquinolone-resistant. Molecular investigations revelead the presence of quinolone resistance genetic markers in 36.36% of ESBL strains. The combined resistance of uropathogenic isolates to fluoroquinolones and extended-spectrum cephalosporins could lead to limitations to available treatment options for UTIs in RTRs.

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Abreviations

RTR = renal transplant recipients
MDROs = multi-drug resistant organisms
MDR = multi drug resistant
ESBLs = extended-spectrum beta-lactamases
UTIs = urinary tract infections
CLSI = Clinical and Laboratory Standards Institute
DDST = double-disk synergy test
PMQR = plasmid-mediated quinolone resistance
QRDRs = quinolone resistance-determining regions
CRE = carbapenem resistant Enterobacteriaceae
CR = carbapenemases
CRG = carbapenem resistance genes

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