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PLASMID-MEDIATED QUINOLONE RESISTANCE AMONG EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCING *ENTEROBACTERIACEAE* FROM BLOODSTREAM INFECTIONS

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The purpose of this study was to determine prevalence and molecular characterization of plasmid-mediated quinolone resistance (PMQR) genes [*qnrA, qnrB, qnrC, qnrD, qnrS, aac(6′)-Ib-cr, qepA,* and *oqxAB*] among extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella* spp. isolates from bloodcultures in Hungary. A total of 103 isolates were tested for quinolone susceptibility by microdilution method and PMQR genes were detected by polymerase chain reaction. About 40 ESBL-producing *E. coli* (39%) and 50 ESBL-producing *Klebsiella* spp. strains (48%) were resistant to ciprofloxacin; 40 ESBL-producing *E. coli* (39%) and 47 ESBL-producing *Klebsiella* spp. strains (45%) were resistant to levofloxacin; and 88 strains including 40 ESBL-producing *E. coli* (39%) and 48 (47%) ESBL-producing *Klebsiella* spp. were resistant to moxifloxacin. Among the 103 ESBL-producing isolates, 77 (75%) isolates (30 *E. coli* and 47 *Klebsiella* spp.) harbored PMQR genes. The most commonly detected gene was *aac(6′)-Ib-cr* (65%). The occurrence of *qnrS* gene was 6%. Interestingly, *qnrA, qnrB, qnrC, qnrD*, and *qepA* were not found in any isolates. Among 77 PMQR-positive isolates, 27 (35.1%) and 1 (1.3%) carried two and three different PMQR genes, respectively. Only *Klebsiella* spp. harbored more than one PMQR genes. Observing prevalence of PMQR genes in the last 8 years, the increasing incidence of *aac(6′)-Ib-cr* and *oqxAB* can be seen. Our results highlight high frequency of PMQR genes among ESBL-producing *Klebsiella pneumoniae* and *E. coli* isolates with an increasing dynamics in Hungary.

Keywords: plasmid-mediated quinolone resistance, *qnr*, *aac(6′)-Ib-cr*, *oqxAB*

Introduction

Fluoroquinolones (FQs) possess an excellent activity against gram-negative bacteria especially against *Enterobacteriaceae* and have been extensively used for

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treatment of infections caused by these pathogens since their introduction in the 1980s [1]. The mode of action of FQs is the inhibition of type II DNA topoisomerases, namely, gyrase and topoisomearse IV [2]. DNA gyrase is the primary target for quinolones in gram-negative bacteria due to higher sensitivity of that enzyme to quinolone inhibition and formation of drug–enzyme–DNA complexes in comparison to the sensitivity of other topoisomerases. Resistance to FQs was explained by accumulation of mutations in the chromosomal genes, encoding DNA gyrase and topoisomerase IV bacterial enzymes, namely, *gyrA, gyrB, parC*, and *parE*. Further resistance mechanisms are the decreased intracellular drug accumulation by upregulation of native chromosomally encoded efflux pump AcrAB or decreased expression of outer membrane porins, e.g., OmpF in *Escherichia coli* [1, 3–5].

Plasmid-mediated quinolone resistance (PMQR) determinants – *qnr* determinants (*qnrA*, *qnr*, *BqnrS*, *qnrC*, and *qnrD*), aminoglycoside acetyltransferase *(6′)*-Ib-cr variant, and specific efflux pumps, namely, *QepA* and *OqxAB* – were discovered as new ways of FQ resistance, mainly in *Enterobacteriaceae.* The acquisition of *qnr* genes leads to low-level FQ resistance, which is meant to be higher minimal inhibitory concentration (MIC) values than the wild-type but still below the internationally approved resistance breakpoints by European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute. In the status of low-level resistance, bacterial strains have 100 times increased mutation frequency (from 10^8 to 10^6) on chromosomal gene (e.g., *gyr*), which facilitates the selection to high-level FQ resistance [6–9]. *PMQR* genes are frequently associated with various beta-lactamase genes, including extended-spectrum beta-lactamases (ESBLs) and narrow spectrum-beta-lactamases, as these resistance determinants are located on same plasmids. Resistance plasmids are usually transferable by conjugation that facilitates their dissemination between different *Enterobacteriaceae* species [9, 10].

In this study, our aim was to determine the PMQR determinants among ESBL-producing *Enterobactericeae* strains from bloodcultures.

Materials and Methods

Bacterial strains

In this study, 103 ESBL-producing *Enterobacteriaceae* isolates from bloodstream infections (49 *E. coli* and 54 *Klebsiella* spp.) of patients treated at intensive care units of Semmelweis University between 2010 and 2014 were collected. Species identification was done by MALDI-TOF/MS (Bruker Daltonik GmbH, Bremen, Germany).

Antibiotic susceptibility

MIC values of nalidixic acid, ciprofloxacin, levofloxacin, and moxifloxacin were determined by broth microdilution methods using EUCAST criteria on all 103 isolates [6]. *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were reference strains for antimicrobial susceptibility testing.

Phenotypic detection of ESBL production

Double-disk synergy tests were performed by placing disks of ceftazidime, cefotaxime, and cefepime at a distance of 20 and 30 mm (center to center) from a disk containing amoxicillin plus clavulanic acid (20/10 μg). A "keyhole" phenomenon was regarded as positive for ESBL production.

Detection of PMQR genes by PCR

Detection of genes encoding PMQR – *qnrA, qnrB, qnrC, qnrD, qnrS, aac (6')- Ib-cr, qepA* and *oqxAB* – was performed on all isolates. A single colony of each test isolate was resuspended in 400-μl double distilled water and was boiled at 100 °C for 15 min. A supernatant was obtained after centrifugation at 13,000 rpm for 15 min and was used as bacterial DNA template in polymerase chain reaction (PCR) assays. The primers for detection of *qnrA, qnrB, qnrC, qnrD, qnrS, aac(6')-Ib-cr, qepA*, and *oqxAB* genes are shown in Table I. PCRs were performed with RedTaq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA), according to manufacturer's instructions with 2 μl of DNA template in a total reaction volume of 30 μl. DNA amplification programs consisted of an initial denaturation step (96 °C for 5 min) followed by 30 cycles of denaturation (96 °C for 30 s), annealing for 30 s (annealing temperature for each primer set is shown in Table I), and extension (72 °C for 1 min), and a final extension of 5 min at 72 °C. Ten microliters of reaction mixture containing PCR product was analyzed by gelelectrophoresis in 0.8% (wt/vol) agarose gel (Sigma-Aldrich) with 120 V for 30 min in electrophoresis system (Bio-Rad, Hercules, CA, USA).

Results

Antibiotic susceptibility

About 40 ESBL-producing *E. coli* (39%) and 50 ESBL-producing *Klebsiella* spp. strains (48%) were resistant to ciprofloxacin; 40 ESBL-producing *E. coli*

Table I. Primers used in this study

Gene	Primer sequence (5′ to 3′)	Size of PCR product (bp)	Annealing temperature (°C)	Reference
qnrA-F	ATTTCTCACGCCAGGATTTG	516	53	[11]
qnrA-R	GATCGGCAAAGGTTAGGTCA			
qnrB-F	ATGACGCCATTACTGTATAA	469	53	[12]
qnrB-R	GATCGCAATGTGTGAAGTTT			
qnrS-F	ACG ACA TTC GTC AAC TGC AA	417	53	[11]
qnrS-R	TAA ATT GGC ACC CTG TAG GC			
qnrC-F	GGGTTGTACATTTATTGAATC	447	50	[13]
qnrC-R	TCCACTTTACGAGGTTCT			
qnrD-F	CGAGATCAATTTACGGGGAATA	582	50	[14]
qnrD-R	AACAAGCTGAAGCGCCTG			
aac(6')Ib-cr-F	TTGCGATGCTCTATGAGTGGCTA	482	54	[15]
aac(6')Ib-cr-R	CTCGAATGCCTGGCGTGTTT			
oqxA-F	CTCGGCGCGATGATGCT	392	57	[16]
oqxA-R	CCACTCTTCACGGGAGACGA			
oqxB-F	TTCTCCCCCGGCGGGAAGTAC	512	64	
oqxB-R	CTCGGCCATTTTGGCGCGTA			
qepA-F	GCA GGT CCA GCA GCG GGT AG	199	60	[17]
qepA-R	CTT CCT GCC CGA GTA TCG TG			

(39%) and 47 ESBL-producing *Klebsiella* spp. strains (45%) were resistant to levofloxacin; and 88 strains including 40 ESBL-producing *E. coli* (39%) and 48 (47%) ESBL-producing *Klebsiella* spp. were resistant to moxifloxacin. The distribution of MIC values of ESBL-producing *E. coli* and *Klebsiella* spp. is shown in Figures 1 and 2.

Detection of PMQR genes

Among 103 ESBL-producing isolates, 77 (75%) harbored PMQR genes, and 30 *E. coli* and 47 *Klebsiella* spp. were detected. The most commonly detected gene was *aac(6′)-Ib-cr* (65%). The occurrence of *qnrS* gene was 6%. Interestingly, *qnrA, qnrB, qnrC, qnrD*, and *qepA* were not found in any isolates.

Among 49 ESBL-producing *E. coli* strains, 29 harbored *aac(6′)-Ib-cr* gene and one carried *qnrS*. In 19 isolates, PMQR genes were not detected.

Among 53 *K. pneumoniae*, 38 harbored *aac(6′)-Ib-cr* gene and five isolates carried *qnrS*. 26 *K. pneumoniae* isolates were positive for *oqxA* and 22 for *oqxB* genes.

Only six isolates – one *E. coli*, four *K. pneumoniae*, and one *K. oxytoca* isolates – harbored *qnrS*. The characteristics of these strains are summarized

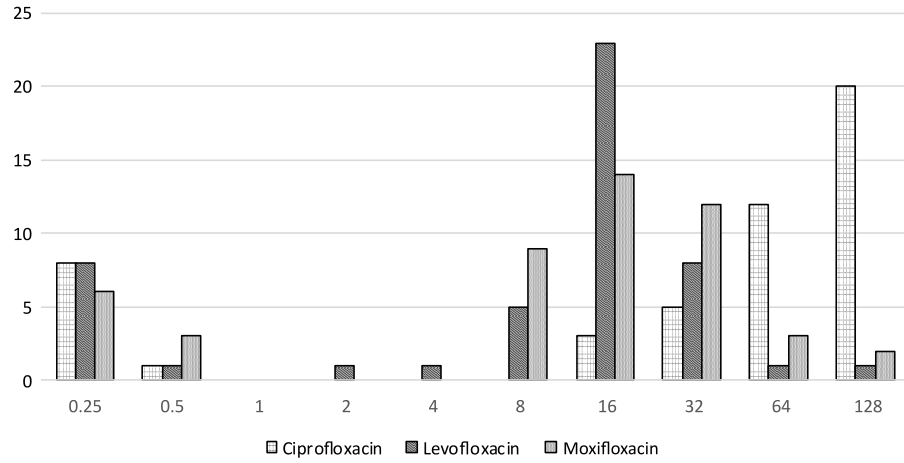

Figure 1. The MIC values (mg/L) of ESBL-producing *E. coli* isolates

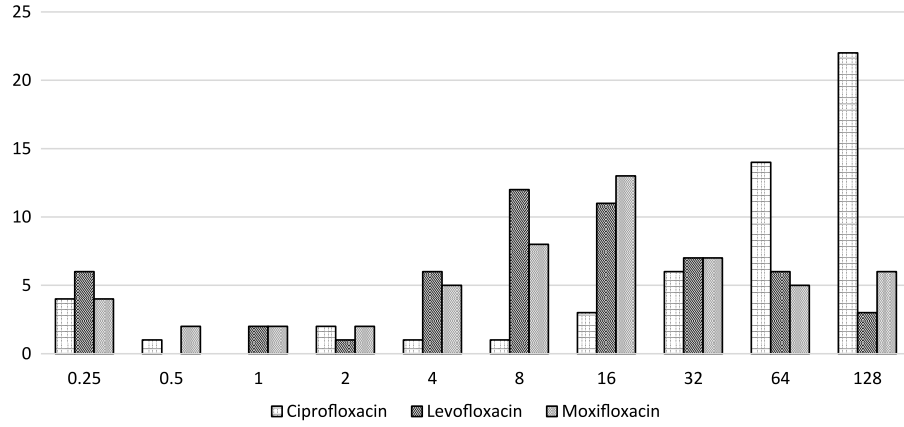

Figure 2. The MIC (mg/L) values of ESBL-producing *Klebsiella* spp.

in Table II. Interestingly, the strain harboring *qnrS* did not contain other PMQR genes.

Among 77 PMQR-positive isolates, 27 (35.1%) and 1 (1.3%) carried two and three different PMQR genes, respectively. Only *Klebsiella* spp. harbored more than one gene. The distribution and coexistence of PMQR genes among the 103 ESBL-producing isolates are shown in Tables III and IV: The distribution of

Table II. Characteristics of *qnrS* harboring strain

Strain	MIC (mg/L)			PMQR genes
	Ciprofloxacin	Levofloxacin	Moxifloxacin	
E. coli 58	>128	>128	>128	*qnrS*
K. pneumoniae 33	>128	>128	>128	*qnrS, oqxA, oqxB*
K. pneumoniae 47	128	32	128	*qnrS*
K. pneumoniae 115	128	8	8	*qnrS*
K. pneumoniae 125	128	64	64	*qnrS*
K. oxytoca 37	<0.25	<0.25	1	*qnrS*

Table III. Distribution of PMQR genes among 103 ESBL producers

Resistance genes	Number (%) of all ESBL producers	Number (%) of ESBL-producing *Klebsiella* spp.
aac(6')-Ib-cr	67 (65%)	38 (70%)
qnrA	0 (0%)	0 (0%)
qnrB	0 (0%)	0 (0%)
qnrC	0 (0%)	0 (0%)
qnrD	0 (0%)	0 (0%)
qnrS	6 (5.8%)	5 (9%)
qepA	0 (0%)	0 (0%)
oqxA	26 (25.2%)	26 (48%)
oqxB	22 (21.4%)	22 (40%)

Table IV. Coexisting PMQR resistance genes among 103 ESBL producers

Coexisting resistance genes	Number (%) of all ESBL producers	Number (%) of ESBL-producing *Klebsiella* spp.
aac(6')-Ib-cr, *oqxA* and *oqxB*	13 (12.6%)	13 (24%)
qnrS, oqxA and *oqxB*	1 (0.97%)	1 (1.8%)
oqxA and *oqxB*	7 (6.8%)	7 (13%)
aac(6')-Ib and *qnrS*	2 (1.9%)	2 (3.8%)
aac(6')-Ib and *oqxA*	3 (2.9%)	3 (5.7%)

ciprofloxacin MIC (mg/L) values of *Klebsiella* spp. harboring no or one or two PMQR genes parallel is shown in Figure 3. There is no correlation between ciprofloxacin MIC values and number of the harbored PMQR genes, as ciprofloxacin MIC values of these strains ranged between 0.25 and 128 mg/L.

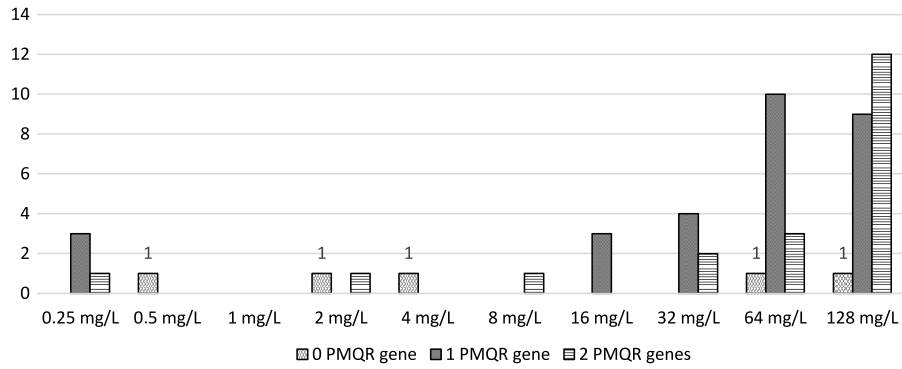

Figure 3. The distribution of ciprofloxacin MIC (mg/L) values of *Klebsiella* spp. harboring no or one or two PMQR genes

Conclusions

This study demonstrated that the most prevalent PMQR gene was *aac (6′)-Ib-cr* (68.8%) followed by *oqxA* (48%), *oqxB* (40%), and *qnrS* (6.4%). Comparing these data to earlier report from Hungary, Szabó et al. [18] showed nearly the same frequency in *qnr* resistance genes among ESBL-producing strains 3% in ESBL-producing *K. pneumoniae* and 1.4% in ESBL-producing *E. coli* isolates. By contrast, *qnrA* gene was not detected in this study, only *qnrS* was observed among bloodculture isolates. On the other hand, *qnrA* has same frequency among urinary tract isolates (unpublished data, personal communication with Dr. Kocsis) compared to earlier published Hungarian data [18]. Frequency of *qnr* genes in *K. pneumoniae* isolates varies in different countries: in China, 65.5% [19]; Malaysia, 48.9% [20] USA, 11.1% [21]; Singapore, 5.2% [22]; Italy, 5.02% [23]; and Brazil, 2.3% [24]. In this study, among *E. coli* and *Klebsiella* spp., no *qnrD* was detected. This is not surprising, as other studies found this resistance determinant mainly in Proteeae tribe (*Proteus* spp., *Morganella* spp., and *Providencia* spp.) of *Enterobacteriaceae* [25, 26].

Interestingly, in this work, the prevalence of *aac(6′)-Ib-cr* increased dramatically from 26.6% to 68.8%, since the first detection in Hungary [18]. Different frequency of *aac(6′)-Ib-cr* among ESBL producers has been reported in several countries, namely, 70.1% in Iran [27] and 16.2% in Spain [28].

OqxAB, as a multidrug efflux pump, confers resistance to quinolone, such as ciprofloxacin, flumequine, norfloxacin, and nalidixic acid, and other antibiotics, such as chloramphenicol and trimethoprim [29]. This study

demonstrated that among ESBL-producing *K. pneumoniae*, a prevalence of 48% and 40% for *oqxA* and *oqxB* genes, respectively, can be seen. Different countries reported various prevalence data: 76.3% for *oqxA* and 74.6% for *oqxB* in Spain [30] and 100% for both genes in China [31]. As in other studies, *qepA* was not present in our ESBL-producing clinical isolates [27, 32].

In this study, there was no correlation between quinolone MIC values and harbored PMQR determinants, as four PMQR harboring strains exhibited susceptible ciprofloxacin MICs, while 45 PMQR-positive strains were resistant to FQs.

In summary, our results of this study reflected considerable frequency of *aac (6′)-Ib-cr* and efflux pump genes among ESBL-positive *K. pneumoniae* clinical isolates in Semmelweis University Clinics. This high frequency of PMQR genes indicated that early detection and routine screening of ESBL-producing *K. pneumoniae* and PMQR carriage seems necessary.

Earlier studies from Hungary demonstrated that diverse fitness cost associated with resistance to FQs allowed the extensive dissemination of major international clones of multiresistant *K. pneumoniae* in healthcare settings [33]. Other data showed that ciprofloxacin MIC values may play a role in dissemination of major *K. pneumoniae* clones [34]. A study demonstrated the need for reduced FQ consumption to prevent the spread of antimicrobial resistant microorganisms among healthcare settings [35].

In addition, it is important to note carbapenemase production of *Enterobacteriaceae*. A study described that among 883 ESBL-positive *Enterobacteriaceae*, 1.6% were carbapenem resistant. For the optimal detection of carbapenem-resistant microorganisms, molecular biological methods are recommended. However, in resource-limited settings, a combination of phenotypic tests can be applied in diagnostic labs [36].

The high prevalence of PMQR determinants should be considered as a wake-up call, because of the latent ability of these genetic platforms, they may recruit novel resistance mechanisms and promote the emergence of multidrug resistant isolates.

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