

PLASMID-MEDIATED QUINOLONE RESISTANCE DETERMINANTS IN ENTEROBACTERIACEAE FROM URINE CLINICAL SAMPLES

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Plasmid-mediated quinolone resistance (PMQR) determinants including, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr*, *oqxAB*, and *qepA*, were investigated in 214 Enterobacteriaceae strains from urine clinical samples. Antimicrobial susceptibility testing for ciprofloxacin, ceftriaxone, and imipenem was performed by broth micro-dilution method. All strains were screened for PMQR genes by PCR. Virulence determinants, namely *afa*, *pap*, *pil*, *sfa/foc*, and *kpsMT* of eight *Escherichia coli* strains proven positive for at least one *qnr* gene, were investigated by PCR. All of the eight investigated strains carried the *pil* gene, showing that P fimbria is a common virulence determinant among *qnr* positive *E. coli*. Out of 214 tested strains, 38 yielded any PMQR determinant, altogether 45 genes were detected namely, 6 *qnrA*, 1 *qnrB*, 2 *qnrD* and 8 *qnrS*, 9 *aac(6′)-Ib-cr*, and 19 *oqxAB*; however, neither *qepA* nor *qnrC* were detected. Notably, 18 *Klebsiella* spp., harbored *oqxAB*, nine *E. coli* were positive for *qnrS* and two *Morganella morganii* yielded *qnrD* resistance determinant. In this study, we demonstrated 17.7% prevalence of PMQR-positive Enterobacteriaceae and first reported *qnrD*-resistance determinant in Hungary. Altogether, 25 PMQR-positive strains were susceptible or low-level resistant to ciprofloxacin with minimum inhibitory concentration (MIC) between 0.06 and 1 mg/L, suggesting that prevalence of PMQR determinants is underestimated and screening among clinical isolates exhibiting reduced susceptibility is necessary. Fluoroquinolone resistance breakpoints of Enterobacteriaceae were revised in 2017 by European Committee of Antimicrobial Susceptibility Testing indicating ciprofloxacin susceptibility only until 0.25 mg/L MIC value.

Keywords: Enterobacteriaceae, quinolone resistance, urinary tract infection

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Introduction

Plasmid-mediated quinolone resistance (PMQR) was first described in 1998 and was named as Qnr determinant [1]. Since then, several lineages of Qnr have been detected as transferable resistance mechanism, namely QnrA, QnrB, QnrC, QnrD, QnrE, QnrS, and QnrVC. To date, numerous alleles of *qnr* genes were identified and listed in www.lahey.com/qnrStudies homepage [2]. Besides Qnr determinants, aminoglycosid-acetyltransferase (6')-Ib-cr was also identified as a plasmid-coded quinolone resistance determinant [3]. Furthermore, QepA and OqxAB efflux pumps also belong to the transferable quinolone resistance mechanisms [4, 5]. Each of the aforementioned resistance determinant confers reduced susceptibility and low-level quinolone resistance in Enterobacteriaceae, which is characterized by ciprofloxacin minimum inhibitory concentrations (MICs) higher than the wild-type phenotype (0.06 mg/L), and reaching the currently accepted resistance breakpoint (0.5 mg/L) by European Committee of Antimicrobial Susceptibility Testing (EUCAST) issued in 2017 [1].

Since the discovery of PMQRs, a worldwide distribution of these determinants has been described in Enterobacteriaceae. The association of PMQRs with beta-lactamases and with various resistance mechanisms was reported from different countries [6]. Several plasmids and mobile genetic elements were described as carriers of PMQR genes and additional resistance determinants. Determinants of *qnrA*, *qnrB*, and *qnrS* are widely disseminated among Enterobacteriaceae. Transferable plasmid coding *qnrB19*, *bla*_{KPC-3}, *bla*_{SHV-11}, *bla*_{TEM-1}, and *aac(6')-Ib* was detected in *Klebsiella pneumoniae* [7]; *qnrS1* and *bla*_{VIM-1} coding conjugative plasmids were described in *Klebsiella oxytoca* [8]; *qnrA1* and *bla*_{VEB} coding transferable plasmids in *Enterobacter cloacae* were detected [9]; plasmids coding for *armA*, *qnrS1*, *aac(6')-Ib-cr*, *bla*_{CTX-M-15}, *bla*_{TEM-1}, and *bla*_{NDM-1} were transferable from *K. pneumoniae* [10]; and conjugative plasmid coding for *qepA*, *rmtB*, and *bla*_{TEM-1} was detected in *Escherichia coli* [11].

In the case of *qnrC*, it was first detected in *Proteus mirabilis* on a transferable plasmid; however, there have been no other reports released about this determinant [12].

First *qnrD* was detected in *Salmonella* spp., but later on, *E. coli*, *P. mirabilis*, *Morganella morganii*, and *Providencia stuartii* were all found to carry this resistance determinant [13–18]. Among clinical isolates, species of *Proteae* tribe commonly carry *qnrD* on small, non-transferable plasmids [16–18]. Most recently, QnrE1 has been detected in *K. pneumoniae*. Studies suggest that *qnrE* was most likely to be located in chromosome of *Enterobacter* spp. and mobilized by ISEcp1 to plasmids of *K. pneumoniae* [19, 20].

Materials and Methods

Strains

A total of 214 non-repetitive Enterobacteriaceae strains were collected between 2013 and 2014 at Semmelweis University, Clinical Microbiology Diagnostic Laboratory from urine clinical isolates, namely 99 *E. coli*, 36 *Proteus* spp., 32 *Klebsiella* spp., 20 *Enterobacter* spp., 15 *Serratia* spp., 6 *Citrobacter* spp., 5 *Morganella* sp., and 1 *P. stuartii*. Identification of strains was performed by matrix-assisted laser desorption ionization time of flight/mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany). Each strain exhibited either susceptible, reduced susceptible, or resistant fluoroquinolone phenotype based on routine diagnostic procedure carried out by disk diffusion method.

Determination of MIC

Ciprofloxacin, ceftriaxone, and imipenem MIC values of strains were determined by broth microdilution method by 96-well microplates and interpreted after EUCAST documents issued in 2016 (www.eucast.org).

PMQR gene detection

Detection of PMQR genes was performed by PCR. DNA preparation was carried out from each tested strain as colonies were incubated at 100 °C for 15 min in a total volume of 0.5 ml ultrapure distilled water (Millipore, Merck KGaA, Darmstadt, Germany) followed by centrifugation of cell suspension at 13,000 rpm on 4 °C. Each PCR mixture contained 1× PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 1.5 mM MgCl₂, 200 mM each deoxynucleotide triphosphate, 20 pmol of each primer, and 1 U of Taq polymerase (Sigma-Aldrich, St. Louis, MO, USA). Primers of this study are listed in Table I. Screening for *qnrA*, *qnrB*, and *qnrS* was carried out by multiplex PCR with specific primer pairs of *qnrA*-, *qnrB*-, and *qnrS* that amplified internal fragments with sizes of 516, 540, and 417 bp, respectively, PCR thermal profile was as follows: 10 min at 95 °C and 32 cycles of amplification consisting of 45 s at 94 °C, 45 s at 53 °C and 1 min at 72 °C, and an additional 10 min at 72 °C for the final extension [21–23]. Simplex PCR for detection of *qnrC* and *qnrD* with specific primers was performed with the following thermal profile 94 °C for 5 min, 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min for 30 cycles; and 72 °C for 10 min, whereas 447 bp of *qnrC* and 550 bp of *qnrD*

Table I. Primers used in the detection of PMQRs

Primer name	Sequence	Reference
qnrA fwd	ATTTCACGCCAGGATTG	[3]
qnrA rev	GATCGGCAAAGGTTAGGTCA	
qnrB fwd	ATGACGCCATTACTGTATAA	[2]
qnrB fwd	GATCGCAATGTGTGAAGTTT	
qnrS fwd	ACGACATTCGTCAACTGCAA	[3]
qnrS rev	TAAATTGGCACCTGTAGGC	
qnrC fwd	GGTTGTACATTTATTGAATC	[12]
qnrC rev	TCCACTTACGAGGTTCT	
qnrD fwd	CGAGATCAATTTACGGGGAATA	[13]
qnrD rev	AACAAGCTGAAGCGCCTG	
qepA fwd	GCA GGT CCA GCAGCG GGT AG	[24]
qepA rev	CTT CCT GCC CGAGTA TCG TG	
oqxA fwd	CTCGGCGCGATGATGCT	[5]
oqxA rev	CCACTCTCACGGGAGACGA	
oqxB fwd	TTCTCCCCGGCGGGAAGTAC	
oqxB rev	CTCGGCCATTTGGCGCGTA	
aac-(6')-Ib fwd	TTGCGATGCTCTATGAGTGGCTA	[25]
aac-(6')-Ib rev	CTCGAATGCCTGGCGTGTIT	

Note: PMQRs: plasmid-mediated quinolone resistances.

were amplified [12, 13]. The *aac(6')-Ib* gene was amplified by PCR with specific primers with following conditions: 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s for 34 cycles to produce a 482-bp product. All *aac(6')-Ib* PCR positive amplicons were further analyzed by digestion with BstF5I (New England Biolabs, Ipswich, MA) to identify *aac(6')-Ib-cr*, which lacks the BstF5I restriction site present in the wild-type gene [24]. A 199-bp fragment of *qepA* was amplified by PCR with *qepA* fwd and rev oligonucleotides with following conditions: initial denaturation at 96 °C for 1 min, followed by 30 cycles of amplification at 96 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. The final extension step was at 72 °C for 5 min [25]. The *oqxAB* resistance determinant was screened by PCR with specific *oqxA* fwd and rev and *oqxB* fwd and rev primers. PCR conditions for *oqxA* were 94 °C for 45 s, 57 °C for 45 s, and 68 °C for 60 s with a cycle number of 34, whereas in the case of *oqxB*, the thermal profile was 94 °C for 45 s, 64 °C for 45 s, and 72 °C for 60 s with a cycle number of 32, whereas 392 bp of *oqxA* and 512 bp of *oqxB* were amplified [5]. DNA fragments were analyzed by electrophoresis in a 1.5% agarose gel (Sigma-Aldrich) at 120 V for 20 min in 1× TAE [40 mM Tris–HCl (pH 8.3), 2 mM acetate, 1 mM EDTA]. Gel was stained by 0.05 mg/L Gelred dye (Biotum) and was evaluated by UV transilluminator.

Detection of virulence determinants of *E. coli*

Tested virulence determinants of *E. coli* were the following: afimbrial adhesins (*afa*), S and F1C fimbriae (*sfa/foc*), pili associated with pyelonephritis (*pap*), K-antigen (*kpsMT*), and P fimbria (*pil*). Detection of virulence determinants of eight *E. coli* strains, previously proven positive for at least one *qnr* gene, was performed by PCR. Colonies of each tested *E. coli* strain were incubated in ultrapure distilled water (Millipore) at 100 °C for 10 min in a total volume of 500 µl followed by centrifugation for 15 min at 13,000 rpm at 4 °C temperature. After centrifugation, 200 ng DNA template of 3 µl of the supernatant was used in PCR along with the following components: 1.25 U Taq DNA polymerase (Sigma-Aldrich), 0.5 µM of each virulence determinant oligonucleotide primer (Table II), 0.2 mM dNTP mix (Sigma), 2.5 mM buffer Mg²⁺ (Sigma), in 50 µl total volume. Amplification was performed with the following protocol: 30 times of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min, followed by an additional elongation at 72 °C for 7 min. Amplicons were investigated by electrophoresis at 120 V for 25 min in a 1.5% agarose gel (Sigma-Aldrich) in a 1× TAE [40 mM Tris–HCl (pH 8.3), 2 mM acetate, and 1 mM EDTA] buffer followed by 15 min of staining in Gelred dye (Biotum) and detected under UV transilluminator.

Results

Of 214 tested strains, 38 carried any of PMQRs alone or in combination and these represent 17.7% prevalence among Enterobacteriaceae. Altogether, 15 strains proved positive to any *qnr* determinant and among them, eight *qnrS*, six

Table II. Primers used in detection of virulence determinants

Primer	Sequence	Reference
<i>afa</i> fwd	5'-GCGGGCAGCAAAGTAAACTCTC-3'	[26]
<i>afa</i> rev	5'-CATCAAGCTGTTTGTCTCGCCCG-3'	
<i>sfa/foc</i> fwd	5'-CTCCGGAGAACTGGGTGCATCTTAC-3'	
<i>sfa/foc</i> rev	5'-CGGAGGAGTAAATTACAAACCTGGCA-3'	
<i>pap</i> fwd	5'-GCAACAGCAACGCTGTTGCATCAT-3'	
<i>pap</i> rev	5'-AGAGAGAGCCACTCTTATACGGACA-3'	
<i>kpsMT</i> fwd	5'-CCATCGATACGATCATTGCACG-3'	[27]
<i>kpsMT</i> rev	5'-ATTGCAAGGTAGTTCAGACTCA-3'	
<i>pil</i> fwd	5'-CATTGCGCTGTAACCCGCC-3'	[28]
<i>pil</i> rev	5'-ATAACACGCCGCCATAAGCC-3'	

Note: *afa*: afimbrial adhesions; *pap*: pili associated with pyelonephritis; *pil*: P fimbria; *sfa/foc*: S and F1C fimbriae; *kpsMT*: K-antigen.

qnrA, two *qnrD*, and a single *qnrB* were detected. Two *E. coli* strains carried both *qnrA* and *qnrS* determinants, although their ciprofloxacin MIC values were still at the wild-type phenotype (0.06 mg/L). Two *M. morganii* strains out of five were positive for *qnrD*, whereas on the other hand, four *P. mirabilis* strains carried a *qnrA* determinant. Altogether, 19 strains (18 *K. pneumoniae* and an *Enterobacter aerogenes*) were positive for both *oqxA* and *oqxB* coding gene of efflux pump. Moreover, 20 strains (13 *E. coli*, 3 *Proteus mirabilis*, 3 *Klebsiella* spp., and 1 *M. morganii*) carried only *oqxA*, while 5 strains were positive only for *oqxB* (four *Enterobacter* spp. and a *K. oxytoca*). In contrast, neither *qepA* nor *qnrC* resistance determinants were found in this study. Among 23 *aac(6)-Ib*, PCR positive strains BstF5I restriction enzyme analysis detected nine *aac(6)-Ib-cr* variant, and these were carried by *E. coli*, *M. morganii*, *Klebsiella* spp., and *Enterobacter* spp. (Table III).

From 38 PMQR positive strains, 25 were under the ciprofloxacin resistance breakpoint of EUCAST issued in 2016 namely, 9 exhibited intermediate-resistant phenotype (MIC 0.5–1 mg/L), 13 were still wild-type (0.06 mg/L), and 3 strains had 0.125 mg/L ciprofloxacin MIC value (Figure 1), but almost all of them were susceptible to ceftriaxone except one *E. coli* and two *K. pneumoniae*. Regarding imipenem MICs, all were found susceptible, only low-level imipenem resistance was common in all *Proteus* spp. and *M. morganii* strains (Table III).

Investigation of virulence determinants showed that *pil* gene, coding the P fimbria virulence determinant was commonly carried by each tested strain. Other virulence factors varied among tested strains (Table IV).

Discussion

Our investigation found various PMQR determinants in a collection of 214 non-repetitive Enterobacteriaceae strains. Of 38 PMQR positive strains, 9 showed intermediate fluoroquinolone resistance phenotype, 13 were still wild-type, and 3 had 0.125 mg/L ciprofloxacin MIC values, and all these 25 strains were still interpreted as susceptible after the EUCAST documents issued in 2016. EUCAST breakpoints in 2016 considered Enterobacteriaceae strains with MIC values under 0.5 mg/L as “susceptible,” on the other hand, MIC over 1 mg/L as “resistant,” and all in between as “intermediate” phenotype. Wild-type fluoroquinolone phenotype was MIC value ≤ 0.06 mg/L. However, from January 1, 2017, these data were revised and declared 0.25 mg/L MIC value as the breakpoint of susceptibility, while resistance was set at 0.5 mg/L. This change correlates well with our findings, as it emphasizes the possibility of the selection of resistant strains at 0.5 mg/L MIC value, which may occur due to presence of PMQRs.

Table III. Plasmid-mediated quinolone resistance (PMQR) determinant positive strains, with ciprofloxacin, ceftriaxone, and imipenem MIC values

Number	Strain	PMQRs	Ciprofloxacin	Ceftriaxone	Imipenem
1	<i>E. coli</i> 199	<i>qnrB</i>	1.0	0.06	0.25
2	<i>E. coli</i> 193	<i>qnrS</i>	0.5	0.06	0.25
3	<i>E. coli</i> 184	<i>qnrS</i>	0.06	0.06	0.5
4	<i>E. coli</i> 180	<i>qnrS</i>	0.125	0.06	0.25
5	<i>E. coli</i> 178	<i>qnrS</i>	2.0	0.06	0.25
6	<i>E. coli</i> 177	<i>qnrS</i>	0.06	0.06	0.25
7	<i>E. coli</i> 175	<i>qnrS, aac(6)-Ib-cr</i>	32	128	0.25
8	<i>E. coli</i> 15	<i>qnrA, qnrS</i>	0.06	0.06	0.125
9	<i>E. coli</i> 38	<i>qnrA, qnrS</i>	0.06	0.06	0.125
10	<i>M. morgani</i> 10	<i>qnrD</i>	2.0	0.06	4.0
11	<i>M. morgani</i> 71	<i>qnrD, aac(6)-Ib-cr</i>	2.0	0.06	4.0
12	<i>P. mirabilis</i> 6	<i>qnrA</i>	4.0	0.06	2.0
13	<i>P. mirabilis</i> 7	<i>qnrA</i>	2.0	0.06	4.0
14	<i>P. mirabilis</i> 40	<i>qnrA</i>	4.0	0.06	4.0
15	<i>P. mirabilis</i> 42	<i>qnrA</i>	0.06	0.06	4.0
16	<i>E. aerogenes</i>	<i>oqxAB</i>	0.125	0.06	0.25
17	<i>K. pneumoniae</i>	<i>oqxAB</i>	8.0	0.25	0.25
18	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.06	0.5	0.25
19	<i>K. pneumoniae</i>	<i>oqxAB</i>	128	0.06	0.5
20	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.06	0.06	0.125
21	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.06	0.06	0.125
22	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.5	0.06	0.125
23	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.06	0.06	0.125
24	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.06	128	0.25
25	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.125	0.5	0.25
26	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.06	0.25	0.25
27	<i>K. pneumoniae</i>	<i>oqxAB</i>	1.0	1.0	0.25
28	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.06	0.06	0.25
29	<i>K. pneumoniae</i>	<i>oqxAB</i>	16	0.125	0.25
30	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.06	0.25	0.125
31	<i>K. pneumoniae</i>	<i>oqxAB</i>	0.5	1.0	0.125
32	<i>K. pneumoniae</i>	<i>oqxAB, aac(6)-Ib-cr</i>	1.0	128	0.25
33	<i>K. pneumoniae</i>	<i>oqxAB, aac(6)-Ib-cr</i>	2.0	0.5	0.25
34	<i>K. pneumoniae</i>	<i>oqxAB, aac(6)-Ib-cr</i>	4.0	0.25	0.5
35	<i>K. oxytoca</i>	<i>aac(6)-Ib-cr</i>	32	1.0	0.25
36	<i>K. pneumoniae</i>	<i>aac(6)-Ib-cr</i>	1.0	0.06	0.25
37	<i>E. kobei</i>	<i>aac(6)-Ib-cr</i>	1.0	0.125	0.5
38	<i>E. cloacae</i>	<i>aac(6)-Ib-cr</i>	1.0	0.25	0.25

Note: All MIC values are in (mg/L).

Furthermore, it should also be noted that numerous strains carry PMQRs at the wild-type fluoroquinolone phenotype with ciprofloxacin MIC 0.06 mg/L, in these cases, PMQR determinants remain latent that can be explained by lack of gene expression.

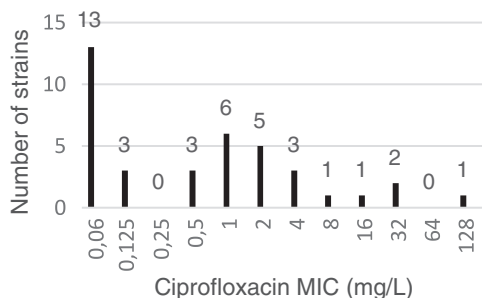


Figure 1. Ciprofloxacin minimum inhibitory concentration (MIC) distribution of 38 PMQR positive strains

Table IV. Distribution of virulence determinants in the tested *E. coli* strains

Strain	afa	pap	pil	sfa/foc	kpsMT
<i>E. coli</i> 15	-	-	+	-	-
<i>E. coli</i> 38	-	+	+	+	+
<i>E. coli</i> 177	-	+	+	+	+
<i>E. coli</i> 178	-	-	+	-	+
<i>E. coli</i> 180	+	-	+	-	+
<i>E. coli</i> 184	-	-	+	+	+
<i>E. coli</i> 193	-	-	+	-	+
<i>E. coli</i> 199	-	+	+	+	+

Note: afa: afimbrial adhesions; pap: pili associated with pyelonephritis; pil: P fimbria; sfa/foc: S and F1C fimbriae; kpsMT: K-antigen.

Our data confirm that ciprofloxacin MIC values alone are not enough to detect resistance; therefore, detection of PMQRs, most importantly *qnr* genes, is to be conducted by molecular biological methods. According to this study, more thorough examination could be considered in case of strains exhibiting reduced susceptibility and intermediate phenotype. In these cases, routine check on presence of PMQRs could be conducted to evaluate possibility of selection of resistant strains. This study found 17.7% prevalence of PMQR-positive Enterobacteriaceae from urine clinical sample and this is the first report of *qnrD* resistance determinant in Hungary. Earlier, investigation from Hungary showed extended-spectrum beta-lactamase (ESBL)-producing *E. coli* and *Klebsiella* spp., with *qnrA*, *qnrB*, *qnrS*, and *aac(6)-Ib-cr* [29, 30]. Although we did not investigate the correlation of PMQRs in ESBL-producing strains, but based on our results, we can say that there is no strong correlation, as we found a total 38 strains being PMQR positive, but among them, only three showed resistance to third generation of cefalosporin.

According to international data, the prevalence of PMQR genes, *qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*, and *qepA* is associated with ESBLs, but actual prevalence of *qnrC* is unknown; however, some data are released about *qnrD* [6, 17, 30].

In this study, we report the first detection of *qnrD* determinant in Hungary, which was found in two *M. morganii* strains that represent around 1% prevalence in Enterobacteriaceae and 40% of *M. morganii*. These data are in good correlation with international data, which demonstrated *qnrD* determinant in clinical isolates among species of Proteaceae tribe (*Proteus* spp., *Providencia* spp., and *M. morganii*) [16–18]. Our results show that P fimbria is a common virulence determinant among *qnr*-positive *E. coli* causing urinary tract infections.

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Conflict of Interest

The authors declare no competing interests.

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