

TRANSFERABLE FLUOROQUINOLONE RESISTANCE IN *ENTEROBACTERIACEAE* AND *PSEUDOMONAS AERUGINOSA* ISOLATED FROM HEMOCULTURES

SHORT COMMUNICATION

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SUMMARY

Background: The main mechanisms causing high-level resistance to fluoroquinolones (FQ) are encoded chromosomally; that includes mutations in genes coding DNA-gyrase, but overexpression of efflux pumps contributes to increased minimum inhibitory concentration (MIC) of FQ as well. However, genes responsible for FQ-resistance may be harboured in transferable/conjugative plasmids. For some time, there was an assumption that resistance to FQ cannot be transferable in conjugation due to their synthetic origin, until 1998, when plasmid-mediated resistance transmission in *Klebsiella pneumoniae* was proved. We aimed to detect the occurrence of transferable FQ-resistance among Gram-negative bacteria isolated from patients in Czech and Slovak hospitals.

Methods: In this study, we tested 236 clinical isolates of Gram-negative bacteria for transferable resistance. Among relevant isolates we performed PCR detection of transferable fluoroquinolone genes (*qnr*).

Results: We have observed transfer of determinants of cephalosporin-resistance, aminoglycoside resistance as well as FQ-resistance (in 10 cases; 4.24%) not only intra-species but inter-species too. The presence of *qnr* gene was detected in two isolates of forty tested (5%). We have also observed that determinants of cephalosporin-resistance and aminoglycoside-resistance were linked to those of FQ-resistance and were transferred en block in conjugation.

Conclusion: We have proved that resistance to fluoroquinolones can be transferred horizontally via conjugation among Gram-negative bacteria of different species and is associated with resistance to other antibiotics.

Key words: fluoroquinolones, resistance, transferable-fluoroquinolone resistance, conjugation, Gram-negative bacteria

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INTRODUCTION

As FQ are antibiotics of synthetic origin, it was predicted that resistance to this group would not evolve and would not be transmissible. Soon it was demonstrated that this assumption was incorrect and in 1997–1998 horizontally transferred resistance to FQ was proved by Gómez-Gómez et al. (1997) and Martínez-Martínez et al. (1998) (1, 2). Since that time, numerous reports of plasmid-mediated FQ-resistance acquisition in different bacterial species were published (3–6). Generally, plasmid-mediated FQ resistance confers only to low-level resistance, as the main chromosomally encoded mechanism – modification of DNA gyrase – is the reason for high-level resistance. Transferable determinants of FQ resistance are represented by *qnr* genes (coding Qnr proteins), *aac(6')-Ib-cr*, *qepA* and *oqxAB*.

Qnr proteins, belonging to pentapeptide-repetitive proteins group, were the firstly discovered transferable elements causing FQ-resistance (2). As yet, five different *qnr* genes were discovered in *Enterobacteriaceae*, those being plasmid-located (3, 7) and

also QnrVC-like protein was revealed in *Vibrio cholerae*, which is not located on plasmid (8).

Discovery of FQ-resistance mediated by *aac(6')-Ib-cr* enzyme was rather surprising, seeing that normally *aac(6')* is responsible for resistance to aminoglycosides (9). The gene *aac(6')-Ib-cr* is only slightly different from *aac(6')-Ib* (2 mutations) and it has acquired ability of ciprofloxacin acetylation. It should be mentioned, that *aac(6')-Ib-cr* contributes only to small increase in MIC levels (5).

Another transferable resistance mechanism is active efflux carried out by efflux pumps QepA and OqxAB (10). QepA pump has a significant structural similarity to transmembrane subunits of MFS (major facilitator superfamily) pumps and it leads to decreased susceptibility to hydrophilic FQ. OqxAB, consisting of two genes *oqxA* and *oqxB*, is localised in one operon. OqxAB belongs to RND (resistance nodulation division) group of pumps and it causes resistance not only to FQ, but also to other agents (10, 11).

The aim of this study was to assess the occurrence of transferable FQ-resistance among Gram-negative bacteria isolated in Slovakia and the Czech Republic.

MATERIAL AND METHODS

Microorganisms

In our study, multiresistant clinical isolates of Gram-negative bacteria isolated from blood of septic patients with positive hemoculture sampled from hospitals in Bratislava, Slovakia, during the period of 2 months (November 2011 – December 2011) were included as well as isolates from the Regional Hospital in Píbram, Czech Republic, collected during the period of 3 years (January 2009 – December 2011). One hundred and ninety-six Czech and forty Slovak samples, respectively, were obtained and included for transfer screening.

Susceptibility Testing

Antibiotic susceptibility to ampicillin, piperacillin, cefuroxime, cefoperazone, ceftazidime, aztreonam, gentamicin, amikacin, tetracycline, trimethoprim/sulfamethoxazole, ampicillin/sulbactam, piperacillin/tazobactam, cefotaxime, cefoperazone/sulbactam, cefepime, meropenem, tobramycin, ciprofloxacin, colimycin, and tigecycline was determined using disc diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (12, 13).

Transferability/Conjugation Testing

Transferability of resistance-determinants was assessed by conjugation by phenotypic method. Strains of rifampin-resistant *Escherichia coli* 3110, rifampin-resistant *Proteus mirabilis* P38, rifampin-resistant *Pseudomonas aeruginosa* 1008, and rifampin-resistant *P. aeruginosa* 1670 were used as recipient strains. *E. coli* strain ATCC 25922 was used as a control strain. Donor strains were incubated overnight in Nutrient broth no. 2 (Biolife Italiana) at 37°C to reach logarithmic phase. After incubation, 1 ml of donor culture was mixed with 1 ml of competent recipient strain culture and cultivated for 20 hours at 37°C. Commixture of donors and recipients was then put on bi-antibiotic plates containing selective antibiotic (rifampin) and one of the following antibiotics: cefotaxime, ceftazidime, cefepime, cefoperazone, meropenem, and ofloxacin (5 µg/mL), which were monitored for transferability of their resistance determinants. After 20 hours of cultivation at 37°C, positivity or negativity of transfer was assessed. Control experiments, with recipient strains and donor strains cultivated alone under the same condition as mixed cultures, were run in parallel to exclude recombinants selection by mutation. Transconjugants, which were proved to acquire at least one resistance determinant, were then analyzed to assess which genes are in connection, with following protocol. Single isolated colony of transconjugants was incubated in Nutrient broth no. 2 (Biolife, Italy) overnight at 37°C and then applied on antibiotic plates according to their original resistance profile. After incubation, positive results were evaluated as a growth on the row of antibiotic plates indicating which resistance determinants are linked to each other and transferred during conjugation together.

Detection of *qnr* Genes

In isolates proved to be able to transfer resistance determinants we performed simplex PCR to detect presence of *qnr*

gene as previously described by Jacoby and colleagues (14). DNA was isolated using spin-column Bacterial DNA Extraction Kit (Ecoli, Bratislava, Slovakia) according to manufacturer's instructions (15). Isolated DNA (2 µl) was used as a template in PCR reaction (50 µl). Master mix (Solis BioDyne, Tartu, Estonia) consisted of 10X buffer BD (0.8 M Tris-HCl, 0.2 M (NH₄)₂SO₄), 25 mM MgCl₂ (to final concentration of 2.5 mM), 20 mM deoxyribonucleotide triphosphates mix (dNTPs to final concentration of 200 µM), forward and reverse primers (0.3 µM of each), DNA polymerase (5U), and ultrapure water for PCR. We used oligonucleotide primers to amplify the *qnr* gene as following: forward 5'-GATAAAGTTTTTCAGCAA-GAGG; and reverse 5'-ATCCAGATCGGCAAAGGTTA. Reaction was run in thermal cycler under the following conditions: initial denaturation for 10 min at 95°C, then 35 cycles of amplification consisting of 1 min at 95°C, 1 min at 54°C and 1 min at 72°C; and 10 min at 72°C for the final extension.

Products were subjected to gel electrophoresis in 2% agarose gel (Serva, Heidelberg, Germany), stained with UltraPower (Bio-Teke, Beijing, China) and the presence of 593 bp product was considered as positive.

RESULTS

Out of 236 clinical isolates obtained, 10 (4.24%) belonging to species *E. coli*, *P. mirabilis*, *Ps. aeruginosa*, *K. pneumoniae*, and *Providencia stuartii* were noticed to be able to transfer their FQ-resistance determinants. During the previous months, we have noticed slowly increasing prevalence of FQ-resistance transferability among clinical isolates (1 case in 2009, 2 cases in 2010, 3 cases in 2011), which altogether made up for occurrence of 6 cases among 196 isolates tested (3.06%) until November 2011. In the last two months of the year 2011 (November – December), the occurrence increased to 10% (4 positives among 40 tested). Details of transfer, genetic analysis and characteristics of transfer-positive strains isolated in 2011 are summarized in Table 1.

Ciprofloxacin minimum inhibitory concentration (MIC) in the transfer-positive isolates ranged from 1 mg/l to > 4 mg/l.

Among 40 isolates collected during the last two months of 2011, we detected the *qnr* gene to be present in two of them (5%).

Resistance to first-generation quinolones (nalidixic acid) was recorded in 140 isolates of 194 tested (72.16%; please note, that *Pseudomonadaceae* were not tested), and high-level resistance (thus to second-generation quinolones, ofloxacin and ciprofloxacin) was recorded in 121 isolates (51.27%).

Ceftazidime and cefepime resistances were the most frequently transferred resistotypes. After the genetic analysis, we found out that ofloxacin-resistance determinants were linked to determinants of cefotaxime, ceftazidime, cefepime, and cefoperazone resistance in 4 isolates, but also association with kanamycin and ticarcillin-resistance determinants was observed. Noteworthy, donor strains were able to transfer their determinants not only to recipients of the same genus, but also inter-species as follows: *P. mirabilis* to *E. coli* and vice versa; and *Ps. aeruginosa* to *P. mirabilis*.

Table 1. Transfer patterns of tested isolates

Isolate characteristics (species/internal number/ origin)	Ciprofloxacin MIC	Recipient strain	Primary transferable resistance	Gene linkage analysis – cotransfer
<i>E. coli</i> /28/hemoculture	>4 mg/l	<i>P. mirabilis</i> P38	Cefotaxim	Cefotaxime + cetazidime + cefoperazone + cefepime + ofloxacin
<i>E. coli</i> /29/hemoculture	>4 mg/l	<i>P. mirabilis</i> P38	Cefoperazone	Cefotaxime + cetazidime + cefoperazone + cefepime + ofloxacin
<i>E. cloacae</i> /30/hemoculture	1 mg/l	<i>E. coli</i> 3110	Ceftazidime	Cefotaxime + ceftazidime + cefoperazone
		<i>P. mirabilis</i> P38	Ceftazidime + cefoperazone	Cefotaxime + ceftazidime + cefoperazone
<i>P. mirabilis</i> /33/hemoculture	4 mg/l	<i>E. coli</i> 3110	Ceftazidime	Ceftazidime + cefoperazone
		<i>P. mirabilis</i> P38	Cefoperazone	Cefotaxime + cetazidime + cefoperazone + cefepime + ofloxacin
<i>Ps. aeruginosa</i> /36/hemoculture	>4 mg/l	<i>P. mirabilis</i> P38	Cefepime	Cefotaxime + cetazidime + cefoperazone + cefepime + ofloxacin

MIC – minimum inhibitory concentration

Table 2. Resistance of *E. coli* to FQ in selected countries of Central Europe (Czech Republic, Austria, Slovakia, Hungary, and Poland). Comparison of ten years data from 2002 and 2011 retrieved from EARS-Net Database

	2002			2011		
	Number of isolates	R	%	Number of isolates	R	%
Austria	472	48	10.17	3,162	704	22.26
Czech Republic	1,586	165	10.40	2,682	630	23.49
Hungary	328	34	10.37	1,213	378	31.16
Poland	133	14	10.53	1,141	312	27.34
Slovakia	214	31	14.49	737	309	41.3
Total	2,33	292	10.8	8,35	2,33	26.1

R – resistant isolates

DISCUSSION

Transferable plasmid-mediated FQ-resistance is an issue of public health concern, whereas such mechanism was long waived as unlikely. Since discovery of plasmid mediated FQ-resistance several studies have been published on this topic. The level of resistance of Enterobacteria and *P. aeruginosa* to FQ is alarming and significantly increasing in Europe including the Central European Region (Table 2). Increase from 10.68% to 26.11% was noticed among *E. coli* by the European Centre for Disease Prevention and Control (ECDC) in that area (16). Considerably high FQ resistance among *E. coli*, *K. pneumoniae* and *Ps. aeruginosa* is reported by ECDC in Slovakia (data from 2011), reaching 41.9%, 70.6% and 58.7%, respectively (17). We can assume that such a high rate of FQ resistance may be associated with their frequent use in clinical practice, as Slovakia belongs to the ‘top 10’ European countries with the highest outpatient use of quinolones (17).

The presented experiments have showed that ofloxacin-resistance can be horizontally transferred during conjugation in Gram-negative bacteria, which is in contrast to previous findings of Kim et al., who did not proved either direct transfer or cotrans-

fer. Therefore, they suggested location of *oqxAB* on nonconjugative plasmid of *E. cloacae* and *K. pneumoniae* (18). We found transfer of ofloxacin resistance determinant during conjugation in 10 isolates, thus plasmid-typing would be advisable.

To our best knowledge, this is the first study to monitor the occurrence of *qnr* genes in Slovak clinical samples. We detected the *qnr* gene in 2 isolates (5%), which indicates that it is not extremely frequent. Thus, other transferable and chromosomally encoded mechanisms are probably contributing to the phenotype of FQ-resistance. Similar results were found in other European countries, e.g. in Poland where the prevalence of *qnr* was 8.3% (19) or in Italy where the prevalence was 5.02% (20). However, our study is biased by relative small range; therefore we suppose further studies to be performed on this issue.

Another finding revealed by our experiments was the fact of inter-species transferability of FQ-resistance determinants. *P. mirabilis* donor was able to transfer FQ resistance not only to recipient strains of *P. mirabilis* but also to recipients *E. coli*, and vice-versa (*E. coli* donors to *P. mirabilis* recipients), which was similar in *Ps. aeruginosa* transferring its determinants to *Proteus sp.*

We also found ofloxacin-resistance determinants being closely linked with those of cephalosporin-resistance and with aminoglycoside-resistance, probably located on the same plasmid, suggesting association between those types of resistances. That is in agreement with findings of Sahn et al., who revealed association of FQ and ceftazidime resistance in *Enterobacteriaceae* and *Ps. aeruginosa* (21).

Briales et al. found that *qnr* and *aac(6′)-Ib-cr* genes, responsible for FQ resistance, were located on plasmid and transferable as well as their association with ESBL production (22). Such results are consistent with our ones presented above. Interestingly, as the others (22, 23), we have also noticed an increase of occurrence of transferable FQ-resistance compared to the previous period (January 2009 – November 2011) from 3.06% prevalence to 10% in the last 2 months of 2011.

CONCLUSIONS

We have showed that resistance to fluoroquinolones can be transferred horizontally via conjugation among Gram-negative bacteria of different species and is associated with resistance to different antibiotic classes – cephalosporins and aminoglycosides. Increasing FQ-resistance is a public health problem considering their wide use on one side, and spreading resistance by plasmids on the other. This emphasizes the need of surveillance of FQ-resistance which should be accompanied by analysis of its transferability and its possible association with other resistance patterns should be kept in investigators' mind. Informing clinicians and keeping them up-to-date about the potential risk of excessive FQ use in practice is of great importance, too.

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Conflict of Interests

None declared

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