Emergence of Enterobacteriaceae co-producing CTX-M-15, ArmA and PMQR in Poland

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Conflict of interest

None declared

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Abstract

Background. Plasmid-mediated extended-spectrum β -lactamases (ESBLs), 16S rRNA methylases and quinolone resistance mechanisms (PMQRs) are well-known agents conferring resistance to more than 1 antimicrobial in its group. The accumulation of these agents poses, therefore, a serious risk to public health.

Objectives. The objective of this study was to investigate the presence of common β -lactamases and 16S rRNA methylases in Qnr-producing Enterobacteriaceae and their genetic relatedness.

Material and methods. We examined 18 Qnr-producing isolates (*Klebsiella pneumoniae* n = 8, *Enterobacter cloacae* n = 6 and *Escherichia coli* n = 4) selected from a collection of 215 ciprofloxacin-resistant strains obtained from patients in a 1030-bed tertiary hospital from 1 March to 31 August 2010. The antibiotics minimum inhibitory concentration (MIC) was determined using E-test. The detection of common β -lactamases, 16S rRNA methyltransferases and PMQR genes was performed using polymerase chain reaction (PCR) and sequencing. Genetic relatedness was assessed with pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

Results. All the isolates tested were susceptible to carbapenems and colistin, while 16 were multidrug-resistant. Thirteen, 2 and 2 isolates carried *qnrB1*, *qnrA1* and *qnrS1*, respectively. Ten of 13 *qnrB1*-positive Enterobacteriaceae also carried genes encoding for *aac(6')-Ib-cr* and at least 1 ESBL. The *bla*_{CTX-M-15} gene was the most common ESBL. The most prevalent combination of genes was *qnrB1*+*aac(6')-Ib-cr*+*bla*_{TEM-1}+*bla*_{CTX-M-15}. Two isolates of *K. pneumoniae* and *E. cloacae* were found to bear multiple extended range resistance traits: ArmA, CTX-M-15, QnrB1, and AAC (6')-Ib-cr. The PFGE showed that most of the isolates exhibited individual DNA patterns, whilst MLST assigned *K. pneumoniae* (n = 8) to 5 sequence types (STs) (ST15, ST323, ST336, ST147, and ST525), *E. coli* (n = 4) to 2 (ST131 and ST1431) and *E. cloacae* (n = 5) to 4 (ST90, ST89, ST133, and the novel ST407).

Conclusions. Our findings reveal the accumulation of resistance traits and their important role in spreading of multiresistant bacteria among hospitalized patients.

Key words: PMQR, ESBLs, MDR, Enterobacteriaceae, Poland

Introduction

Currently, the global spread of multidrug resistant (MDR) bacteria is considered a major public health concern. In the last decade, we have witnessed a dramatic increase in the number of MDR Enterobacteriaceae. Data from the European Antimicrobial Resistance Surveillance Network (EARS-Net)¹ indicates that a majority of the *Escherichia coli* and *Klebsiella pneumoniae* had combined resistance to third generation cephalosporins, aminoglycosides and fluoroquinolones. Furthermore, the combined resistance has increased significantly also in Poland since 2010, especially for *K. pneumoniae*, with levels ranging from 23% to 52.5% in 2013.¹ However, data on molecular characteristics of MDR Enterobacteriaceae simultaneously resistant to the aforementioned antimicrobial agents is still insufficient in Poland.

Because of their broad-spectrum antimicrobial activity, fluoroquinolones are commonly used agents in clinical and veterinary medicine to treat a wide variety of microbiological infections. The stepwise mutations in gyrase and topoisomerase IV subunits are recognized as the most common mechanism of quinolone resistance in Enterobacteriaceae. Moreover, plasmid-mediated quinolone resistance (PMQR) determinants were also reported in MDR strains as contributing to the increase of fluoroquinolones resistance.² We previously³ described the coexistence of PMQR determinants (*qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*) and mutations in *gyrA* and *parC* among fluoroquinolone-resistant clinical Enterobacteriaceae.³

It is worth noting that previous studies have shown the association of PMQR-encoding genes with other antibiotic resistance genes, such as extended-spectrum β -lactamases (ESBL), including CTX-M-15 or plasmid-mediated AmpC genes.^{2,4–6} In addition, PMQRs may also be co-transferred with plasmid-mediated 16S rRNA methylase-encoding genes.⁷ However, data on coexistence of PMQRs with the other resistance traits in Enterobacteriaceae collected in Poland is lacking.

In the present study, we investigated the presence of ESBLs, pAmpC and 16S rRNA methylases in Qnr-producing Enterobacteriaceae from clinical sites in Poland, and determined their genetic relatedness. We described the Qnr-producing Enterobacteriaceae strain with the accumulation of plasmid-mediated, broad range resistance traits: ArmA, CTX-M-15, QnrB1, and AAC (6')-Ib-cr that, to the best of our knowledge, has not been yet reported from Poland.

Material and methods

Study population

A retrospective study was conducted of 18 Qnr-producing Enterobacteriaceae (*K. pneumoniae* n = 8, *Enterobacter cloacae* n = 6 and *E. coli* n = 4) found among the previously

described 215 fluoroquinolone-resistant clinical isolates collected from 2017 inpatients in a 1030-bed tertiary hospital in a period from 1 March to 31 August 2010.³

Phenotypic and genotypic investigation of resistance

Minimum inhibitory concentrations (MICs) to 11 antibiotics were determined using E-test Strips (bioMérieux, Marcy-l'Etoile, France) and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (<http://eu-cast.org>). For the Qnr-producing isolates, amplification of genes encoding β -lactamases of the most common families was performed with primers for *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}.⁸ The multiplex polymerase chain reaction (PCR) with family-specific primers which detected the plasmid-mediated AmpC genes *bla*_{MOX}, *bla*_{FOX}, *bla*_{EBC}, *bla*_{ACC}, *bla*_{DHA}, and *bla*_{CIT} was performed as described previously.⁹ The characterization of 16S rRNA methyltransferase genes (*armA*, *rmtA*, *rmtB*, and *rmtC*) was done in isolates with resistance to amikacin with PCR using primers as described previously.¹⁰ Furthermore, the DNA sequencing of genes encoding for PMQRs, the selected β -lactamases and 16S rRNA methyltransferase ArmA was performed. The nucleotide sequences of the PCR products were determined using conventional Sanger method. All the DNA sequences were compared with sequences in the GenBank database using the BLAST algorithm (<http://www.blast.ncbi.nlm.nih.gov>).

Molecular typing

The genetic relatedness of tested isolates was studied by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The PFGE was conducted using *Xba*I endonuclease. The PFGE patterns were analyzed using BioNumerics software v. 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Sequence types (STs) were determined using 7 highly conserved housekeeping genes according to appropriate MLST schemes.^{11–13} The MLST Databases available at <http://mlst.warwick.ac.uk/mlst/dbs/Ecolia>; <http://pubmlst.org/ecloacae/> and <http://bigsdweb.pasteur.fr> were used for assigning each sequence type.

Plasmid isolation and electrophoresis

The plasmid DNAs from Qnr-producing isolates was extracted using the NucleoBond PC20 kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. Plasmid size was determined with extraction and electrophoresis using a 0.8% agarose gel (Prona Plus, Burgos, Spain) in Tris-borate-EDTA (TBE) buffer. *Klebsiella pneumoniae* DM0269 containing 50 kb, 90 kb and 180 kb, respectively, were used as molecular weight plasmid calculation.¹⁴

Results

Multidrug resistance and genotyping of PMQR-producing isolates

The majority of tested isolates were MDR, with resistance to antimicrobial agents showed in Table 1. Additional resistance was found to the following: amoxicillin and clavulanic acid (94%; R >8 mg/L), cefotaxime (72%; R >2 mg/L), ceftazidime (72%; R >4 mg/L), gentamicin (67%; R >4 mg/L), ciprofloxacin (100%; R >1 mg/L), trimethoprim/sulfamethoxazole (78%; R >4 mg/L), and chloramphenicol (72%; R >8 mg/L). All isolates were susceptible to carbapenems and colistin.

Sequencing analysis showed that *qnrB1*, *qnrA1* and *qnrS1* were found in 13, 2 and 2 isolates, respectively. Ten of 13 *qnrB1*-positive Enterobacteriaceae also carried genes encoding for *aac(6')-Ib-cr* and at least 1 ESBL (Fig. 1). Among them, the most prevalent combination was *qnrB1+aac(6')-Ib-cr+bla_{TEM-1}+bla_{CTX-M-15}*, which was detected in 6 isolates, and the *bla_{CTX-M-15}* gene was the most common in all the *QnrB1*-producing isolates (61%). Furthermore, 2 *QnrB1*- and *CTX-M-15*-producing isolates (No. 088 and No. 216) possessed additionally the *armA* gene coding for 16S rRNA methyltransferase ArmA. In contrast, *pAmpC* genes were not detected in any of isolates tested.

All the tested isolates were distinguished by the PFGE with similarity ranging from 50% to 92% (Fig. 1). Four clonal groups were distinguished, each encompassing strains

with the PFGE profiles similarity ≥80%. Two clonal groups (A and B) were found in *K. pneumoniae* and single clonal groups (C and D) occurred in *E. coli* and *E. cloacae*, respectively. By the multilocus sequence typing (MLST), 5 STs were identified for *K. pneumoniae*: ST15, ST323, ST336, ST147, and ST525, 2 for *E. coli*: ST131 and ST1431, and 4 for *E. cloacae*: ST90, ST89, ST133, and the novel ST407. Isolates belonging to the same PFGE clonal group belonged also to the same ST. With the single exception (clonal group C), isolates belonging to the same clonal group shared the same resistance traits. In the clonal group C, 2 *E. coli* isolates (No. 155 and No. 169) of ST131 with 83% of PFGE patterns similarity revealed different resistance traits (Fig. 1). These 2 isolates revealed multiple plasmids (5 and 6), including both large and small plasmids (Fig. 2).

Discussion

A significant increase in MDR Enterobacteriaceae has been reported worldwide.¹ The occurrence of MDR is usually concomitant with the acquisition of fluoroquinolone resistance.² According to EARS-Net data, in Poland from 2010 to 2013 fluoroquinolone resistance rates for *E. coli* remained almost constant (>25%) but significantly increased from 33% to 70.1% for *K. pneumoniae*.¹ What is noteworthy is that the horizontally transferable PMQRs, which are considered to support the development of the high-level

Table 1. Antimicrobial minimum inhibitory concentrations for the 18 *Qnr*-producing Enterobacteriaceae

No.	Species	Specimen	Minimum inhibitory concentration [mg/L]										
			AMC	CTX	CAZ	IPM	MEM	GEN	CIP	TGC	CST	SXT	CML
088	Kpn	blood	64	32	4	0.190	0.016	>1024	16	1	0.750	>32	12
036	Kpn	urine	32	0.190	0.750	0.125	0.016	1	128	3	0.750	2	64
060	Kpn	urine	64	32	12	0.125	0.023	64	1,024	1.500	0.500	>32	>256
158	Kpn	urine	16	48	>256	0.125	0.047	256	128	2	0.380	>32	16
159	Kpn	urine	24	>256	128	0.190	0.047	0.500	16	0.500	0.380	>32	6
215	Kpn	urine	32	>32	256	0.032	0.125	64	128	0.500	0.500	>32	16
091	Kpn	intubation tube swab	24	>256	64	0.250	0.064	32	64	1.500	0.380	>32	6
209	Kpn	tracheotomy tube swab	16	0.500	12	0.032	0.003	0.750	64	0.750	0.380	0.250	12
003	Ecl	urine	>256	>256	64	0.190	0.016	128	2	1	0.190	>32	>256
092	Ecl	urine	32	>256	16	0.250	0.047	32	16	1.500	0.380	>32	>256
110	Ecl	urine	>256	12	64	0.250	0.032	8	4	0.250	0.380	>32	>256
118	Ecl	urine	>256	>256	>256	0.250	0.032	48	64	3	0.380	>32	>256
214	Ecl	urine	32	>256	24	0.190	0.023	32	1.500	1	0.380	>32	6
216	Ecl	urine	>256	>256	>256	0.500	0.380	256	64	2	0.500	>32	16
169	Ec	blood	16	0.125	0.380	NT	0.016	12	64	0.250	0.500	0.064	16
014	Ec	urine	16	0.047	0.125	0.190	0.012	0.750	64	0.190	0.380	>32	4
064	Ec	urine	1.50	0.016	0.064	0.190	0.016	0.380	64	0.190	0.250	0.047	2
155	Ec	urine	24	32	8	0.125	0.016	0.500	256	1	0.500	>32	>256

Kpn – *Klebsiella pneumoniae*; Ecl – *Enterobacter cloacae*; Ec – *Escherichia coli*; AMC – amoxicillin-clavulanic acid; CTX – cefotaxime; CAZ – ceftazidime; IPM – imipenem; MEM – meropenem; GEN – gentamicin; CIP – ciprofloxacin; TGC – tigecycline; CST – colistin; SXT – trimethoprim/sulphamethoxazole; CML – chloramphenicol; NT – not tested.

Fig. 1. Characterization of 18 Qnr-producing Enterobacteriaceae isolates. The dendrogram based on pulsed-field gel electrophoresis (PFGE) ST – sequence type; PMQR – plasmid-mediated quinolone resistance.

fluoroquinolone resistance, are frequently reported to spread together with ESBLs on the same plasmid.^{4–6,15} In this study, we screened clinical Qnr-producing Enterobacteriaceae for the presence of multiple antimicrobial resistance genes. The majority of tested isolates possessed the PMQR determinants – QnrB1 and AAC (6')-Ib-cr – and were identified mainly in combination with CTX-M-15 as the most common ESBL. However, we found no co-existence of Qnr genes and plasmid-mediated AmpC β-lactamases determinants. It is well-known that CTX-M-15-producing isolates are commonly multiresistant to various antibiotic agents, among them fluoroquinolones or aminoglycosides.⁴ Furthermore, the coexistence of QnrB1 and CTX-M-15 was also frequently observed in other European countries.^{4,5} Notably, our data shows that in Poland, the aforementioned combination of resistance traits was extended by the 16S rRNA methyltransferase ArmA that confers the high-level resistance to almost all clinically relevant aminoglycosides. Moreover, the herein reported resistance triangle (QnrB1, CTX-M-15 and ArmA) was found in 2 Enterobacteriaceae species. Notably, one of them contained an additional PMQR gene: *aac(6')-Ib-cr*. Taking into account the relatively low number of isolates tested in this study, the incidence of 2 isolates with the aforementioned resistance triangle may reveal only “the tip of iceberg”. Thus, our findings may argue for the need of further specific monitoring of resistance. Interestingly, the coexistence of QnrB, CTX-M-15 and ArmA

in *K. pneumoniae* has been reported in China.⁷ In Europe, the coexistence of QnrA, CTX-M-15 and ArmA was reported in *K. pneumoniae* in Switzerland, which also co-produced CMY-16 together with carbapenemases OXA-48 and NDM-1.¹⁶ In Poland, we have also reported *K. pneumoniae* co-producing *Klebsiella pneumoniae* carbapenemase (KPC) with ArmA and CTX-M-3 ESBL.¹⁴ To the best of our knowledge, the presented study is the first report on clinical Enterobacteriaceae isolates co-producing PMQR, CTX-M-15 and ArmA in Poland. In this study, we identified FQ-resistant isolates belonging to different STs possessing CTX-M-15. We found *K. pneumoniae* isolate (No. 088) and *E. cloacae* isolate (No. 216) to bear QnrB1, CTX-M-15 and ArmA, and belong to ST323 and ST89, respectively. Although there is not enough data to consider these 2 STs epidemic, in our study we also found *K. pneumoniae* of ST15, ST336, ST147, and *E. coli* of ST131. These STs have been reported in many countries and, therefore, are considered international clones.^{17,18} In addition, *K. pneumoniae* ST15 and ST336 have not been reported in Poland yet. Interestingly, in this study we found *K. pneumoniae* ST15 and ST336 isolates with 2 PMQRs (QnrB1 and AAC (6')-Ib-cr) and 2 ESBLs (CTX-M-15 and SHV-11 or SHV-28) accompanied with TEM-1 β-lactamase. We also observed *E. coli* ST131 isolate No. 155 co-producing PMQR (QnrB1 and AAC (6')-Ib-cr), TEM-1 and CTX-M-15. Interestingly, this isolate revealed

Fig. 2. Plasmids profiles

83% PFGE-profile similarity with other ST131 isolate No. 169 that shared only QnrS1 and TEM-1. Notably, both these isolates differed by plasmid content that may reflect the dynamics of plasmid-mediated resistance traits exchange in clinical settings. Moreover, we found the novel *E. cloacae* ST 407 co-producing QnrB1 and AAC (6')-Ib-cr together with CTX-M-15. What is worthy of note is that all the aforementioned PMQR-producing isolates have been previously found to have mutations in quinolone resistance-determining regions (QRDR) *gyrA* and *parC*.³

In conclusion, the presented study provides baseline information on the “successful clones” possessing plasmid-mediated resistance genes providing resistance to most of clinically relevant group of antimicrobial agents including β -lactams, aminoglycosides and fluoroquinolones. It is also worth noting that fluoroquinolones (mainly ciprofloxacin) are able to induce bacterial SOS response to DNA damage and potentially enhance the transmission of resistance traits by promoting horizontal dissemination of various antibiotic resistance determinants, and increase the frequency of mutants.¹⁹ The herein reported coincidence of PMQRs with other resistance traits, often multiple and broad-range plasmid-mediated mechanisms, may suggest a significant contribution of fluoroquinolone therapy in increasing the MDR phenotype in clinical Enterobacteriaceae strains.

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