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Co-existence of plasmid-mediated quinolone resistance determinants and mutations in *gyrA* and *parC* among fluoroquinolone-resistant clinical Enterobacteriaceae isolated in a tertiary hospital in Warsaw, Poland

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ABSTRACT

Plasmid-mediated quinolone resistance (PMQR) determinants and the distribution of mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* were investigated in 215 ciprofloxacin-resistant (MIC > 1 mg/L) clinical Enterobacteriaceae collected during a 6-month prospective study in a tertiary hospital in Warsaw, Poland. PMQR determinants were present in 49 isolates (22.8%), among which *aac(6′)-Ib*-cr and *qnrB*1 predominated (85.7% and 26.5%, respectively). Mutations in *gyrA* and *parC* QRDRs were detected among 89.8% of isolates (MIC ≥ 4 mg/L). Changes in Ser-83, Ala-84 and Asp-87 in GyrA and Ser-80 and Glu-84 in ParC were detected. Five isolates with ciprofloxacin MICs in the range 1.5–16 mg/L were found to have unaltered QRDRs, with PMQR as the only fluoroquinolone (FQ) resistance trait detected. The remaining 44 PMQR-positive isolates were found to carry altered QRDRs. Three substitutions (two in GyrA and one in ParC) were detected in 23 isolates, whilst 8 isolates carried four mutations (two in GyrA and two in ParC). One isolate of *Klebsiella pneumoniae* with two amino acid substitutions in the ParC QRDR in the presence of *aac(6′)-Ib*-cr and *qnrB*1 had a ciprofloxacin MIC of 16 mg/L. The results presented here show that FQ resistance in these clinical Enterobacteriaceae is a complex interplay between PMQR determinants and mutations in *gyrA* and *parC* rather than a single stepwise accumulation of mutations in the gyrase and topoisomerase subunits. In addition, these results show the role of PMQR determinants in promoting QRDR mutations and the acquisition of high-level FQ resistance in clinical settings.

1. Introduction

Enterobacteriaceae are common pathogens causing nosocomial and community-acquired infections. Multidrug-resistant Enterobacteriaceae (MRE) have been reported worldwide [1]. MRE are often resistant to fluoroquinolones (FQs). FQs are important synthetic antimicrobial agents widely used in clinical and veterinary medicine. In the past few years, resistance to FQs has increased across the globe [1], thereby limiting available therapeutic options or resulting in treatment failure. A variety of FQ resistance mechanisms and/or their accumulation within one isolate have been reported in clinical strains. High-level resistance to FQs [minimum inhibitory concentration (MIC) ≥ 32 mg/L] is considered a consequence of mutations involving chromosomal genes encoding two enzymes, namely DNA gyrase and topoisomerase IV. Both enzymes are essential for bacterial DNA replication [2]. DNA gyrase is composed of two subunits (GyrA and GyrB) and DNA topoisomerase IV is also composed of two subunits (ParC and ParE). ParC is homologous to GyrA, whereas ParE is homologous to GyrB. In Gram-negative bacteria, DNA gyrase is considered the primary target for FQs [2,3]. Amino acid alterations and their accumulation in DNA gyrase and topoisomerase IV predominantly occur within the quinolone resistance-determining region (QRDR) in *Escherichia coli* between amino acid positions 67 and 106 [2]. Alterations in both GyrA and ParC often confer high-level

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resistance and are reported more frequently than alterations in GyrB or ParE [2]. In *E. coli*, substitutions at Ser-83 and Asp-87 in GyrA and at Ser-80 and Glu-84 in ParC are the most common mutations observed among FQ-resistant strains [2].

Moreover, several plasmid-mediated quinolone resistance (PMQR) mechanisms have been shown to reduce susceptibility to FQs. Qnr proteins, AAC(6′)-Ib-cr and QepA are PMQR determinants that have been identified in different species of Enterobacteriaceae worldwide [3,4]. The Qnr proteins protect DNA from quinolone binding to topoisomerases. To date, five types of Qnr proteins (QnrA, QnrB, QnrC, QnrD and QnrS) with numerous variants have been described (http://www.lahey.org/qnrStudies/). Another PMQR determinant is a variant of aminoglycoside acetyltransferase AAC(6′)-Ib. The AAC(6′)-Ib-cr enzyme is able to acetylate kanamycin, tobramycin and amikacin but is also responsible for reduced susceptibility to hydrophilic quinolones such as ciprofloxacin or norfloxacin [4]. This variant differs from wild-type AAC(6′)-Ib by two amino acid substitutions at codons 102 (Trp→Arg) and 179 (Asp→Tyr), which both appear to be required to confer reduced susceptibility to FQs [4]. The QepA determinant, a major facilitator efflux pump, confers decreased susceptibility to hydrophilic quinolones [4]. Although the PMQR determinants are considered to provide only a low level of resistance, their presence may stimulate mutations in genes encoding for DNA gyrase and topoisomerase IV [3,4]. It is noteworthy that PMQR determinants and mutations in the QRDRs are often found together in clinical strains of Enterobacteriaceae with high-level quinolone resistance [5–7].

Data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) [1] indicate an increase in resistance to ciprofloxacin among invasive Enterobacteriaceae strains, which is a matter of concern. However, little is known about the prevalence of PMQR determinants and the diversity of DNA gyrase and topoisomerase IV mutations in clinical isolates of Enterobacteriaceae in Poland. Thus, the objective of this study was to determine the prevalence of PMQR determinants and to investigate the distribution of mutations in the QRDRs of *gyrA* and *parC* among ciprofloxacin-resistant (MIC > 1 mg/L) clinical isolates of Enterobacteriaceae collected during a 6-month study in a tertiary hospital in Warsaw, Poland.

2. Materials and methods

2.1. Bacterial isolates and fluoroquinolone susceptibility testing

A collection of 215 ciprofloxacin-resistant (MICs ranging from 1.5 mg/L to >1024 mg/L), non-duplicate clinical isolates of Enterobacteriaceae obtained from 2017 inpatients who were screened from 1 March 2010 to 31 August 2010 in a 1030-bed tertiary hospital in Warsaw was examined for FQ resistance mechanisms. The isolates were most frequently isolated from urine (n = 146), wound (n = 40) and blood (n = 11). The isolates comprised *E. coli* (n = 101), *Klebsiella pneumoniae* (n = 75), *Enterobacter cloacae* (n = 25), *Proteus mirabilis* (n = 8), *Citrobacter freundii* (n = 3), *Morganella morganii* (n = 2) and *Serratia marcescens* (n = 1). Species identification and antimicrobial susceptibility testing were undertaken with a VITEK® 2 system (bioMérieux, Marcy-l'Étoile, France) in a routine laboratory. Resistance to ciprofloxacin was the first selection criterion for inclusion in the study. All ciprofloxacin-non-susceptible (MIC ≥ 2 mg/L) Enterobacteriaceae isolates detected by the commercial system were confirmed as resistant to ciprofloxacin, norfloxacin and sparfloxacin (MP Biomedicals, Eschwege, Germany) by MIC determination using the agar dilution method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. In addition, the Etest method was used to determine the MICs of moxifloxacin. The MIC results for ciprofloxacin, norfloxacin and moxifloxacin were interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (http://www.eucast.org/clinical_breakpoints/) and isolates with a MIC > 1 mg/L were classified as resistant to FQs. The MIC for each clinical isolate was measured at least twice.

2.2. Detection of resistance genes and Sanger DNA sequence analysis

All FQ-resistant Enterobacteriaceae isolates were screened for the presence of PMQR determinants [*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib* and *qepA*] by PCR amplification using primers and conditions as described previously [9–13]. All of the PCR products for PMQR genes were confirmed by direct DNA sequencing. In addition, the PCR product for *aac(6′)-Ib*-cr was confirmed by enzymatic digestion with *Bts*CI (Thermo Scientific, Vilnius, Lithuania). The presence of 272-bp and 210-bp DNA fragments indicated *aac(6′)-Ib*, whereas an undigested fragment was indicative of *aac(6′)-Ib*-cr. Furthermore, PCR and DNA sequencing of the QRDRs of *gyrA* and *parC* were performed using previously described primers [14]. DNA sequences of the QRDRs of *gyrA* and *parC* genes were compared with the QRDRs of wild-type reference strains of *K. pneumoniae*, *E. coli*, *E. cloacae* and *P. mirabilis* (GenBank accession nos. **AF052258**, **AF052254**, **AF052256** and **AM942759**, respectively, for *gyrA*; and **AF303641**, **NC000913**, **D88981** and **AM942759**, respectively, for *parC*). Sequence alignment and analysis were performed online using the BLAST program (http://www.ncbi.nlm.nih.gov) and CLC Sequence Viewer v.6.9.1. (CLC Inc., Aarhus, Denmark).

2.3. Genetic relatedness

The PMQR-harbouring isolates were analysed by pulsed-field gel electrophoresis (PFGE) as described previously [15] using a CHEF-DR II System (Bio-Rad Laboratories Inc., Hercules, CA) and endonuclease *Xba*I (Thermo Scientific) with a switching time of 3–30 s for 24 h at 14 °C and a voltage gradient of 6.0 V/cm. PFGE patterns were analysed using BioNumerics software v.6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity clustering analyses were performed using UPGMA (unweighted pair group method with arithmetic mean) and Dice correlation coefficient with a tolerance of 1.2%. PFGE typing was performed twice.

3. Results

3.1. Prevalence of plasmid-mediated quinolone resistance determinants among fluoroquinolone-resistant Enterobacteriaceae

PMQR determinants were present in 49 (22.8%) of the 215 ciprofloxacin-resistant isolates tested. PMQRs were observed more frequently in *K. pneumoniae* (29/75; 38.7%) than in *E. coli* (13/101; 12.9%), *E. cloacae* (6/25; 24.0%) or *P. mirabilis* (1/8; 12.5%) (Table 1). The *aac(6′)-Ib*-cr was the most common PMQR determinant detected in 42 (85.7%) of the 49 isolates (Table 1). The *qnr* genes were found in 18 isolates (36.7%), including *qnrB* that was identified more frequently (13; 26.5%) than *qnrA* and *qnrS* (3; 6.1% each); only one isolate (no. 64) was positive for both *qnrA* and *qnrS* (Table 1). Moreover, *qnr* and *aac(6′)-Ib*-cr were detected in combination in 11 isolates (22.4%). In contrast, *qnrC*, *qnrD* and *qepA* were not detected in any of the isolates tested.

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