Detection of plasmid-mediated quinolone resistance in clinical isolates of Enterobacteriaceae strains in Hamadan, West of Iran

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Received 16 July 2016; revised 15 November 2016; accepted 29 November 2016
Available online 19 December 2016

Keywords
Enterobacteriaceae; Antibiotic resistance; Plasmid; Quinolone resistance; Fluoroquinolone

Abstract
Plasmid mediated quinolone resistance (PMQR) determinants have arisen as a significant concern in recent years. The aim of this study was screening of resistant-clinical isolates to fluoroquinolone antibiotics and detection of \( qnr \) and \( aac(6')-Ib-cr \) genes.

For this purpose we collected 100 fluoroquinolone-resistant Enterobacteriaceae which were from 3 hospitals in Hamadan, west provinces of Iran, between October 2012 and June 2013. The all samples were identified by biochemical tests and confirmed by PCR method. Antimicrobial susceptibility to 14 antimicrobial agents including levofloxacin and ciprofloxacin were determined by disk diffusion methods and ciprofloxacin MIC was obtained by broth microdilution method as Clinical Laboratory Standards Institute (CLSI) recommendations. The isolates were screened for the presence of \( qnrA \), \( qnrB \), \( qnrS \) and \( aac(6')-Ib-cr \) genes using PCR assay. Among the screened isolates, 64 strains (64%) of \( \text{Escherichia coli} \), 23 strains (23%) of \( \text{Klebsiella pneumoniae} \), 13 strains (13%) of \( \text{Proteus mirabilis} \) were collected as quinolone-resistant isolates. Out of 100 isolates, two (2%) were positive for \( qnrS \), seventeen (17%) isolates were positive for \( qnrB \) and we did not find \( qnrA \) gene in any of the isolates. There were also 32 positive isolates for \( aac(6')-Ib-cr \) determinant. We described the
1. Introduction

Enterobacteriaceae is a rod-shaped Gram-negative bacteria, many members of which are a normal part of the flora. They are considered as one of the most common human pathogens and frequently cause several diseases such as urinary tract, cystitis, pyelonephritis, septicemia, pneumonia, peritonitis, meningitis and infections associated with prosthetic devices such as catheters (Aslani and Alikhani, 2009; Haghighatpanah et al., 2016; Nordmann et al., 2011; Patel et al., 2009; Paterson and Bonomo, 2005; Sedighi et al., 2016). Quinolone antibiotics are broad-spectrum antibiotics and very important in the treatment of a wide range of diseases, especially urinary tract infections. Large range of activities, and the low side effects, as well as acceptable oral absorption, made these antibiotics as the first-line drugs to treat a variety of infections. The most common quinolone family is ciprofloxacin (Mandell, 2005; Shahcheraghi et al., 2013). These antibiotics tend to bind tightly to the bacterial enzymes (DNA gyrase or topoisomerase IV), and inhibit the bacterial growth. Aminoglycoside and quinolone antibiotics are used to treat infections caused by many genera and species of Enterobacteriaceae family. Over the past three decades, resistance to quinolones and aminoglycosides among Enterobacteriaceae family has increased significantly. One of the most important reasons which has been reported worldwide is the incidence of plasmid-mediated resistance genes (Briales et al., 2012; Alikhani et al., 2013).

Fluoroquinolone resistance is mainly caused by mutations in chromosomal genes encoding the quinolone targets, such as DNA gyrase and topoisomerase IV, but in the past few decades, plasmid-mediated quinolone resistance (PMQR), has been increasingly reported in most parts of the world (Briales et al., 2012; Hooper and Rubinstein, 2003). Generally, three mechanisms of plasmid-mediated quinolone resistance have been described: (i) qnr proteins that protect the quinolone targets, (ii) aac(6’)-Ib-cr enzyme that acetylate aminoglycosides, ciprofloxacin and norfloxacin, (iii) Efflux pumps associated with QepA which excretes hydrophobic fluoroquinolones (Amin and Wareham, 2009; Yamane et al., 2008; Strahilevitz et al., 2009; Robicsek et al., 2006a).

In the present study, we investigated the prevalence of the PMQR determinants including qnrA, qnrB, qnrS and aac (6’)-Ib-cr in fluoroquinolone-resistant Enterobacteriaceae isolates that were obtained from clinical specimens. However, No previous survey has considered clinical isolates in our area for the presence of aac (6’)-Ib-cr determinant.

2. Materials and methods

2.1. Bacterial isolates

In total, 100 clinical isolates of non-replicate Enterobacteriaceae strains collected between October 2012 and June 2013 were selected for the study based on resistance to ciprofloxacin (zone diameter ≤15 mm) and/or reduced susceptibility/resistance to levofloxacin (zone diameter ≤13 mm). Specimens included in the study were from inpatients admitted at hospitals and from outpatients. All collected isolates were non-susceptible to ciprofloxacin. Samples were cultured on MacConkey agar. Then isolates were recognized by biochemical tests like: TSI, KIA, SIM, Simon citrate, MR-VP (Mahon et al., 2011). Our detection was confirmed by the presence of rpoB gene in all isolates by PCR. Previous studies showed that the rpoB gene can be used as the most promising target for detection of Enterobacteriaceae by PCR amplification (Fazzeli et al., 2012).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the broth microdilution and disk diffusion methods following Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2013; Safari et al., 2015). The tested antimicrobial agents were: ciprofloxacin (Sigma, Steinheim, Germany) by broth microdilution; and cefepime (30 µg), ceftoxime (30 µg), ceftazidim (30 µg), aztreonam (30 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), kanamycin (30 µg), doxycyclin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), tigecycline (15 µg), trimethoprim/sulfamethoxazole (25 µg) (MAST, UK) using the disk diffusion method.

2.3. Screening for qnr and aac(6’)-Ib-cr genes

All clinical isolates were grown for 18 h at 37 °C in MacConkey agar and DNA was extracted by SDS-Proteinase K phenol chloroform method as described (Farajnia et al., 2013). The qnrA, qnrB and qnrS genes were screened using a multiplex PCR-based technique (Yang et al., 2013) and the presence of aac(6’)-Ib-cr was detected using single PCR, producing a 482-bp product (Park et al., 2006) (Table 1). The Comparison of aac(6’)-Ib-cr-positive and aac(6’)-Ib-cr-negative isolates with antibiotics resistant were shown in Table 5.

2.4. Statistical analysis

Statistical analysis was performed using chi-square test with SPSS software (version 16). A P value of ≤0.05 was considered to indicate statistical significance.

3. Results

Among the screened isolates, 64 strains of Escherichia coli (64%), 23 strains of Klebsiella pneumoniae (23%), and 13 strains of Proteus mirabilis (13%) were collected as fluoroquinolone-resistant isolates. Most of quinolone-resistant strains, (ii) strains collected between October 2012 and June 2013 were associated with QepA which excretes hydrophobic fluoroquinolones, (iii) Efflux pumps associated with qnrA which excretes hydrophobic fluoroquinolones (Farajnia et al., 2013). The Comparison of aac(6’)-Ib-cr-positive and aac(6’)-Ib-cr-negative isolates with antibiotics resistant were shown in Table 5.

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resistant strains were obtained from urine and tracheal samples with 48% and 36%, respectively. The other samples were collected from blood culture (4%), bed sore (2%), stool culture (1%), wound culture (8%) and throat culture (1%).

3.1. Antimicrobial susceptibility

Antibiotic susceptibility testing by disk diffusion showed that the 100 isolates were all susceptible to meropenem (100%). The highest sensitivity after meropenem was obtained with imipenem (92%) and ertapenem (82%). Resistance was most often observed with cefotaxime (97%), trimethoprim/sulfamethoxazole (90%), aztreonam (84%), doxycycline (77%), kanamycin (75%) and cefepime (61%) (Table 2). The ciprofloxacin minimum inhibitory concentrations (MIC) of 100 isolates were tested by broth microdilution (Table 3). Among the isolates, 9 strains were intermediate to ciprofloxacin but all of the qnr and aac(6’)-Ib-cr positive isolates were resistance to ciprofloxacin.

3.2. Screening for the qnr and aac(6’)-Ib-cr genes

The qnr genes were detected in 19/100 (19%) of fluoroquinolone resistance-Enterobacteriaceae by PCR. The qnrB gene was present in 11 (47.8%) of K. pneumoniae, 4 (6.25%) of E. coli and 2 (15.38%) of P. mirabilis. The qnrS gene was present in 1 (4.34%) of K. pneumoniae and 1 (7.69%) of P. mirabilis. We did not find qnrA gene in any of the isolates (Table 4).

The aac(6’)-Ib-cr gene was detected in 32 (32%) of isolates. We found 13 (56.5%) of K. pneumoniae, 11 (17.2%) of E. coli and 8 (61.53%) of P. mirabilis positive for this gene (Table 4).

4. Discussion

Over three past decades, bacterial resistance to quinolones has increased in clinical isolates. According to our results, fluoroquinolone-resistant isolates showed multidrug resistance to other antimicrobial agents like cefazidime, cefotaxime, cefepime, kanamycin, amikacin, aztreonam, doxycycline and trimethoprim/sulfamethoxazole, that it may involve other
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Table 5 Comparison of aac(6’)-Ib-cr-positive and aac(6’)-Ib-cr-negative isolates with antibiotics resistant.

| Antibiotics       | Negative No (%) | Positive No (%) | Total | P value |
|-------------------|-----------------|-----------------|-------|---------|
| Kanamycin         |                 |                 |       |         |
| R                 | 45 (66.2)       | 30 (93.8)       | 75 (75)| 0.716   |
| I                 | 6 (8.8)         | 0 (0)           | 6 (6) |         |
| S                 | 17 (25)         | 2 (6.3)         | 19 (19)|         |
| Ciprofloxacin     |                 |                 |       |         |
| R                 | 65 (95.6)       | 32 (100)        | 97 (97)| 0.079   |
| S                 | 3 (4.4)         | 0 (0)           | 3 (3) |         |
| Levofloxacin      |                 |                 |       |         |
| R                 | 57 (83.8)       | 28 (87.5)       | 85 (85)| 0.573   |
| I                 | 4 (5.9)         | 2 (6.3)         | 6 (6) |         |
| S                 | 7 (10.3)        | 2 (6.3)         | 9 (9) |         |

 mechanisms, such as chromosomal mutation in genes encoding for DNA gyrase (Yang et al., 2013; Shaheen et al., 2013), efflux pumps and producing AmpC β-lactamases (Gu et al., 2004) but these isolates still are susceptible to carbapenem antibiotics.

Previous studies suggested that the quinolone resistance rates in uropathogenic E. coli (UPEC) were high (84.2% antimicrobial resistance against nalidixic acid) because quinolones are the first choice of urinary tract infection (Muhammad et al., 2011), but in this study, K. pneumoniae had the highest fluoroquinolone resistance rates among Enterobacteriaceae spp. In the present study, 100 fluoroquinolone-resistant strains were examined for the presence of genes that naturally increases resistance to quinolones. In this study, we detected 17 qnrB and 2 qnrS genes but we did not find qnrA gene in any of the isolates and it is probably associated with the geographical distribution of qnr genes. The qnr genes were more prevalent among Klebsiella spp. (12/23, 52.2%) than among E. coli (4/64, 6.25%), as previously described in other studies conducted in France (Porrel et al., 2006), the United States (Robicsek et al., 2006b), Spain (Lavilla et al., 2008), China (Jiang et al., 2008) and Norway and Sweden (Karaha et al., 2010). In previous study, the prevalence of qnr genes was much lower in E. coli isolates (4.8%) than in other species of clinical isolates of the Enterobacteriaceae in China, however, the prevalence of aac(6’)-Ib-cr appeared to be lower in Enterobacter cloacae isolates (9.3%) than in isolates of the other species tested. qnrA gene was not found in any of isolates (Yang et al., 2008).

To our best knowledge, this is the first study on the prevalence of aac(6’)-Ib-cr plasmid-mediated quinolone resistance in Hamadan city, and present findings suggest that aac(6’)-Ib-cr gene was more prevalent than qnr genes. The aac(6’)-Ib-cr gene was detected most often in Klebsiella spp. (13/23, 56.5%) than among E. coli (11/64, 17.2%). In the present study, there is no isolates which carried 2 types of qnr genes but 15 isolates with aac(6’)-Ib-cr carried qnrS (one strain of Klebsiella and one strain of Proteus) and qnrB (seven isolates of Klebsiella, four isolates of E. coli and two isolates of Proteus), simultaneously. There was a relationship between the presence of qnrA, -B, or -S genes and aac(6’)-Ib-cr. aac(6’)-Ib-cr was detected only in 15/19 (78.9%) of qnr-positive isolates but in 17/81 (20.9%) of the qnr-negative isolates (P 0.0001 by a two-tailed Fisher’s exact test), indicating that the qnr genes and aac(6’)-Ib-cr can circulate dependently. A comparable prevalence was reported from China where the prevalence of the aac(6’)-Ib-cr variant among E. coli and K. pneumoniae isolates was 55.2% among qnr-positive isolates but only 6% among qnr-negative isolates (Jiang et al., 2008). The reason is not understood yet, but it is known that some plasmids can carry both aac(6’)-Ib-cr and qnr genes (Robicsek et al., 2006b). According to previous studies, the cr variant of aac(6’)-Ib-cr encodes an enzyme that had slightly reduced efficiency in acetylation of kanamycin (Robicsek et al., 2006c), aac(6’)-Ib-cr confers resistance to kanamycin but not to gentamicin (Robicsek et al., 2006b). So we analyzed the relationship between aac(6’)-Ib-cr and susceptibility to ciprofloxacin, levofloxacin and kanamycin (Table 2). The results of this study confirmed that there is no relationship between aac(6’)-Ib-cr and susceptibility to ciprofloxacin, levofloxacin and kanamycin (P > 0.05).

This study showed that the prevalence of plasmid-mediated quinolone resistance due to the qnr and aac(6’)-Ib-cr genes was high among fluoroquinolone-resistant clinical isolates of Enterobacteriaceae in Hamadan. Fluoroquinolone resistance is mainly caused by mutations in chromosomal genes encoding the quinolone targets, such as DNA gyrase and topoisomerase IV. So PMQR is not the only resistant mechanism to quinolone. In our study, 41 fluoroquinolone-resistant clinical isolates (41%) were positive for plasmid-mediated quinolone resistance genes and the other strains (59 isolates) had probably the other resistant mechanisms that are not investigated in this study. Among 3 important species (E. coli, Klebsiella and Proteus spp.), the prevalence of qnr appeared to be much lower in E. coli isolates (6.25%) than in other species of clinical isolates of the Enterobacteriaceae. However, the prevalence of aac(6’)-Ib-cr appeared to be lower to (17.2%) in E. coli isolates. qnrA gene was not found in large numbers of resistant clinical isolates of Enterobacteriaceae spp. Infections caused by qnr-positive isolates might subsequently enhance the selection of resistant mutants and increase the risk of therapeutic failure.

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