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Original Article

Prevalence of *qnr* Genes in Extended-Spectrum β-Lactamase Producing *Klebsiella pneumoniae* Isolated from Clinical Urine Specimens in University Teaching Hospitals, Iran

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Background and Aims: Extended-spectrum β-lactamase (ESBL) producing gram negative bacteria are resistant to penicillins, narrow and extended-spectrum cephalosporins, and aztreonam; also they are frequently resistant to trimethoprim-sulfamethoxazole, aminoglycosides, and quinolones. This study aimed to investigate the prevalence of plasmid-mediated quinolone resistance (PMQR) determinants *qnr*A, *qnr*B and *qnr*S in ESBL producing *Klebsiella pneumoniae* isolates.

Materials and Methods: In this descriptive-sectional study, 130 *Klebsiella pneumoniae* isolates were collected from urine specimens and identified by convectional biochemical tests from December 2013 to August 2014,. Antimicrobial susceptibility testing was performed by disk diffusion method (Kirby-Bauer). The presence of ESBLs was confirmed by combination disk tests. E.test method was used for determination of ceftazidime and ciprofloxacin minimum inhibitory concentration (MIC). *qnr* genes were investigated by multiplex polymerase chain reaction and after sequencing, indexed in Genbank database.

Results: Out of 130 isolates, 46 (35.4%) isolates were identified as ESBL producers, considering all, the highest rate of resistance belonged to amoxicillin, cefotaxime and ceftriaxone (each one 100%) and the lowest rate of resistance was for meropenem and ertapenem (each one 4.3%). 45 (97.8%) isolates were resistant to ceftazidime (MIC ≥ 16) and 24 (52.2%) isolates were resistant to ciprofloxacin (MIC≥4). *qnr*B and *qnr*S genes were detected in 21 (45.7%) and 7 (15.2%) isolates, respectively. 7 (15.2%) isolates were positive for both *qnr*B and *qnr*S genes. *qnr*A was not detected.

Conclusions: With respect to the high prevalence of *qnr* genes in ESBL producing *Klebsiella pneumoniae* isolates, quinolones and beta-lactam agents should be used with caution.

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Introduction

Klebsiella pneumoniae is a gram negative rod belonged to Enterobacteriaceae and considered as one of the most important causes of urinary tract infections (UTIs) and pneumonia in immunosuppressed patients with underlying diseases [1, 2]. Since the extended-spectrum cephalosporins are often used to treat infections caused by these bacteria, resistance to these antimicrobial agents have increased. Extended-spectrum β-lactamase (ESBL) producing bacteria are resistant to penicillins, narrow and extended-spectrum cephalosporins, and aztreonam and frequently resistant to trimethoprim-sulfamethoxazole, aminoglycosides and quinolones [3]. The emergence of the close relationship between resistance to quinolones and other antimicrobial agents, particularly beta-lactams and aminoglycosides is a major problem in the treatment of these infections [4]. Quinolone resistance has increased among human and animal isolates in the past three decades [5]. Quinolone resistance in Enterobacteriaceae family is mainly the result of chromosomal mutations in genes coding for DNA gyrase and topoisomerase IV, changes in outer membrane or expression of efflux pumps [6]. In addition to chromosomal mutations, plasmid-mediated quinolone resistance (PMQR) has emerged around the world [7]. Three mechanisms have been described for PMQR: quinolone targets protection by Qnr proteins, the Aac(6)-Ib-cr enzyme that acetylates aminoglycosides, ciprofloxacin and norfloxacin, OqxAB and QepA plasmid-mediated efflux pumps that are responsible for moving antibiotics out of the cells [5, 8]. For the first time, PMQR was reported in a clinical isolate of *Klebsiella pneumoniae* from the USA in 1998 [9]. *qnr* genes encode the proteins that bind to DNA gyrase and topoisomerase IV and protect the DNA [7]. Five Qnr proteins have been identified that include QnrA, QnrB, QnrS, QnrC and QnrD [5, 10]. Qnr proteins have been reported from various species of the Enterobacteriaceae family around the world and six variants of QnrA have been known (QnrA1 to QnrA6). Two other genes that are responsible for resistance to quinolones are *qnr*B and *qnr*S, which encode proteins QnrB (six variants) and QnrS (with two variants) [7].

ESBL producers are usually resistant to multiple drugs. In most cases, genes with different resistance mechanisms are located on the same plasmids that ESBL genes are. So, in some ESBL producing isolates resistance to quinolones, aminoglycosides and trimethoprim-sulfamethoxazole has been seen [3]. According to the high prevalence of ESBL producing bacteria which most of them are multi-drug resistance isolates and lack of sufficient studies about the prevalence of *qnr* genes in Iran, so this study aimed to investigate the prevalence of PMQR determinants *qnr*A, *qnr*B and *qnr*S in ESBL producing *Klebsiella pneumoniae* isolates.

Materials and Methods

Isolation and identification of bacteria

In this descriptive-sectional study, 130 isolates of *Klebsiella pneumoniae* were collected from urine specimens of hospitalized patients with

UTIs in teaching hospitals of Yazd Shahid Sadoughi and Karaj Universities, from December 2013 to August 2014. Urine samples were cultured on blood agar and eosin methylene blue agar (EMB) and after 24 hours of incubation at 37°C, mucoid colonies were identified by convectional biochemical tests, including fermentation of sugars in TSI medium (glucose and lactose fermenter), indole production and motility in SIM medium (indole negative and nonmotile), the way of glucose fermentation in MR-VP medium (MR negative and VP positive), growth in simmon citrate medium (citrate-positive) and urease production in urea medium (urease-positive) (all media from Merck, Germany) [11]. The protocol was approved by Ethics Committee of Shahid Sadoughi university of medical sciences, Yazd, Iran.

Antimicrobial susceptibility test

Antimicrobial susceptibilities were determined by standard disk diffusion method (Kirby-Bauer) according to the CLSI recommendations [12]. The tested antibiotics were: Amoxicillin (25 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), cefoxitin (30 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), imipenem (10 µg), meropenem (10 µg), ertapenem (10 µg), gentamicin (10 µg) and tetracycline (30 µg) (all antibiotic disks from Mast, UK).

The evaluation of minimum inhibitory concentration (MIC)

MIC of ceftazidime and ciprofloxacin were determined using the E-test method (all E-test strips from Liofilchem, Italy) [13]. The results were interpreted according to the CLSI guidelines [12]. *Escherichia coli* ATCC 25922 was used for quality control.

Screening and confirmation of ESBL production

The isolates that were resistant to at least one of the third-generation cephalosporins such as ceftazidime, cefotaxime, and ceftriaxone were confirmed by combination disk method [12]. Cefotaxime (30 µg) and ceftazidime (30 µg) disks with and without clavulanic acid (10 µg) were used (all disks from Mast, UK). A≥5 mm enhancement in zone diameter for ceftazidime/ clavulanate (30/10 µg) and cefotaxime/ clavulanate (30/10 µg) compared to ceftazidime (30 µg) and cefotaxime (30 µg) disks were defined as a positive result. *Escherichia coli* ATCC 25922 were used as negative controls and *Klebsiella pneumoniae* ATCC 700603 as a positive control.

DNA extraction

Salting out method was used for DNA extraction. Briefly, bacteria were cultivated in tryptic soy broth (Merck, Germany) 18 hours prior to the extraction procedures. 1 ml of fresh bacterial suspension in tryptic soy broth was poured in 1.5 ml microtube, then washed for three times with phosphate buffered saline. The bacterial pellet was suspended in 500 µl NET buffer (Nacl 50mM, Ethylenediaminetetraacetic acid 10 mM, Tris-Hcl 50 mM) and SDS with an end concentration of 1% and incubated at 56°C for one hour. For DNA purification, 300 µl of 6 M NaCl was added and centrifuged. The supernatant was transferred into a 1.5 ml microtube and absolute ethanol was added for precipitation. The washing step was performed

by using ethanol 70%. The pellet was diluted in 100 µl sterile distilled water and stored at -20°C.

Detection of *qnr* genes

Multiplex polymerase chain reaction (PCR) assay was performed to determine the presence of the *qnr*A, *qnr*B and *qnr*S genes in the ESBL producing isolates using thermo cycler (Quant Biotech, UK) and Taq DNA Polymerase 2X Master Mix (Ampliqon, Denmark). The specific primers, which were used for amplification have been shown in table 1 [14]. The reaction was performed with end concentrations of 1X PCR master mix, 8 pmol of each primer and 100 ng of template DNA in a total volume of 20 µl. PCR conditions were as follow: initial denaturation step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 60s, annealing at 53°C for 60s, extension at 72°C for 60s and a final extension at 72°C for 5 min. PCR products were analyzed on 2% agarose gel alongside 50 bp DNA ladder by electrophoresis.

Table 1. The primers used for PCR and DNA sequencing

Primer name	Nucleotide sequence	Amplicon size (bp)
***qnr*A**	5′ ATTTCTCACGCCAGGATTTG 3′ 5′ GATCGGCAAAGGTTAGGTCA 3′	516 bp
***qnr*B**	5′ GATCGTGAAAGCCAGAAAGG 3 5′ ACGATGCCTGGTAGTTGTCC 3′	469 bp
***qnr*S**	5′ ACGACATTCGTCAACTGCAA 3′ 5′ TAAATTGGCACCCTGTAGGC 3′	417 bp

Sequencing

PCR products were sequenced to confirm and the results were analyzed by using BLAST [15, 16]. The sequences of *qnr*B and *qnr*S were submitted to the GenBank database.

Statistical analysis

The study data were analyzed using SPSS software version 20 (SPSS Inc, Chicago, IL, USA).

Results

In this study, 130 *Klebsiella pneumoniae* isolates were collected from urine specimens of patients with UTIs. The results showed that 54 (41.5%) isolates were resistant to at least one of the third generation cephalosporins. 54 (41.5%), 52 (40%) and 52 (40%) isolates were resistant to ceftazidime, cefotaxime and ceftriaxone, respectively. 54 extended spectrum cephalosporins resistant isolates were confirmed by combination disk method, and ESBL production was found in 46 (85.2%) isolates. From total 130 isolates, 35.4% were ESBLs producers (Fig. 1). The highest rate of antibiotic resistance of ESBLs producing isolates were belonged to amoxicillin, cefotaxime, and ceftriaxone (each one 100%) and the lowest rate of resistance was for meropenem and ertapenem (each one 4.3%) (Table 2). This survey showed high resistance to other antibiotic families such as fluoroquinolones. The rate of resistance to the ciprofloxacin was 52.2%.

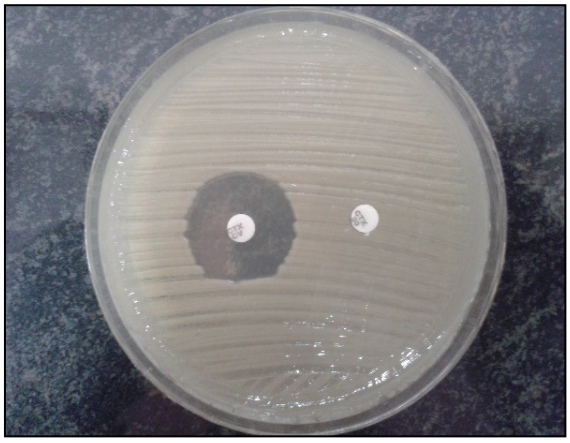

Fig. 1. Confirmatory combination disk method, the appearance of ESBL producing isolate.

Table 2. Susceptibility pattern of ESBL producing isolates.

Antibiotic	Resistant No (%)	Intermediate No (%)	Sensitive No (%)	Total No (%)
Amoxicillin	46 (100)	0 (0)	0 (0)	46 (100)
Cefotaxime	46 (100)	0 (0)	0 (0)	46 (100)
Ceftriaxone	46 (100)	0 (0)	0 (0)	46 (100)
Ceftazidime	45 (97.8)	0 (0)	1 (2.2)	46 (100)
Cefepime	42 (91.3)	1 (2.2)	3 (6.5)	46 (100)
Gentamicin	31 (67.4)	0 (0)	15 (32.6)	46 (100)
Imipenem	30 (65.2)	12 (26.1)	4 (8.7)	46 (100)
Tetracycline	26 (56.5)	0 (0)	20 (43.5)	46 (100)
Ciprofloxacin	24 (52.2)	1 (2.2)	21 (45.7)	46 (100)
Nalidixic acid	24 (52.2)	1 (2.2)	21 (45.7)	46 (100)
Norfloxacin	22 (47.8)	2 (4.3)	22 (47.8)	46 (100)
Cefoxitin	9 (19.6)	3 (6.5)	34 (73.9)	46 (100)
Ertapenem	2 (4.3)	5 (10.9)	39 (84.8)	46 (100)
Meropenem	2 (4.3)	4 (8.7)	40 (87)	46 (100)

The MIC of ceftazidime and ciprofloxacin were investigated for ESBL producing isolates and the results were summarized in table 3.

The results showed that 45 (97.8%) isolates were resistant to ceftazidime (MIC≥16) and 24 (52.2%) isolates were resistant to ciprofloxacin (MIC≥4).

Table 3. MIC of ceftazidime and ciprofloxacin for ESBL producing isolates.

Antibiotic	**Ceftazidime**			**Ciprofloxacin**		
MIC range	**0.016- 256 (µg/ml)**			**0.002- 32 (µg/ml)**		
Sensitivity	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
MIC (µg/ml)	≥ 16	8	≤ 4	≥ 4	2	≤ 1
No (%)	45 (97.8)	0 (0)	1 (2.2)	24 (52.2)	1 (2.2)	21 (45.7)

From 46 ESBL producing isolates, 21 (45.7%) isolates had at least one of the *qnr* genes. *qnr*B and *qnr*S were detected in 21 (45.7%) and 7 (15.2%) isolates, respectively, while *qnr*A was not detected. 7 (15.2%) isolates were positive for both *qnr*B and *qnr*S genes (Fig. 2).

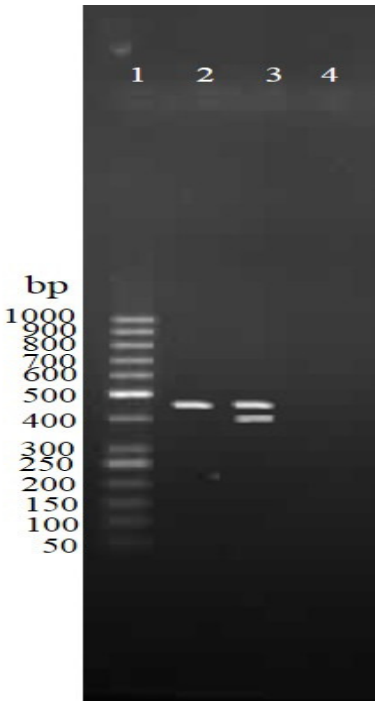

Fig. 2. Evaluation of *qnr*B and *qnr*S genes PCR products by agarose gel electrophoresis. Lane 1: 50 bp DNA ladder; Lane 2: *qnr*B; Lane 3: *qnr*B and *qnr*S; Lane 4: negative control.

Nucleotide sequence accession number

The sequences of *qnr*B and *qnr*S have been indexed in Genbank database and assigned accession numbers KT315571, KT315572 for *qnr*B and KT315570, KT315573 for *qnr*S.

Discussion

The indiscriminate use of antibiotics not only inhibits bacteria, but also causes antibiotic resistance. ESBL enzymes have increased in the recent years substantially and ESBL producing bacteria due to inactivation a wide range of beta-lactam drugs, especially third generation cephalosporins, have faced treatment with many problems. The emergence and spread of these bacteria are often due to the widespread use of broad-spectrum beta-lactams. Patients in Intensive Care Units (ICUs) because of weakened immune system are mainly more prone to getting infected with these organisms [17]. ESBL producing *Klebsiella pneumoniae* isolates are important virulence factors that causing infections in hospitalized patients. Since these isolates show resistance to beta-lactam and other antibiotic drugs, they are often difficult to treat. These isolates can easily transfer resistance genes to other by plasmid [18]. The cause of this resistance may be associated with the coordinated expression of several resistance mechanisms [6]. The

plasmids that carry genes for ESBLs may also carry *qnr* genes for resistance to quinolones. Recently, a large number of studies have reported that the *qnr* genes are often encountered in ESBL-producing isolates [19-21]. In the current study, 54 (41.5%) isolates were resistant to at least one of the third generation cephalosporins and ESBL production was found in 46 (85.2%) isolates. Similar to our findings, Nasehi et al reported that 41% of *Klebsiella pneumoniae* isolated from different clinical specimens were resistant and 96% of them were identified as ESBL producers [22]. This study showed that from 130 isolates, 46 (35.4%) isolates were positive for ESBLs production, while Mirsalehian et al detected 76.7% ESBL positive rate among *Klebsiella pneumoniae* isolated from different clinical specimens in ICUs in Tehran [17]. This difference indicates the more widespread prevalence of ESBLs producing isolates in ICUs. In Mexico, *Klebsiella pneumoniae* isolated from different clinical specimens were analyzed by combination disk method and 35.9% of isolates were ESBLs producers [23].

Nineteen percent of *Klebsiella pneumoniae* isolated from community-acquired UTIs in Colombia were ESBLs producers, which were lower than our results [24]. So, this difference shows that stay in the hospital and the use of broad-spectrum cephalosporins widely can increase ESBL producing *Klebsiella pneumoniae* infections.

In this study, 21 (45.7%) and 7 (15.2%) of ESBL producing isolates were positive for *qnr*B and *qnr*S, respectively. *qnr*A was not identified in any isolate. 21 (45.7%) isolates had at least one of the *qnr* genes. In china, Jiang et al detected *qnr*A, *qnr*B and *qnr*S in 8.1%, 4% and 4% of ESBL producing *Klebsiella pneumoniae* isolates, respectively [25].

Wang et al found *qnr*A, *qnr*B and *qnr*S determinants in 2.4%, 6.1% and 15.1% of *Klebsiella pneumoniae* strains producing ESBL or AmpC-type β-lactamase isolated from pediatric hospitals in China, respectively [6]. The prevalence of *qnr*S was in agreement with our findings. In Korea, Kim et al reported 40.5% of ESBL producing *Klebsiella pneumoniae* isolates had at least one of the *qnr* genes and *qnr*A was not identified, which is similar to our findings. *qnr*B4 and *qnr*S1 were detected in 33.3% and 7.1% of isolates [3].

PMQR causes low-level resistance to fluoroquinolones and it increases when these isolates exposure to them [26]. So, the differences in the results of various studies are related to the pattern of consumption and prescription of antibiotics and how to use different tools for infection controls in hospitals in various geographical regions.

Conclusion

The results of this study show a high prevalence of *qnr* genes in ESBL producing *Klebsiella pneumoniae* isolates. Since these genes reside on transmissible plasmids and can spread quickly, it is recommended that antimicrobial susceptibility tests are performed before treatment and the results are reported to the clinicians and infection control committees. Advance drug resistance surveillance and molecular characteristics of ESBL producing isolates are necessary to guide the appropriate antibiotic use.

Conflict of interest

All authors declare that there is no conflict of interest.

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