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Quinolone Resistance Determinants *qnr*, *qep*, and *aac(6')-Ib-cr* in Extended-Spectrum B-Lactamase Producing *Escherichia coli* Isolated From Urinary Tract Infections in Tehran, Iran

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Abstract

Background: Prevalence of plasmid mediated quinolone resistance in *Escherichia coli* clinical isolates is a serious problem in developing countries.
Objectives: The aim of this study was to investigate quinolone resistance determinants among Extended-Spectrum B-Lactamases (ESBL)-producing *E. coli* recovered from patients with nosocomial urinary tract infection.
Methods: A total of 290 *E. coli* isolates, obtained from patients with UTI, were included in this study. The phenotypic confirmatory test for ESBL production was performed by double disc synergy test and combined disk diffusion test. Kirby-Bauer Disk diffusion method was performed to test susceptibilities of ESBL-producing *E. coli* isolates to 15 antimicrobial agents. Isolates were screened for the presence of ESBL and plasmid-mediated quinolone resistance genes by polymerase chain reaction (PCR).
Results: In the present study, 290 (71.7%) *E. coli* strains were recovered from 410 hospitalized patients with UTI, 51.7% of which were found to have ESBL positive results. In vitro, the susceptibilities of ESBL-producing *E. coli* strains showed that all isolates were resistant to amoxicillin and penicillin and resistance rates to other of antibiotics differed from 40% to 96%. Among the 150 ESBL positive isolates, frequencies of *aac(6')-Ib*, *oqxA*, *oqxB*, *qnrA*, *qnrB*, *qnrS*, and *qepA* were 74.7%, 8%, 4%, 3.3%, 1.3%, 2%, and 2.7%, respectively. Coexistence of *bla* CTX-M, *bla* TEM, *bla* CTX-M, and *aac(6')-Ib* were the most widely distributed resistance genotypes.
Conclusions: The data of the present study revealed the high prevalence of plasmid-mediated quinolone resistance genes among ESBL-producing *E. coli* in the hospitals. The *bla* CTX-M genes were found to be the dominant ESBL-encoding gene.

Keywords: Extended-Spectrum B-Lactamase (ESBL), Plasmid-mediated Quinolone Resistance, Beta Lactamase (PMQR), Urinary Tract Infection (UTI)

1. Background

Urinary tract infection (UTI) is one of the most frequent types of nosocomial infections and probably effects nearly one-half of all people during their lifetime. Many clinicians are commonly encountered with UTIs in developing countries. The emergence of extended-spectrum β-lactamase (ESBL), as an important cause of transferable multidrug resistance in gram-negative bacteria, particularly *E. coli*, is a health problem throughout the world (1). The production of beta lactamase as the predominant cause of resistance to β-lactam antibiotics among bacteria is mostly mediated by acquisition of beta lactamase genes located on mobile genetic elements, such as plasmids or transposons (2). According to several studies, Plasmid-Encoded Temoneira (TEM), Sulfhydryl Variable (SHV) and Cefotaximase (CTX-M) are the most prevalent ESBLs, which are classified in class A ESBLs, yet the distribution of ESBL genotypes could vary according to the antimicrobial agents used in each hospital or local community (3). Moreover, quinolone resistance in *E. coli* clinical isolates became a serious problem in developing countries as well as in developed countries, since the introduction of quinolones for treatment of UTI caused by *E. coli* for human bacterial infections (4). For years, quinolone resistance was thought to be only the result of chromosomal mechanisms including mutations in DNA gyrase and topoisomerase genes quinolone resistance-determining regions (QRDRs), active export of the drugs via efflux pumps and also decreased permeability related to porin loss (5). Recently, studies have shown that plasmid-mediated quinolone resistance (PMQR) plays an important role in the development of resistance to quinolone. The first report of PMQR mechanisms was in a *Klebsiella pneumonia* strain from the United States, during year 1998 (6).

To date, at least 3 types of PMQR determinants, includ-

ing qnr families, *aac(6')-Ib-cr*, and active efflux pumps have been described in clinical isolates (7). The pentapeptide-repeat family Qnr proteins (QnrA, QnrB, QnrS, QnrC, and QnrD) protect DNA gyrase and topoisomerase IV from quinolone inhibition (6). The *aac(6')-Ib-cr* gene encodes an aminoglycoside acetyltransferase that modifies not only aminoglycosides but also ciprofloxacin and norfloxacin (5). QepA, an efflux pump belonging to the major facilitator subfamily, is related to a decrease in susceptibility to hydrophilic fluoroquinolones (7). OqxAB, a multidrug efflux pump, is related to reduced fluoroquinolone susceptibility and resistance to multiple agents (6, 7). However, in addition to chromosomal mutations, the horizontal transfer of plasmids carrying quinolone resistance determinants plays an important role in increasing rates of resistance to quinolone (6, 8). The existence of multiple resistance genes on the same plasmid and their transfer between ESBL-producing clinical isolates is a great concern. Although the clinical relevance of PMQR is unknown, it has been frequently reported that some PMQR genes are thought to be linked with ESBL production and some are often found to be located on the same plasmid, so that some reports showed an association of *qnr* genes with ESBL and AmpC beta-lactamase (9, 10). Nowadays, rapid dissemination of resistance to β-lactams and quinolones among *E. coli* clinical isolates has been described, which finally lead to problems for the treatment of infections (11). There are still a few reports on the prevalence of PMQR genes and their different types in ESBL-producing *E. coli* isolated from patients with nosocomial UTI in the area.

2. Objectives

The present study aimed at providing insights in to the understanding of the distribution and dissemination of PMQR determinants among ESBL-producing *E. coli*, recovered from patients with nosocomial UTI in Iran.

3. Methods

3.1. Study Setting and Bacterial Isolates

A total of 290 non-duplicated *E. coli* clinical isolates from the 410 urine specimens of hospitalized patients with UTI, during a period of 13 months from January 2015 to January 2016, were included in this study. Nosocomial UTI was confirmed through clinical examination conducted by a physician. All of the enrolled cases had a history of nosocomial UTI. All the urine specimens after transportation to the laboratory were processed immediately. Isolates were identified by characteristic morphology of colony, gram stain, and routine standard biochemical tests. Colony count semi-quantitative method was performed according to surface streak procedure using calibrated loops. The cultured plates were incubated under aerobic conditions at 37°C for 24 to 48 hours. Measurements equal or more than 105 CFU/mL were considered as positive UTI test results (12). Confirmed samples as *E. coli* isolates were stored in Tryptic Soy Broth (TSB; Merck, Germany) containing 20% glycerol at -70°C for further studies.

3.2. Extended-Spectrum B-Lactamase Confirmatory Test

The phenotypic confirmatory test for ESBL production was performed by double disc synergy test (DDST) and combined disk diffusion test (CDDT), according to the clinical and laboratory standards institute (CLSI) criteria for ESBL screening (13). All the isolates were also screened for *bla* genes (SHV, TEM, and CTX-M) using polymerase chain reaction (PCR).

3.3. Double Disc Synergy Test (DDST)

Double disc synergy test was done using cefotaxime (30 μg) and ceftazidime (30 μg) with and without clavulanic acid (10 μg) discs on mueller-hinton agar (MHA, Oxoid, United Kingdom), 25 mm apart from each other. The organisms were considered to be producing ESBL when the zone of inhibition was equal or more than 5 mm for either antimicrobial agent tested with clavulanic acid versus its zone when tested without clavulanic acid (14).

3.4. Combined Disc Diffusion Test (CDDT)

Combined disc diffusion test was done using both ceftazidime (30 μg) disc alone and in combination with clavulanic acid (30 μg/10 μg). Discs were placed 25 mm apart from each other. Zone of inhibition diameter more than or equal to 5 mm, for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone, was interpreted as ESBL production (15). *Escherichia coli* ATCC 25922 was used as the quality control.

3.5. Antimicrobial Susceptibility Testing

Kirby-Bauer disk diffusion method was performed to test susceptibilities of ESBL-producing *E. coli* isolates to 15 antimicrobial substances, according clinical laboratory and standards institute (CLSI) recommendations (13). The antimicrobials tested were aztreonam (AT 30 μg), gentamicin (GEN 10 μg), ciprofloxacin (CIP 5 μg), amikacin (AK 30 μg), ceftazidime (CZX 30 μg), imipenem (IMP 10 μg), cefotaxime (CTX 30 μg), piperacillin (PI 100 μg), cefoxitin (CX 30 μg), cephalexin (CN 30 μg), co-trimoxazole (COT 25 μg), amoxicillin (AMX 30 μg), penicillin (P 10 μg), norfloxacin (NX 10 μg), and nalidixic acid (NA 30 μg). Antibiotic disks

used in this research were supplied by HiMedia laboratories Pvt, Ltd., Mumbai, India. *Escherichia coli* ATCC 25922 was used as the quality control strain in susceptibility testing. Multidrug-resistance (MDR) was defined as resistance to at least 3 or more unrelated antibiotics (16). The confirmed samples as *E. coli* were stored at -70°C in Tryptic soy broth (TSB; Merck, Germany), containing 20% glycerol and were subjected to molecular identification.

3.6. DNA Extraction and Polymerase Chain Reaction Assay

The DNA template for the PCR process was extracted from a pure colony of an overnight growth of *E. coli* isolates on Luria-Bertani agar (Oxoid, UK), using the QIAamp DNA isolation kit (Qiagen, Hilden, Germany), precisely according to the manufacturer's recommendations. The concentration of the total extracted DNA was assessed by a spectrophotometer.

All isolates were screened for the presence of ESBL and PMQR genes by PCR amplification using primers listed in Table 1. The amplicons were visualized under ultraviolet light after separation by 1.2% agarose gel electrophoresis at 80 V for 2 hours and staining with ethidium bromide. Both strands of the purified amplicons were sequenced and compared with genes in the GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm their identities.

3.7. Statistical Analysis

Statistical analysis was performed using the SPSS software for Windows, version 17.0 (SPSS Inc., Chicago, IL).

4. Results

4.1. Prevalence of Extended-Spectrum B-Lactamase-Producing Escherichia coli Isolates

In this study, 290 (70.7%) *E. coli* strains were recovered from 410 hospitalized patients with UTI. The age of patients was equally distributed in 74.7% female and 25.3% male sample (M: F ratio was 0.3). The patient's age ranged from 9 months to 75 years old with a median of 43 years. The highest occurrence of nosocomial UTI was in the age group of 35 to 50 years (48%). The prevalence of ESBL-positive *E. coli* isolates in nosocomial UTIs was 51.7% by DDST and CDDT phenotypic methods.

4.2. Antimicrobial Drug Susceptibility

The result of disk-diffusion susceptibility testing indicated high frequency of the resistance to the majority of antimicrobial agents. All isolates were resistant to amoxicillin and penicillin. The susceptibility rates of isolates to other antibiotics were as follows, co-trimoxazole 96%, cephalexin 88%, ceftazidime 86%, cefotaxime 80%, ciprofloxacin 80%, nalidixic acid 78%, cefoxitin 74.7%, norfloxacin 70%, gentamicin 65.4%, piperacillin 41.3%, and aztreonam 40%. Overall, 109 (72.7%) and 132 isolates (88%) were highly susceptible to amikacin and imipenem, respectively. Of the 150 isolates, 130 (86.7%) were MDR. The predominant resistance profile among the isolates was resistance to 10 antibiotics (18.7%), followed by 7 antibiotics (16.7%), and 5 antibiotics (15.3%). Out of 150 ESBL-positive *E. coli*, 55 isolates (36.7%) were simultaneously resistance to nalidixic acid, ciprofloxacin, and norfloxacin.

4.3. Prevalence of bla and Plasmid-Mediated Quinolone Resistance Genes

In total, out of 150 ESBL-positive isolates, 120 isolates (80%) harbored *bla* CTX-M followed by *bla* TEM (70.7%) and *bla* SHV (50.7%), respectively. The *aac(6')-Ib* was the most prevalent plasmid-mediated mechanism of quinolone resistance in Iran. Among the 150 ESBL-positive isolates, *aac(6')-Ib* was detected in 112 isolates (74.7%), *oqxA* in 12 isolates (8%), *oqxB* in 6 isolates (4%), *qnrA* in 5 isolates (3.3%), and *qnrB* (1.3%), *qnrS* (2%) and *qepA* in 4 isolates (2.7%), which were confirmed by sequencing of the PCR products. Out of 10 *qnr*-positive isolates, 8 isolates were resistant to nalidixic acid and ciprofloxacin, whereas 2 isolates were resistant to nalidixic acid and susceptible to ciprofloxacin. Two isolates carried both *oqxA* and *oqxB* genes and were resistant to norfloxacin, cefotaxime, co-trimoxazole, ciprofloxacin, and nalidixic acid. Coexistence of ESBL and PMQR genes was identified in 107 (71.3%) *E. coli* isolates. Coexistence of *bla* CTX-M and *aac(6')-Ib* was the most widely distributed resistance genotype, which was observed in 40 (26.7%) isolates. The distribution of ESBL-encoding genes and PMQR genes among 150 ESBL producers is shown in Table 2.

5. Discussion

The widespread use of beta lactam and quinolone antibiotics for treatment of *E. coli* infections has recently led to the spread of resistance to these antibiotics. The increase of resistant isolates to beta lactam and quinolone antibiotics is a great concern for the empirical treatment of nosocomial infections and public health because these traits can be horizontally-transmitted to other isolates by plasmids (18-21). In the recent years, increase of ESBL-producing *E. coli* isolates has been reported from different parts of the world, particularly Asia (3). Several studies have suggested that distribution of ESBL-producing *E. coli* isolates and ESBL genotypes vary according to the studied area. In Turkey, 84% (22), Portugal 67.9% (23), Colombia 11.7% (24) and Japan 20.4% (25) of *E. coli* isolates were re-

Table 1. Polymerase Chain Reaction Primers Used to Detect bla Genes and Plasmid-Mediated Quinolone Resistance Genes in This Study

Target Gene	Nucleotide Sequence	Size of the Amplified Product (bp)	Reference
blaTEM	5´-TCGGGGAAATGTGCGCG-3´	972	(17)
	5´-TGCTTAATCAGTGAGGCACC-3´		
bla SHV	5´-GGGTTATTCTTATTTGTCGC-3´	615	(3)
	5´-TTAGCGTTGCCAGTGCTC-3´		
bla CTX-M	5´-ACGCTGTTGTTAGGAAGTG-3´	857	(3)
	5´-TTGAGGCTGGGTGAAGT-3´		
aac (6')-Ib	5´-TTGCGATGCTCTATGAGTGGCTA-3´	482	(18)
	5´-CTCGAATGCCTGGCGTGTTT-3´		
qepA	5´-CTGCAGGTACTGCGTCATG-3´	403	(4)
	5´-CGTGTTGCTGGAGTTCTTC-3´		
oqxA	5´-GACAGCGTCGCACAGAATG-3´	339	(19)
	5´-GGAGACGAGGTTGGTATGGA-3´		
oqxB	5´-CGAAGAAAGACCTCCCTACCC-3´	240	(19)
	5´-CGCCGCCAATGAGATACA-3´		
qnrA	5´-AGAGGATTTCTCACGCCAGG-3´	619	(19)
	5′-GCAGCACTATKACTCCCAAGG-3´		
qnrB	5′-GATCGTGAAAGCCAGAAAGG-3´	469	(20)
	5′-CGATGCCTGGTAGTTGTCC-3´		
qnrS	5´GCAAGTTCATTGAACAGGCT-3´	428	(4)
	5′-TCTAAACCGTCGAGTTCGGCG-3´		

ported to be ESBL-producing strains, while 55.6% of the isolates of the present study were ESBL. These differences may be associated with type and volume of samples, design of the study, and drug regimens in different geographical regions. The results of antibiotic susceptibility test revealed that all isolates were resistant to amoxicillin and penicillin and the majority of the isolates were resistant to beta lactam, as well as quinolone. The findings were in accordance with recent data (11). This is an alarm for clinicians, suggesting that prescription of these antibiotics must be changed. Although predominant genotypes of ESBLs in different studies were diverse, new CTX-M β-lactamases have emerged as prevalent ESBL worldwide type. With regards to the different types of ESBL, as expected, CTX-M enzymes were the most prevalent type of ESBL (80%) followed by TEM (70.7%) and SHV (50.7%). The high prevalence of CTX-M gene in the current study was consistent with other studies worldwide. In a study conducted in Portugal, CTX-M-producer strains were the most prevalent type of ESBL (76%) among bacteria isolated from UTIs (26). In another study done in Lithuania, the prevalence of CTX-M among *E. coli* (96%) and *K. pneumoniae* (71 %) isolates was very high (27). In a number of studies, the predominant genotypes of ESBLs were diverse, for instance in Italia, Portugal, and Turkey the most predominant ESBL genotype was TEM. The findings of the current study about CTX-M confirm the theory that CTX-M enzymes are replacing SHV and TEM enzymes as the prevalent ESBL type.

Quinolone, as a major antibacterial component, is often administered with other antimicrobials, most notably beta lactams, for treatment of *E. coli* infections especially in patients with UTI. As previously mentioned, PMQR genes play an important role in resistance to quinolones because of their horizontal transfer (28). The existence of resistance to quinolone in ESBL-producing isolates has been previously reported (29). In the present study, percentage of quinolone resistant isolates (68%) was substantially higher than those in Spain (19.4%) (30), Italy (44%) (31), and France (30%) (32). Warburg et al. showed that resistance to fluoroquinolone during a periods of 14 years in nosocomial isolates of *E. coli* has increased from 11.3% to 46.6%, and also reported that from 1991 to 1997, no isolate had *aac(6')-Ib-cr*, whereas from 1998 onwards, 7.1% of the isolates had *aac(6')-Ib-cr* (32). High levels of resistance ob-

Table 2. Distribution of Extended-Spectrum Β-Lactamases (ESBL)-Encoding Genes and Plasmid-Mediated Quinolone Resistance Genes among 150 ESBL-Producers

Coexisting Resistance Genes	No (%)
***bla* CTX-M**	120 (80)
***bla* TEM**	106 (70.7)
***bla* SHV**	76 (50.7)
aac(6´)-Ib	112 (74.7)
qepA	4 (2.7)
qnrA	5 (3.3)
qnrB	2 (1.3)
qnrS	3 (2)
oqxA	12 (8)
oqxB	6 (4)
***bla* CTX-M and *bla* TEM**	60 (40)
bla* CTX-M and *aac(6')-Ib	40 (26.7)
***bla* CTX-M and *bla* SHV**	10 (6.7)
bla* CTX-M, *bla* TEM, *bla* SHV and *aac(6´)-Ib	5 (3.3)
bla* CTX-M, *oqxA*, *oqxB*, *qnrA	3 (2)
bla* TEM, *bla* SHV and *aac(6´)-Ib	15 (10)
bla* TEM and *aac(6´)-Ib	15 (10)
***bla* TEM and *bla* SHV**	11 (7.3)
bla* SHV and *aac(6´)-Ib	25 (16.7)
bla* SHV and *aac(6´)-Ib*, *oqxA	4 (2.7)
aac(6´)-Ib*, *qnrA*, *qnrS	2 (1.3)
aac(6´)-Ib*, *oqxA*, *qepA	3 (2)

served in the current study could be associated with acquired resistance genes. In this study, the dominant PMQR gene was *aac(6')-Ib* (74.7%) followed by *oqxA* (8%), *qnr* (6.7%), *oqxB* (5.3%), and *qepA* (2.7%). These isolates were present in both quinolone resistance and susceptible isolates. High frequency of *aac(6')-Ib* among ESBL producers has been reported previously (32). In a study conducted on 514 clinical *E. coli* isolates in China, Zhou et al. exhibited that the dominant PMQR gene was *aac(6')-Ib* (49.2%) (28). However, our findings support earlier suggestions of a linkage between *aac(6')-Ib-cr* and CTX-M ESBLs (32).

In 2011, Briales et al. showed that out of 382 isolates of ESBL-producing *E. coli* and *K. pneumoniae*, 14 isolates (3.7%) were positive for qnr genes (3 qnrA1, 5 qnrB like and 6 qnrS1) and 62 isolates (16.2%) were positive for the mutant variant of *aac(6')-Ib -cr*. According to this study, in Spain, the *aac(6')-Ib-cr* gene was the most prevalent PMQR gene in *E. coli* and *K. pneumoniae*-producing ESBL (30).

In a study conducted on quinolone resistance amongst *E. coli* strains isolated from UTI by Firoozeh et al., (2013) frequencies of qnrA and qnrB in nalidixic acid-resistant isolates were 12.1% and 7.8% and also in ciprofloxacin-resistant isolates, these were 22.2% and 14.3%, respectively (33). However, in the present study, the prevalence of *qnrA* and *qnrB* was 3.3% and 1.4% respectively. This difference may be due to the selected *E. coli* population.

OqxAB, as a multidrug efflux pump, confers resistance to quinolone and other agents in clinical isolates. This gene has also been found in *E. coli* isolated from pigs, chicken, and farm workers (34). In this study, it was determined that the oqxAB gene is present in 20 (13.3%) isolates, which was higher than those in China (6.6%), Sweden (1.8%), and South Korea (0.4%) (21). The high rates of *oqxAB* gene in this study might support the speculation that misuse and abuse of antibiotics and even close contact with domestic animals could be the presumptive cause for dissemination of oqxAB gene. This is consistent with other researches, which found that *qepA* was rarely present in *E. coli* clinical isolates (35-37). In the current study, *qepA* was detected in 2.7% of isolates.

In summary, this study revealed the high prevalence of PMQR genes in ESBL-producing *E. coli* at the studied hospitals. The *bla* CTX-M genes were found to be the dominant ESBL-encoding gene. Early detection and routine screening of ESBL-producing *E. coli* for PMQR carriage is very important for the prevention of the development of newer determinants in the studied area.

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