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Dissemination of Extended-Spectrum β-Lactamases and Quinolone Resistance Genes Among Clinical Isolates of Uropathogenic *Escherichia coli* in Children

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Background: Urinary tract infection (UTI) is one of the most common childhood bacterial infections and *Escherichia coli* is the major pathogen. Producing β-lactamase enzymes are the most common mechanism of bacterial resistance.
Objectives: This study aimed to determine the prevalence of Extended-Spectrum β-Lactamases (ESBLs) and Quinolone Resistance *(qnr)* genes in *E. coli* strains isolated from UTIs.
Materials and Methods: In this study, a total of 120 isolates of *E. coli* from urinary tract infections of the children were collected at Besat Hospital in Hamadan, Iran, from October 2010 to October 2011. The bacterial isolates were identified by standard biochemical methods. Antimicrobial susceptibilities were determined by disk diffusion method, and ESBLs-producing was confirmed phenotypically using the double-disk synergy (DDS) test. The presence and identification of ESBLs and *qnr* genes were determined by Polymerase Chain Reaction (PCR).
Results: The highest sensitivity was seen to imipenem (96.7%), amikacin (92.5%), nitrofurantoin (93.3%), ofloxacin (81.7%), gentamicin norfloxacin (70.8%), and ciprofloxacin (79.2%). In contrast, the highest rate of resistance was seen to co-trimoxazole (77%) and nalidixic acid (40.9%). The results showed that 6 (2.18%) and 4 (1.12%) isolates of ESBL-producing *E. coli* were positive with respect to having *qnrB* and *qnrS* genes, respectively. No isolates was found to have *qnrA*.
Conclusions: CTX-M was the most prevalent ESBL genotype in uropathogenic *E. coli* (UPEC) isolated from UTI. In addition, a high frequency of *qnr* genes among ESBL-producing *E. coli* was identified in this study. In order to avoid treatment failures, we recommend using phenotypic and molecular methods to diagnose these enzymes and *qnr* genes.

Keywords: Quinolones; Antibiotic Resistance; *Escherichia coli*

1. Background

Urinary tract infection (UTI) is one of the most common childhood bacterial infections and also the second common infection among different societies. It is also an important factor in creating scar and progressive destruction of renal structure, chronic renal failure, poor growth, urinary stones, and hypertension in children (1). *Escherichia coli* is the most important opportunistic pathogen causing more than 80% of urinary tract infections (2). β-Lactam antibiotics have been widely used to treat *E. coli* infections; however, treatment of UTIs has become increasingly problematic. The incidence rate of antibiotic resistance towards these antibiotics is growing every day in the world (3). Producing β-lactamase enzymes are the most common mechanism of bacterial resistance. Extended-spectrum β-lactamases (ESBLs)-producing bacteria have usually multi-drug resistance, because most of the times, the genes related to the other resistive mechanisms have been also placed on the same plasmid carrying the genes encoding ESBLs (4-6).

Fluoroquinolones are wide spectrum potent drugs, used in treating a wide range of infections. Although fluoroquinolones are not approved for use in persons under 18 years of age, with increasing resistance to cephalosporins, they can be used in critically ill patients with Gram-negative infections and in children with complicated urinary tract infections (7). Their extensive use has been associated with raising level of quinolone resistance. The principle mechanism of quinolone resistance was attributed to the chromosomal mutations in DNA gyrase, DNA topoisomerase IV, or active efflux pumps. However, a plasmid-mediated quinolone resistance gene encoding a pentapeptide repeat protein (*qnr*) was discovered in 1998 (8).

A range of plasmid-mediated quinolone resistance determinants, including *qnr*, have been reported global in clinical isolates of *Enterobacteriaceae* (9, 10). These *qnr* genes are frequently associated with ESBLs (9). Various studies around the world have shown that the releasing *qnr* genes are among the bacterial isolates. Gene *qnrA* causes quinolone resistance and Minimum inhibitory concentration (MIC) increases the fluoroquinolones up to 32 times in *E. coli*. This gene has been reported from all over the world. The other *qnrB* and *qnrS* plasmid resistance genes are also detected in *Enterobacteriaceae*, which have the amino acid sequence similarity of 41% and 60% with *qnrA* (10-12). Resistance of Gram-negative bacteria to the extended-spectrum β-lactam antibiotics has expanded rapidly in last two decades. This resistance is largely attributed to the plasmids, containing genes encoding ESBLs. Since the quinolone resistance genes are also located on plasmid.

2. Objectives

We studied the prevalence of ESBLs and frequency of *qnrA*, *qnrB,* and *qnr*S genes in *E. coli* strains isolated from UTIs in children.

3. Materials and Methods

This cross-sectional descriptive-analytic study was done on 120 urine samples collected from children suffering from UTI at Besat hospital (children's ward), Hamadan, Iran from October 2010 to October 2011. Midstream urine collection method was performed for toilet-trained children and catheter or suprapubic aspiration was used for non-toilet-trained children. Conventional biochemical tests were used for bacterial identification. The ethics committee of the Hamadan University of Medical Sciences approved the study protocol (No.: 891224115). Inclusion criteria were children younger than 12 years suspected to UTI at Besat hospital and the exclusion criteria were as follows: 1) the patients who had received antibiotic before doing urine sample, 2) The patients that in their urine culture, more than one type of microorganism had grown and sampling was done by urine bag.

3.1. Antimicrobial Susceptibility

Antimicrobial susceptibilities were determined by disk diffusion agar assay (13) to co-trimoxazole (25 μg), amikacin (30 μg), gentamicin (10 μg), ceftriaxone (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), cefixime (5 μg), cefotaxime (30 μg), ceftazidime (30 μg), cefpodoxime (10 μg), azteroname (30 μg), nitrofurantoin (300 μg), and imipenem (10 μg) (India HIMEDIA company). The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (14). The quality control of the study was done by *E. coli* ATCC 25192.

3.2. Determination of Extended-Spectrum β-Lactamases-Producing Strains by the Combined Disk Method

In order to identify ESBL-producing strains, the double-disk synergy (DDS) test was carried out on Mueller-Hinton agar (Himedia CO, India) with disks, containing ceftazidime (30 μg), cefotaxime (30 μg), cefpodoxime (30 μg), and ceftriaxone (30 μg) placed respectively on the MH agar to a distance of 20 mm from combined disks of ceftazidime/clavulanic acid (30.10 μg), cefotaxime/clavulanic acid (30.10 μg), cefpodoxime/clavulanic acid (30.10 μg), and ceftriaxone/clavulanic acid (30.10 μg). The zone diameters were read using the CLSI assay. A difference of ≥ 5 mm between the inhibition zone diameters of either of the cephalosporin disks and their respected cephalosporin-clavulanate disk is considered to be phenotypic confirmation of ESBLs production (14).

3.3. DNA Extraction

DNA of ESBL or non-ESBL -producing or quinolone-resistant strains of *E. coli* was extracted by boiling method as per the guidelines (15). PCR amplification of *bla* genes, including *bla-CTX- M*, *bla-SHV*, *bla-TEM*, and *qnr* genes (*qnrA*, *qnrB*, *qnrS*) were performed with the specific primers (16, 17) listed in Table 1. ESBL-positive and ESBL-negative isolates were screened by multiplex PCR for *qnr* genes. PCR products were analyzed by electrophoresis in a 1.5% agarose gel, containing SYBR safety at 100 V for 30 min.

Table 1. The Primers Used in This Study

Target	Primer Sequence('3 → '5)	Size, bp	References
bla-CTX-M	TCTTCCAGAATAAGGAATCCC	909	(17)
	CCGTTTCCGCTATTACAAAC		
Bla-SHV	CTTTACTCGCTTTATCG	868	(17)
	TCCCGCAGATAAATCAC		
bla-TEM	ATGAGTATTCAACATTTCCG	931	(17)
	CCAATGCTTAATCAGTGAGC		
qnrA	AGAGGATTTCTCACGCCAGG	580	(18)
	TGCCAGGCACAGATCTTGAC		
qnrB	GGMATHGAAATTCGCCACTG	264	(18)
	TTTGCYGYYCGCCAGTCGAA		
qnrS	GCAAGTTCATTGAACAGGGT	428	(18)
	TCTAAACCGTCGAGTTCGGCG		

3.4. Data Analysis

Data analyzed by SPSS v.16 using McNemar and Chi-square tests. P ≤ 0.05 is regarded as significant at 95% confidence interval.

4. Results

Susceptibility testing: In this study, 120 strains of Uropathogenic *E. coli* (UPEC) isolated from the urine samples of children with UTIs were studied. Of 120 isolates, 25% (n = 30) were sensitive to co-trimoxazole, 92.5% (n = 111) to amikacin, 62.5% (n = 75) to ceftriaxone, and 50.8% (n = 61) to nalidixic acid (Table 2). The results demonstrate that 41 strains (34.2%) were resistant to three or more antibiotics and are known as the strains with multidrug resistance (MDR).

4.1. Prevalence of Extended-Spectrum β-Lactamases in Urinary E. coli Isolates

The Double-disk synergy test (DDST) detected ESBLs in 33 (27.3%) of isolates which means 87 (71.9%) were non-ESBLs *E. coli* isolates. For comparison of resistance prevalence to quinolones in ESBL-producing *E. coli* strains and ESBL-nonproducing strains, intermediate resistance rate of isolates were considered as resistance (Table 3).

Statistical analysis of the results revealed that there is a significant relation between resistance to ciprofloxacin and nalidixic acid in ESBL producing isolates (P < 0.05). However, the prevalence rate of resistance to ofloxacin and norfloxacin in strains of ESBL-producing was more than the strains of non-producing ESBLs. After doing statistical analysis the significant relation was not confirmed.

4.2. Detection of β-Lactamase and qnr genes by Polymerase Chain Reaction

A total of 120 UPEC were tested for bla-CTX-M, *bla-SHV,* and *bla-TEM* genes. Our results showed that 66.7% (n = 80), 20.8% (n = 25), and 40.8% (n = 49) of *E. coli* isolates harboring *bla-CTX-M*, *bla-SHV,* and *bla-TEM*, respectively (Table 4). Among the investigated *E. coli* isolates, 11.7% (14 out of 120) contained *qnr* genes, of which, *qnrB* (n = 8, 57.1%) and *qnrS* (n = 6, 42.9%) were detected. The *qnrA* gene was not found in any strains. The distribution of *qnr* containing isolates in ESBL-producing and quinolone-resistant strains are shown in Table 5. Based on the statistical analysis about strains resistant to quinolones, the results of Chi-squared and TauKendal tests proved the existence of a significant relation between the quinolone resistance and presence of *qnr* genes (P < 0.05).

Table 2. Frequency Distribution of Resistance and Antibiotic Sensitivity in Strains of Uropathogenic *E. coli* [a]

Antibiotic	Resistance	Intermediate	Sensitive
Cefixime	41 (34.2)	5 (4.2)	74 (61.6)
Ceftazidime	42 (35)	8 (6.7)	70 (58.3)
Cefotaxime	43 (35.8)	7 (5.8)	70 (58.3)
Ceftriaxone	39 (32.5)	6 (5)	75 (62.5)
Cefpodoxime	37 (30.8)	11 (9.2)	72 (60)
Imipenem	3 (2.5)	1 (0.8)	116 (96.7)
Azteroname	40 (33.3)	12 (10)	68 (56.7)
Gentamicin	21 (17.5)	2 (1.7)	97 (80.8)
Amikacin	7 (5.8)	2 (1.7)	111 (92.5)
Co-trimoxazole	85 (70.8)	5 (4.2)	30 (25)
Nalidixic acid	49 (40.9)	10 (8.3)	61 (50.8)
Nitrofurantoin	0 (0)	8 (6.7)	112 (93.3)
Ciprofloxacin	(15) 18	(5.8)7	(.279)95
Norfloxacin	18 (15)	5 (4.2)	97 (80.8)
Ofloxacin	17 (14.2)	5 (4.2)	98 (81.7)

[a] Data are presented as No. (%).

Table 3. Comparison of Resistance to Quinolones in Extended-Spectrum β-Lactamases-Producing and Non- Extended-Spectrum β-Lactamases-Producing *E. coli* strains [a]

Isolates	Quinolones							
	Ciprofloxacin (CF)		Ofloxacin (OF)		Nalidixic Acid (NA)		Norfloxacin (NX)	
	S	R	S	R	S	R	S	R
ESBL-producing	19 (57.6)	14 (42.4)	19 (57.6)	14 (42.4)	16 (48.5)	17 (51.5)	20 (60.6)	13 (39.4)
Non-ESBL-producing	76 (87.4)	11 (12.6)	79 (90.8)	8 (9.2)	63 (72.4)	24 (27.6)	77 (88.5)	10 (11.5)

[a] Data are presented as No. (%).

5. Discussion

Urinary tract infection is one of the most common childhood infections. The prevalence of UTIs in childhood is 3% - 5% (1). There are various studies about the *E. coli* antibiotic resistance in different parts of the world. For example, Datta et al. stated that the rates of resistance on 87 isolates of *E. coli* to ampicillin, amikacin, cephalexin, ceftazidime, cefotaxime, ceftriaxone, co-trimoxazole, gentamicin, ofloxacin, and ciprofloxacin were 95%, 41%, 94%, 78%, 87%, 87%, 94%, 64%, 93%, and 16%, respectively (18). The spread of ESBL-producing bacteria has been strikingly rapid worldwide, indicating that continuous monitoring system and effective infection control measures are absolutely necessary. In this study, the prevalence of ESBL-producing *E. coli* by DDST method is 27.3%; whereas in 2003, this rate was reported at 28.1% in Lebanon, which is very close to the results of this study (19). Shayanfar et al. (20) in Tehran reported that 28.6% of *E. coli* isolates are ESBL-producing, which is very close to the result of our study (27.3%). Different and disturbing rates have been presented from different places of the world. For example, in a hospital in Poland the rate of ESBL-producing was reported at 92.2% (21) and in some hospitals in Turkey and Spain was 69.14% and 98.4% (22, 23) respectively. In hospitals of Korea in 2005 this rate was equal to 84.3% (24). The reason of differences can be due to prolonged staying of the patients in hospital (21), inappropriate and excessive use of antibiotics (22), duration of hospitalization, invasive diagnostic or therapeutic procedures, type of cephalosporins, and test method.

In the present research, our statistics suggest that CTX-M (66.7%) could be the most prevalent, followed by *bla-TEM* (40.8%) and *bla-SHV* (20.8%). In Pakistan, the frequency of *CTX-M* gene was reported at 100% (25) and in 2011, Yazdi et al. reported it at 87.1% (26). The rate of *bla-TEM* gene in Belgium was reported at 44% (27) and in 2009 in France it was 52.9% (28), which was close to our statistics. The frequency of *bla-SHV* is higher compared with the results of the studies done by Jonas Bonnedahl et al. in France as 11.7% (28) or Hussain et al. in Pakistan as 15.4% (25). In 2011, Yazdi et al. reported the frequency of this gene as 70.6% (26) and in 2008 in Brazil it was equal to 67.8%, which is higher than its frequency in our area.

Table 4. Molecular Features of β-Lactamase Genes in strains of UPEC (n = 120) [a]

Genotype	Isolates
CTX-M.TEM.SHV	13 (10.8)
CTX-M.TEM	25 (20.8)
CTX-M.SHV	8 (6.7)
TEM.SHV	2 (1.7)
CTX-M	34 (28.3)
TEM	9 (7.5)
SHV	2 (1.7)

[a] Data are presented as No. (%).

Table 5. Characteristics of *E. coli* Strains, Containing *qnr* Genes in ESBL-Producing and Quinolone-Resistant Strains

Isolates Number	ESBL Producing	Quinolone-Resistant	Status of Quinolone-Resistant				*qnrA*	*qnrB*	*qnrS*
			CF	OF	NA	NX			
1	+ [a]		S	S	S	S			+
2	+		S	S	S	S		+	
3	+	+	S	S	R	S		+	
4	+	+	R	R	R	R			+
5	+	+	R	R	R	R			+
6	+	+	R	R	R	R		+	
7	+	+	R	R	R	R		+	
8	+	+	S	S	R	S		+	
9	+	+	R	R	R	R			+
10	+	+	R	R	R	R		+	
11		+	R	R	R	R		+	
12		+	R	R	R	R			+
13		+	S	R	S	S			+
14		+	S	S	R	S		+	

[a] Indicates that it was positive.

Our results showed that 49 (40.9%), 18 (15%), 18 (15%) and 17 (14.2%) cases of the isolates were resistant to nalidixic acid, ciprofloxacin, norfloxacin, and ofloxacin, respectively. In the present study, the comparison of resistance patterns in ESBL-producing and -nonproducing *E. coli* demonstrated that the resistance of ESBL-producing bacteria is high and these results were similar to the study of Padmini and associates. We can explain that the plasmids carrying ESBLs genes may also transfer the genes resistant to non β-lactam antibiotics to the other bacteria (29). In this study, by using multiplex-PCR method, we examined the prevalence of *qnr* genes in strains of ESBL-producing *E. coli* and also the strains resistant to quinolones. As a result, the 10 strains (30.3%) of ESBL-producing *E. coli* had *qnr* genes from which, 6 strains (18.2%) possesd *qnrB* gene and 4 strains (12.1%) had *qnrS* gene. In this study, of 14 positive isolates for *qnr* genes, 8(57.1%), 9 (64.3%), 8 (57.1 %), and 11 (78.6.1%) were resistant to Ciprofloxacin (CF), Ofloxacin (OF), Norfloxacin (NX) and Nalidixic acid (NA), respectively. Determinants of *qnr* can confer reduced susceptibility to fluoroquinolones or low-level quinolone resistance.

In 2007, for the first time in the Middle East, two studies were carried out on the prevalence rate of *qnr* genes (17, 30). Oktem et al. in Turkey studied on 78 enterobacterial clinical isolates (including 34 *E. coli* and 44 *Klebsiella pneumonia*), which all were ESBLs (+) and 5 isolates (6.3%) were diagnosed to have *qnrA* gene. The *qnrB* and *qnrS* genes were not detected (30). Cattoir et al. in Kuwait examined 64 ESBL-producing enterobacterial isolates, including 29 *E. coli*, 19 *K. pneumonia*, 9 *Proteus Mirabilis*, 4 *Enterobacter Cloacae*, 3 *Enterobacter aerogenes*, 2 *Citrobacter freundii*, and 1 *Serratia marcescens* and could find only the *qnrB* gene just in 3 isolates (2 strains of *E. Cloacae* and 1 strain of *C. frondy*). In none of *E. coli* strains, *qnrA*, *qnrB,* and *qnrS* genes were found (17). In 2010, Bouchakour et al. studied on 39 enterobacterial isolates of ESBL-producing bacteria (including 16 *E. coli*, 14 *Klebsiella spp*, 8 *E. Cloacae,* and 1 *P. Mirabilis*) by multiplex-PCR technique in order to find *qnr* genes. They found that in 14 isolates (36%), there were *qnr* genes (*qnrA*: 10.25%, *qnrB*: 23.07%, and *qnrS*: 2.56%), which based on the type of bacteria, 3 strains (18.7%) were from isolates of *E. coli* (1 *qnrA*, 1 *qnrB* and 1 *qnrS*), 5 strains (62.5%) from isolates of *E. Cloacae* (4 *qnrB* and 1 *qnrA*), and 6 strains (50%) from isolates of *K. pneumonia* (4 *qnrB* and 2 *qnrA*) (31).

In this study, the *qnrS* gene has been identified for the first time in Iran. Although because of the harmful side effects of quinolones in children, hospitals do not recommend these drugs for children, the prevalence of these genes was high in samples of this study. Probably, the high prevalence of *qnr* genes in children is not related to the high use of quinolones; but may result from the transfer of these genes from adults or other sources. Also in this study, the prevalence rate of *qnr* genes in quinolone-resistant strains was also determined in 12 strains (26.1%), which 5 strains (10.9%) had *qnrB* gene and 7 strains (15.2%) had *qnrS* gene. In 2003, Wang et al. by studying on 213 isolates (including 146 *E. coli* and 67*K. pneumonia*) resistant to ciprofloxacin (separated from pediatric ward of hospitals in Shanghai, China, by multiplex-PCR technique) found that 7.5% (4) of *E. coli* isolates and 11.9% (8) of *K. pneumonia* isolates contained *qnr* genes. Among these, the *qnrA* gene in 8 strains were resistant to ciprofloxacin (5.5%), and *qnrB* as well as *qnrS* genes each in 5 strains (3.4%) were resistant to ciprofloxacin (5). In this study, 26.1% of *E. coli* strains resistant to quinolones had *qnr* genes that in Wang study it was higher. This high prevalence can be due to non-promiscuous use of antibiotics in adults and horizontal transfer of the strains containing these genes to children.

In summary, our study showed that frequency of *bla-CTX-M was* higher than *bla-TEM* and *bla-SHV* in ESBLs-producing *E. coli*, and also according to the similar studies in different places of the world, we can conclude that the prevalence rate of *qnr* genes is unfortunately very high in our country and more caution is required in prescribing and using antibiotics. We found a significant association between resistance to quinolones in ESBL-producing isolates and presence of *qnr* genes ($P < 0.05$). Based on these results, using fluoroquinolones is not recommended in the children with complicated urinary tract infections because of ESBLs-producing *E. coli* and carbapenems can be used in these situations instead. Our data highlight the importance of detecting ESBLs, and *qnr* determinants in the area. The incidence of antimicrobial resistance genes such as ESBLs and *qnr* genes should also be monitored constantly.

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