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## Asian Pacific Journal of Tropical Biomedicine

journal homepage: [www.elsevier.com/locate/apjtb](http://www.elsevier.com/locate/apjtb)Original article <http://dx.doi.org/10.1016/j.apjtb.2015.09.022>Prevalence of multi-drug resistant uropathogenic *Escherichia coli* in Potohar region of PakistanIhsan Ali<sup>1, #</sup>, Zara Rafaque<sup>1, #</sup>, Safia Ahmed<sup>1</sup>, Sajid Malik<sup>2</sup>, Javid Iqbal Dasti<sup>1\*</sup><sup>1</sup>Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan<sup>2</sup>Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan

## ARTICLE INFO

## Article history:

Received 3 Aug 2015

Received in revised form 26 Aug, 2nd

revised form 28 Aug 2015

Accepted 20 Sep 2015

Available online 10 Nov 2015

## Keywords:

Fluoroquinolone-resistant

Uropathogenic *E. coli*

Multi-drug resistant

Extended spectrum beta-lactamase

## ABSTRACT

**Objective:** To scrutinize patterns of multi-drug-resistant uropathogenic *Escherichia coli* (UPEC) strains and particularly of fluoroquinolone-resistance this is an alternative choice for the treatment of urinary tract infections.

**Methods:** Bacterial samples ( $n = 250$ ) were collected from out-patients from August 2012 to August 2014 Islamabad. Antibiotic susceptibility profiling and determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations were performed according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2012). Genes, *qnrA*, *qnrB* and *qnrS* were identified by DNA amplification and sequencing.

**Results:** The highest percentage of UPEC isolates were resistant to co-trimoxazole (82%) followed by cephalothin (80%), 2nd Gen, 3rd Gen and 4th Gen cephalosporins, respectively. Resistance against gentamicin, amikacin remained 29% and 4%. For other drugs including nitrofurantoin, tetracycline, carbapenem and beta-lactam inhibitors remained below 10%. Altogether, 59% of the isolates were resistant to at least three antibiotics including one fluoroquinolone. Overall, MICs for ciprofloxacin remained ( $MIC \geq 256 \mu\text{g/mL}$ ) and for levofloxacin ( $MIC \geq 16 \mu\text{g/mL}$  and  $32 \mu\text{g/mL}$ ). No significant differences were observed regarding MIC values of extended spectrum  $\beta$ -lactamase (ESBL) and non-ESBL producers. For *qnrS* and *qnrB* positive isolates MICs remained above  $32 \mu\text{g/mL}$ . Prevalence of UPEC was significantly higher among females and 40% of the isolates were ESBL producers.

**Conclusions:** Higher percentages of ESBL producing UPEC were associated with urinary tract infections. Moreover, the majority of these isolates were multi-drug resistant and fluoroquinolone-resistant.

## 1. Introduction

Uropathogenic *Escherichia coli* (*E. coli*) (UPEC) is one of the most common cause of urinary tract infections (UTIs) worldwide. A substantial amount of surveillance data indicates the prevalence of trimethoprim-sulfamethoxazole (TMP-SMX) resistant-UPEC strains [1–3]. Eventually, fluoroquinolone has become an alternative therapeutic choice for the treatment [3].

However, recently the prevalence of fluoroquinolone-resistance among uropathogenic strains has also been reported in different regions [1–3]. Particularly, in the Asian-Pacific region and in India, the prevalence of extended spectrum  $\beta$ -lactamases (ESBL) producing fluoroquinolone-resistant uropathogenic strains is significantly higher [4].

Fluoroquinolone-resistance is conferred by different molecular mechanisms including chromosomal mutations in DNA gyrase and/or topoisomerase IV, porins and/or efflux pumps genes. In addition, members of the pentapeptide repeat family of proteins encode plasmid-mediated quinolones resistance. The first member of this family of proteins is known as QnrA, which was identified in USA two decades earlier [5]. Several other genetic determinants belonging to this pentapeptide repeat family of proteins include QnrB, QnrC, QnrD and QnrS which were identified in subsequent years [5]. These molecular factors have wide distribution and were mostly reported in

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Peer review under responsibility of Hainan Medical University.

Foundation Project: Partly supported by the research grant of Higher Education Commission Pakistan (No-3782) and internal research funds of Quaid-i-Azam University Islamabad.

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ESBL producing Enterobacteriaceae [4]. Exceptionally, some of the homologues of Qnr-like proteins were also reported in other bacterial species including *Mycobacterium*, *Enterococcus*, *Listeria*, *colistridia* and recently, a new member of the same protein family was identified in *Stenotrophomonas maltophilia*, annotated as Smqnr [4].

Global epidemiological studies indicate the prevalence of multi-drug resistant (MDR) ESBL producing Enterobacteriaceae both in the community acquired and healthcare facility associated infections [6–10].

Previously, from Asian regions, several studies focused on the prevalence of ESBL producing UPEC, which is known to be associated with 90% of the community acquired and approximately 50% of the nosocomial UTIs worldwide [11]. In Pakistan, over the last decade, an increase in resistance against quinolones has been witnessed in other bacterial species [12–16]. However, not much is known about the fluoroquinolone-resistance in ESBL producing UPEC strains and its co-relation with plasmid encoded *qnr* genes.

## 2. Materials and methods

### 2.1. Bacterial strains and antimicrobial agents

Bacterial isolates under scrutiny ( $n = 250$ ) were collected from out-patients of the Pakistan Institute of Medical Sciences Islamabad during the period of August 2012 to August 2014. The experimental work was approved by the Ethical Committees of the hospital and in compliance with recommendations of the Ethical Committees Committee; privacy was maintained regarding patient data. Patients were of different age groups and from different geographical locations across the country. Available clinical history and associated symptoms were registered for the record. The identification of UPEC was performed according to the standard microbiological and biochemical protocols [17]. Antibiotic susceptibility profiling was performed by using Muller-Hinton agar (Oxide, England), according to the Clinical and Laboratory Standards Institute (CLSI) guideline provided in year 2013 [18]. The antibiotic discs were purchased from Bioanalyse (Turkey). The names and concentrations of antibiotic discs are as follows; TMP-SMX (1.25 µg TMP, 23.75 µg SMX), ciprofloxacin (5 µg), sparfloxacin (10 µg), norfloxacin (10 µg), levofloxacin (5 µg), cefpime (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefixime (5 µg), cefuroxime (30 µg), cephalothine (30 µg), gentamicin (10 µg), amikacin (30 µg), and augmentin: amoxicillin-clavulanic acid (30 µg), nitrofurantoin (300 µg), sulzone: cefoperazone-sulbactam (105 µg), tigecycline (15 µg), minocycline (30 µg), meropenem (10 µg), aztreonam (30 µg), tazocin: tazobactam-piperacillin (110 µg). To confirm the prevalence of (ESBL) producing isolates, phenotypic tests were performed by using discs of amoxicillin-clavulanic acid, cefotaxime, ceftazidime and meropenem (aztreonam). For testing antibiotic susceptibility Muller-Hinton agar (Oxide, England) was used by following the Kirby Bauer disc-diffusion method as described in CLSI guidelines [18].

### 2.2. Determination of MICs and MBCs for levofloxacin and ciprofloxacin

Antibacterial activity of levofloxacin and ciprofloxacin were determined by measuring minimum inhibitory concentrations (MICs). For these measurements micro-dilution broth method was used as described by CLSI guidelines [18]. Briefly, an

adjusted inoculum of the test organism was inoculated into Muller-Hinton broth (Oxoid, Basingstoke, UK) containing two fold serial dilution of an initial antibiotic solution, each well contained approximately  $5 \times 10^5$  CFU/mL. Bacterial cultures were tested against several antibiotic dilutions: 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg/mL. The cultures were incubated for 18 h at 37 °C, prior to make any observations and upon inhibition of visible growth, the lowest concentrations of antimicrobial agents responsible for inhibition were considered MICs. By definition, minimum bactericidal concentration (MBC) was considered to be the concentration of drug at which 99.9% reduction in the colony forming unit has been recorded when comparing with the original inoculums. Determination of MBCs was carried out by plating 0.01 mL from the tube having no visible growth on Muller-Hinton agar plate (Oxide, England) after 18–24 h of incubation at 37 °C and was defined as the concentration that reduced the colony forming units up to 99.9% in comparison to original inoculums. The strain of *E. coli* ATCC 25922 was used as quality control. The breakpoints for the resistance against ciprofloxacin were 4 mg/L and for levofloxacin 8 mg/L according to the CLSI guidelines [18].

### 2.3. DNA extraction and amplification of *qnrA*, *qnrB* and *qnrS* genes

Bacterial DNA extraction was performed by phenol-chloroform method [19] and the qualitative analysis of the DNA was performed by directly visualizing on agarose gel (Sigma, Germany). After confirmation, eighty-five quinolones resistant strains were screened for the presence of *qnrA*, *qnrB* and *qnrS* genes by using previously reported sets of primers and PCR conditions [19].

### 2.4. DNA sequencing of *qnrA*, *qnrB* and *qnrS* genes

The amplified products were purified by using (Gel Band Purification Amersham, USA) DNA sequencing was done by automated DNA sequencing system (ABI 3130, Perkin-Elmer Applied Biosystems, Foster city, California). The electropherogram of sequenced DNA was analyzed by Chromas 2.23 (Technelysium, Queensland, Australia). Protein and nucleotide sequence similarity were analyzed by using BLAST (Basic Local Alignment Search Tool 138.0). DNA sequences were submitted to GenBank database which were assigned following accession numbers KR698789, KR698790, KR698791, KR698792, KR698793.

### 2.5. Statistical analysis

The antibiotic resistance data was recorded in MS-Excel and the statistical analysis of the data was performed through GraphPad Prism, version 5 and statistical significance between different parameters was checked by *Chi*-square test, *t*-test and ANOVA at a cut-off value of  $P < 0.05$ .

## 3. Results

### 3.1. Prevalence of antibiotic resistance among UPEC

Altogether, 148 samples were confirmed as UPEC, antibiotic resistance was checked against cephalosporins (1st Gen, 2nd

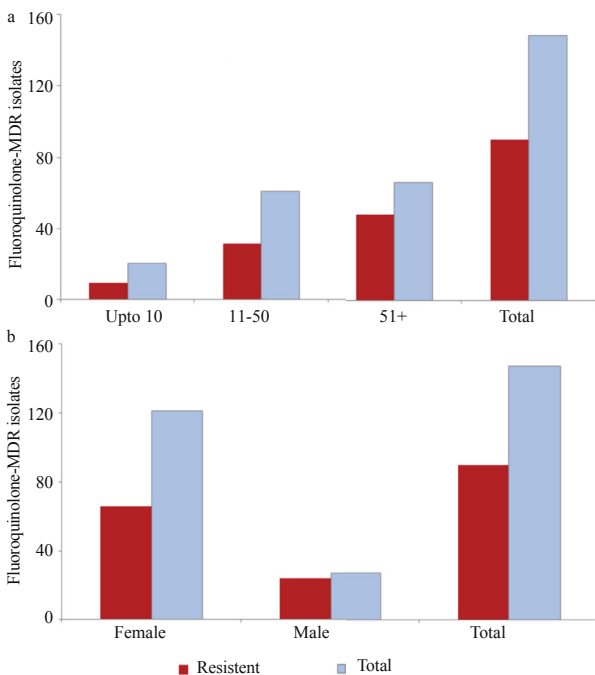
Gen, 3rd Gen and 4th Gen) aminoglycosides, carbapenems, quinolones, nitrofurantoin, monobactams, sulfonamides, gly-cylcyclines, tetracycline and beta-lactamase inhibitors. The highest percentage of isolates was resistant to co-trimoxazole (82%) and cephalosporin (80%), 1st Gen, cephalothin that was followed by other generation of cephalosporin (Table 1). Seventy-one percent of the tested isolates were resistant to amoxicillin-clavulanic acid. Resistance against gentamicin remained 29% while against amikacin 4%. The percentage of resistance against other antibiotics including nitrofurantoin, tetracycline, carbapenem and β-lactam inhibitors remained below 10% (Table 1). A total of 59% of the isolates showed resistant to at least three antibiotics including fluoroquinolone

and were therefore named as fluoroquinolone MDR (Figure 1a, b). The overall rate of resistance towards fluoroquinolone remained 61% (n = 91). Individually, the rate of resistance against ciprofloxacin, sparfloxacin, levofloxacin and norfloxacin remained 60%, 59%, 58% and 57%, respectively. Overall, the prevalence of UPEC was significantly higher among female patients in comparison to the male patients (Figure 1b). However, distribution of the fluoroquinolone-resistant isolates was found to be significantly higher among the male patients  $P < 0.05$  (Figure 2a). Observed patterns of fluoroquinolone-resistant MDR isolates are shown among different age groups of the patients (Figure 1a). Overall, higher numbers of isolate were MDR (Figures 3 and 4). The distribution of the MDR isolates among different genders and age groups is shown in (Figure 5a, b). Out of all tested isolates 40% of the isolates (n = 59) were ESBL producers while remaining (n = 89) isolates were non-ESBL producers.

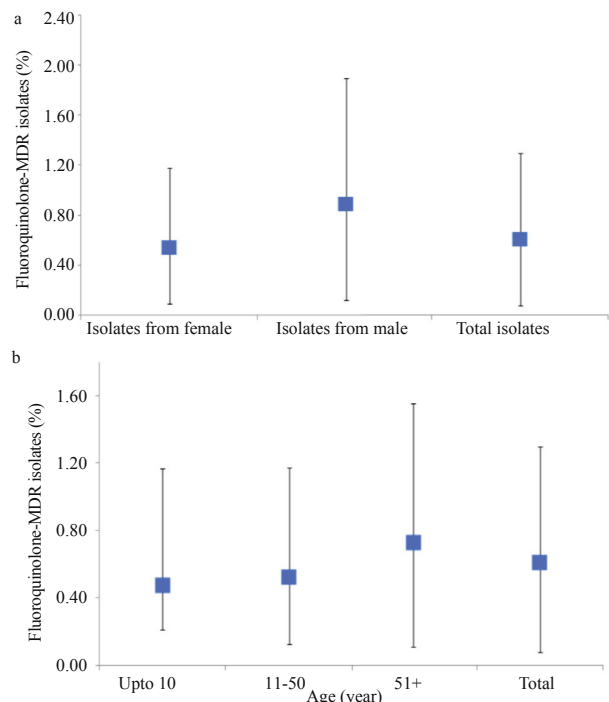
**Table 1**

Numbers and percentages of antibiotic resistant UPEC isolates. n (%).

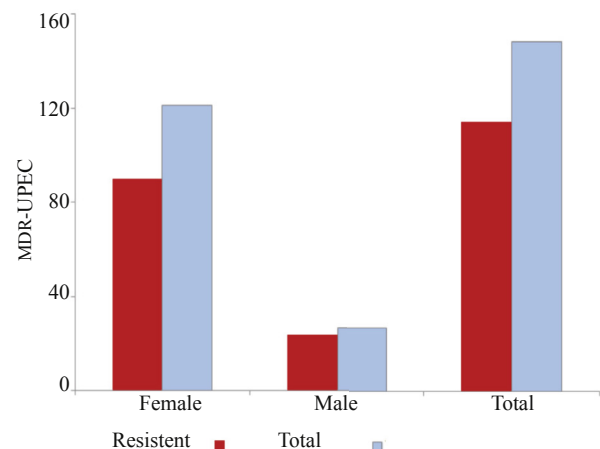
Antibiotic	Resistant isolates	ESBL producers
Ciprofloxacin	90 (60.8)	46 (51.1)
Sparfloxacin	89 (60.1)	46 (51.6)
Levofloxacin	91 (61.4)	46 (50.5)
Norfloxacin	87 (58.7)	46 (52.8)
Amikacin	6 (4.0)	2 (33.3)
Gentamicin	43 (29.0)	19 (44.1)
Aztreonam	80 (54.0)	53 (66.2)
Cephalothine	119 (80.0)	58 (48.7)
Cefixime	94 (63.5)	58 (61.7)
Cefuroxime	92 (62.1)	58 (63.0)
Cefotaxime	94 (63.5)	57 (60.6)
Ceftazidime	91 (61.4)	57 (63.0)
Cepfime	86 (58.1)	55 (64.0)
Tigecycline	1 (0.6)	1 (100.0)
Minocycline	13 (8.7)	11 (85.0)
Piperacillin tazobactam	7 (4.7)	2 (29.0)
Sulzone	11 (7.4)	7 (64.0)
TMP-SMX	122 (82.0)	57 (47.0)
Meropenem	2 (1.3)	1 (50.0)
Nitrofurantoin	9 (6.0)	4 (44.4)
Fosfomycin	15 (10.0)	6 (40.0)
Amoxicillin-clavulanic acid	105 (71.0)	57 (54.2)



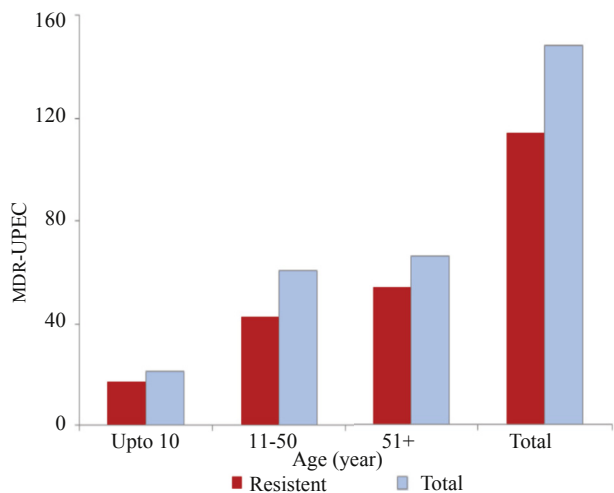
**Figure 1.** Age (a) and gender (b) wise prevalence of fluoroquinolone-MDR UPEC.



**Figure 2.** Age (a) and gender (b) wise distribution of fluoroquinolone-MDR UPEC.



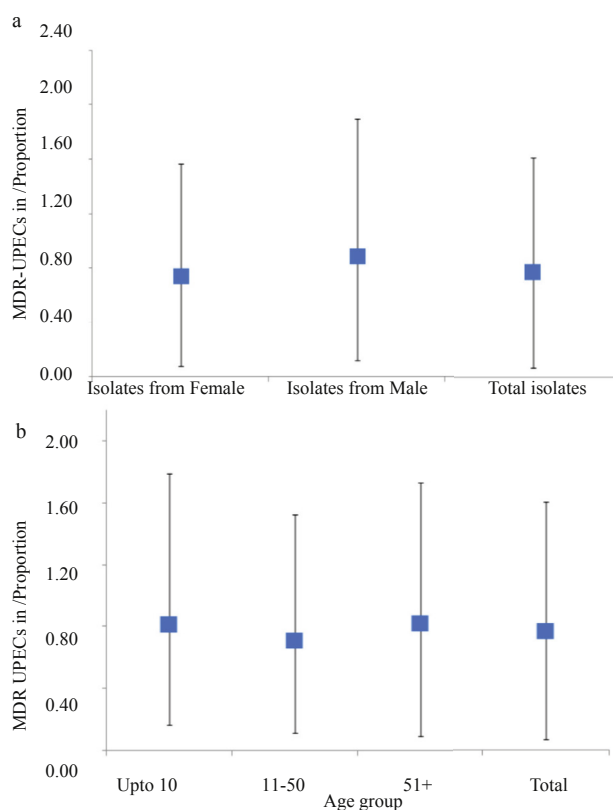
**Figure 3.** Overall prevalence of MDR-UPEC in male and female patients.



**Figure 4.** Overall prevalence of MDR-UPECs in different age groups.

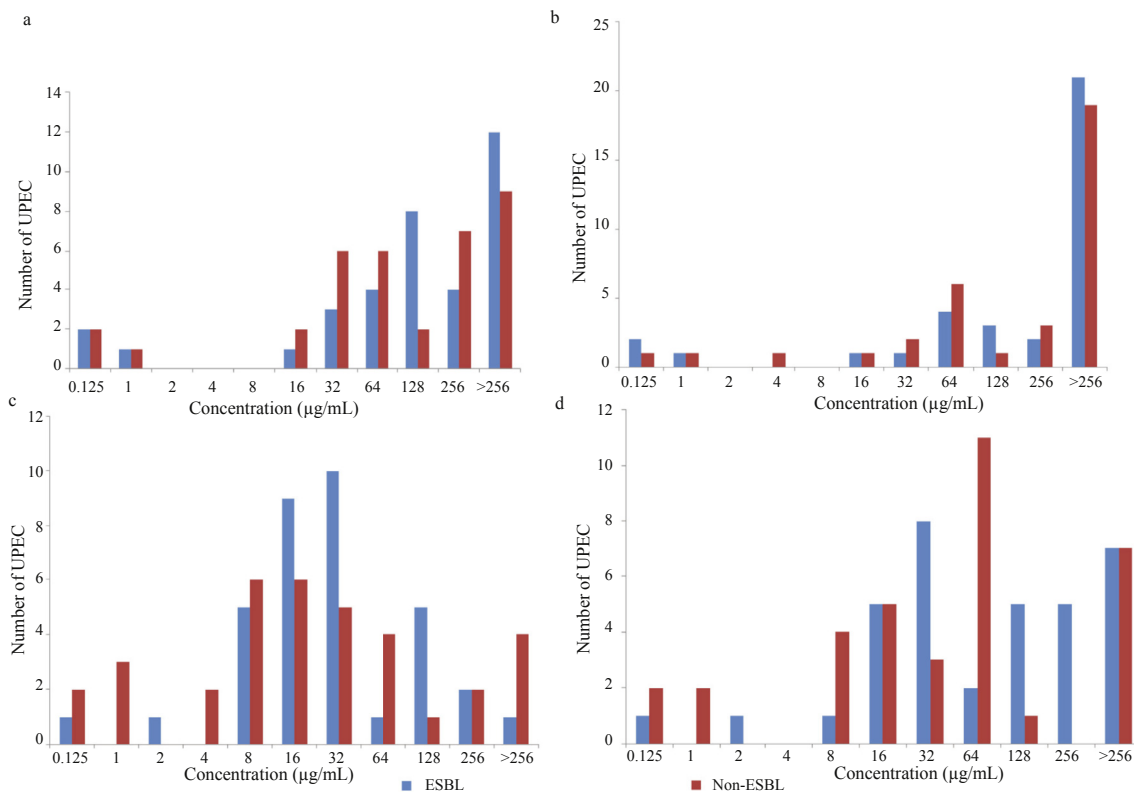
### 3.2. Measurement of MICs

Two equal subset of the selected isolates each ( $n = 35$ ) ESBL producers and ( $n = 35$ ) non-ESBL producers were tested to determine MIC of ciprofloxacin and levofloxacin, two most commonly prescribed fluoroquinolone antibiotics. Bacterial isolates were tested against different concentrations of antibiotics ranging from 0.125  $\mu\text{g/mL}$  to 256  $\mu\text{g/mL}$ . Overall, MIC values for ciprofloxacin remained significantly higher ( $\text{MIC} \geq 256 \mu\text{g/mL}$ ) for both ESBL producers and non-ESBL producers (Figure 6a). In case of levofloxacin however, MIC values for the majority of the isolates fall between ( $\text{MIC} \geq 16 \mu\text{g/mL}$  and  $32 \mu\text{g/mL}$  as shown in Figure 6c). Similarly, MBCs for both antibiotics (ciprofloxacin and levofloxacin) were determined for both ESBL producers and



**Figure 5.** Overall distribution of MDR-UPEC among male and female patients (a) and among different age groups (b).

non-ESBL producers. Overall, the majority of isolates both ESBL producers and non-ESBL producers showed MBC value  $\geq 256 \mu\text{g/mL}$  for ciprofloxacin (Figure 6b). In contrast to ciprofloxacin for levofloxacin, MBC value remained 64  $\mu\text{g/}$



**Figure 6.** Ciprofloxacin MICs (a) and MBCs (b), and levofloxacin MICs (c) and MBCs (d) of ESBL and non-ESBL UPEC.

mL for the majority of ESBL producers and 32 µg/mL for non-ESBL producing isolates (Figure 6d). Overall, the differences between ESBL producers and non-ESBL producers regarding their MICs and MBCs values were not statistically significant. The mean distribution of MIC-ciprofloxacin, MBC-ciprofloxacin, MIC-levofloxacin and MBC-levofloxacin is shown (Figure 7). An independent comparison between mean values of MIC-ciprofloxacin and MBC-ciprofloxacin, MIC-levofloxacin and MBC-levofloxacin confirmed significant differences ( $P < 0.0001$ ). Spearman's rank correlation was established between MIC and MBC values of ciprofloxacin and levofloxacin that showed that these differences were not more than fourfold in any case (Figure 8a, b). Similarly, the

difference between MIC-ciprofloxacin and MIC-levofloxacin were statistically significant ( $P < 0.0001$ ).

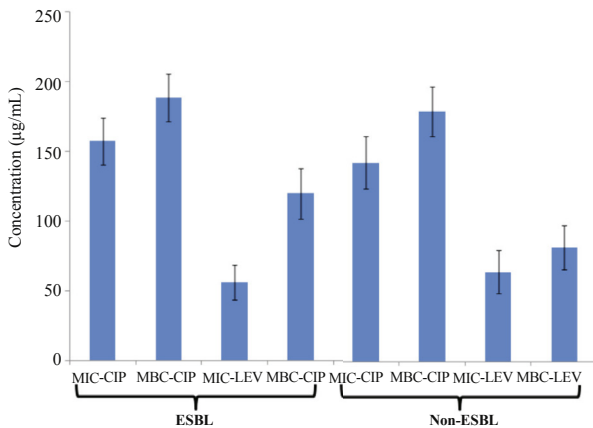
### 3.3. Detection and DNA sequencing of *qnrA*, *qnrB* and *qnrS* genes

All the fluoroquinolone-resistant isolates ( $n = 91$ ) were screened for the presence of plasmid-mediated quinolones resistance genes out of which 2 (2.35%) were found to carry *qnrB* gene while three (3.53%) were found to carry *qnrS* gene. None of the sample was positive for *qnrA* gene. Overall, MICs for *qnrS* and *qnrB* positive isolates remained above 32 µg/mL.

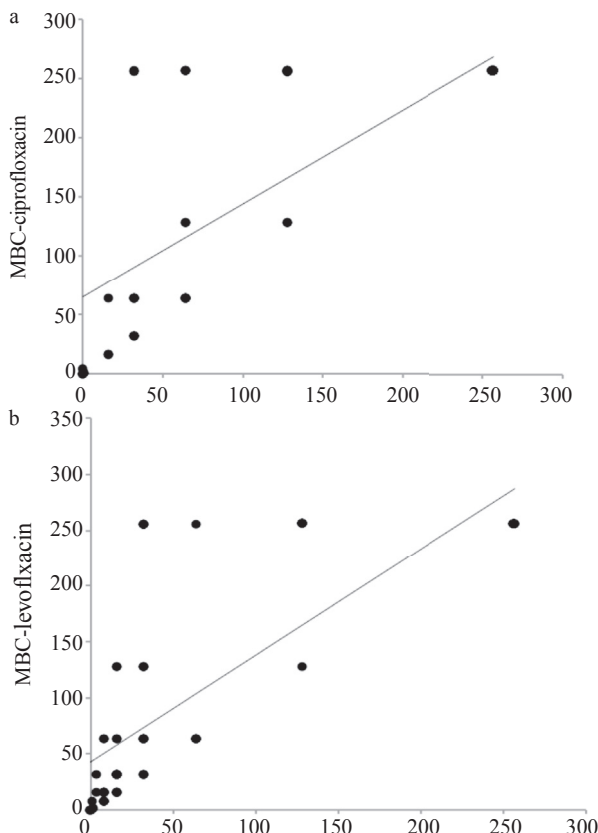
## 4. Discussion

In this study, the highest percentage of isolates was resistant to TMP-SMX which was widely used as a first treatment choice for UTI infections [20]. For this class of antibiotics, similar trends have previously been noticed [1,2]. Due to the higher percentage of resistance, in some countries use of TMP-SMX has become limited. For example, German national guidelines do not recommend this agent as a first choice for the treatment of uncomplicated cystitis [21]. Since TMP-SMX resistance is associated with the development of concomitant resistance to other antibiotics thereof, limited use of TMP-SMX may help to sustain its effectiveness over the long run. Other than TMP-SMX, uncomplicated acute cystitis in female patients is treated with nitrofurantoin, however it is not recommended for the patients with pyelonephritis [22]. Overall rate of resistance against nitrofurantoin remained low in this study, therefore, our finding reiterates that nitrofurantoin is a suitable empiric choice for the treatment of acute cystitis in this region.

Along with other antibiotics, resistance against fluoroquinolone is of particular interest because it is frequently recommended for the treatment of complicated cystitis in female patients. In this study, the overall prevalence of fluoroquinolone-resistant isolates is higher in female patients. Vice versa, given the small numbers of male UTI cases, overall distribution of fluoroquinolone-resistant isolates is higher among male patients. Fluoroquinolones are also used as a first choice for the treatment of UTIs in men, mainly because of certain advantages of this antibiotic over co-amoxiclav, particularly in terms of its pharmacokinetic properties [23,24]. Taken together, in this study 59% of the isolates were fluoroquinolone-resistant, indicating considerable constraints on treatment options particularly for complicated cystitis. ESBL producing fluoroquinolone-resistant uropathogenic strains have significantly been increased in the Asian-Pacific region and particularly in India [4]. Upon scrutiny, 40% of the isolates were ESBL producers in this study and equal numbers of both ESBL producers and non-ESBL producers were tested to determine MICs of ciprofloxacin and levofloxacin, both are most commonly prescribed fluoroquinolone across Pakistan. For ESBL producers, resistance to monobactams and all four generations of cephalosporin ranged from 54% to 80.4% while resistance to co-amoxiclav remained 71%. Furthermore, the majority of isolates were resistant to all three classes of antibiotics. It is noteworthy, that frequent uses of cephalosporin are linked with the selection of ESBL producers in different regions [25].



**Figure 7.** Mean distribution of MICs and MBCs of ciprofloxacin and levofloxacin.



**Figure 8.** Spearman's rank correlation established between MIC and MBC values of ciprofloxacin (a) and levofloxacin (b).



Upon measurement of MICs and MBCs of ciprofloxacin and levofloxacin, no significant differences were observed regarding ESBL and non-ESBL producing isolates, though MICs values for both groups remained significantly higher. In case of both antibiotics, MICs and MBCs differences did not exceed more than three folds, generally for bactericidal drugs like fluoroquinolone, MBCs do not exceed more than fourfold of MICs. Overall, ciprofloxacin (MIC  $\geq$  256  $\mu\text{g}/\text{mL}$ ) MICs values were significantly higher. In case of levofloxacin, MIC values remained between MIC  $\geq$  16  $\mu\text{g}/\text{mL}$  and 32  $\mu\text{g}/\text{mL}$ . Fluoroquinolones have particular pharmacodynamic properties as they exhibit bactericidal activity which is concentration-dependent and have extended post antibiotic effects. However, it is important to note that determination of MICs and MBCs *in vitro* may provide insights about the antimicrobial activity of drugs, however such findings have the least significance regarding the treatment of infected patients, mainly because fluctuating level of drug in the serum of the treated patients and the numbers of bacteria at site of infection *in vivo* may pose completely different scenario. Furthermore, during *in vitro* measurement of MICs or MBCs bacterial growth is not static that is normally the case *in vivo*. Nevertheless, *in vitro* determination of MICs and MBCs based on breakpoints differentiates two populations (*i.e.* susceptible and resistant).

In this study, *qnrB* and *qnrS* genes were associated with low level of fluoroquinolone-resistance. Previously, several independent reports confirmed that Qnr-positive ESBL producer isolates conferred low level resistance to quinolones particularly because the key function of the Qnr proteins is to protect the gyrase proteins. However, resistance mediated by these molecular factors may facilitate co-selection of plasmid encoded resistance to other antibiotics [26–28]. Conclusively, the problem of antibiotic resistance has gained unprecedented momentum worldwide, therefore important measures are mandatory to ensure rational and well-informed use of antibiotics as are taken in European Union. Overall, this report indicates a higher prevalence of MDR and ESBL UPEC in this region and prevalence of *qnr* genes in UPEC is linked with the moderate level of fluoroquinolone-resistance.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

The work was partly supported by the research grant of Higher Education Commission Pakistan (No.-3782) and internal research funds of Quaid-i-Azam University Islamabad.

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