

Quinolone Resistance Detection by PCR-RFLP and Multiplex-PCR among Extended-Spectrum β -Lactamase Producing *Enterobacteriaceae*

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Abstract

Quinolone resistance limits the therapeutic potential for the Extended-Spectrum β -lactamase (ESBL) producing *Enterobacteriaceae*. The aim of this study was to investigate the most common mechanisms of quinolones resistance in ESBL-producing *Enterobacteriaceae* using both phenotypic and genotypic methods. Out of 1766 clinical isolates collected between October 2012 and September 2013, 219 *Enterobacteriaceae* clinical isolates were ESBL producers, nalidixic acid and ciprofloxacin resistant were identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF/MS) and the Minimal Inhibitory Concentration (MIC) values of ciprofloxacin and nalidixic acid was detected before and after addition of phenylalanine-arginine β -naphthylamide (Pa β N) efflux pump inhibitor. Thirty three isolates were selected for screening of the plasmid-mediated fluoroquinolone resistance (PMQR) genes; (*qnr*, *aac(6')-Ib-cr* and *qepA*) and the efflux pump genes (*oqxAB* genes) by multiplex PCR. Whereas *GyrA* and *ParC* genes mutations were detected by PCR-RFLP assay. The Pa β N changed the MIC values of 28 isolates. The *GyrA* gene mutation was detected in 24/33 (72.7%), while the *parC* gene mutation was detected in 3/33 (9.1%). *Qnr*-genes were detected in 13/33 (39.4%), *aac(6')-Ib* gene was detected in 24/33 (72.7%). *qepA* gene was detected in only one *Klebsiella pneumoniae* isolate. Finally the *oqxA* gene was detected in 16/33 (48.5%) of the studied isolates. The present study indicated that the Pa β N was an effective phenotypic screening method for quinolones resistance efflux pump; moreover PCR-RFLP offered simple and rapid method for detection of ciprofloxacin-resistance that could be useful for clinical diagnosis and epidemiological studies

Introduction

Enterobacteriaceae are among the most important etiological factors of nosocomial and community acquired infections. The most commonly isolated pathogens are *Klebsiellapneumoniae*, *Escherichia coli* and *Enterobacter* spp. *Salmonella* and *Shigella* spp [1]. The prevalence of Gram-negative microorganisms that are resistant to different antibiotics and the resultant deficit in antibiotics that emphasizes the urgent need for novel therapeutic agents for the treatment of Gram-negative infectious agents [2]. Beta-lactams and fluoroquinolones constitute the main therapeutic agent of choices to treat infections caused by these pathogens. However, the development of resistance to these compounds, especially, by Gram negative microorganisms constitutes a noticeable success of bacterial survival [3,4,5]. Quinolones resistance complicates the treatment of infections caused by ESBL-producing *Enterobacteriaceae* strains [6]. The most important mechanisms involved in quinolones resistance in *Enterobacteriaceae* are (i) accumulation of chromosomal mutations primarily in the quinolone resistance-determining regions (QRDRs) of the target genes, *gyrA* which encodes DNA and *parC* which encodes topoisomerase IV [7]. (ii) Up regulation of the native efflux pumps which decreases the intracellular drug accumulation [8] alone or in addition to decreased expression of outer membrane proteins [9]. Three plasmid-mediated fluoroquinolone resistance (PMQR) mechanisms have been described; including (i) the *Qnr* (*qnrA*, *qnrB*, *qnrS*, *qnrC* and *qnrD* proteins, (ii) the *aac(6')-Ib-cr* enzyme, and, (iii) *QepA* and *oqxAB* plasmid-mediated efflux pumps [5]. ESBL-producing isolates are commonly associated with PMQR genes in

Enterobacteriaceae [6]. Use of efflux pump inhibitors is a unique anti-resistance approach that can return activity for different families of antibiotics. However, their clinical use is difficult due to the toxicity, stability, selectivity and bioavailability of available molecules [10]. Phenylalanine-arginine β -naphthylamide (Pa β N) is a well-studied efflux pump inhibitor (EPI) that is routinely combined with fluoroquinolone antibiotics. As a result of the competition between Pa β N and the antibiotic, Pa β N is extruded outside by the efflux pump and the antibiotic reaches its effective concentration inside the cell [11]. The aim of this study was to investigate the most common mechanisms of quinolones resistance in ESBL-producing *Enterobacteriaceae* clinical isolates using both phenotypic method by testing the effect of Pa β N (EPI) and genotypic methods by using multiplex-PCR to detect plasmid mediated (PMQR) and PCR-RFLP assay to detect mutations in the quinolone-resistance determining

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regions of *gyrA* and *parC*. The detection of mutations associated with reduced susceptibility to fluoroquinolones by PCR-RFLP is well thought-out in our country as it serves as a specific, rapid, inexpensive, and simple testing alternative to sequencing assays.

Material and Methods

Ethics statement

The present study was approved by the Faculty of Medicine Cairo University Hospital, Egypt. Written informed consent was not necessary for this retrospective study, as it was part of our standard microbiological routine. Patient data were anonymous for the purposes of this analysis, and all confidential patient information was protected in accordance with Egyptian law.

Hospital setting

The study was conducted at Cairo University Hospital, which serves patients in Cairo (Egypt) and provides medical and surgical care in all medical specialties. The study took place from October 2012 to September 2013. The study conforms to the relevant regulatory standards and is in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI) guidelines.

Clinical isolates (selection and identification)

Out of 1766 clinical bacterial isolates collected from October 2012 to September 2013, a total of 219 *Enterobacteriaceae* clinical isolates were ESBL- producers, nalidixic acid and ciprofloxacin resistant. All isolates were collected from inpatients admitted at Kasr Al-Ainy Cairo University Hospital, Egypt. All 219 isolates were processed and identified by the standard procedures of the Clinical and Laboratory Standards Institute guidelines [12] and were identified to the species level using MALDI-TOF/MS with score values > (1.9) using the Bruker software microflex RTC version 3.1 and then stored in brain heart infusion broth with 15% glycerol at -60°C until further analysis.

Phenotypic detection of Quinolones resistance

All 219 *Enterobacteriaceae* isolates were subjected to phenotypic detection of quinolones resistance.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disc diffusion method (Modified Kirby-Bauer technique) using Muller Hinton agar, aerobic incubation at 35°C for 16–18 h. Antimicrobial discs, Imipenem (10 mcg), Meropenem (10 mcg), Gentamicin (10 mcg), Ciprofloxacin (5 mcg), Amikacin (30 mcg), Cotrimoxazole (25 mcg), Cefepime (30mcg), Cefotaxime (30mcg), Cefotaxime+Clavulanic acid (30/10mcg), Aztreonam(30mcg), Ceftazidime (30 mcg), Ceftazidime + Clavulanic acid(30/10 mcg), Amoxicillin-Clavulanic acid (20/10mcg), and Cefoxitin (30 mcg) were obtained from Oxoid Co. (Oxoid Limited, Basingstoke, Hampshire, England). Phenotypic screening and confirmation of Extended-Spectrum β -Lactamase/Extended-Spectrum β -Lactamase/ESBL production was done using the combined disc tests [12].

Minimal Inhibitory Concentration

Minimal inhibitory concentration of nalidixic acid (NA) and ciprofloxacin (CIP) was determined by broth microdilution method following CLSI guidelines 2012. CIP and NA MICs were measured

in microdilution plates with a final volume of 200 μ l/well. 100 μ l Mueller-Hinton broth was added in each well plus an extra 74.4 μ l only in the first well and 25.6 μ l ciprofloxacin from a 2 mg/ml stock solution. Serial dilutions were done and 100 μ l of bacterial inoculum with 105 CFU/ml was added in each well, obtaining ciprofloxacin concentration of 128 μ g/ml in the first well and 0.06 μ g/ml in the last one. In case of Nalidixic acid 10.24 μ l was added in the first well from a 5 mg/ml stock solution. After serial dilutions and addition of bacterial inoculum with 105 CFU/ml in each well; Nalidixic acid concentrations of 128 μ g/ml in the first and 0.06 μ g/ml in the last wells were obtained. The lowest antibiotic concentration that inhibits the growth was considered as the MIC value.

Phenylalanine-arginine β -naphthylamide (Pa β N) efflux pump inhibitor

According to the CLSI (2010) recommendations, before and after addition of P β NA (Sigma-Aldrich Co, St Louis, MO, USA) the lowest antibiotic concentration that inhibits the growth was considered as the MIC value. One hundred μ l of Mueller-Hinton broth containing the bacterial suspensions was added to the wells of a sterile microdilution plate. By adding 50 μ l of suitable concentrations of CIP and NA to the first line of wells, serial dilutions were performed. 20 μ l constant P β NA concentration solution was added (20 μ g/ml) to each well. At least four fold decreases in the MIC values of ciprofloxacin and nalidixic acid was evaluated as the presence of the efflux pump [13].

Genotypic detection of Quinolones resistance

Out of a total of 219 isolates, 28 isolates showed MIC changes after addition of P β NA, 5 isolates were added as a negative control. A total of 33 isolates were subjected to PCR assay of the PMQR genes, as well as PCR amplification of the QRDRs of *gyrA* and *parC*, followed by RFLP to detect mutations in *gyrA* and *parC*.

DNA extraction

Extraction of DNA was performed using the QIAamp DNA mini kit (50) (QIAGEN GmbH, Hilden, Germany Cat. No.51304) according to manufacturer instructions.

PCR amplification

PCR amplifications of the quinolone resistance determining regions (QRDRs) of *gyrA* and *parC* were carried out using the primers listed in Table 1. The primer used to amplify a 344-bp fragment containing the QRDR of the *gyrA* gene. The conditions were 94°C for 60 sec, 55°C for 45 sec, and 72°C for 60 sec for 30 cycles. For *ParC* gene, amplification was carried out for 35 cycles at 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec to amplify a 168 bp fragment [14]. PCR products were submitted to electrophoresis on a 1.5% agarose gel containing ethidium bromide to visualize the amplified bands under UV.

Restriction Fragment Length Polymorphism (RFLP)

All PCR products positive for *gyrA* and *parC* genes were further analyzed by digestion with Hinf I (Thermo scientific FastDigest restriction enzyme, Fermentas-Lithuania) to identify mutations [15]. The wild-type *gyrA* contains the artificial Hinf I cleavage site. Consequently, Hinf I digests the amplified 344-bp products to produce two fragments for *gyrA* 210 and 272 bp respectively, and also for the wild type *parC* HinfI digest 168 PCR product for 32 and 65bp respectively. Ten μ l of each amplification product was mixed

No.	gene	sequence
1	<i>qnrA Fwd</i>	5-ATT TCT CAC GCC AGG ATT TG-3
	<i>qnrA Rev</i>	5-GAT CGG CAA AGG TTA GGT CA-3
2	<i>qnrB Fwd</i>	5-GAT CGT GAA AGC CAG AAA GG-3
	<i>qnrB Rev</i>	5-ACG ATG CCT GGT AGT TGT CC-3
3	<i>qnrS Fwd</i>	5-ACG ACA TTC GTC AAC TGC AA-3
	<i>qnrS Rev</i>	5-TAA ATT GGC ACC CTG TAG GC-3
4	<i>aac(6)-Ib Fwd</i>	5-TTG CGA TGC TCT ATG AGT GGC TA-3
	<i>aac(6)-Ib Rev</i>	5-CTC GAA TGC CTG GCG TGT TT-3
5	<i>qepA Fwd</i>	5-GCA GGT CCA GCA GCG GGT AG-3
	<i>qepA Rev</i>	5-CTT CCT GCC CGA GTA TCG TG-3
6	<i>oqxA Fwd</i>	5-CTC GGC GCG ATG ATG CT-3
	<i>oqxA Rev</i>	5-CCA CTC TTC ACG GGA GAC GA-3
7	<i>oqxB Fwd</i>	5- TTC TCC CCC GGC GGG AAG TAC-3
	<i>oqxB Rev</i>	5- CTC GGC CAT TTT GGC GCG TA-3
8	<i>gyrAWF</i>	5-AAA TCT GCC CGT GTC GTT GGT-3
	<i>gyrAWR</i>	5-GCC ATA CCT ACG GCG ATA CC-3
9	<i>parCWF</i>	5-CTG AAT GCC AGC GCC AAA TT-3
	<i>parCWR</i>	5-GCG AAC GAT TTC GGA TCG TC-3

Table 1 : Primers used in the study.

with 1 μ L of Hinf I, 2 μ L of 10 X fast Digest green buffers and 17 μ L of water and incubated at 37 C for 5 min. Ten micro-liters of the digested fragments were run in 3% agarose gel. The gel was stained with ethidium bromide and the DNA bands were visualized with UV transilluminator. DNA ladder (50 pb and 25 bp) (Promega1) was used as a molecular weight marker.

Detection of Plasmid Mediated Quinolones Resistance Determinants (PMQR)

Screening of the five PMQR determinants was carried out by two sets of multiplex PCR amplification, one for *qnrA*, *qnrB*, and *qnrS* and the other for *aac (6')-Ib* and *qepA* [14].Also Detection of *oqxAB* was performed as described elsewhere[16].

Statistical analysis

The resulting data was analyzed using SSPS version 13 program. Nominal data was expressed as frequency and percentage and compared using Chi square test. The Pvalue < 0.01 was considered significant.

Results

Out of 1766 clinical *Enterobacteriaceae* isolates, 219 (12.4%) isolates were ESBL producers and quinolone resistant, including; *Escherichia coli* 98 (44.7%), *Klebsiella pneumoniae* 92 (42%), *Salmonella enterica* 8 isolates plus one isolate *Salmonella enteritidis*, one isolate *Salmonella choleraesuis* and one isolate *Salmonella bongori* total 11 (5%), one isolate *Enterobacter ludwigii* plus two isolates *Enterobacter. cloacae* total 3 (1.4%), *Proteus mirabilis* 8 (3.65%) and *Shigella flexneri* 2 (0.9%). The most common site of isolation was the urine specimens 112 (51.1%) (The P-value was significant P < 0.01), wound specimens 65 (29.6%), sputum specimens 16 (7.3%) followed by stool specimens

13 (5.9%). The distribution of clinical isolates according to the clinical specimens are shown in Table 2. The presence of co-resistance among different classes of antibiotic families was significant (The P-value is 0.000346) except for carbapenems and polymyxin classes. In our study minimum inhibitory concentrations (MICs) of ciprofloxacin antibiotic and nalidixic acid before and after addition of efflux pump inhibitor Pa β N resulted in reduction of MIC in total 28 isolates. In *E. coli* isolates adding Pa β N resulted in reduction in MIC by more than 2 log₂ dilution in 9 samples for ciprofloxacin antibiotic and in 4 samples for nalidixic acid (The P-value is 0.145483, the result is not significant at P < 0.10). While addition of Pa β N to *Klebsiella pneumoniae* isolates resulted in reduction of MIC by more than 2 log₂ dilution in 8 isolates for ciprofloxacin antibiotic and in 5 isolates for nalidixic acid (P-Value is 0.997521, the result is not significant at P < 0.10). Moreover addition of Pa β N to the *Enterobacter spp* isolates resulted in reduction of MIC by more than 2 log₂ dilution in 2 samples for both ciprofloxacin and nalidixic acid antibiotic (P-value is 1, the result was not significant at P < 0.10). Whereas addition of Pa β N to the *Proteus mirabilis* isolates resulted in a reduction of MIC by more than 2 log₂ dilution in 2 samples for ciprofloxacin and in 2 samples for nalidixic acid antibiotic, the result is also insignificant at P < 0.10. Also addition of Pa β N to the *Salmonella spp* isolates resulted in reduction of MIC by more than 2 log₂ dilution in 6 samples for both ciprofloxacin and nalidixic acid antibiotic by a percent 100% (The result is not significant at P < 0.10). Finally addition of Pa β N to the *Shigella flexneri* isolates resulted in reduction of MIC by more than 2 log₂ dilution in the 1 samples for both ciprofloxacin and nalidixic acid antibiotic by a percent 100% (The P-value is 0.252656, The result is not significant at P < 0.10). Thirty three isolates (28 of them showed MIC changes after addition of P β NA in addition to 5 isolates added as a negative control) were selected for further PCR work up, including PMQR genes, as well as PCR amplification of the QRDRs of *gyrA* and *parC*, followed by RFLP to detect mutations in *gyrA* and *parC*. By PCR-RFLP assay *gyrA* gene mutation was detected in 24/33 (72.7%), while *par C* gene mutation was detected in 3/33 (9.1%).

The prevalence of 85.7% of PMQR genes and *qnr*-genes were identified in 13/33 (39.3%) of the clinical isolates as shown in (Figure 1), one isolate contained *qnrA* gene only, one isolate contained the *qnrB* gene only, one isolate contained both *qnrA* and *qnrS* genes and two isolates contained both *qnrB* and *qnrS* genes. *Aac(6')-Ib* gene was detected in 72.7 % (24/33) of the clinical isolates as shown in (Figure 2). The prevalence of *aac(6')-Ib-cr* was 72.7% (8/11) among *E. coli*, 88.8%(8/9) among *Klebsiella pneumoniae*, 66.6% (2/3) among *Enterobacter spp.*, 66.6% (2/3) among *Proteus mirabilis* and 66.6% (4/6) among *salmonella spp* clinical isolates. *oqxA* gene was detected in 51.5 % (16/33) of the clinical isolates. The prevalence was 18.1% (2/11) among *E. coli*, 66.6% (2/3) among *Enterobacter spp.*, 33.3% (1/3) among *Proteus mirabilis* and 50% (3/6) among *Salmonella spp*. *Klebsiella pneumoniae* isolates showed positive results for *oqxA* gene by 100% (8/9). Whereas *oqxB* gene was detected in 21.2 % (7/33) of the clinical isolates. *QepA* gene was detected in only one *Klebsiella pneumoniae* isolate which was highly resistant to both antibiotics before and after the addition of Pa β N as shown in (Figure 3).

All *E. coli*, *Enterobacter*, *Proteus* and *Salmonella* isolates showed negative results for the *OqxB* gene. Isolates of positive PCR results for efflux pump genes *oqxAB* showed MIC changes after addition of efflux pump inhibitor Pa β N in 88.2% (15/17) for ciprofloxacin and 70.5% (12/17) for nalidixic acid. The results of multiplex-PCR and PCR-RFLP for the tested *Enterobacteriaceae* clinical isolates are presented in Table 3.

Isolate	Ciprofloxacin (mg/ml)		Nalidixic acid (mg/ml)		Isolate	Ciprofloxacin (mg/ml)		Nalidixic acid (mg/ml)	
	- P β NA	+ P β NA	- P β NA	+ P β NA		- P β NA	+ P β NA	- P β NA	+ P β NA
E1	> 146.66	> 146.66	> 146.66	> 146.66	E45	> 146.66	> 146.66	> 146.66	> 146.66
E2	> 146.66	> 146.66	> 146.66	> 146.66	E46	> 146.66	> 146.66	> 146.66	> 146.66
E3*	73.33	<2.291	> 146.66	> 146.66	E47	> 146.66	> 146.66	> 146.66	> 146.66
E4	> 146.66	> 146.66	> 146.66	> 146.66	E48	> 146.66	> 146.66	> 146.66	> 146.66
E5	<2.291	<2.291	> 146.66	> 146.66	E49	> 146.66	73.33	> 146.66	146.66
E6	> 146.66	> 146.66	> 146.66	> 146.66	E50	> 146.66	<2.291	> 146.66	> 146.66
E7	> 146.66	> 146.66	> 146.66	> 146.66	E51	> 146.66	> 146.66	> 146.66	> 146.66
E8	> 146.66	> 146.66	> 146.66	> 146.66	E52	> 146.66	<2.291	> 146.66	<2.291
E9	73.33	73.33	> 146.66	> 146.66	E53	> 146.66	> 146.66	> 146.66	> 146.66
E10	> 146.66	> 146.66	> 146.66	> 146.66	E54	> 146.66	> 146.66	> 146.66	> 146.66
E11*	> 146.66	146.66	> 146.66	> 146.66	E55	146.66	146.66	> 146.66	> 146.66
E12	73.33	73.33	> 146.66	> 146.66	E56	> 146.66	> 146.66	> 146.66	> 146.66
E13	> 146.66	> 146.66	> 146.66	> 146.66	E57	146.66	146.66	> 146.66	> 146.66
E14	> 146.66	> 146.66	> 146.66	> 146.66	E58	> 146.66	> 146.66	> 146.66	> 146.66
E15	> 146.66	> 146.66	> 146.66	> 146.66	E59*	73.33	73.33	> 146.66	> 146.66
E16	73.33	73.33	> 146.66	> 146.66	E60	> 146.66	> 146.66	> 146.66	> 146.66
E17	> 146.66	<2.291	> 146.66	<2.291	E61	> 146.66	> 146.66	> 146.66	> 146.66
E18	> 146.66	> 146.66	> 146.66	> 146.66	E62	> 146.66	> 146.66	> 146.66	> 146.66
E19	> 146.66	> 146.66	> 146.66	> 146.66	E63	73.33	73.33	> 146.66	> 146.66
E20*	> 146.66	> 146.66	> 146.66	> 146.66	E64	> 146.66	> 146.66	> 146.66	> 146.66
E21	> 146.66	> 146.66	> 146.66	> 146.66	E65	> 146.66	> 146.66	> 146.66	> 146.66
E22	> 146.66	> 146.66	> 146.66	> 146.66	E66	> 146.66	> 146.66	> 146.66	> 146.66
E23	> 146.66	> 146.66	> 146.66	> 146.66	E67	> 146.66	> 146.66	> 146.66	> 146.66
E24	> 146.66	> 146.66	> 146.66	> 146.66	E68	> 146.66	> 146.66	> 146.66	> 146.66
E25	> 146.66	> 146.66	> 146.66	> 146.66	E69	> 146.66	> 146.66	> 146.66	> 146.66
E26*	> 146.66	9.1663	> 146.66	> 146.66	E70	> 146.66	> 146.66	> 146.66	> 146.66
E27	> 146.66	> 146.66	> 146.66	> 146.66	E71	> 146.66	> 146.66	> 146.66	> 146.66
E28	> 146.66	> 146.66	> 146.66	> 146.66	E72	> 146.66	> 146.66	> 146.66	> 146.66
E29	> 146.66	> 146.66	> 146.66	> 146.66	E73	> 146.66	> 146.66	> 146.66	> 146.66
E30	<2.291	<2.291	> 146.66	> 146.66	E74	> 146.66	> 146.66	> 146.66	> 146.66
E31	> 146.66	> 146.66	> 146.66	> 146.66	E75	73.33	73.33	> 146.66	> 146.66
E32	> 146.66	> 146.66	> 146.66	> 146.66	E76	146.66	146.66	> 146.66	> 146.66
E33	> 146.66	> 146.66	> 146.66	> 146.66	E77*	> 146.66	9.1663	> 146.66	36.665
E34	> 146.66	> 146.66	> 146.66	> 146.66	E78*	> 146.66	36.665	> 146.66	146.66
E35	> 146.66	> 146.66	> 146.66	> 146.66	E79	> 146.66	> 146.66	> 146.66	> 146.66
E36	> 146.66	> 146.66	> 146.66	> 146.66	E80	> 146.66	> 146.66	> 146.66	> 146.66
E37	> 146.66	> 146.66	> 146.66	> 146.66	E81	> 146.66	> 146.66	> 146.66	> 146.66
E38	> 146.66	> 146.66	> 146.66	> 146.66	E82*	> 146.66	36.665	> 146.66	> 146.66
E39	146.66	146.66	> 146.66	> 146.66	E83	> 146.66	> 146.66	> 146.66	> 146.66
E40	> 146.66	> 146.66	> 146.66	> 146.66	E84	> 146.66	> 146.66	> 146.66	> 146.66
E41	> 146.66	> 146.66	> 146.66	> 146.66	E85	> 146.66	> 146.66	> 146.66	> 146.66
E42	> 146.66	> 146.66	> 146.66	> 146.66	E86*	> 146.66	> 146.66	> 146.66	> 146.66
E43	> 146.66	> 146.66	> 146.66	> 146.66	E87	> 146.66	> 146.66	> 146.66	> 146.66
E44	> 146.66	> 146.66	> 146.66	> 146.66	E88	> 146.66	> 146.66	> 146.66	> 146.66

Table (2): MIC changes of ciprofloxacin and nalidixic acid before and after addition of PA β N for 98 *E.coli* isolates.

Isolate	Ciprofloxacin (mg/ml)		Nalidixic acid (mg/ml)		Isolate	Ciprofloxacin (mg/ml)		Nalidixic acid (mg/ml)	
	_ P β NA	+ P β NA	_ P β NA	+ P β NA		_ P β NA	+ P β NA	_ P β NA	+ P β NA
K1	75	2.3438	>75	>75	K47*	>75	2.3438	>75	37.5
K2	18.75	<1.17	>75	<1.17	K48	>75	>75	>75	>75
K3*	>75	2.3438	>75	9.375	K49	>75	>75	>75	>75
K4	9.375	<1.17	37.5	37.5	K50*	>75	>75	>75	>75
K5	9.375	<1.17	37.5	37.5	K51	>75	>75	>75	>75
K6	9.375	9.375	37.5	37.5	K52	>75	>75	>75	>75
K7	2.3438	<1.17	>75	<1.17	K53	4.6875	4.6875	37.5	37.5
K8	>75	>75	>75	>75	K54	75	75	>75	>75
K9	>75	9.375	>75	>75	K55	>75	>75	>75	>75
K10	>75	>75	>75	>75	K56	>75	>75	>75	>75
K11	37.5	<1.17	>75	<1.17	K57	75	75	>75	>75
K12	>75	>75	>75	>75	K58	4.6875	4.6875	>75	>75
K13	>75	9.375	>75	>75	K59	75	75	>75	>75
K14	>75	>75	>75	>75	K60	18.75	18.75	>75	>75
K15	>75	>75	>75	>75	K61	75	37.5	>75	75
K16	9.375	9.375	37.5	37.5	K62*	>75	18.75	>75	75
K17*	>75	>75	>75	>75	K63	75	75	>75	>75
K18	75	75	>75	>75	K64	>75	>75	>75	>75
K19*	>75	18.75	>75	18.75	K65	>75	>75	>75	>75
K20	>75	>75	>75	>75	K66	>75	>75	>75	>75
K21	>75	>75	>75	>75	K67	>75	>75	>75	>75
K22	>75	>75	>75	>75	K68	>75	>75	>75	>75
K23	>75	>75	>75	>75	K69	>75	>75	>75	>75
K24	>75	>75	>75	>75	K70	>75	>75	>75	>75
K25	>75	>75	>75	>75	K71*	75	37.5	>75	<1.1718
K26*	>75	75	>75	>75	K72	2.3438	2.3438	4.6875	4.6875
K27	>75	>75	>75	>75	K73	>75	>75	>75	>75
K28	>75	>75	>75	>75	K74	>75	>75	>75	>75
K29	>75	>75	>75	>75	K75	75	75	>75	>75
K30	>75	>75	>75	>75	K76	9.375	9.375	>75	>75
K31	>75	>75	>75	>75	K77	>75	>75	>75	>75
K32	>75	>75	>75	>75	K78	>75	>75	>75	>75
K33*	>75	9.375	>75	>75	K79	>75	>75	>75	>75
K34	>75	75	>75	>75	K80	>75	>75	>75	>75
K35	>75	>75	>75	>75	K81	>75	>75	>75	>75
K36	>75	>75	>75	>75	K82	>75	>75	>75	>75
K37	>75	>75	>75	>75	K83	>75	>75	>75	>75
K38	4.6875	4.6875	>75	>75	K84	>75	>75	>75	>75
K39	75	75	>75	>75	K85	9.375	9.375	>75	>75
K40	<1.17	<1.17	<1.17	<1.17	K86	>75	>75	>75	>75
K41	>75	>75	>75	>75	K87	>75	>75	>75	>75
K42	>75	>75	>75	>75	K88	>75	>75	>75	>75
K43	18.75	<1.17	>75	<1.17	K89	>75	>75	>75	>75
K44	>75	>75	>75	>75	K90	>75	>75	>75	>75
K45	<1.1718	<1.1718	<1.1718	<1.1718	K91	>75	>75	>75	>75
K46	>75	>75	>75	>75	K92	75	75	>75	>75

Table : MIC changes of ciprofloxacin and nalidixic acid before and after addition of PA β N for 92 *Klebsiella* spp. isolates.

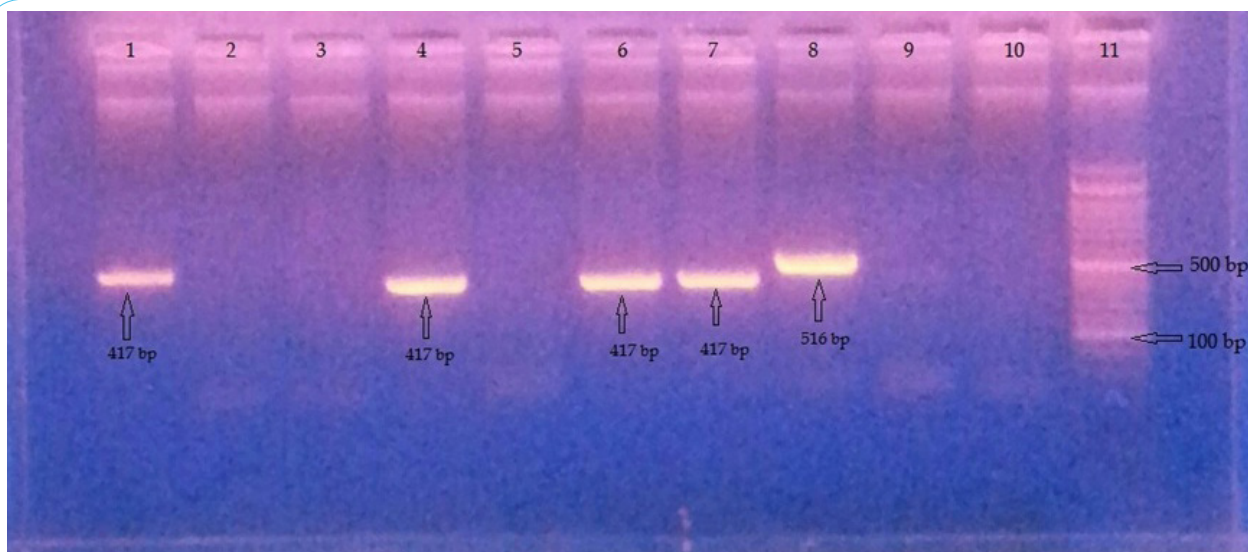


Figure 1: Shows agarose gel electrophoresis of multiplex PCR of *qnrA*, *qnrB* and *qnrS* genes. Lane 1-10: represent the clinical isolates expression of *qnrA* gene at 516 bp., *qnrB* gene at 469 bp and *qnrS* at 417 bp, Lane 11 represent marker.

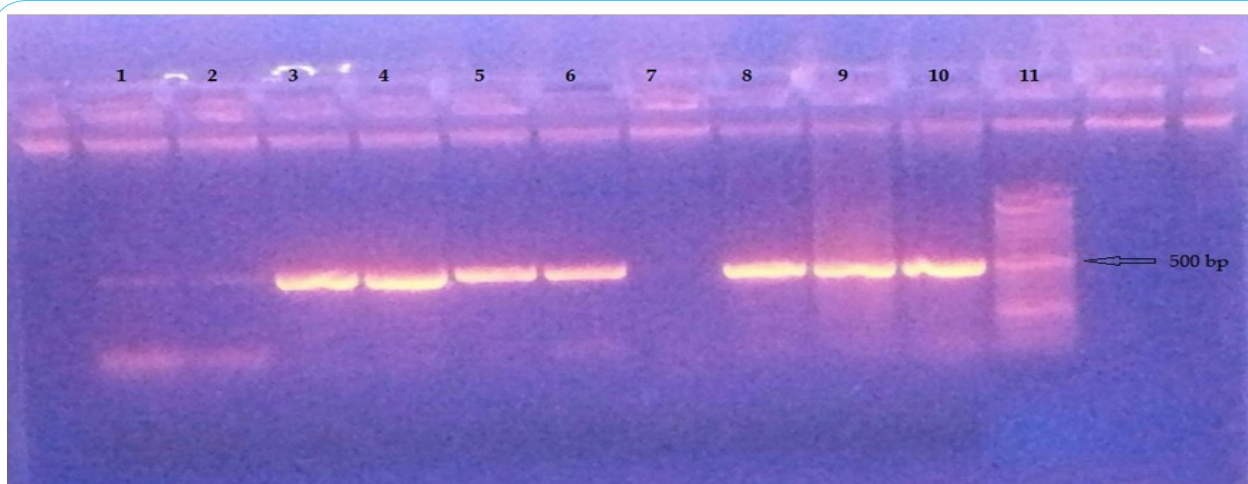


Figure 2: Shows agarose gel electrophoresis of the PCR. Lane 1-10: represent the clinical isolates expression of *Aac(6)-Ib* gene at 482 bp., Lane 11 represent marker.

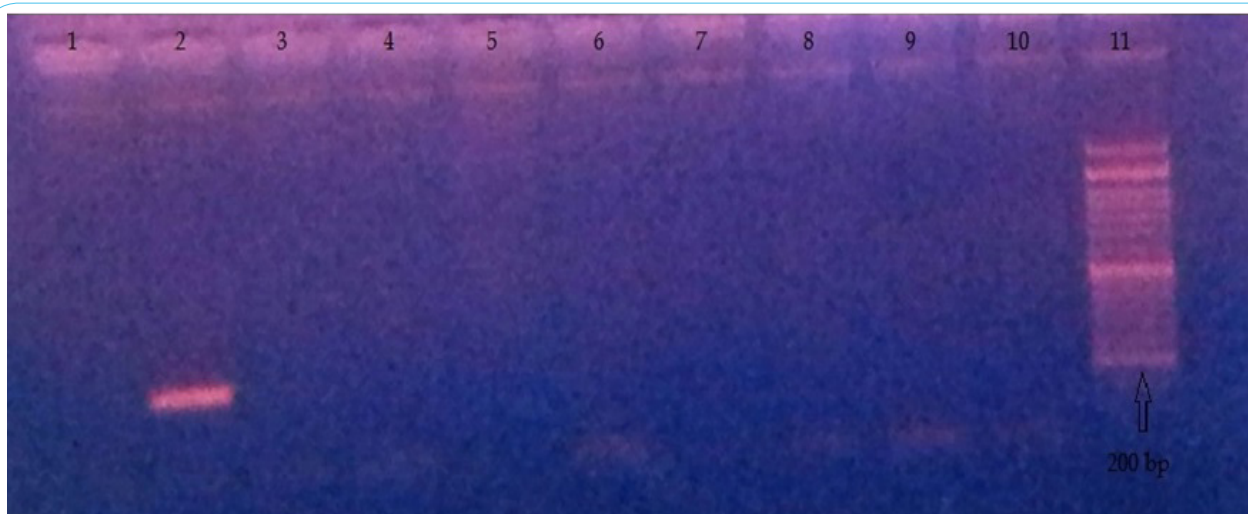


Figure 3: Shows agarose gel electrophoresis of the PCR. Lane 1-10: represent the clinical isolates expression of *qepA* gene at 199 bp., Lane 11 represent marker.

Isolate	Ciprofloxacin (mg/ml)		Nalidixic acid (mg/ml)	
	- P β NA	+ P β NA	- P β NA	+ P β NA
En1*	73.33	<1.1457	> 146.66	<1.1457
En2*	146.66	<1.1457	> 146.66	<1.1457
En3	18.33	<1.1457	> 146.66	<1.1457
En4	> 146.66	> 146.66	> 146.66	> 146.66
En5	> 146.66	> 146.66	> 146.66	> 146.66
En.6	> 146.66	> 146.66	> 146.66	> 146.66
En7	> 146.66	> 146.66	> 146.66	> 146.66
En8*	<1.1457	<1.1457	<1.1457	<1.1457
P1	36.665	9.1663	> 146.66	9.1663
P2	73.33	9.1663	> 146.66	> 146.66
P3*	> 146.66	73.33	> 146.66	> 146.66
P4	73.33	73.33	> 146.66	> 146.66
P5	36.665	<1.1457	73.33	<1.1457
P6*	> 146.66	62.5	> 146.66	> 146.66
P7*	> 146.66	73.33	> 146.66	> 146.66

Table 4: MIC changes of ciprofloxacin and nalidixic acid before and after addition of PA β N for 8 *Enterobacter*, 8 *Proteus*.

* isolates which selected for PCR screening

Discussion

The occurrence of antibiotic-resistant bacteria of all genera has been on the rise. Efflux pumps are transport proteins involved in the extrusion of toxic substrates (including virtually all classes of clinically relevant antibiotics) from within cells into the external environment. The emergence and spread of MDR *Enterobacteriaceae* that cause human infections is increasing worldwide. The most common problematic pathogens in this family are: *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter spp.* They are identified mostly in hospital settings but also as a source of community-acquired infections [1]. Urinary tract infections (UTI) are among the most common bacterial infections in various parts of the world with high medical costs. In this study the most common site of isolation was the urine 112 (51.1%) [17].

In this study, the MICs of ciprofloxacin antibiotic and nalidixic acid antibiotics before and after addition of efflux pump inhibitor Pa β N were measured in 219 strains with fixed concentration of P β NA (20 μ g/ml). Changes were detected in only 28 isolates. Helaly et al. [18] found that by increasing the concentration of PA β N, there is progressive reduction in MIC of ciprofloxacin and levofloxacin which is the same for concentration 25 and 50 μ g/ml (8- and 16- fold for ciprofloxacin and levofloxacin, respectively), while at concentration of 100 μ g/ml a more reduction in the MIC is noticed reaching 16-fold for ciprofloxacin and 32-fold for levofloxacin (64-fold for E30). While

Isolate	gyrA	parC	qnrA	qnrB	qnrS	Aac(6)-Ib	qepA	oqxA	oqxB
K26	+	+	-	-	-	+	-	+	-
K50	+	+	+	-	+	+	+	+	+
K17	+	+	-	+	+	+	-	+	-
En1	+	+	-	+	+	+	-	+	-
En2	+	+	-	-	+	+	-	+	-
P3	+	+	-	-	-	+	-	+	-
K47	+	+	-	-	-	-	-	+	+
K33	+	+	-	-	+	+	-	+	+
K62	+	+	-	-	-	+	-	+	+
K71	+	+	-	-	+	+	-	+	+
K3	+	+	-	-	+	+	-	+	+
E50	-	+	+	-	-	+	-	-	-
E11	+	+	-	-	-	+	-	+	-
E26	+	+	-	-	-	-	-	-	-
E78	+	+	-	-	-	-	-	-	-
E82	+	+	-	-	-	-	-	-	-
E59	+	+	-	-	-	+	-	-	-
E86	+	+	-	-	-	+	-	-	-
E20	+	+	-	-	-	+	-	-	-
E77	+	+	-	-	-	+	-	+	-
P6	-	+	-	-	-	-	-	-	-
P7	-	+	-	+	-	+	-	-	-
E3	+	+	-	-	-	+	-	-	-
K19	+	+	-	+	-	+	-	+	+
E96	+	+	-	-	-	+	-	-	-
En8	-	-	-	-	-	-	-	-	-

Table 5: PCR screening results for Plasmid-mediated quinolone resistance genes and efflux pump genes.

Lunn et al. [19] reported that when the MICs of ciprofloxacin were measured in the presence of PA β N, ten of the nalidixic acid resistant isolates showed a 1.3- to 2-fold decrease, whereas no change was detected in any of the other seven isolates. In all but two isolates (MIC of 0.094 μ g/ml), susceptibilities to ciprofloxacin remained decreased in the presence of PA β N. Glatz [20] observed that at least four-fold reduction in ciprofloxacin MICs was found in the presence of PA β N in 79% of representative isolates; at least eight fold reductions in ciprofloxacin MICs in the presence of PA β N (PA β N+) was detected in 37% of representative isolates. In another study conducted by Lavigne et al. [21] on five patients presented imipenem susceptible *E. aerogenes* strains, then intermediate or resistant isolates. They observed that the PA β N addition reduced the MICs for Ofloxacin in all strains. Another study carried out by Yedekci et al. [13] noted changes in nalidixic acid and ciprofloxacin MIC values in the presence of fixed concentration of PA β N (20 pLg/ml).

All the 33 strains were subjected to PCR-RFLP with the same PCR primers and restriction enzymes. PCR amplification of *gyrA* and *parC* was successful for all 33 clinical strains including the positive controls which generated products with the expected amplicon sizes of 344 and 168 bp. Our PCR-RFLP method is simple because the PCR primers and restriction enzymes are identical and the method is compatible with all 6 *Enterobacteriaceae* species. Therefore, the level of quinolones resistance for all 6 *Enterobacteriaceae* species may be determined without previous species identification from clinical specimens. The PCR-RFLP method provided results within 5 h. Since plasmid-mediated quinolone resistance (PMQR) was first described in 1998, four types of PMQR determinants have been identified: *qnr*, *aac(6)-Ib-cr*, *qepA*, and *oqxAB* [22]. The *qnr* genes are transferable genes that confer low-level quinolone resistance by protection of topoisomerase, *qnrA* had an additive effect of a 10-fold increase in the minimum inhibitory concentration (MIC) whatever the number of topoisomerase mutations, and *qnrS* was additive to *qnrA* with a further 2- to 10-fold increase in the MIC [23].

In Egypt a study carried out by Hassan et al. [24] who found that out of 30 ESBL producers *E. coli* isolates, 8 (26.6%) were positive for *qnr* genes, and the *qnrA1*-, *qnrB1* and *qnrS1*-type genes were detected alone or in combination in 5 (16.6%), 7 (23.3%) and 5 (16.6%) isolates, respectively. Recently, the association of *aac(6)-Ib-cr* with genes encoding the beta-lactamase CTX-M-15 or other ESBLs has been reported [25]. The *qepA* gene, together with the *qnr* family and *aac(6)-Ib-cr*, is the third recently detected plasmid-borne determinant of resistance to the fluoroquinolones. These genes confer only low-level resistance, but their presence could potentially facilitate evolution of the bacterial host toward higher levels of resistance by mutational alterations in the target type II topoisomerases [26]. *qepA* gene was detected in only one *Klebsiella* isolate in this study which was highly resistant to both antibiotics before and after the addition of the efflux pump inhibitor Pa β N. *oqxA/OqxB* is highly prevalent in diverse MDR *K. pneumoniae* of human origin. This efflux pump may be an important factor contributing to the MDR profile of *K. pneumoniae*, and to its versatility as a zoonotic and nosocomial colonizer [27]. Among the 83 *K. pneumoniae* strains, Taherpour and Hashemi [28] found that 48 (57.5%) were ESBL positive. The prevalence of both *oqxA* and *oqxB* detected in *K. pneumoniae* was high: 50 (60.2%) and 50 (60.2%), respectively. In this study, fosfomycin and tigecycline were more active than other antibiotics. Isolates positive for both *oqxA* and *oqxB* were regarded as *oqxAB* positive as the *oqxAB* is encoded by *oqxA* and *oqxB* in the same operon [22].

Conclusion

The presence of co-resistance among different classes of antibiotic families was significant among the ESBL-producing *Enterobacteriaceae*. The Pa β N was an effective phenotypic screening method for quinolones resistance. The data obtained suggests the wide occurrence of PMQR genes in clinical ESBL-producing ciprofloxacin resistant *Enterobacteriaceae* isolates. Also the present study provides sufficient data suggesting that PCR-RFLP methodology is a simple and rapid method (it can be performed within 5 hours) for the detection of ciprofloxacin-resistant strains useful for clinical diagnosis and epidemiological studies. Routine surveillance of microbial population to determine the extent of antibiotic resistance should be conducted to provide suitable treatment guidelines.

Competing Interests

The author declares that he has no competing interests.

Author Contributions

All authors contributed to, designed, and coordinated the study.

Study concept and design: MK, DI, and SM. Microbiological analysis of the samples: RE, DI, SE and SJ.

Acquisition of data: RE, DI, and SE. Analysis and interpretation of data: SJ, RE, DJ, SE, and SM.

Drafting of the manuscript: GK, RE, DI, and SE. Critical revision of the manuscript for important intellectual content, RE, DI, SE, and GK.

Study supervision: MK, RE, DI, SA, SJ, and SE. All authors read and approved the final manuscript.

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