International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 9, Issue 8, 2017, pp.-924-929.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

Research Article

FREQUENCY OF PLASMID-MEDIATED QUINOLONE RESISTANCE DETERMINANTS *QNR* AND *QEPA* AMONG CLINICAL ISOLATES OF *Escherichia coli* and *Klebsiella pneumoniae* PRODUCING EXTENDED- SPECTRUM β-LACTAMASES FROM SAUDI ARABIA INTENSIVE CARE UNITS

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Received: July 29, 2017; Revised: August 16, 2017; Accepted: August 17, 2017; Published: August 28, 2017

Abstract- The aim of this study is to investigate the plasmid mediated quinolone resistance (PMQR) determinants (*Qnr*-like, *QepA* genes) and the relationship between PMQR and ESBL in ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from ICU patients in Saudi Arabia. A total of 34 ESBL producing *E. coli* and *K. pneumoniae* isolates were collected from different ICUs in a tertiary care hospital and identified by double disk synergy test and phenotypic confirmatory disk diffusion test. *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ genes were investigated for their presence in the phenotypic positive ESBL producing Gram negative isolates. In addition, fluoroquinolone phenotypically identified resistant strains were tested for the presence of PMQR genes; *QnrA, QnrB, QnrS, QepA using PCR assay.* The relationship between *ESBL production and fluoroquinolone* resistance was then studied. MIC determination revealed that 100%and 88.2 % of the strains were resistant to nalidixic acid and *ciprofloxacin*, respectively. *BlaCTX* gene was seen in 50% of the isolates while blaSHV gene was detected in only one isolate of each. No *blaTEM gene* was found among the *K. pneumoniae* isolates while15.3% ESBL producing E. coli harbored *the blaTEM* gene. Qnr genes were detected in 4 strains and all of them carried *QnrB*-like gene (3 *E. coli,* 1 K. *pneumoniae),* while no *QnrA* neither *QnrS* could be detected. All *Qnr* strains showed ESBL phenotype. Moreover, the 34 isolates showed the presence of *QepA* gene in 6 *E. coli* and 1 *K. pneumoniae.* Only one *K. pneumoniae* isolate shared both the *Qnr* and the *QepA* gene. The association between one or more of the ESBL harboring genes and *Qnr* determinants was found in 7 isolates (6 *E. coli* and 1 *K. pneumoniae).* Although the *Qnr* genes carriage rate was found low among those strains, there was a clear correlation between *PMQR* genes and *β-lactamase* genes, as well as a high resistance rate against ciprofloxacin.

Keywords- ICU, PMQR, Fluoroquinolones, ESBL

Citation: Nermin H. Ibrahim, et al., (2017) Frequency of Plasmid-Mediated Quinolone Resistance Determinants *Qnr* and *QepA* Among Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae* Producing Extended- Spectrum β-Lactamases from Saudi Arabia Intensive Care Units. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 9, Issue 8, pp.-924-929.

Academic Editor / Reviewer: Dr Aakash Shah

Introduction

Multidrug resistance in Gram-negative rods has become a major health problem worldwide challenging the appropriate use of antibiotics. Most of the resistance mechanisms involved in the β-lactam resistance are connected to the production of β -lactamases, including clavulanic-acid inhibited extended-spectrum β -lactamases (ESBLs) [1]. The incidence of ESBLs has increased dramatically and is now considered a large global concern [2,3]. Documented risk factors accompanying higher incidence of infection with ESBLs include misuse of antimicrobials; using of devices; residence in an ICU; severity of illness and prolonged hospital stay [4-6]. ICU patients are specifically more prone to no-socomial infections as the normal skin and mucosal defenses against infections are commonly compromised by the use of invasive devices. Many of these infections are attributed to Gram negative bacilli invasion, particularly, ventilator-associated pneumonia and catheter-associated urinary tract infections [7,8].

ESBL production was frequently associated with *K. pneumoniae*, however, plasmid mediated ESBL is transferable to other genera and can be found in *E. coli* and other *Enterobacteriaceae* [2,4,9,10]. ESBLs are classically encoded on large plasmids (80 to 300 kb), which can be transferrable among bacterial species [2]. Due to the plasmid-mediated nature of ESBL transfer, one of the major concerns is the co-harboring of an ESBL with fluoroquinolone resistance.

Fluoroquinolones mechanisms of resistance typically involved DNA target mutations; however, since 1998, Plasmid-mediated quinolone resistance (PMQR) has arisen in Enterobacteriaceae particularly among extended-spectrum β-lactamase (ESBL). These fluoroquinolone transferable genes involve the *Qnr* group (*QnrA, QnrB, QnrS, QnrC, and QnrD*), their protein products were found to protect the DNA gyrase and topoisomerase IV from inhibition by quinolones. These genes have been identified worldwide with a frequent association with clavulanate expanded-spectrum β-lactamases and plasmid-mediated cephalosporinases [11] as well as the aminoglycoside acetyltransferase allele, *aac (6')-Ib-cr*, involved in acetylating and consequently dropping the activity of norfloxacin and ciprofloxacin, and the fluoroquinolone-specific efflux pump protein, *QepA*, capable of pumping fluoroquinolones out of bacterial cells [12-14].

ESBL producing *Klebsiella pneumoniae* and *Escherichia coli* are amongst the most common pathogens causing hospital acquired infections (HAIs); especially in ICUs. There is a very limited data concerning the association between quinolone resistance and resistance to other antimicrobial agents, particularly β -lactams in these Gram negative rods isolated in Saudi Arabia. Therefore, it was important to determine the prevalence of ESBL production among *K. pneumoniae* and *E. coli* by using a phenotypic disc diffusion assay. The genes responsible for ESBL productions were also assessed among the tested isolates namely; *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$. Moreover, the presence of fluoroquinolone resistance genes was studied among the tested *E. coli* and *K. pneumonia* yields to assess the correlation between the presence of *Qnr* and *QepA* genes and the ESBL-production from *Enterobacteriaceae* isolated from ICU units in Saudi Arabian hospitals.

Materials and Methods

Specimens and isolate identification

Specimen collection

The study included eighty-six Gram negative isolates collected from ICU patients from a tertiary care hospital, Riyadh, Saudi Arabia. The isolates were collected after informed consent obtained from the patients or their guardians according to Ethics Committees of University Hospitals. The study was performed between May 2015 and December 2016. The age range of patients was from 1-71years old. These clinical isolates were obtained from urine samples, blood cultures, lower respiratory tract specimens, surgical wound sepsis and catheters. They were selected for the study based on reduced susceptibility/resistance to β-lactams and ciprofloxacin.

Growth on MacConkey and EMB Agar.

The Gram-negative isolates were re-inoculated onto MacConkey Agar and EMB agar (Oxoid, Hampshire, UK) plates, incubated at 37^{0}C for 24–48 h and Lactose fermentation was recorded, oxidase test and the standard biochemical tests were performed and recorded. Furthermore, API 20E identification kits (bioMerieux SA, Marcy l'Etoile, France) were employed for confirming their species [15-17].The isolates were maintained in brain-heart infusion broth supplemented with 16% (w/v) glycerol at -80°C until use.

Antimicrobial susceptibility testing / Screening for ESBL enzyme production and Fluoroquinolone Resistance

Disc diffusion method

Disk diffusion method was performed according to the Clinical Laboratory Standards Institute (CLSI) guidelines to test for the antibiotic susceptibility of the isolates [18].

Gram-negative isolates' colonies under test were suspended in sterile saline and adjusted to a 0.5 McFarland standard corresponding to 1.5 × 10^8 CFU/mL. Aliquots of 100 µL of each suspension were uniformly spread onto Muller Hinton Agar (MHA) plates. The tested antimicrobial disks were: Ceftriaxone (CRO 30 µg), Aztreonam (ATM 30 µg), Ceftazidime (CAZ 30 µg), Cefotaxime (CTX 30 µg)and Ciprofloxacin (CIP 5 µg). All the antibiotic discs were purchased from Oxoid (Oxoid, Hampshire, UK).

Clavulanic acid (1000 µg/ml) stock solution was freshly prepared and used to prepare either Ceftazidime/clavulanic acid (CAZ /CL 30/10 µg) and Cefotaxime/ clavulanic acid (CTX /CL 30/10 µg). Double disk synergy test (DDST) was performed to screen for ESBLs [19] followed by phenotypic confirmatory disk diffusion test (DDT) [20] according to CLSI guidelines.

Escherichia coli ATCC 25922 was used as a negative control and *Klebsiella pneumoniae* ATCC 700603 as an ESBL positive control [21]. Plates were incubated aerobically at 35^{0}C for 24h. The experiments were performed in triplicate.

Minimum Inhibitory Concentration (MIC) Determination of Nalidixic Acid (NA) and Ciprofloxacin (CIP)

Broth micro-dilution method was used to determine MICs for NA and CIP (Sigma-Aldrich, Switzerland) for the selected strains. Their values were evaluated according to the Clinical and Laboratory Standards Institute. The *E. coli* ATCC 25922 was included as quality control strain [22].

Plasmid DNA Extraction, Genes Amplifications and Identification

Preparation of plasmid DNA

Luria-Bertanii broth (5ml) (Oxoid, Hampshire, UK) was inoculated with a single colony from each organism taken from MacConkey's agar plates then incubated for 20 h at 37º C. 1.5ml of the overnight culture was then centrifuged at 12,000 rpm for 5 min and the cells were harvested. Plasmid DNA were then isolated by alkaline lysis method using Plasmid mini kit-12123 (Qiagen, Germany) according to the manufacturer's instructions [23].

PCR amplification of *β-lactams* encoding genes

Three ESBL genes were investigated for their presence in 34 phenotypic positive ESBL Gram negative isolates namely; *bla*$_{CTX-M}$, *bla*$_{SHV}$, *bla*$_{TEM}$ using a uniplex PCR assay. The used primers were designed using oligonucleotide primers specific for each of the three tested genes by Eurofins MWG Operon (Germany) as shown in [Table-1].

Table-1 *Target ESBL genes and primers used for strain characterization*.

Target	Primer	Sequence (5´-3´)	Product size (bp)	Reference
bla$_{CTX-M}$	MU1 -F	ATG TGC AGYACC AGT AAR GT	544	[24]
	MU2 -R	TGG GTRAAR TAR GTS ACC AGA		
bla$_{SHV}$	SHV-F	ATT TGT CGC TTCTTT ACT CGC	1018	[24]
	SHV-R	TTT ATG GCG TTACCT TTG ACC		
bla$_{TEM}$	TEM-F	CATTTCCGTGTCGCCCTTATTC	800	[25]
	TEM-R	CGTTCATCCATAGTTGCCTGAC		

The primers amplification DNA fragments were 544, 1018 and 800 bp for *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$, respectively [24,25]. A single reaction mixture contained 1µl of plasmid DNA extract, 1µl of 10µM of each primer, and 45 µl of PCR Super Mix (Invitrogen, USA) in a final volume of 50 µl. The reactions were run using a thermal cycler (MycyclerTM ,BioRad, USA) under the following conditions: heating at 94°C for 5 min; followed by 30 cycles of 94°C for 30s, 55°C for 30s and 72°C for 50 sec; and a terminal cycle of 72°C for 10 min for *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{TEM}$ amplification [26,27] .

In each PCR run, *K. pneumoniae* ATCC 700603 was used as a positive control for ESBL production and *E. coli* ATCC 25922 was added as a negative control. A 100 bp and 1 Kb DNA ladder size markers were used. 1.5% agarose gels (Bio Gene, USA) containing 1X tris-acetate EDTA (Bio gene, USA) and 0.5 µg of ethidium bromide/ml (Biogene USA) were used to separate the resulting PCR products. The gels were then visualized under UV transilluminator (BioRad, USA) and photographed with BioRadGel documentation system (BioRad, USA).

Fluoroquinolone Resistance

Fluoroquinolone phenotypically identified resistant strains were tested for the presence of certain plasmid-mediated quinolone resistance (PMQR) genes conferring resistance in the *E. coli* or *K. pneumoniae* isolates under test using PCR amplification assay.

Four tested genes were investigated using oligonucleotide primers specific for each of the tested genes by Eurofins MWG Operon (Germany) as depicted in [Table-2] [28,29]

PCR was performed on the extracted DNA using PCR Super Mix (Invitrogen, USA). PCR product size of 700, 120, 800 and 218 were for *QnrA, QnrB, QnrS and QepA*, respectively.

Amplification was performed with a Mycycler TM Thermal cycler (BioRad, USA): 30 cycles of PCR were done, with one cycle of denaturation (1 min at 94°C), annealing (1 min at 55°C) and extension (1 min at 72°C). A 100 bp DNA ladder was used as a size marker.PCR products were run on 1.5% agarose gels, detected by ethidium bromide staining, visualized under ultraviolet light and photographed.

Table-2 *Plasmid-mediated quinolone resistance (PMQR) primers for the targeted genes used for strain characterization.*

Target	Primer	Sequence (5´-3´)	Product size (bp)	Reference
QnrA	QnrA-F	TTC AGC AAG ATT TCT CA	700	[28]
	QnrA-R	GGC AGC ACT ATT ACT CCC AA		
QnrB	QnrB-F	CCT GAG CGG CAC TGA ATT TAT	120	[28]
	QnrB-R	GTT TGC TGC TCG CCA GTC GA		
QnrS	QnrS-F	CAA TCA TAC ATA TCG GCA CC	800	[28]
	QnrS-R	TCA GGA TAA ACA ACA ATA CCC		
QepA	QepA-F	GCAGGTC CAGCAGCGGGTAG	218	[29]
	QepA-R	CTTCCTGCCCGAGTATC GTG		

Results

The study group consisted of 86 patients who were hospitalized in different ICU units in Tertiary Care Hospitals, Riyadh, Saudi Arabia. Sixty-six (76.7%) *Escherichia coli* were isolated from different cases; 22 of them were from the MICU, 18 from SICU, 14 from CICU, 6 from PICU and 6 from NICU. *Klebsiella Pneumoniae* isolates were recovered from 20 cases (23.2%), 6 from MICU, 6 from NICU, 4 from PICU and 4 from CICU. No *K. Pneumoniae* isolates were recovered from SICU. These isolates were obtained from urine samples (50.6%), blood cultures (24.1%), lower respiratory tract specimens (11.9%), surgical wound sepsis pus aspirates (3.6%) and other samples (9.8%).

Antibiotic resistance profile of the tested isolates showed that most of the Gram-negative isolates were resistant to β-lactam antibiotics tested [Table-3]. All the 86 isolates were resistant to both aztreonam and cefotaxime. Resistance profile to ceftriaxone and ceftazidime was 98% and 91%, respectively. Fourteen out of 20 (70%) *K. pneumoniae* isolates were resistant to ciprofloxacin while in case of *E. coli* the percentage of resistance to ciprofloxacin was 85. [Table-3]

Table-3 *Antibiotic resistance profile of the bacterial isolates.*

Antibiotic	*E. coli* n=66, (%)	*K. pneumoniae* n=20, (%)	Total n=86, (%)
CRO 30 µg	64, (97)	20, (100)	84, (98)
ATM 30 µg	66, (100)	20, (100)	86, (100)
CAZ 30 µg	58, (88)	18, (90)	78, (91)
CTX 30 µg	66, (100)	20, (100)	86, (100)
CIP 5 µg	56, (85)	14, (70)	70, (81)

Phenotypic assessment was performed in all 86 isolates to identify the production of ESBL by double disk synergy test and consequently confirmed by disk diffusion test. Out of the 86 Gram negative isolates obtained from the different ICU units, 34 (22.1%) were ESBL producing strains; 26 (76.5%) were *E. coli* isolates while 8 (23.5%) *K. Pneumoniae* isolates were considered ESBL by expansion in zone diameter to 3-5mm for either antimicrobial agent tested in combination with clavulanic acid (10 mg) against their zones when tested alone.

MIC determination of nalidixic acid and ciprofloxacin against the selected ESBL producing strains revealed that all the strains tested either *K. Pneumoniae* or *E. coli* were resistant to nalidixic acid since their MIC values were > 8µg/mL while only 30 out of 34 strains were resistant to ciprofloxacin having MIC values of ≥0.125µg/ml.

The selected 34 isolates were then subjected to PCR to identify the presence of *β-lactams* encoding genes; *blaCTX-M, blaSHV* and *blaTEM*. *BlaCTX* gene was seen in 50% either of *E. coli* (13 isolates) or *K. pneumoniae* (4 isolates) as seen in [Table-4] while *blaSHV* gene was detected in only one isolate of each (3.9% and 12.5% for *E. coli* and *K. pneumoniae*, respectively). On the other hand, no *blaTEM* gene was found among the *K. Pneumoniae* isolates. Nevertheless, the *blaTEM* gene was detected in 4 ESBL producing *E. coli* yields (15.3%) [Table-4]. Fourteen out of the 34 isolates have none of the genes causing β- lactamase activity (data not shown).

Relationships between ESBL genotypes and fluoroquinolones resistance phenotypes are shown in [Table-5]. Out of the 17 Gram negative isolates that carry the *blaCTX* plasmid gene, 12 and 17 isolates were resistant to ciprofloxacin and nalidixicacid, respectively. Both isolates that carry the *blaSHV* gene were resistant to nalidixic acid while only one of them was resistant to ciprofloxacin with MIC, ≥0.125 µg/mL while 3 (75%) of the isolates that carry the *blaTEM* gene were resistant to ciprofloxacin.

Table-4 *Frequency of different genes responsible for the ESBL production among the selected isolates.*

Tested gene	*E. coli* (26) Number of isolates, (%)	*K. pneumoniae* (8) Number of isolates, (%)
blaCTX	13, (50)	4, (50)
blaSHV	1, (3.9)	1, (12.5)
blaTEM	4, (15.3)	0, (0)

The presence of *QnrA, QnrB, QnrS*, and *QepA* genes was investigated in the ESBL-positive isolates by PCR using specific primers. Assessment of *Qnr* genes revealed 4 positive isolates all of which were carried on*QnrB*-like gene with the expected size (3 *E. coli*, 1 *K. pneumoniae*), while, there were neither *QnrA* nor *QnrS* detected among the tested yields [Table-6]. The carriage rate of *Qnr* among *E. coli* isolates was higher than in *K. pneumonia*. All *Qnr* strains showed ESBL phenotype.

Table-5 *Distribution of ESBL genotypes among quinolone resistant strains phenotypically*

GENOTYPE	CIP Resistance n (%)	NA Resistance n (%)
***blaCTX*(n=17)**	12 (71)	17(100)
***blaSHV*(n=2)**	1(50)	2 (100)
***blaTEM*(n=4)**	3(75)	4(100)

Furthermore, the 34 analyzed isolates showed the presence of *QepA* gene with the expected size in 7 strains (6 *E. coli*, 1 *K. pneumoniae*). Only one *K. pneumoniae* isolate shared both the *Qnr* and the *QepA* gene. Again, the rate of *QepA* gene carriage was higher in *E. coli* than *Klebseilla.* [Table-6]. However, the association between one or more of the ESBL harboring genes and *Qnr* determinants was found in 7 isolates (6 *E. coli* and 1 *K. pneumoniae*) (data not shown).

Table-6 *Frequency of different genes responsible for the quinolone resistance among the selected isolates.*

Tested gene	*E. coli* (26) Number of isolates, %	*K. Pneumoniae* (8) Number of isolates, %
QnrA	0,0	0,0
QnrB	3,11.5	1,12.5
QnrS	0,0	0,0
Qep A	6, 23	1,12.5

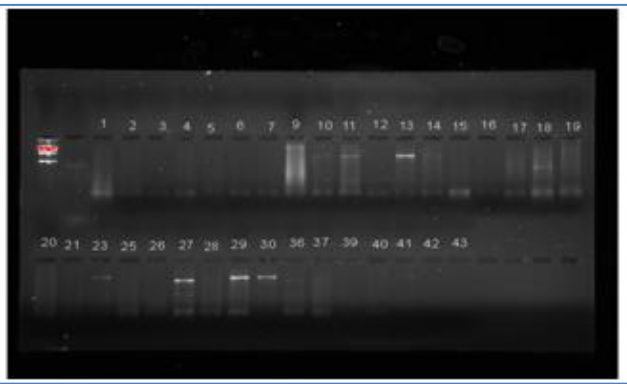

Fig-1 Detection of *QepA* gene. Uniplex PCR for *QepA*: 1.5 % Agarose gel showing products of PCR amplification from 34 clinical isolates *QepA* (218 bp). DNA ladder (1st lane), molecular size standard is 100 bp.

Discussion

Gram negative *Enterobacteriaceae* are commonly associated with hospital-acquired infections among patients in Intensive Care Units (ICUs) [7,8]. In the last decade, increasing dominance of ESBL producing Gram negative bacteria have been reported in clinical settings and surveillance studies [30,31]. Organisms that possess extended spectrum β- lactamases are usually multi drug resistant, hydrolyze aztreonam as well as third-generation cephalosporins thus rendering them useless [32]. ESBL-producing *Enterobacteriaceae*, particularly *Klebsiella* spp. and *Escherichia coli*, have been linked to diverse clinical diseases ranging from colonization to wide variety of infections. Furthermore, several nosocomial epidemics have been reported among patients in intensive care facilities due to ESBL-producing organisms [33].

In the current study, phenotypic characterization of *E. coli and K. pneumoniae* ESBL producers isolated from different ICUs was performed, from tertiary care hospitals in Riyadh, Saudi Arabia during 2015-2016. Out of the 86 Gram negative isolates obtained, (26 out of 66) 33.3% and (8 out of 20) 40% among isolated *E. coli* and *K. Pneumoniae*, respectively were found to be ESBL producers. This shows that there has been an increased level in the prevalence of ESBL producing strains among these species within the Kingdom of Saudi Arabia since Somily *et al*. [34] in 2014, they reported that the frequency of ESBL-producing *E. coli* and *K. pneumoniae* has ranged between 4.8% and 15.8%, this also concurred with what has been documented in different regions in the Kingdom [35-38]. This rapid increase in the prevalence of ESBL Gram negative pathogens in this study can be attributed to the exposed environment of the ICU and the fact that the antimicrobial resistance is high in patients in ICUs than in patients in other hospital departments [39-42]. However, comparable findings were obtained by Rodriques *et al.* [43], who reported that 53% of the nosocomial Gram-negatives were found to be ESBL producers. While, even higher percentages were found in a study done by Suhkla *et al.* [44], who reported ESBL production in 72 % of the Gram-negative pathogens isolated.

Within different *Enterobacteriaceae* species ESBL genes are often carried on plasmids, which can be transferred horizontally among bacteria of either the same or closely related species [45,46]. Important gene families within the group of ESBLs include *blaCTX-M*, *blaTEM* and *blaSHV* among many others [47]. The most frequent ESBLs produced by *Enterobacteriaceae* were found to be (CTX-M-1 and CTX-M-9) in Saudi Arabia, (CTX-M-9, CTX-M-14, CTX-M-15) in China (CTX-M-14, CTX-M-15 and SHV-12) in Korea [48-51]. Furthermore, in India, Sekar *et al* [52] found *blaCTX-M* gene in 14/39 selected clinical isolates. In addition, many studies recorded that *blaCTX-M-15* as the most prevalent CTX-M type found in ESBL-producing *E. coli* strains worldwide [53,54]

These findings agreed with the present study, where, among the screened ESBL isolates, the prevalence of *blaCTX-M* was 50% either in *E. coli* or *K. Pneumoniae* while it was 3.9% for *E. coli* and 12.5% for *K. Pneumoniae* and 15.3% for *E. coli* and 0% for *K. Pneumonia* for *blaSHV* and *blaTEM*, respectively [Table-4]. Fourteen out of the 34 isolates have none of the genes causing β- lactamase activity (data not shown), indicating the inaccuracy of phenotypic detection of ESBL by typical susceptibility testing and the need of molecular methods for detection of probable other mechanisms of ESBL production.

From the 1980s onward, quinolones were used to treat infections at multiple body sites [55]. They were introduced as an appropriate substitute to other antibacterials that have been compromised by resistance. The target of fluoroquinolones in a bacterial cell are the DNA gyrase and topoisomerase IV enzymes [56] that are responsible for DNA replication and relaxation of negative super coils of the bacterial chromosomes and subsequent cell division [57,58]. Reduced susceptibility to the quinolones by interfering with this process is caused by Plasmid Mediated Quinolone Resistance genes (PMQR) [59]. These resistance genes can be transferred between bacteria and presence of these genes result in a reduced susceptibility to ciprofloxacin [60,61]. A large family within this group of PMQR genes is the *Qnr* genes [62]. Moreover, efflux pumps are also incriminated as part of the resistance mechanisms. Two variants are encoded by genes that were described to be plasmid mediated. One is the quinolone specific efflux pump A *(QepA*) [63], another is the multi-drug exporting pump *OqxAB*[64].

Qnr genes have been extensively reported through South and East Asian countries [65]. In the present data, we reported the distribution of PMQR determinants among a collection of 34 *Enterobacteriaceae* clinical ICU isolates in Riyadh, Saudi Arabia, including *Qnr* and *QepA* genes [Table-6]. Overall, PMQR genes were detected in 11 (26 %) out of 34 isolates, comprising 4 *Qnr* and 7 *QepA* genes. *Qnr* genes were detected in 11.8 % of the isolates, all of them carried *QnrB*-like gene. This percentage was higher than a study done by Chenxi Han *et al.* where *Qnr* genes were detected in 4.1% of the isolates which were isolated from pediatric patients and rather more than another report from Europe [66], Sweden [67] and Korea [68].These differences in *Qnr* prevalence can be explained by the difference in environmental conditions, the sample size or the low level of quinolone usage in pediatrics and hence the acquisition of its resistance genes.

QnrS was found be more frequently detected than *QnrA*in clinical *Enterobacteriaceae* isolates in a recent European survey. [69]. That was not the case among our isolates, since; neither *QnrA* nor *QnrS* could be detected. These findings concurred with Liassine *et al*. [70], where no variants of *QnrS* were reported in their study, proposing that *QnrS* is not the cause for flouroquinolone-resistant human isolates in Switzerland. They also found that *Qnr B* was the most prevalent *Qnr* determinant among their *Enterobacteriacae* isolates. Similarly, Only *Qnr B* was detected in the isolates under test.

In this work, the *Qnr* genes were less prevalent among *E. coli* (11.5 %, 3/26) than among *Klebsiella* spp. (12.5%, 1/8), which agrees with the results obtained by El-Malki *et al.* [11] and to previous studies conducted in France [69], the United States [71], Spain[72], and China [73].

Although 30 out of the 34 ESBL isolates were ciprofloxacin resistant, the *Qnr* genes were only detected in 4 isolates while *QepA* gene was only found in 7 isolates (6 *E. coli*, 1 *K. pneumoniae*). Furthermore, only one *K. pneumoniae* isolate shared both the *Qnr* and the *QepA* gene. This suggests that the presence of the horizontally-acquired genes may cause elevated floroquinolnes' MICs in isolates that carried these genes. It is therefore possible that other horizontally acquired resistance genes that was not done in this study such as *OqxAB*or *aac(6')-Ib*, over-active efflux, or even newer mechanisms are the cause for their high MIC values.

In this study, out of the 34 isolates, 30 isolates (88%) showed resistance simultaneously to β-lactam and quinolones (data not shown) and all 26 of the ESBL-producing *E. coli* isolates displayed a MDR phenotypes [Table-3]. Out of the ESBL-*E. coli* strains, 34.6% harbored at least one PMQR determinant. This finding confirms the significant association rate of *Qnr* genes with ESBL genes, which agrees with many other studies [63].Previous surveys conducted in Europe described fewer PMQR determinants in ESBL-*E coli* isolates than found in the current study (10% in Norway and Sweden and 19% in Spain) [74-76].

A high percentage of *Qnr*gene was found among ESBL-producing enterobacterial species as described by many researchers [69,77-79]. The *Qnr* gene confers partial resistance to fluoroquinolones by protecting DNA gyrase from the effects of fluoroquinolones [79]. InChenxi Han e*t al.* study [65],the distribution of *Qnr* gene among ESBL *E. coli* isolates showed that *QnrS* was the most prevalent (2.1%), followed by *QnrB* (1.0%), and *QnrA*(1.0%). These results were comparable to Jiang's report, where the prevalence of *QnrA, QnrB, and QnrS* was 1.9%, 1.5%, and 1.9%, respectively [73]. Nevertheless, in this study, *QnrB* was the most prevalent gene among the tested ESBL-producing isolates. In addition, *Qnr S* was not found among all the tested ESBL isolates, and that contradicts with HayatoOkade *et al.*[80] who reported that *QnrS* was significantly related to ESBL genes, such as *blaCTX* or *blaSHV* and that associations of *QnrS1-blaCTX-M14* or *blaSHV-2* have also been reported in China [81].

In the current work, the association between one or more of the ESBL harboring genes and *Qnr* determinants was found in only 7 isolates (6 *E. coli* and 1 *K. pneumoniae*) (data not shown). These findings signify that the distribution and prevalence rate of plasmid-mediated Qnr genes differs within populations and according to the sample source and the geographical regions.

Although, *Qnr* genes were thought to be the emerging PMQR determinant in CTX-M-producing strains isolated in France [77], the current findings suggest that *Qep*

A rather than *Qnr*, is the spreading PMQR determinant in ESBL-*E coli* isolates.
It has been reported that the *Qnr* gene co-existed with *blaCTX−M* and *blaSHV*alleles [73]. This coincided with the current investigation, as 50% of the isolates in which *Qnr* genes were detected contained *blaCTX−M*-like genes. Moreover, 72% of the isolates harboring *Qep A* genes carried *blaCTX−M* and /or *bla TEM* like genes (data not shown). Another interesting finding in this work was that *QnrB, Qep A, blaCTX* and *bla SHV* were co-harbored in a single *K. pneumoniae* isolate (K311) (data not shown). Although fluoroquinolone resistance is not typically encoded on plasmids, plasmid-mediated resistance may encourage the development of high-level resistance under antimicrobial pressure [79,82]. Therefore, the co-expression of a *Qnr* gene and *Qep A* genes on plasmids carrying ESBLs may encourage the development of chromosomal -mediated resistance.

Conclusion and Recommendations

In conclusion, the current investigation demonstrates that resistance to the studied drugs is already common and may occur through multiple mechanisms. Increased spread of ESBL-producing organisms in ICUs where there is significant antimicrobial pressure, may preferentially select organisms that have acquired resistance to multiple classes of antimicrobials. This rise in fluoroquinolone resistance in ESBL-producing *E. coli* or *K. Pneumoniae* infections may rapidly demolish the usefulness of the two presently most accepted antibiotic choices for therapy, b-lactams and floroquinolones leading to few low-cost substitutes for managing infections due to Gram-negative organisms. Furthermore, horizontally-acquired resistance to the quinolones, and seemingly other drug resistance may be present on mobile elements that could be conveyed among pathogens. Sometimes multiple genes are responsible for production of ESBLs in a single isolate. *QepA* was found to be the predominant PMQR determinant in ESBL-producing *E. coli* or *K. Pneumoniae* isolated in ICU, Riyadh, Saudi Arabia and most of the strains that harbored *blaCTX-M* gene carried one or more of the PMQR determinant.
The current study carries significant findings that high light the mechanisms of resistance among ESBL producing Gram negative bacilli in the ICUs in Saudi Arabia and may guide the rational use of empirical antibiotic treatment. Though, it may have some limitations as not including potentially important factors like prior antibiotic exposure, ICU length of stay, or devices' use.
Future studies should seek to precise different PMQR genes' functions and relationship with antibiotics' resistance. Furthermore, plasmid's carriage of these PMQR genes should be assessed by conjugation tests while the identification of different mutations in gyrase and topoisomerase IV enzymes due to the presence or absence of these genes should be confirmed by sequencing.

Acknowledgement / Funding

The authors extend their appreciation to the Deanship of Scientific Research at KingSaud University for funding this work through research group NO (RGP-1438-003).

Author Contributions

All authors contributed equally to the work.

Conflict of Interest: There is no conflict of interest.

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