Detection of *qnr* genes in ESBLs producing and non-producing coliforms

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ABSTRACT

Background and objectives: The discovery of plasmid-mediated quinolone resistance (PMQR) in the late 1990's added a new dimension to quinolone resistance. During the last years, the occurrence of extended-spectrum-β-lactameses (ESBLs) and (PMQR) within coliforms group has gained particular attention. The objective of this study was to determine the prevalence of plasmid-mediated fluoroquinolone resistance genes in ESBL-producing coliforms

Materials and method: One hundred and seventy three clinical samples collected from both gender , (110) from urinary tract infections (UTIs), and (63) from patients with diabetic foot infections (DFIs) who visited or admitted to AL- Salam General Teaching Hospital and AL- Wafa'a center for diabetic patients in Mosul city of Ninavah province / Iraq from April 2013 to February 2014.The isolates were identified to species level depended on morphological, biochemical and physiological tests and confirmed by RapID^{TM} ONE system (Remel \ USA).

Results: The results showed the majority of them were resistant to most antibiotics. Resistance was observed most often to nalidixic acid (85.9%)followed by norfloxacin (70.5%) and ciprofloxacin (67%). All ciprofloxacin resistant isolates were tested for their ability to produce the extended spectrum β-lactamase (ESBL) enzymes using the double disk synergy test (DDST). Out of the total (57) ciprofloxacin resistant coliforms tested, (66.7%) were ESBL producers. Ciprofloxacin resistant coliforms species were conducted for PCR to investigate the presence of *qnr* genes. Out of (57) ciprofloxacin resistant spp. *qnrB* gene (469 bp) was detected in 14(24.6%) spp. while none of the species had *qnrA* and *qnrS* genes in our study. The results also revealed that *qnrB* gene was found in all (100%) of ESBL-producing *E. coli* and *K. pneumoniae* spp.

Keywords: Ciprofloxacin resistance, *qnr* genes, Coliforms

INTRODUCTION

Quinolones are widely used to treat clinical infections in both in and out patients ; therefore a survey of quinolone resistance would be especially useful. Their use now accounts for about (11%) of overall prescriptions of antimicrobials in human medicine and one of them , ciprofloxacin is the most used antibiotic in the world (Lyonga *et al*., 2014 and Tarchouna *et al*., 2015).

Quinolone resistance was for a long time considered to be entirely mediated by mutations in chromosomal genes encoding quinolone targets (that is , DNA gyrase and topoisomerase IV) and/or in regulatory genes of outer-membrane proteins or efflux pumps . Plasmid carrying *qnr* genes have been found to transmit quinolone resistance . These genes encode pentapeptide repeat proteins that block the action of ciprofloxacin on bacterial DNA gyrase and topoisomerase IV (Rushdy *et al*., 2013 and Al - Marjani *et al*., 2015).

The discovery of plasmid-mediated quinolone resistance (PMQR) in the late 1990's added a new dimension to quinolone resistance. During the last years , the occurrence of extended-spectrum-β-lactameses (ESBLs) and (PMQR) within coliforms group has gained particular attention . ESBL and *qnr* genes can be carried either on the same plasmid or on different plasmids within the same isolate (Kim *et al*., 2010 and Raei *et al*., 2014). Therefore , plasmid-mediated resistance to fluoroquinolones among ESBL-producing coliforms is alarming as it facilitates therapy failure (Vali *et al*., 2015).The objective of this study was to determine the prevalence of plasmid-mediated fluoroquinolone resistance genes in ESBL-producing coliforms.

MATERIAL AND METHOD

Bacteriological samples

A total of 173 bacteriological samples were collected from patients (110 urine samples and 63 diabetic foot infection samples) of both gender who visited or admitted to AL- Salam General Teaching Hospital and AL- Wafa'a center for diabetic patients in Mosul city of Ninavah province / Iraq from April 2013 to February 2014 . Samples were transported quickly by sterile transport media and sterile cotton swabs to the laboratory for culturing on

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MacConky agar and Blood agar . The isolates were identified to species level depended on morphological, biochemical and physiological tests and confirmed by RapID™ ONE system (Remel \ USA).

Detection of Extended - Spectrum β - Lactamase (ESBLs)

The test was done by using the double disk synergy test (Ahmed *et al* ., 2013). The organism to be tested was spread onto a Mueller - Hinton agar plate as in the standard disk diffusion method . Four antibiotic disks are used including Amoxycillin / Clavulanic acid 20 / 10 µg / disk , Ceftazidime , Ceftriaxone , and Cefotaxime 30 µg / disk for each once . The Amoxycillin / Clavulanic acid disk was placed in the center of the plate , while the three antibiotics disks were placed at distances of 30 mm (edge to edge) from it . The plate was incubated at 37°C for 18 0 24 hours , if an enhanced zone of inhibition between either of the cephalosporin antibiotics and the Amoxycillin / Clavulanic acid disk occurred , the test was considered positive .

Detection of quinolone resistance isolates

The bacterial isolates were tested for their susceptibility to ciprofloxacin 10 µg / disk , norfloxacin 10 µg / disk **,** and nalidixic acid 30 µg / disk by using standard disk diffusion method (Bauer *et al*.,1966). All isolates which were resistant to ciprofloxacin are suspected to harbor *qnr* genes (Pakzad *et al* ., 2011) .

DNA extraction

Genomic DNA was extracted from all ciprofloxacin resistant isolates using Wizard Genomic DNA purification kit supplemented by (Promega \ USA) according to manufacture instructions. The purity and concentration of genomic DNA were measured using Biodrop spectrophotometer.

Agarose gel electrophoresis

The method described by Sambrook and Ruseel (2001), was used for prepare horizontal agarose gel electrophoresis for genomic DNA and PCR product . Agarose at concentration of 0.7gm / 100ml was prepared for genomic DNA , and 1.2gm / 100 ml for PCR product .

Table (1):Primer used for the amplification of *qnr* genes .

Genes	Oligonucleotides (5`→ 3`)	Product size	Reference
qnrA	**F** ATTTCTCACGCCAGGATTTG **R** GATCGGCAAAGGTTAGGTCA	516 bp	Pakzad *et al* ., (2011)
qnrB	**F** GATCGTGAAAGCCAGAAAGG **R** ACGATGCCTGGTAGTTGTCC	469 bp	
qnrS	**F** ACGACATTCGTCAACT GCAA **R** TAAATTGGCACCCTGTAGGC	417 bp	

Table (2): The PCR reaction components (25µl) for genes amplification .

Component	Volume (µl)
GoTaq Green Master Mix (2X)	12.5
Nuclease Free Water	9.5
DNA Template	2
Forward Primer (10 picomoles)	0.5
Reverse Primer (10 picomoles)	0.5
Total volume	25

Table (3). Program conditions for amplification of *qnr* genes .

Stage	Temperature °C	Time (min.)	Cycle number
Initial denaturation	94	3	1
Denaturation	94	1	32
Annealing	53	1	
Extension	72	1	
Final extension	72	7	1
Hold	4	3	1

Figure (1): Identification of *Citrobacter freundii* by RapID™ ONE panel kit

Table (4): Number and percentage of coliforms spp. isolated from two types of infection

Coliforms	Type of infection		Total
	UTIs No. (%)	**DFIs** No. (%)	No.(%)
E. coli	28 (50.9)	17 (56.7)	45 (52.9)
K. pneumoniae	17 (30.9)	8 (26.7)	25 (29)
Ent. cloacae	2 (3.7)	0 (0)	2 (2.4)
Ent. aerogenes	5 (9.1)	0 (0)	5 (5.9)
C. freundii	0 (0)	2 (6.7)	2 (2.4)
Serratia marcescens	2 (3.7)	3 (10)	5 (5.9)
Serr. fonticola	1 (1.8)	0 (0)	1 (1.2)
Total	55 (64.7)	30 (35.3)	85 (100)

Table (5): Fluoroquinolones resistance among coliforms according to types of infection

Type of infections	Bacterial isolates	Antibiotics		
		CIP No (%)	NOR No. (%)	NA No.(%)
UTIs	*E. coli* (n=28)	19 (67.9)	21 (75)	24 (85.7)
	K. pneumoniae (n = 17)	14 (82.4)	14 (82.4)	16 (94.1)
	Ent. cloacae (n = 2)	0 (0)	0 (0)	1 (50)
	Ent. aerogenes (n = 5)	3 (60)	3 (60)	4 (80)
	Serr. marcescens (n = 2)	1 (50)	1 (50)	2 (100)
	Serr. fonticola (n = 1)	0 (0)	0 (0)	1 (100)
DFIs	*E. coli* (n = 17)	11 (64.7)	12 (70.6)	15 (88.2)
	K. pneumoniae (n = 8)	5 (62.5)	5 (62.5)	6 (75)
	C. freundii (n = 2)	2 (100)	2 (100)	2 (100)
	Serr. marcescens (n = 3)	2 (66.7)	2 (66.7)	2 (66.7)
Total	N = 85	57 (67%)	60 (70.5%)	73 (85.9%)

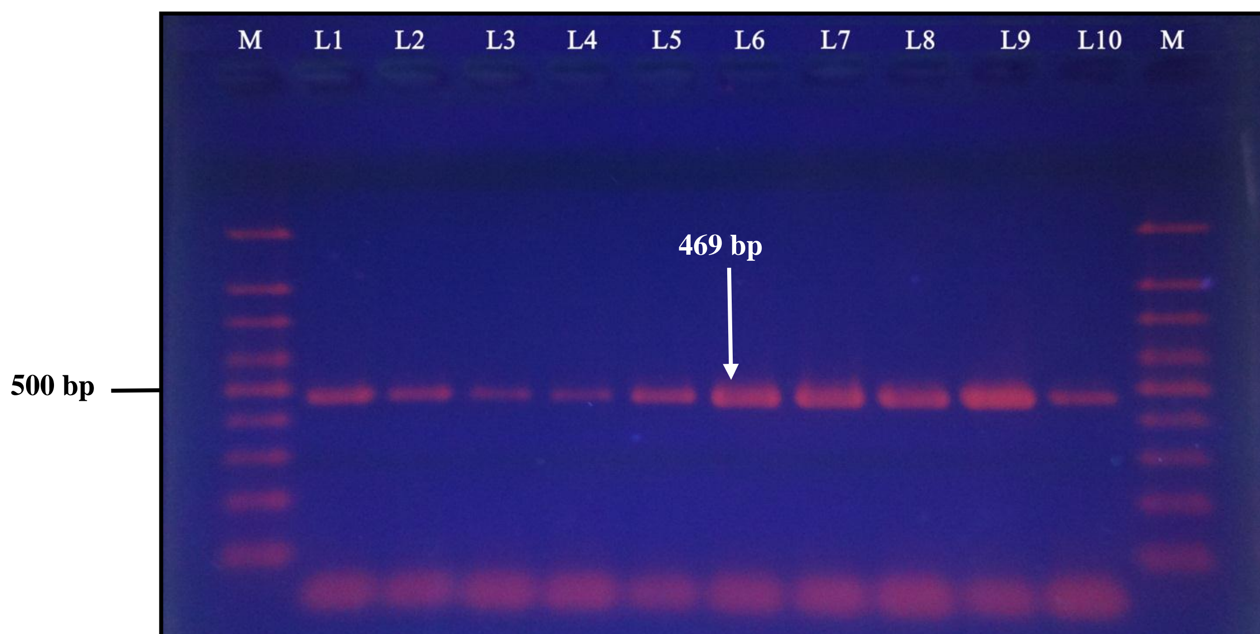
Table (6): Number and percentage of ESBLs production among ciprofloxacin resistant coliforms according to types of infection

Type of infection	Ciprofloxacin resistant isolates	Total No.	Positive ESBL No. (%)	Negative ESBL No. (%)	P values
UTIs	*E. coli*	19	16 (84.2)	3 (15.8)	0.003*
	K. pneumoniae	14	8 (57.1)	6 (42.9)	0.593
	Ent. aerogenes	3	0 (0)	3 (100)	0.317
	Serr. marcescens	1	0 (0)	1 (100)	1.000
DFIs	*E. coli*	11	9 (81.8)	2 (18.2)	0.035*
	K. pneumoniae	5	4 (80)	1 (20)	0.180
	C. freundii	2	1 (50)	1 (50)	1.000
	Serr. marcescens	2	0 (0)	2 (100)	0.564
Total		57	38 (66.7%)	19 (33.3%)	0.012*

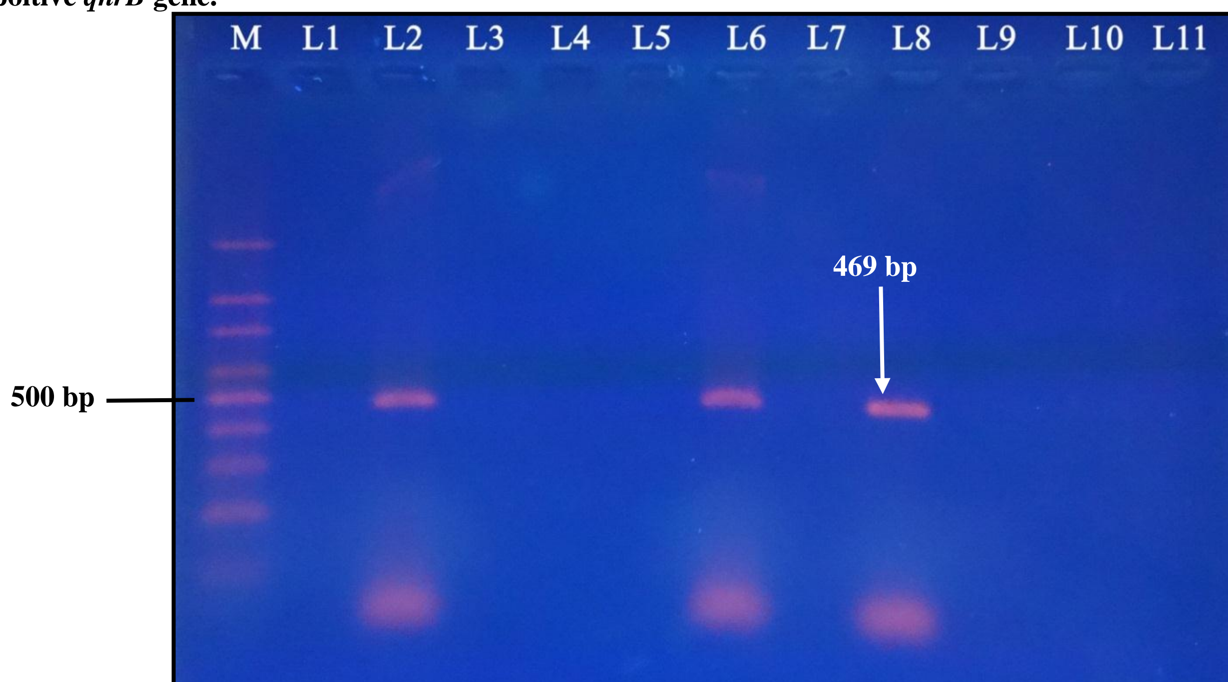
* Sig. : $P \leq 0.05$.

Table (7) : Number and percentage of *qnr* genes in ciprofloxacin resistant isolates of coliforms

Ciprofloxacin resistant coliforms	*qnr* gene types		
	qnrA N. (%)	*qnrB* N. (%)	*qnrS* N. (%)
E. coli (n = 30)	0(0)	10 (33.3)	0(0)
K. pneumoniae (n = 19)	0(0)	3 (15.8)	0(0)
Ent. aerogenes (n = 3)	0(0)	1 (33.3)	0(0)
Serr. marcescens (n = 3)	0(0)	0(0)	0(0)
C. freundii (n = 2)	0(0)	0(0)	0(0)
Total (n = 57)	0(0)	14 (24.6)	0(0)



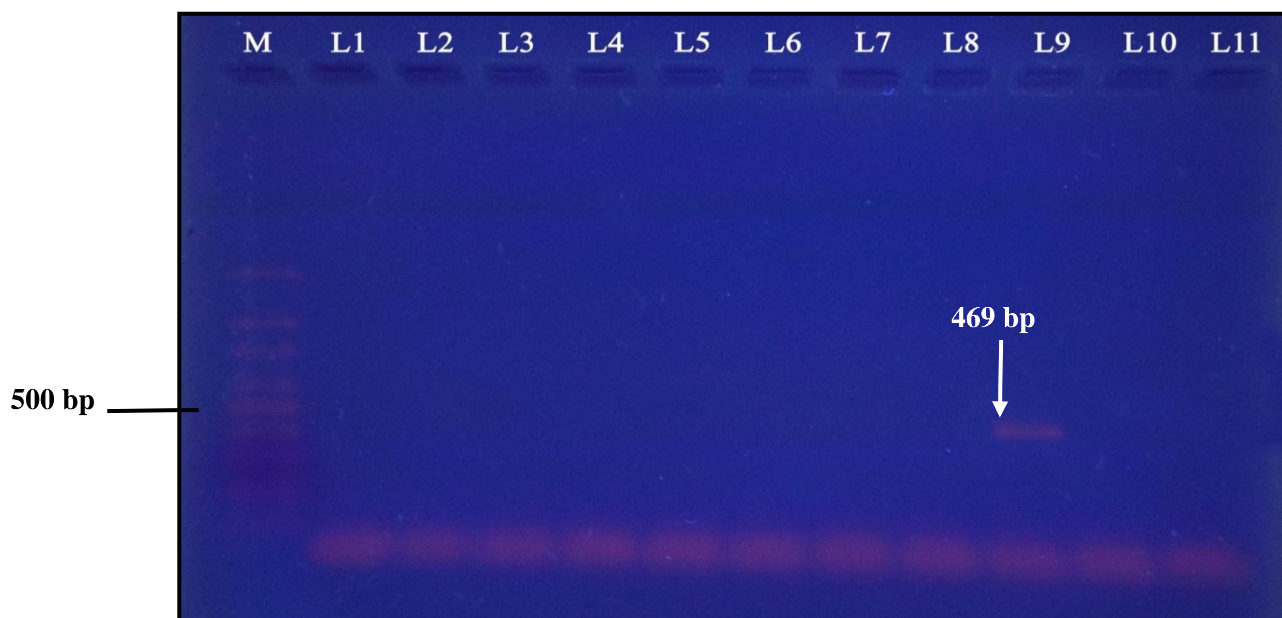
Figure(2): Electrophoresis of PCR amplification of *qnrB* gene in ESBL- *E. coli* with expected product length 469bp in (1.2%) agarose gel. M lane, left and right (100bp ladder). Lane 1 – 10 poitive *qnrB* gene.



Figure(3): Electrophoresis of PCR amplification of *qnrB* gene in ESBL- *K. pneumoniae* with expected product length 469bp in (1.2%) agarose gel. M lane (100bp ladder). Lane 2,6,and8 positive *qnr B* gene.

Table (8): Number and percentage of *qnr* genes in ESBLs producing and non-producing coliforms.

qnrB positive isolates	ESBLs N. (%)	non-ESBLs N. (%)
E. coli (n = 10)	10 (100%)	0 (0)
K. pneumoniae (n = 3)	3 (100%)	0 (0)
Ent. aerogenes (n = 1)	0 (0)	1 (100)
Total n = 14	13 (92.9)	1 (7.1)



Figure(4): PCR amplification of *qnrB* gene in non-ESBL coliforms with expected product length 469bp in (1.2%) agarose gel. M lane (100bp ladder). Lane 9 positive *qnrB* gene in *Ent. aerogenes*

DISCUSSION

Primers used

Primers sequences were taken from previous articles(Table 1) . All primers were synthesized by Alpha DNA company, CANADA.

Polymerasechain reaction (PCR) assay

PCR reaction

All PCR reactions were performed in 25µl volumes in Eppendorf tube . Laminar flow cabinet with UV lamp was used for the preparation of reaction mixture , all the reaction components were prepared separately in ice and used with optimum concentration . The PCR reactions components are shown in (Table 2) .

Detection of *qnrA* , *qnrB* , and *qnrS* genes

PCR conditions program depending on Pakzad et al ., (2011) , with some modification was used for detection of fluoroquinolones resistance genes as shown in (Table 3) .

Statistical analysis

All statistical analysis was conducted using the Statistical Package for Social Sciences (S.P.S.S.) version 19 from IBM Company, USA. The $x2$ test was used for statistical comparison of groups , values < 0.05 were regarded as significant .

RESULT

The present study was carried out in AL-Salam General Teaching Hospital and one center for Diabetic Patients at Mosul City , between April 2013 and February 2014 . Out of the total (173) samples collected from both gender , (110) from urinary tact infections (UTIs),and(63) from patients with diabetic foot infections(DFIs).

Identification of coliforms bacteria was first made by the bacteriological methods, biochemical tests and RapID™ ONE panel kit was used for accurate identification of isolates at species level (Figure 1). The result showed that the isolated bacteria belonged to five genera ; the number and percentage of coliforms isolated from UTIs and DFIs are listed in (Table 4). The total number of coliform bacteria was (85) isolates , of which UTIs isolates were more frequently encountered (55; 64.7%) than the DFIs isolates (30;35.3%) . Among the isolates , *Escherichia coli* was the predominant isolates 45 (52.9%) from clinical samples, 28 (50.9%) were UTIs samples , while 17 (56.7%) were DFIs samples followed by *Klebsiella pneumoniae*

which isolated from 25 (29%) samples 17 (30.9%) were UTIs samples , and only 8 (26.7%) were DFIs samples**.**

The most important coliforms are *E. coli* , *Klebsiella* , *Enterobacter* , and *Citrobacter* . They can cause many infections like UTIs and wound infections (Dhakal *et al* ., 2008 and Basavzraj and Jyothi , 2015) . In the current study , *E. coli* (50.9%) represented the predominant isolate from UTIs . This finding agrees with other reports in which *E. coli* was the commonest pathogens isolated from patients with UTIs (Dishvarian , 2010 and Zaki and Elewa, 2015).

Fluoroquinolones resistance pattern of coliforms isolates

Quinolones constitute an important and highly used group of antimicrobial drugs in human and veterinary medicine for a wide variety of microbial infections . Because of wide clinical use , bacterial isolates resistant to quinolone and fluoroquinolone are emerging and spreading rapidly , especially in coliform bacteria (Guo *et al* ., 2010 and Saranya *et al* ., 2013) . The susceptibility of (85) coliforms spp. against quinolone agents were studied to evaluate the pattern of their resistance using disk diffusion method .

Out of (85) bacterial isolates , 73 (85.9%) isolates were resistant to nalidixic acid , whereas 60 (70.5%) , and 57 (67%) isolates were resistant to norfloxacin and ciprofloxacin respectively(Table 5) . This finding was relatively close to that of Ogbolu *et al* .,(2012) who found that the resistance rate of nalidixic acid among gram-negative bacilli was(98.3%) , while that for ciprofloxacin was (76.5%) . Many reports have indicated that the widespread use of fluoroquinolons is contributing to the increasing percentages of fluoroquinolone insusceptible bacterial strains , including coliforms (Nambo-odiri *et al*., 2011 and Majumdar *et al*., 2012).

However , the proportion of *E. coli* and *K. pneumoniae* isolates that were isolated from UTIs showed highest resistance to fluoroquinolones when compared to those isolated from DFIs .This may be because fluoroquinolones are preferred as initial agents for empiric therapy in UTI, or due to their excellent activity against the pathogens which are commonly encountered in UTIs (Sureshkumar *et al* ., 2012) .

Phenotypic Extended - Spectrum β - lactamase (ESBLs) detection

The spread of ESBL-producing bacteria has been strikingly rapid worldwide , indicating that continues monitoring systems and effective infection control measures are absolutely required. All ciprofloxacin resistant isolates were tested for their ability to produce the ESBL enzymes using the double disk synergy test (DDST) . This test depends on the synergism that occurs between the clavunate and the cephalosporine , where the clavunate destroys the ESBL and open the way for cephalosporine to exert it's action on bacteria (Ahmed *et al*., 2013).

Out of total (57) ciprofloxacin resistant coliforms tested , 38 (66.7%) isolates were ESBL producers , while the remaining 19 (33.3%) isolates were non-ESBL producers . In the current study , we reported a high incidence of ESBL production among ciprofloxacin – resistant *E. coli* strains isolated from UTIs and DFIs (84.2%), and (81.8%) respectively, followed by *K. pneumoniae* isolates (57.1%) and (80%) respectively . Similar to this high result was reported in a study conducted in Nigeria by Alo *et al*., (2012) who found that (80%) of ciprofloxacin resistant *E. coli* and *K. pneumoniae* were ESBLs producers . While in contrast , Tolun *et al*., (2004) who revealed that only (5.1%) of the ciprofloxacin-resistant *K. pneumoniae* strains produced ESBL .

It is worthy to note that no ESBL production was observed among the isolates of *Ent. aerogenes* and *Serr. marcescens*, whereas only 1 (50%) of the ciprofloxacin-resistant *C. freundii* was ESBLs producers as shown in (Table 6). In contrast , locally other studies are detected the production of ESBL by *Ent. aerogenes* and *Serr. marcescens* isolates (Lafi and Mohammed , 2012 and Tuwajj , 2014) .

Molecular detection of *qnrA* , *qnrB* , and *qnrS* genes by PCR

Polymerase Chain Reaction (PCR) technique has been used to amplify *qnr* genes, which may be responsible for flouroquinolones resistance . The PCR analysis showed that among the (57) ciprofloxacin resistant isolates , the *qnr* genes screened in the present study was present in a total of 14/57 (24.6%) isolates as shown in (Table 7). The *qnrB* gene (469bp) was detected in 10(33.3%) *E. coli* , in 3(15.8%) *K. pneumoniae* , and in 1(33.3%) *Ent. aerogenes* isolates as shown in(Figures 2, 3, and 4). Non of the isolates had *qnrA* and *qnrS* type genes in our study .

The prevalence of plasmid-mediated quinolone resistance (PMQR) determinant was investigated in many countries . Tarchouna *et al* , (2015) found that (32%) of *E. coli* strains

isolated from different clinical samples in a Tunisian hospitals were positive for the presence of *qnr* genes, and only (12.5%) of them were carried *qnrB* gene . Another study conducted in Egypt by EL-Mahdy (2015) who revealed that (53%) of *E. coli* , *K. pneumoniae* , and *Enterobacter* spp. were positive for the *qnr* genes . In China , Wang *et al*.,(2008) found that the prevalence rates of *qnr* genes among ciprofloxacin-resistant isolates of *E. coli* and *K. pneumoniae* were (7.5%) and (11.9%) respectively , these rates are lower than our results . Differences in distribution of the *qnr* genes may be attributed to difference in geographical area , or may be due to difference in selection criteria (EL-Mahdy , 2015).

The relation between *qnrB* gene and ESBLs producing and non-producing coliforms:

Resistance to quinolone and fluoroquinolone is often associated with ESBL-producing organisms as well as association with integron carrying *qnr* genes(Saboohi *et al*., 2014) The *qnrB* gene was found in all (100%) of ESBL-producing *E. coli* and *K. pneumoniae* isolates as shown in (Figures 2 and 3). This is because the *qnr* genes are usually associated with the same mobile genetic elements as those of ESBL genes (Pasom *et al*., 2013) . In Kuwait , Vali *et al*.,(2015) revealed that (78%) of *K. pneumoniae* isolates were positive for *qnrB* gene . Several reports have detected a positive correlation between *qnrB* gene and the ESBL production (Pakzad *et al*., 2011 and Goudarzi *et al* ., 2015).

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