Detection of Plasmid-Mediated Quinolone Resistance Genes in Clinical and Environmental Hospital Isolates of Klebsiella pneumoniae in Al-Najaf City pneumoniae Klebsiella

الكشف عن جينات المقاومة البلازميدية لمضادات الكوينولون المعزولة من الحالات السريرية وبيئة المستشفيات في مدينة النجف الاشرف

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الخلاصة :
الهدف : التحري عن الجينات البلازميدية المسببة لمقاومة لمضادات الكوينولون Plasmid-Mediated Quinolone Resistance (PMQR) في بكتريا *Klebsiella pneumoniae* المقاومة لمضادات الكوينولون المعزولة من حالات سريرية وبيئة المستشفيات .
المنهجية : تم جمع195 سريرية مختلفة و 50عينة بيئية من ثلاث مستشفيات رئيسة في محافظة النجف الاشرف. شخصت بكتريا *Klebsiella pneumoniae* اعتماداً" على الاختبارات الزرعية والكيموحيوية التقليدية فضلا عن استخدام نظام Api 20-E .تم التحري عن مقاومة مضادات الكوينولون مظهريا عن طريق تنميتها في وسط الماكونكي المدعم بمضاد السبروفلوكساسين بتركيز 10 مايكروغرام/مل ومن ثم اختبرت حساسية العزلات المقاومة للسبروفلوكساسين تجاه 18مضاداً حيوياً تابعة لأصناف مختلفة بطريقة الانتشار بالقرص لكيربي-باور.كما تم الكشف عن وجود جينات المقاومة البلازميدية(*qnrB, qnrS aac (6 ')-Ib-cr,* و *qepA*) في عزلات بكتريا *K. pneumoniae* المقاومة لمضادات الكوينولون باستعمال تقنية Multiplex polymerase chain reaction .
النتائج : أظهرت النتائج عائدية 89 عزلة لبكتريا *K. pneumoniae* من مجموع245 عينة سريرية وبيئية، بدت 38 % من العزلات(34) مقاومة لمضاد السبروفلوكساسين (10 مايكروغرام/مل).بينت نتائج اختبار الحساسية للمضادات الحيوية امتلاك جميع العزلات(34) قيد الدراسة لصفة المقاومة للعديد من المضادات الحيوية Multidrug resistant اذ قاومت على الاقل لاربعة عشر مضاد حيوي اما نتائج الكشف عن جينات مقاومة الكوينولون البـــلازميديــة فكان الجين *aac (6 ')-Ib-cr* هوالأكثر شيوعا فقد ظهر في 14 عزلة بنسبة (41.18%) لوحده او مع الجين *qnrS* وكانت 2.94% من العزلات حامله لجيني *aac (6 ')-Ib-cr* و*qepA* و 8.82 % من العزلات حاملة لثلاث جينات *aac (6 ')-Ib-cr* و*qepA* و*qnrS* في حين ظهرالجين *qnrB* في عزلة واحدة فقط (29.41%) كان مصدرها خمج الجروح.
الاستنتاج : انتشار واسع لجينات المقاومة البلازميدية لمضادات الكوينولون *qnrB* and *aac (6 ')-Ib-cr, qepA, qnrS* . ان هذه الدراسةهي أول تقرير للتحري عن الطفرات في الجينات البلازميدية بين عزلات *K.pneumoniae* البيئية والسريرية في العراق .
التوصيات : ضرورة اجراء دراسات لمعرفة مدى انتشار المقاومة الكروموسومية و المقاومة البلازميدية لمضادات الكوينولون في *K.pneumoniae* وبقية انواع البكتيريا المرضية الشائعة .

Abstract
Aim of study : This study aimed to detected the presence of the plasmid mediated quinolone resistance genes in quinolone resistant *Klebsiella pneumoniae* isolates from clinical and environmental hospital samples.
Methodology : A total of 195 clinical samples of different sources and 50 environmental hospital samples were collected from three main hospitals in Al-Najaf city .*K. pneumoniae* was identified depending on cultural and traditional biochemical tests, then confirmed by API 20E system. Phenotype detecting of quinolone resistance in *K. pneumoniae* isolates were carried out by growing on MacConkey agar supplemented with 10µg/ml Ciprofloxacin. Antibiotic susceptibility performed by disk diffusion. Quinolones resistant isolates were selected for molecular study for detecting *aac (6 ')-Ib-cr, qepA, qnrS* and *qnrB* as plasmid mediated resistance genes using Multiplex polymerase chain reaction.
Results: Eighty-nine isolates were identified as .*K.pneumoniae*.Thirty–four(38%) resist ciprofloxacin(10µg/ml) and were resistant to at least fourteen antibiotics to which they are tested. Hence, all isolates were considered to be multidrug resistant (MDR). The results of detection the plasmod mediated antibiotic resistance genes revealed the widely distribution *aac (6 ')-Ib-cr* gene alone or combined with *qnrS* gene in 14 (41.18%)

isolates ,or with *qepA* (2.94%) and 8.82% of bacterial isolates carried *aac (6 ')-Ib-cr, qepA* and *qnrS* whereas only one isolates (29.41%) that caused wound infection showed the presence of *qnrB* gene.
Conclusions : High prevalence MDR *K. pneumoniae* harbouring PMQR mediated by *aac(6`)-Ib-cr* and *qnrS* . This study is the first trail to detect PMQR genes in clinical and environmental isolates of *K.pneumoniae* in Iraq.
Recommendations: Further studies are necessary to understand the dissemination of plasmid mediated genes (*qnr, aac(6`)-Ib-cr* and *qepA* gene) and chromosomal resistance among *K.pneumoniae* and other common pathogenic bacteria.
Keyword: *Klebsiella pneumoniae,* plasmid mediated quinolone resistance, *qnrS, qnrB, aac(6`)-Ib-cr* ,*qepA*,and Gram negative bacteria

INTRODUCTION

Fluoroquinolones have been frequently prescribed as empirical therapy against most hospital and community infections due to increased appearance of multiple drug resistant Gram negative bacteria including *Klebsiella pneumoniae* and to the disease severity [(1)] .With extensive clinical use of quinolones. Fluoroquinolone resistance has been a problem in clinical medicine for its limiting of available agents in the treatment of many types of infection [(2)].

Quinolone resistance in the family Enterobacteriaceae is mostly attributed to the accumulation of mutations in the bacterial enzymes targeted by: DNA gyrase and DNA topoisomerase IV[(3,4)]. In addition Active efflux systems (acrAB-TolC) resulting in decreased intracellular accumulation of fluoroquinolones in *K.pneumoniae* [(4)].Morever,Plasmid-mediated quinolone resistance (PMQR) with the potential for horizontal transfer has been described along with three mechanisms: (i) a quinolone-protective mechanism encoded by the *qnr* genes[(3)]; (ii) a modifying enzyme, *aac(60)-Ib-cr* and (iii) an efflux pump encoded by the *qepA* gene[(5,6)]. Plasmid-encoded quinolone resistance determinants confer low-level resistance, but their presence could potentially facilitate the evolution of the bacterial host toward higher levels of resistance by mutational alterations in type II topoisomerases[(4)]. *K. pneumoniae* strains represent an incredibly great epidemic potential and are one of the major sources of horizontally spreading antimicrobial resistance[(2)].Fluoroquinolone resistant *K.pneumoniae* constitutes one of the most common Gram-negative bacteria showing multiple antibiotic resistance worldwide[(2,3,5,6)]. There is little information regarding in the occurrence of PMQR genes in Iraq . This study aimed to investigate occurrence of PMQR genes in ciprofloxacin resistant *K.pneumoniae* by multiplex PCR technique.

MATERIALS AND METHODS

Sample collection:

A total of 245 samples were collected from different source that include urinary tract infection(89), Wound infection(54), Burn infection(32) and female genital tract infection(23),in addition to 50 swabs were taken from environmental hospital samples(operations and burn wards) during November , 2011 to February, 2012 from three hospitals :Al-Sadr Teaching, Al-Hakeem, and Al-Manathera in the Al-Najaf City.

Isolation and identification

Clinical and environmental hospital samples were cultured onto MacConkey agar and incubated for 18-24 h at 37^{o}C. All lactose-fermenting isolates were tested by morphologic characteristics and standard biochemical tests according to MacFaddin, (2000) [(7)]. Confirmation of *K.pneumoniae* was conducted using API20E system.

Phenotypic Detection of Fluoroquinolones Resistance

Preliminary screening of *K. pneumoniae* isolates resistance to ciprofloxacin was carried out using pick and patch method on MacConkey agar supplemented with 10 µg/ml ciprofloxacin , incubated at 37˚C for 18-24h .

Antibiotics Susceptibility Test

Antibiotic Susceptibility of ciprofloxacin resistant *K. pneumoniae* isolates to 18 antibiotics (carbencillin (100µg), amoxillin-clavulanic acid (20/10µg), cephalothin (30µg), ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), cefoxitin (30µg), imipenem (10µg), meropenem (10µg), azteronam (30µg), tobramycin (10µg), trimethoprim (5µg), nitrofurantoin (300µg), naldixicacid (30µg), norfloxacin (5µg) from Bioanalyse, Turkey.levofloxacin (10µg) and gatifloxacin (5µg) from Himedia, India) was detected according to the Clinical and Laboratory Standards Institute (CLSI) protocol[8] by standard diffusion method on Mueller Hinton Agar (Himedia, India) .

Polymerase Chain Reaction Protocols

a) PCR Mixture and thermocycling conditions

Total DNA of Fluoroquinolon resistant isolates of *K.pneumonia*e was extracted using DNA Extraction Mini Kit(Genidi/ korea) according to manufactures' instructions.

The occurrence of PMQR genes *(qnrB, qnrS, aac(6ˉ)-Ib-cr* and *qepA*)in Fluoroquinolones resistant *K.pneumonia*e isolates were detected via multiplex PCR procedures using the following oligonucleotide primers (Biocorp, Canada) (Promiga): *qnrB* /F (5'-GATCGTGAAAGCCAGAAAGG -3'), *qnrB/*R(5'- ATGAGCAACGATGCCTGGTA - 3')[9] ; *qnrS*/F (5'- GCAAGTTCATTGAACAGGGT -3') R (5' - TCTAAACCGTCGAGTTCGGCG-3'); *aac(6ˉ)-Ib-cr/* F (5'- TTGCGATGCTCTATGAGTGGCTA -3')[10]; *aac(6ˉ)-Ib-cr/* R (5' - CTCGAATGCCTGGCGTGTTT - 3')[11]; *qepA* /F (5'- AACTGCTTGAGCCCGTAGAT - 3')and *qepA* / R (5' - GTCTACGCCATGGACCTCAC - 3')[12].

Multiplex PCR was performed using fast multiplex pcr kit (Kapa-USA) as follows: in an Eppendorf reaction tube, 25 µl master mix was prepared for each test .A master mix contained the following components (according to the manufacturer instruction):

12.5 µl fast Multiplex master mix;0.75 µl of 20 µM/µl each upstream and downstream ;1.5 µl Nuclease free distilled water (Promega-USA) and 2 µl of DNA template.The cycling was performed using protocol comprising an initial denaturing step at 95°C for 3 minutes, followed by 30 cycles of 95°C for 15 seconds,60°C for 30 seconds and 72°C for 45 sec 12.and final extension at72c̊ for10 minutes.Final hold step4˚ C.

b) Agarose Gel Electrophoresis

Agarose gel electrophoresis for pcr products parallel to a molecular marker (promega/ USA effective size range: 100 to 1500 bp) was performed for detecting and evaluating size products. Finally, the gel was photographed using Biometra gel documentation system .

RESULTS

Isolation and identification

From a total of 32 burn swabs, 54 wound swab, 23 vaginal swabs and 86 urine samples collected from three hospitals in al-Najaf City. *Klebsiella pneumoniae* was isolated from 56.25% (18) burn swabs, 55.5% (30) wound swab, and 24.4% (21) urine samples, while no klebsiella isolate was detected among vaginal swabs. Twenty isolates (40%) of *K. pneumoniae* were identified in hospital environmental samples.

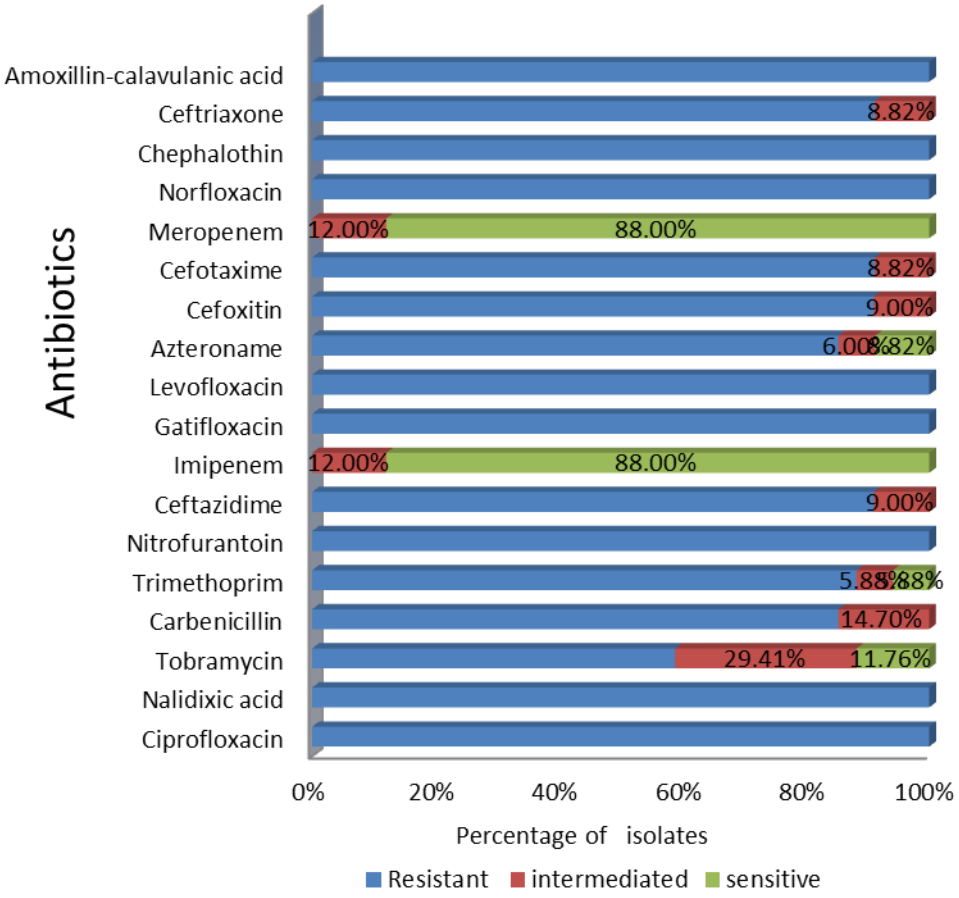
Primary detection of fluoroquinolone(ciprofloxacin) resistance phenotype

Table (1) Fluoroquinolone resistant Klebsiella pneumoniae isolates obtained from clinical and environmental hospital samples

Source of samples	No. *K. pneumoniae* isolates	No.(%)Ciprofloxacin Resistant *K. pneumoniae* isolates
Clinical samples	Urine	12 (13.48)
	Wound	7 (7.86%)
	Burn	9 (10.11%)
Environmental hospital samples		6 (6.74%)
Total		34(38.2%)

Table (1)shows that out of 89 *K. pneumoniae* isolates, 34(38 %) isolates exhibited flouroquinolone resistance phenotype.

Antibiotics Susceptibility



Figure(1):Antibiotics susceptibility profile of 34 isolates of quinolone resistant Klebsiella pneumoniae recovered from three hospitals in Al-Najaf City

Our study showed that all ciprofloxacin resistant *K.pneumoniae* isolates were resistant to all quinolones antibiotic tested (Levofloxacin, Gatifloxacin, Norfloxacin, Nalidixic acid) and Multiple antibiotic resistance which showed highly resistance to at least more than 14 antibiotic while 88% isolates were susceptible to Carbapenem (Imipenem and Meropenem)as shown in Figure (1).

Detection of Plasmid-Mediated Quinolone Resistance Genes

Table (2): Distribution of plasmid-mediated quinolones resistance genes among Klebsiella pneumoniae clinical and environmental hospital isolates(N=34)

Plasmid-Mediated Quinolone Resistance Genes	Clinical Isolates		Environmental Hospital Isolates		Total	
	No	%	No	%	No	%
aac(6´)-Ib-cr	10	29.41	4	11.67	14	41.18
aac(6´)-Ib-cr) - *qnrS*	12	32.29	2	4.65	14	41.18
qnrB	1	29.41	-	-	1	2.94
aac(6`)-Ib-cr-qepA-qnrS	3	8.82	-	-	3	8.82
aac(6`)-Ib-cr- qepA	1	29.41	-	-	1	2.94
Total of -ve isolates	1	29.41	-	-	1	2.94
Total	28	82.35	6	17.64	34	100

Table(2) shows significant high percentage (97%) of fluroquinolone resistant *Klebsiella pneumoniae* isolates were found to carry PMQ genes. Ninty –four percent (32/34) of quinolones resistant *K. pneumoniae* were found to carry *aac(6`)-Ib-cr* gene either alone(14/34 isolates) or in combination(18/34 isolates).

Table (3): Distribution of plasmid-mediated quinolones resistance genes according to the source of Klebsiella pneumoniae clinical isolates(N=28)

Plasmid-Mediated Quinolones Resistance Genes	UTI		Burn		Wound		Total No. (%)	
	No	%	No	%	No	%	No	%
aac(6´)-Ib-cr	3	8.82	2	4.65	5	14.7	10	29.41
aac(6´)-Ib-cr) - *qnrS*	5	14.7	6	17.64	1	29.41	12	32.29
qnrB	-	-	-	-	1	29.41	1	29.41
aac(6`)-Ib-cr-qepA-qnrS	3	8.82	-	-	-	-	3	8.82
aac(6`)-Ib-cr- qepA	1	29.41	-	-	-	-	1	29.41
Total of -ve isolates	-	-	1	29.41	-	-	1	29.41
Total	12	32.35	9	26.47	7	20.58	28	82.35

Plasmid mediated quinolone resistance gene , *qepA* was detected in four isolates , collected from urinary tract infections ,listed in table (3). *qnrS*, has been amplified in17 isolates collected from different clinical and environmental sources in combination either with *aac(6`)-Ib-cr* gene or with both *aac(6`)-Ib-cr* and *qnrS* genes

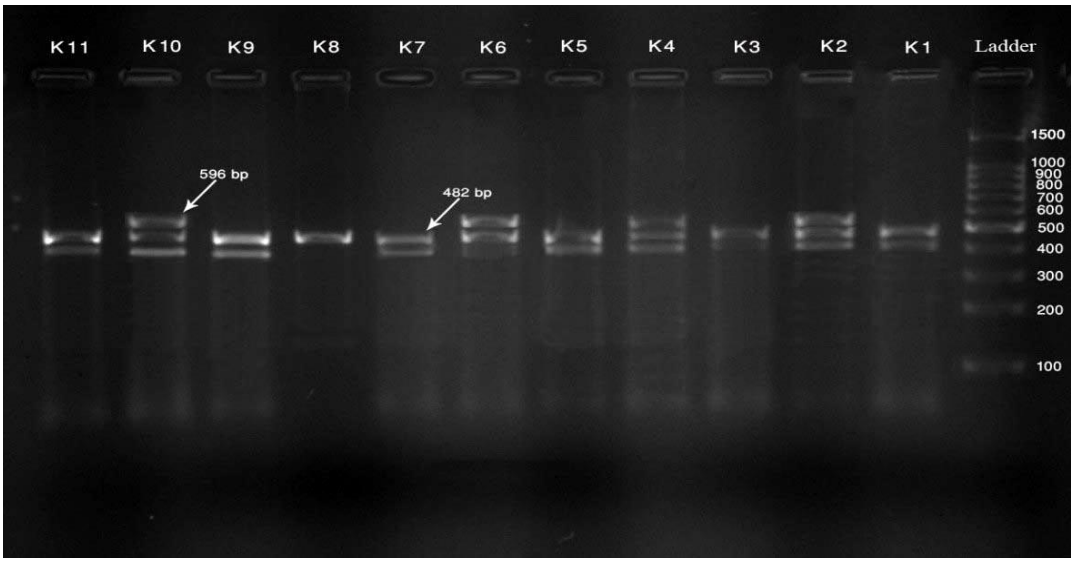
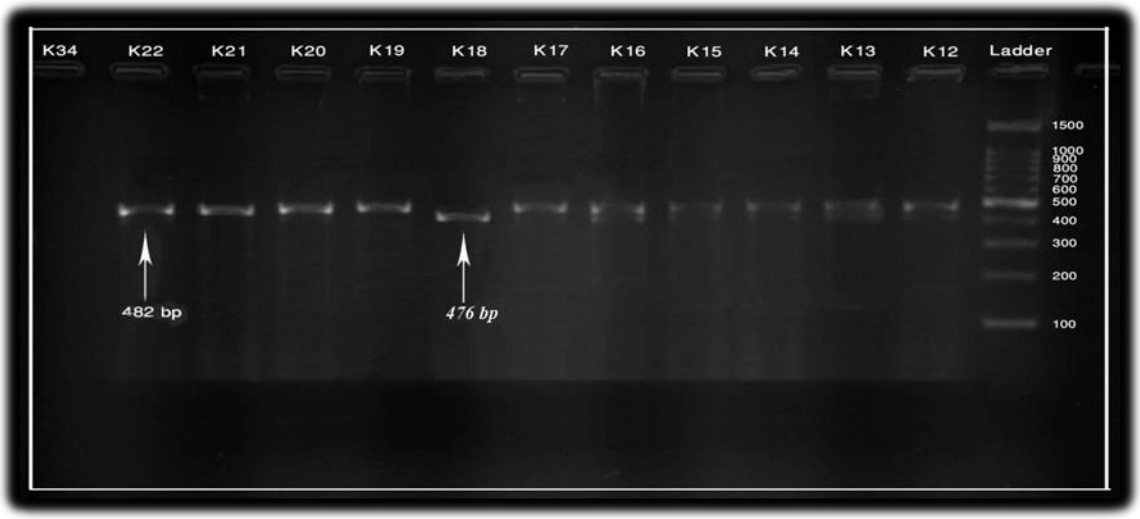


Figure (2) : Ethidium bromide stained agarose gel of PCR products of *qepA*(596bp), *aac(6')-Ib-cr*(482bp), *qnrS*(428bp), *qnrB*(476bp) genes from *Klebsiella pneumoniae* extracted DNA. The electrophoresis performed at 60volt for 2h .Lane Ladder : 100bp standard size references marker. Lane (K1-11) : *K.pneumoniae* isolates positive for *aac(6')-Ib-cr* gene Lane (K2-4,6,10) *K. pneumoniae* isolates positive for *qepA* gene.Lane (K1,2-4,5,7,9,10,11) *K. pneumoniae* isolates positive for *qnrS* gene



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This figure reveals *qnrB* gene positive isolates that collected from wound at the Lane K18 *K. pneumoniae* isolate positive for *qnrB* gene. Lane (K12,13,14,15,16,18,19,20,21 and22) : *K. pneumoniae* isolates positive for *aac(6')-Ib-cr* gene.

DISCUSSION

In the present study *K.pneumoniae* constituted 35.38% of Clinical samples (18 (56.25%) burn swabs, 30 (55.5%) wound swab, and 21 (24.4%)urine samples. Twenty isolates (40%) of *K. pneumoniae* were identified in environmental hospital samples.

This study is relatively in agreement with a study carried out in General Al-Nasseriyia Hospital by Nakkash and Al-Husseiny (2008) that isolated 99 isolates of *K. pneumoniae*

represented by 61 isolates from surgical wound infections and 38 from hospital environment[13] . and a study by al-Al-Sehlawi (2012) showed that 39.16% *Klebsiella*.spp isolates obtained from clinical samples and environmental hospital samples in the Holly Najaf city[14]. While, Fayroz-Ali (2012) found that *Klebsiella*.spp was the second most common (16.8%) organism isolated from urine samples from patients after *E.coli*, [15]and Al Rediany (2012) detected *Klebsiella* spp. in 20 samples(16%) of burn infection and 105 (84%) samples from wound infection in Al- Najaf hospitals[16].

The wide spread for *Klebsiella* spp. may be due to the ability of this bacteria to survive under unsuitable environmental conditions since it has a thick polysaccharide capsule, and different mechanisms of antibiotic resistance [17].

The percentage of *K. pneumoniae* is variable in the different studies, this may be attributed to drug overuse, difference of diagnostic methods and the hospital policy in management of such cases. Moreover, geographic climatic and hygienic factors may also be correlated with the relative variability of results among different area[18].

Antibiotic susceptibility test showed that all ciprofloxacin resistant isolates considered to be multidrug resistant (MDR) they resist to at least fourteen antibiotic to which they are tested but Carbapenems (imipenem and meropenem) were the most efficient antibiotics against *K. pneumoniae* isolates due to high susceptibility rate. This is an expected result in *K. pneumoniae* which recorded in local studies ([14,16]) while was lower than that reported by other local studies which reported that the susceptibility of *K.pneumoniae* isolates collected from clinical and environmental samples to imipenem was (100%) [16,19,20].

The increase in the rate of quinolone resistance is high in Najaf hospitals [19,20] and the resistance may be multi-factorial, this resistance may be result from the tendency for bacteria to develop resistance and subtherapeutic concentrations of the drug [15].

In present study, the PCR technique had been confirmed that PMQR genes were found with remarkably high percentage (33/34, 97.11%) in quinolone resistant *K. pneumoniae* isolates that constituted 92.86% (27/28) of clinical isolates and 100%(6/6) of hospital environmental isolates.

A variant of aminoglycoside amnoacetyl transferase enzyme(*aac(6´)-Ib-cr*) is capable to modify ciproflocxacin and reducing its activity has been reported to be widely prevalent around the world and to be circulated with *qnr* genes or and *qepA* gene[5,21].There is substantial increase in quinolone resistance associated with *aac(6´)-Ib-cr* gene are found in14 (41.14%), *K.pneumoniae* isolates, and this gene shows a combination with *qnrS* and *qepA* 13 (38.32%), 1(29.41%), respectively. In addition to combination *aac(6´)-Ib-cr* with *qepA* and *qnrS* 3(8.82).

The percentage of *qnrs* positive isolates agreed with Chines study by Cai *et al*. (2011) that identified *qnrS* in 18.9% of 37*K. pneumoniae* isolates but *qnrB* gene was not detected in their study. [22]

Out of 64 *Enterobacteriaceae* isolates collected from Kuwait hospitals, 3 (4.7%) were positive for a *qnrB* gene[10]. In Japan, six *qnrB* genes were detected in *K. pneumoniae, K.oxytoca, Escherichia. coli, Citrobacter freundii, Proteus mirabilis* and *P.fluorescence* from zoo reptiles and falcons[23]. In Chennai , *qnrB* gene was detected in 48% of 23 multi drug resistant isolates of *K. pneumoniae* [5].But in our study amplification product of *qnrB* gene was observed in one isolates .this may be associated to the geographical distribution of *qnr* genes.

Jacoby and his colleagues (2009) found that 36.4% (4 /11) isolates were carried *aac(6´)-Ib-cr* gene. plasmid mediated quinolon resistance (PMQR) genes have already been detected in all populated continents and in most clinically common Enterobacteriaceae. Among these genes, *aac(6')-Ib-cr* seems to be more prevalent [24] .

Plasmid-mediated antibiotic resistance plays a significant role in the spread and increase fluoroquinolone resistance in most clinically common Enterobacteriaceae strains worldwide[1]. in Iraq , PMQR gene was previously recorded in clinical isolates of *E. coli* in Al-Najaf city [15]. There are little information in the distribution PMQR genes in *K.pneumoniae*, to our knowledge this is the first report of PMQR genes associated with *aac(6´)-Ib-cr,qnr* and *qepA* in *K.pneumoniae* from clinical and environmental hospital specimens.

CONCLUSIONS

High prevalence of multidrug resistant *K.pneumoniae* harbouring PMQR mediated by *aac(6`)-Ib-cr* and *qnrS* . This is the first report from Iraq demonstrating plasmid-mediated quinolone resistance (PMQR) mediated by *qnr* genes( *qnrS*and *qnrB* ) , *aac(6`)-Ib-cr*, and and *qepA* genes in *K. pneumoniae*.

RECOMMENDATIONS:

1. There is an urgent need for surveillance studies to evaluate the clinical seriousness of spreading of multidrug resistance *Klebsiella pneumoniae* at the level of republic of Iraqi's hospitals
2. Further studies are necessary to understand the dissemination of plasmid mediated genes (*qnr*, *aac(6`)-Ib-cr* and *qepA* gene)and QRDR genes in *K. pneumoniae* and other common pathogenic bacteria to guide appropriate clinical treatment in the future.

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