Open Access **Baghdad Science Journal** **P-ISSN: 2078-8665**
2020, 17(2):406-414 **E-ISSN: 2411-7986**

DOI: http://dx.doi.org/10.21123/bsj.2020.17.2.0406

Prevalence of Quinolones Resistance Proteins Encoding Genes (*qnr* genes) and Co-Resistance with β-lactams among *Klebsiella pneumoniae* Isolates from Iraqi Patients

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Received 17/2/2019, Accepted 13/10/2019, Published 1/6/2020

Abstract:

This study investigated the prevalence of quinolones resistance proteins encoding genes (*qnr* genes) and co-resistance for fluoroquinolones and β-lactams among clinical isolates of *Klebsiella pneumoniae*. Out of 150 clinical samples, 50 isolates of *K. pneumoniae* were identified according to morphological and biochemical properties. These isolates were collected from different clinical samples, including 15 (30%) urine, 12 (24%) blood, 9 (18%) sputum, 9 (18%) wound, and 5 (10%) burn. The minimum inhibitory concentrations (MICs) assay revealed that 15 (30%) of isolates were resistant to ciprofloxacin (≥4µg/ml), 11 (22%) of isolates were resistant to levofloxacin (≥8 µg/ml), 21 (42%) of isolates were resistant to ertapenem (≥8 µg/ml), 18 (36%) of isolates were resistant to imipenem (4- ≥16µg/ml), 43 (86%) of isolates were resistant to ceftriaxone (4- ≥64 µg/ml), 42 (84%) of isolates were resistant to ceftazidime (16-64 µg/ml), and 40 (80%) of isolates were resistant to cefepime (4- ≥16µg/ml). The results revealed that all fluoroquinolone resistant *K. pneumoniae* isolates were resistant for β-lactams that used in this study. Genotypic detection of *qnr* genes revealed that *qnrS* and *qnrB* were found in 38 (76%) and 18 (36%) of *K. pneumoniae* isolates, respectively. On the other hand, *qnrA*, *qnrC*, and *qnrD* were not found among *K. pneumoniae* isolates. DNA sequencing of *qnrB* gene revealed that the presence of silent and missense mutations that may have led to increase the resistance values of MICs for ciprofloxacin and levofloxacin. These variants were registered in NCBI at the accession numbers LC373260 and LC381730. The phylogenetic tree of *qnrB* variants showed a significant deviation of these variants from *K. pneumoniae* species. The spread of *qnr* genes among clinical isolates of *K. pneumoniae* and high association observed between resistance to fluoroquinolones and β-lactams have led to a major threat to public health through development of MDR *K. pneumoniae*.

Key words: *Klebsiella pneumoniae*, Mutations, Phylogenetic tree, *qnr* genes.

Introduction:

Klebsiella pneumoniae is a gram negative, rod shaped, encapsulated, and non-motile bacterium, which belongs to the family Enterobacteriaceae (1). It is an opportunistic pathogen that can colonize mucosal surfaces of human and causes serious nosocomial and community acquired infections include urinary tract infections (UTIs), pneumonia, bacteremia, liver abscess, cystitis, and wound and burn infections (2). The development of resistance mechanisms of *K. pneumoniae* against most common usage antibiotics has led to emergence of multidrug resistant (MDR) *K. pneumoniae* that associated with high rates of morbidity and mortality due to the limited options of clinical treatment (3).

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Fluoroquinolones are one of the last resorts of antimicrobial options used to treat infections of *K. pneumoniae*. These agents act by preventing the synthesis and replication of the bacterial DNA by targeting topoisomerases (DNA gyrase and topoisomerase IV) (4). The resistance of *K. pneumoniae* for fluoroquinolones is partially attributed to harbor *qnr* genes, which encode quinolones resistance proteins that prevent fluoroquinolones to bind with their topoisomerases target (5). *qnrA* was first determined in a multiresistant *K. pneumoniae* strain in USA in 1994 and then became widely spread throughout world. The detection of *qnrA* was then followed by determination of *qnrS*, *qnrB*, *qnrC*, and *qnrD*, respectively (5). Although *qnr* genes confer low resistance against fluoroquinolones, they enhance selection of chromosomal mutations in the topoisomerases encoding genes *gyrA* and *parC*, leading to increase resistance of *K. pneumoniae* (6).

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P-ISSN: 2078-8665
E-ISSN: 2411-7986

qnr genes are usually carried on plasmids that can transfer horizontally, ensuring the wide dissemination of resistance to fluoroquinolones, so that *K. pneumoniae* can survive in the face of these agents (4,5). Furthermore, *qnr* genes can be found on the chromosome (5).

The presence of *qnr* genes together with β-lactamases encode genes on the same plasmid provides cross-resistance for both agents, leading to emergence of extremely drug resistant (XDR) *K.pneumoniae* (3).

This study aims to investigate the prevalence of quinolones resistance proteins encoding genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*) among clinical isolates of *K.pneumoniae* and association between *qnr* genes with resistance to fluoroquinolones and β-lactams.

Materials and Methods:

Collection of Samples:

A total of 150 clinical samples (urine, blood, sputum, wounds, and burns) were collected from patients who suffered from different cases, including UTIs, bacteremia, pneumonia, burns, and wounds infections from different hospitals in Baghdad. The collection of samples was done according to instructions of the ethics committee at the Ministry of Health in Baghdad according to the official approval that numbered 31864.

Bacterial Isolation and Identification:

The clinical samples were cultured on MacConkey agar, Blood agar, Eosin Methylene Blue agar (Oxoid, UK), and CHROMagar Orientation (Pioneer, France). All culture media were incubated at 37°C for 24 hrs (7). Then, the pure colonies were selected and identified by performing biochemical assays, including oxidase, catalase, and IMVIC tests (7). The Vitek 2 Compact System (BioMerieux, France) was used for confirmation of identified bacteria (8).

Minimum Inhibitory Concentrations (MICs):

The MICs were determined using Vitek 2 Compact System against seven antibiotics, including Ciprofloxacin, Levofloxacin, Ceftazidime, Ceftriaxone, Cefepime, Ertapenem, and Imipenem (8). The results were interpreted according to CLSI (9).

DNA Extraction:

The Wizard Genomic DNA Purification Kit (Promega, USA) was used to extract DNA from bacterial isolates according to information of manufacturing company. The concentrations and purity of DNA were measured using Nanodrop (Biogroup, UK) (10).

Determination of Genotyping of *qnr* Genes:

The extracted DNA from *K. pneumoniae* isolates was screened for *qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS* using primers (Alpha DNA, USA) that reported in Table 1. The lyophilized product of these primers was dissolved in sterilized deionized distilled water (ddDW) (Promega, USA) to obtain 100 pmol/µl and then diluted to 10 pmol/µl according to information of manufacturing company. The Polymerase Chain Reaction (PCR) was performed using Thermal Cycler (BioRad, USA) at a volum of 20 µl, which consists of 10 µl Go Taq Green Master Mix (Promega, USA), 2 µl template DNA, 1 µl F-primer, 1 µl R-primer, and 6 µl ddDW (11). The PCR of *qnr* genes was done under the optimal conditions that reported in Tables 2,3,4,5, and 6, respectively (12,13,14).

Table 1. Sequence of Primers used in this study.

Genes	Primer Sequence (5´- 3´)	Size (bp)	Reference
qnrA	F:AGAGGATTTCTCACGCCAGG R:TGCCAGGCACAGATCTTGAC	580	12
qnrB	F:GATCGTGAAAGCCAGAAAGG R:ACGATGCCTGGTAGTTGTCC	469	13
qnrC	F:GGGTTGTACATTTATTGAATCG R: CACCTACCCATTTATTTTCA	307	14
qnrD	F:CGAGATCAATTTACGGGGAATA R:AACAAGCTGAAGCGCCTG	533	14
qnrS	F:GCAAGTTCATTGAACAGGGT R:TCTAAACCGTCGAGTTCGGCG	428	12

qnr: quinolone resistance protein.

Table 2. The Optimal Conditions for amplifying *qnrA* by PCR.

PCR Steps	Temperature (°C)	Time (min)	Cycles' Number
Initial Denaturation	95	15	1
Denaturation	95	1	
Annealing	55	1	30
Extension	72	5	
Final Extension	72	5	1
Hold	4	∞	-

Table 3. The Optimal Conditions for amplifying *qnrB* by PCR.

PCR Steps	Temperature (°C)	Time (min)	Cycles' Number
Initial Denaturation	95	5	1
Denaturation	94	1	
Annealing	55	1	30
Extension	72	2	
Final Extension	72	5	1
Hold	4	∞	-

Table 4. The Optimal Conditions for amplifying *qnrC* by PCR.

PCR Steps	Temperature (°C)	Time (min)	Cycles' Number
Initial Denaturation	95	15	1
Denaturation	95	1	
Annealing	55	1	30
Extension	72	5	
Final Extension	72	5	1
Hold	4	∞	-

Table 5. The Optimal Conditions for amplifying *qnrD* by PCR.

PCR Steps	Temperature (°C)	Time (min)	Cycles' Number
Initial Denaturation	95	15	1
Denaturation	95	1	
Annealing	56	1	30
Extension	72	5	
Final Extension	72	5	1
Hold	4	∞	-

Table 6. The Optimal Conditions for amplifying *qnrS* by PCR.

PCR Steps	Temperature (°C)	Time (min)	Cycles' Number
Initial Denaturation	95	15	1
Denaturation	95	1	
Annealing	56	1	30
Extension	72	5	
Final Extension	72	5	1
Hold	4	∞	-

Gel Electrophoresis:

The PCR products were run on 1% agarose gel stained with 0.5 µg/ml ethidium bromide in 1X TAE buffer (Promega, USA) using DNA ladder (100-1500 bp) supplied by Promega (USA), as a marker of DNA size. The electrophoresis was performed at 100 V for 80 min. The UV-Transilluminater (Major Science, Taiwan) was used for observation of PCR products under 320 nm UV light (15).

DNA Sequencing:

The sequencing of two PCR products that referred to the *qnrB* was done by Genetic Analyzer (Macrogen Inc., South Korea). The result was compared with the reference database available in the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) using BioEdit program version 7.1 (DNASTAR,Madison,WI,USA) (http://bioedit.software.informer.com/7.1/) (16). The variations were translated into amino acid sequences using expasy online program (http://web.expasy.org/translate/) (17).

Phylogenetic Tree Construction:

The observed PCR amplicons variants of *qnrB* genetic loci were compared with the neighbor

homologous sequences using NCBI-BLASTn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The blast results of the observed variants were aligned and constructed using Clustal Omega and Simple Phylogeny Tools, respectively (https://www.ebi.ac.uk/Tools/msa/clustalo/). A full inclusive tree, including the observed variants was visualized, as a polar cladogram using Figtree Tool (http://tree.bio.ed.ac.uk/software/figtree/).

Results and Discussion:

Fifty isolates were identified as *K. pneumoniae* (Table 7). The bacteria were found at high prevalence in urine 15 (30%) followed by blood 12 (24%), whereas sputum and wounds were obtained 9 (18%) isolates, and finally burns were obtained 5 (10%) isolates. Several studies revealed that the most common site of *K. pneumoniae* infections is UTIs followed by bloodstream infections, pneumonia, and burns and wounds infections (2,18,19). Other studies collected *K. pneumoniae* from other cases, including pus, stool, cerebrospinal fluid, and catheters (18,19,20, 21,22).

Table 7. Distribution of *Klebsiella pneumoniae* isolates.

Samples	Isolates No.(%)
Urine	15 (30)
Blood	12 (24)
Sputum	9 (18)
Wounds	9 (18)
Burns	5 (10)
Total	50 (100)

The results of MICs listed in Table 8 exhibited that *K. pneumoniae* isolates showed high resistance to third and fourth generation cephalosporins. It was found that 43 (86%) of *K. pneumoniae* isolates were resistant to ceftriaxone with MIC 4- ≥64 µg/ml, 42 (84%) of isolates were resistant to ceftazidime with MIC 16-64 µg/ml, and 40 (80%) of isolates were resistant to cefepime with MIC 4-≥16 µg/ml. On the other hand, *K. pneumoniae* isolates exhibited low resistance for carbapenems, in which 21 (42%) of *K. pneumoniae* isolates were resistant to ertapenem with MIC ≥8 µg/ml and 18 (36%) of isolates were resistant to imipenem with MIC 4- ≥16 µg/ml. The production of extended spectrum beta-lactamases (ESBLs) and AmpC- β-lactamases, in addition to carbapenemases is responsible for evolving high resistance to cephalosporins and carbapenems (23). The emergence of carbapenemases represents a great problem in modern medicine due to confer resistance not only for carbapenems, but also for non β-lactams, particularly aminoglycosides and fluoroquinolones (23,24).

Table 8. MICs Values of *Klebsiella pneumoniae* isolates.

Antibiotic	Resistant Values of MICs (µg/ml)	Resistant Isolates No.(%)
Ceftriaxone	4- ≥64	43 (86)
Ceftazidime	16-64	42 (84)
Cefepime	4- ≥16	40 (80)
Ertapenem	≥8	21 (42)
Imipenem	4- ≥16	18 (36)
Ciprofloxacin	≥4	15 (30)
Levofloxacin	≥8	11 (22)

K. pneumoniae isolates exhibited lower resistance against fluoroquinolones than β-lactams (cephalosporins and carbapenems). Consequently, fluoroquinolones were more effective against *K. pneumoniae* isolates. The results revealed that 15 (30%) of *K. pneumoniae* isolates were resistant to ciprofloxacin with MIC ≥4 µg/ml, while 11 (22%) of isolates were resistant to levofloxacin with MIC ≥8 µg/ml. The acquisition of plasmid mediated quinolone resistance (PMQR) enables *K. pneumoniae* to exhibit low resistance against fluoroquinolones through three mechanisms include protection of topoisomerases by the action of Qnr proteins, acetylation of fluoroquinolones by expression of *aac(6')-Ib-cr*, and extruding of fluoroquinolones through the action of efflux pumps OqxAB and QepA (3,5,6).

Association between resistance to fluoroquinolones and β-lactams revealed that all fluoroquinolones resistant *K. pneumoniae* isolates were resistant for β-lactams. This may indicate the co-existence of *qnr* genes and β-lactams resistance encoding genes on the same plasmid, leading to confer co-resistance for both agents (3,5). This contributes to emergence of XDR *K. pneumoniae* that represents a great challenge in medicine with the limited options of clinical treatment (3).

Screening of *qnr* genes revealed that *qnrS* was most prevalent. It was found in 38 (76%) of *K. pneumoniae* isolates with 428 bp (Fig.1). Moreover, *qnrB* was detected in 18 (36%) of *K. pneumoniae* isolates with 469 bp (Fig.2). On the other hand, *qnrA*, *qnrC*, and *qnrD* were not found among *K. pneumoniae* isolates. Although these genes have low prevalence, they can easily spread among clinical isolates of *K. pneumoniae* and even to other species by horizontal gene transfer because these genes are mainly carried on plasmids. (3,5,6).

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P-ISSN: 2078-8665
E-ISSN: 2411-7986

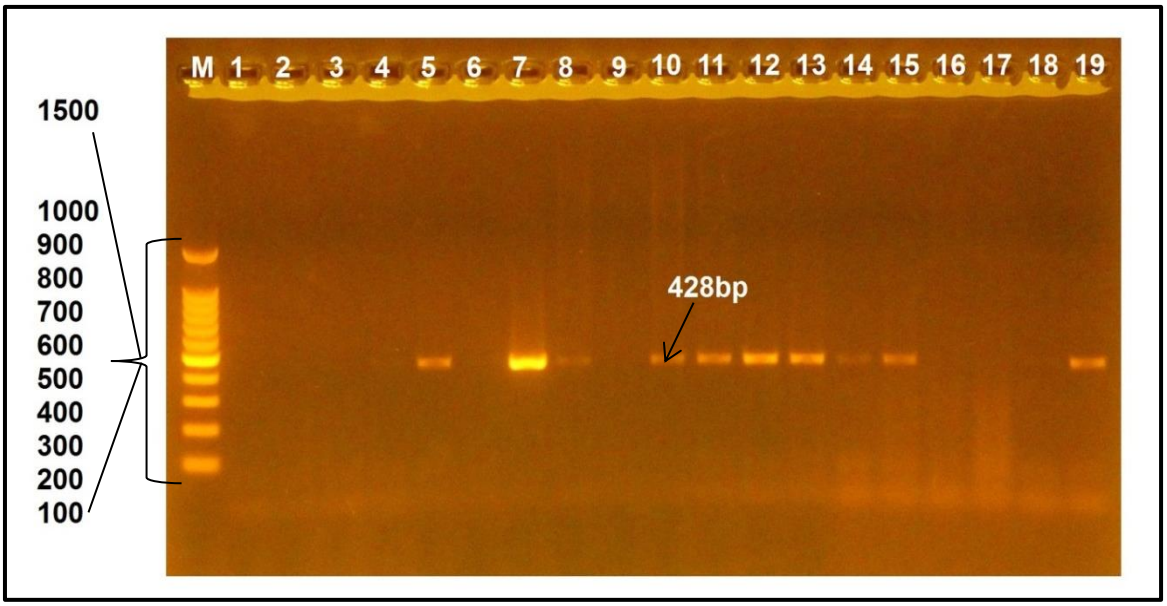

Figure 1. Gel Electrophoresis of PCR products showed *qnrS* gene with 428 bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500 bp). Lanes 5,7,8,10,11,12,13,14,15,19 represent *K. pneumoniae* PCR positive isolates.

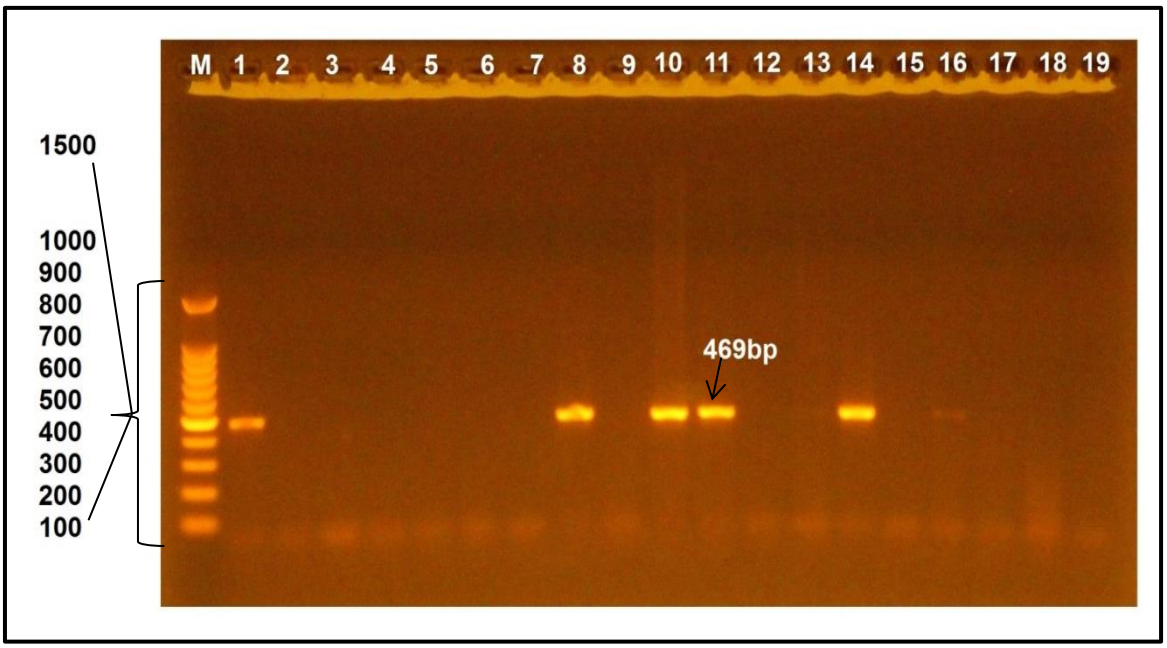

Figure 2. Gel Electrophoresis of PCR products showed *qnrB* gene with 469 bp on 1% agarose at 100V/80min. Lane M: DNA ladder (100-1500 bp). Lanes 1,8,10,11,14,16 represent *K. pneumoniae* PCR positive isolates.

Correlation between phenotype (MIC) and genotype (PCR) of fluoroquinolones resistance revealed that 17 (34%) of *K. pneumoniae* isolates were resistant for ciprofloxacin and/or levofloxacin, of these 15 (88.23%) isolates possessed *qnrS* and/or *qnrB*. On the other hand, 33 (66%) isolates were susceptible for ciprofloxacin and levofloxacin, in spite of most of these harbored *qnrS* and/or *qnrB*. This can be explained due to *qnr* genes confer low resistance in which increased MIC values did not exceed the breakpoint of susceptibility that mentioned in the CLSI (5,6).

DNA Sequencing of *qnrB* exhibited that the presence of point mutations in both selected isolates (qnrB-A and qnrB-B) (Fig.3). Some point mutations were observed to be transition, which led to substitution of pyrimidine with another pyrimidine or purine with another purine and others were transversion, which led to replacement of pyrimidine with purine and vice versa (25,26). The translation of protein revealed that some point mutations didn't affect on the amino acid sequences

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P-ISSN: 2078-8665
E-ISSN: 2411-7986

(silent mutations), but others led to replacement of one amino acid with another (missense mutations), as illustrated in Table (9) (25,26). These mutations may have elevated MICs values of ciprofloxacin and levofloxacin. The mutant DNA sequences of qnrB-A and qnrB-B were deposited in NCBI at the accession numbers LC373260 and LC381730.

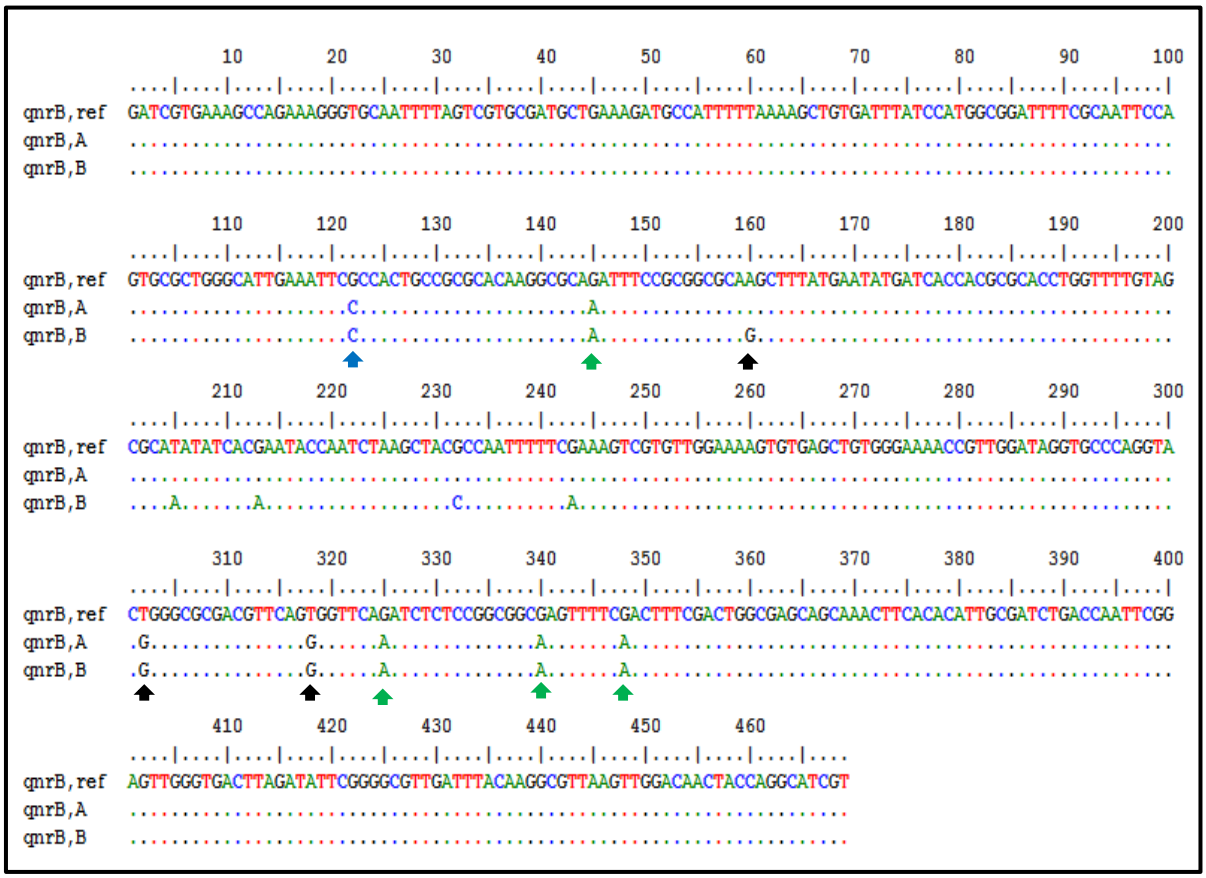

Figure 3. DNA Sequences Alignment of the observed native strains with their corresponding reference sequences of the 469 bp amplicon of the *qnrB* gene (GenBank accession number CP025143.1). Each substitution SNP was highlighted according to its position in the PCR products.

Table 9. The observed mutations in the DNA sequence of *qnrB* gene and amino acid substitution of *Klebsiella pneumoniae* isolates (qnrB-A and qnrB-B).

Isolate	DNA base substitution (Native>Allele)	Position in the reference DNA	Type of substitution	Type of point mutation	Amino acid substitution
qnrB-A , qnrB-B	G>C	158441	Transversion	Missense	R>P
qnrB-A , qnrB-B	G>A	158464	Transition	Missense	D>N
qnrB-B	A>G	158479	Transition	Missense	S>*G
qnrB-B	T>A	158524	Transversion	Missense	Y>N
qnrB-B	G>A	158532	Transition	Silent	-
qnrB-B	G>C	158551	Transversion	Missense	*A>P
qnrB-B	G>A	158562	Transition	Silent	-
qnrB-A , qnrB-B	T>G	158621	Transversion	Missense	L>R
qnrB-A , qnrB-B	T>G	158637	Transversion	Missense	S>R
qnrB-A , qnrB-B	G>A	158644	Transition	Missense	D>N
qnrB-A , qnrB-B	G>A	158659	Transition	Missense	E>K
qnrB-A , qnrB-B	G>A	158667	Transition	Silent	-

G:guanine, C:cytosine, A:adenine, T:thymine, R:arginine, P:proline, D:aspartic acid, N:asparagine, S:serine, *G:glycine, Y:tyrosine, *A:alanine, L:leucine, E:glutamic acid, K:lysine.

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P-ISSN: 2078-8665
E-ISSN: 2411-7986

Phylogenetic tree (Fig.4) indicated the presence of at least twenty three species that harbored *qnrB* variants sequence. The observed high number of point mutations that noticed in both *qnrB* variants had led to a significant deviation from *K. pneumoniae* species. However, both *qnrB* variants were located near *K. pneumoniae* portion, in which the majority of *K. pneumoniae* species were localized. Nevertheless, such mutations were still confined within *Citrobacter freundii* and *K. pneumoniae* without deviated toward other distant organisms. This tree had given nearly high biodiversity of variable organisms. This may indicate a potential evidence about the restricted power of *qnrB* based tree in yielding a final determination of the current local *K. pneumoniae* isolates.

In conclusion, although *qnr* genes provide low resistance against fluoroquinolones, they enhance chromosomal mutations in *gyrA* and *parC*, leading to evolve high resistance for these agents. The high association observed between resistance to β-lactams and fluoroquinolones has led to a major threat to public health through development of MDR *K. pneumoniae*.

Acknowledgment:

Special Thanks go to the laboratory staff of Hospitals of Medical City in Baghdad for their assistance during the collection of samples.

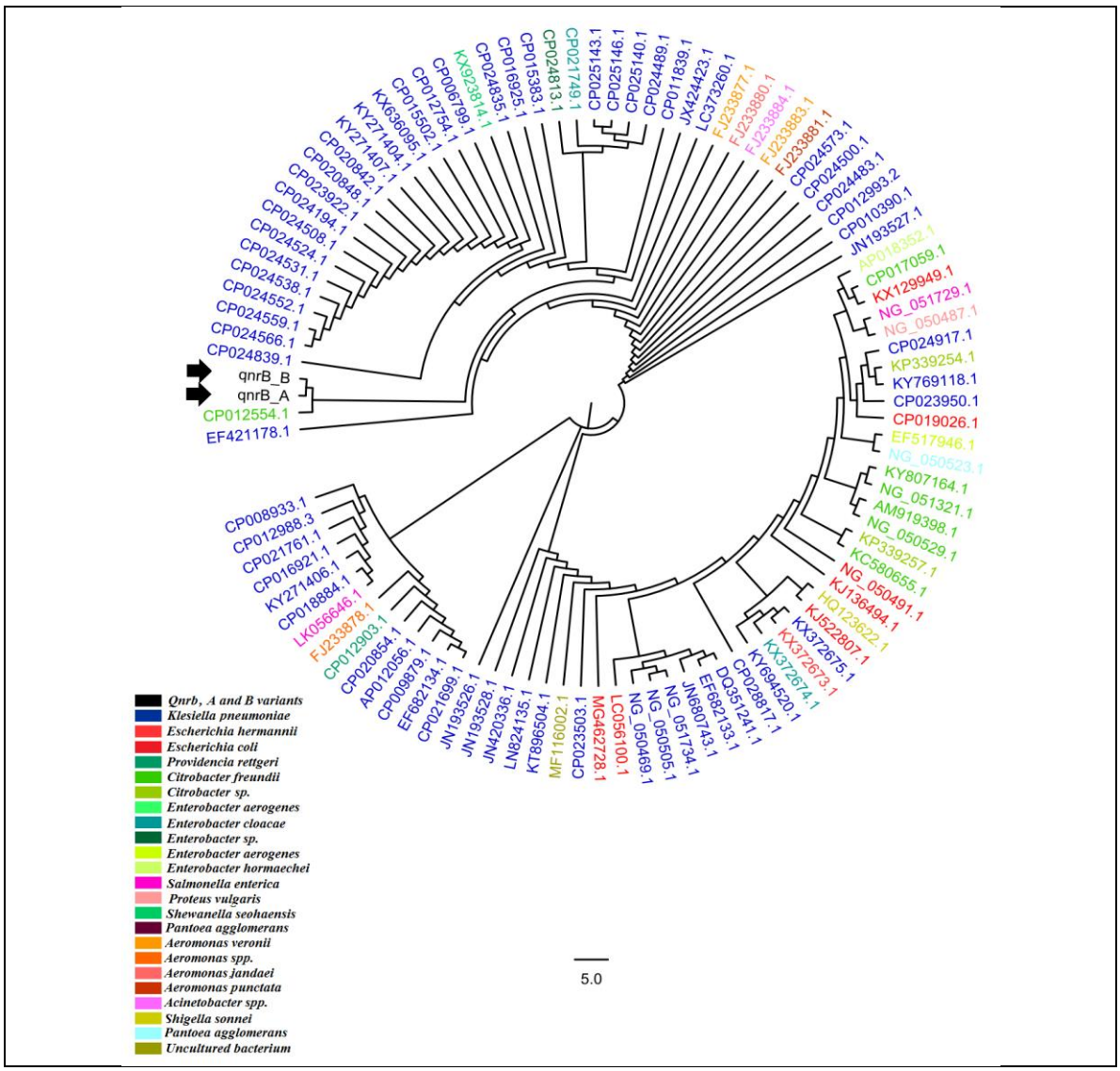

Figure 4. Phylogenetic Tree of the 469 bp variants of *qnrB* genetic fragments for *K. pneumoniae* local isolates. The black color refers to the sequenced two variants, while other colors refer to other referring NCBI deposited species. All the mentioned numbers referred to Genbank accession numbers of each referring species. The number "5.0" refers to the degree of scale range among the comprehensive tree categorized organisms.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

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التحري عن الجينات المشفرة لبروتينات مقاومة الكينولينات (جينات *qnr*) و المقاومة المشتركة مع البيتالاكتام في عزلات *Klebsiella pneumoniae* من المرضى العراقيين

مصطفى سهيل مصطفى **رنا مجاهد عبدالله**

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الخلاصة:

لقد تحرت هذه الدراسة عن انتشار الجينات المشفرة لبروتينات مقاومة الكينولينات (جينات *qnr*) والمقاومة المشتركة لمضادات الفلوروكينولينات و البيتالاكتام في عزلات سريرية لبكتيريا *Klebsiella pneumoniae* . تم الحصول على 50 عزلة من بكتريا *K. pneumoniae* من 150 عينة سريرية اذ شخصت حسب الصفات المظهرية و الكيموحيوية. جمعت العزلات من عينات سريرية مختلفة شملت الادرار 15 (30%) و الدم 12 (24%) و القشع 9 (18%) و الجروح 9 (18%) و الحروق 5 (10%). اظهرت نتائج التراكيز المثبطة الدنيا MICs ان15 (30%) عزلة كانت مقاومة لمضاد ciprofloxacin (≤4 مايكروغرام\مل) و 11 (22%) عزلة مقاومة لمضاد levofloxacin (≤8 مايكروغرام\مل) و 21 (42%) عزلة مقاومة لمضاد ertapenem (≤8 مايكروغرام\مل) و 18 (36%) عزلة مقاومة لمضاد imipenem (4 -≤16 مايكروغرام\مل) و43 (86%) عزلة مقاومة لمضاد ceftriaxone (4-≤64 مايكروغرام\مل) و 42 (84%) عزلة مقاومة لمضاد ceftazidime (16-64 مايكروغرام\مل) و40 (80%) عزلة مقاومة لمضاد cefepime (4-≤16 مايكروغرام\مل). اظهرت النتائج ان جميع عزلات بكتريا *K. pneumoniae* المقاومة للفلوروكينولينات كانت مقاومة للبيتالاكتام المستخدمة في هذا البحث. اظهر التشخيص الجيني لجينات *qnr* ان الجينين *qnrS* و *qnrB* كانا موجودين في 38 (76%) و 18 (36%) من عزلات بكتريا *K. pneumoniae* على التتالي. من جهة اخرى اظهرت النتائج عدم وجود الجينات *qnrA* و *qnrC* و *qnrD* في عزلات بكتريا *K. pneumoniae* . اظهر تحليل تتابع الحامض النووي DNA للجين *qnrB* وجود طفرات صامتة و طفرات انحشار والتي من الممكن ان تكون قد ادت الى زيادة في قيم المقاومة للتراكيز المثبطة الدنيا MICs لكل من المضادين ciprofloxacin و levofloxacin . تم تسجيل هذه التغايرات في موقع NCBI تحت الارقام التسلسلية LC373260 و LC381730 . ان الشجرة التطورية لتغايرات الجين *qnrB* اظهرت ان هناك انحراف ملحوظ لهذه التغايرات عن النوع *K. pneumoniae* . ان انتشار جينات *qnr* ضمن العزلات السريرية لبكتيريا *K. pneumoniae* والارتباط العالي الملاحظ في المقاومة لكل من الفلوروكينولينات و البيتالاكتام قد ادى الى تهديد كبير للصحة العامة من خلال تطور بكتيريا *K. pneumoniae* ذات المقاومة المتعددة للمضادات الحيوية.

الكلمات المفتاحية: *Klebsiella pneumoniae* ، الطفرات ، الشجرة التطورية ، جينات *qnr*.