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ORIGINAL RESEARCH

Detection of gyrA and parC Mutations and Prevalence of Plasmid-Mediated Quinolone Resistance Genes in *Klebsiella pneumoniae*

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Background and Aim: Recently, the extensive use of quinolones led to increased resistance to these antimicrobial agents, with different rates according to the organism and the geographical region. The aim of this study was to detect the resistance rate of *Klebsiella pneumoniae* Iraqi isolates toward quinolone antimicrobial agents, to determine genetic mutations in *gyrA* and *parC*, to screen for efflux-pump activity, and to screen the presence of plasmid-mediated quinolone resistance (PMQR) genes.

Methods: Forty-three *K. pneumoniae* isolates were confirmed phenotypically and genotypically by Vitek 2 system and species specific primers by PCR using the targeting *rpo* gene followed by sequencing. Antibiotic susceptibility test was carried out using disc diffusion method. Quinolone resistant isolates were subjected to ciprofloxacin MIC testing, and cartwheel method to screen for efflux pump activity. The presence of the plasmid mediated quinolone resistance genes *qepA, qnrB, qnrS*, and *aac(6)Ib* was tested by PCR. Sequencing of *gyr*A and *par*C was performed.

Results: We observed a high rate of resistance to ceftriaxone, gentamicin ciprofloxacin, and levofloxacin. Low rate of resistance was detected against amikacin and azithromycin. Ciprofloxacin MIC results revealed that 96.1% of the isolates had MICs >256 µg/mL, 83.4% had MICs >512 µg/mL while 34.6% had MIC >1024 µg/mL. Testing of isolates against ciprofloxacin mixed with EtBr at various concentrations resulted in decreased resistant. Sequencing results showed that Ser83Leu was the most common mutation in *gyr*A that was observed in all quinolone resistant isolates, followed by Asp87Asn. Ser80Ile mutation in *par*C was observed in 77.7% of the tested isolates. The prevalence of PMQR genes was 92.5% *aac (6)-Ib*, 51.8% *qnr*B, 40.7% *qep*A, and 37% *qnr*S.

Conclusion: Quinolone resistance is common in *K. pneumoniae* isolates in Baghdad. The frequent mutation in *gyr*A and *par*C, and the presence of PMQR genes is alarming.

Keywords: MDR *K. pneumoniae*, quinolones resistance gene, PMQR

Introduction

Due to the rapid emergence and spread of resistance to antibiotics, it is urgent to monitor the use of antibiotics and determine the ways of treatment to minimize the random and miss use of antibiotics.[1–5] Therefore, it is very important to continue studying genes that responsible for the resistance to different antibiotics in pathogenic bacteria; including genes that responsible for quinolone resistance.[6–11]

Klebsiella pneumoniae (K. pneumoniae) is a leading cause of hospital and community-acquired infections.[12,13] Quinolones are commonly used and effective antibiotics for

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treatment of multidrug resistant bacteria.[14,15] However, clinical isolates of *K pneumoniae* that are resistant to multiple antibiotics, including fluoroquinolones have frequently been described.[16–18] *K. pneumoniae* can resist antibiotic action through several mechanisms including drug inactivation, target alteration, increased efflux pump activity, and decreased cell permeability.[19] Resistance to quinolones is mediated mainly by mutations in the quinolone resistance-determining regions (QRDR), particularly in *gyr*A and *par*C genes, which leads to structural changes in DNA gyrase and/or topoisomerase IV, that reduces the affinity to fluoroquinolones.[20] Additional mutation can occur in genes that regulate efflux pumps activity.[21]

In the past, quinolones resistance was believed to be mediated by bacterial chromosomal mutations till 1998 when the researchers discovered plasmid mediated genes in *K. pneumoniae*, named "*qnr*" encoding to pentapeptide repeat family which play a role in binding and protecting DNA gyrase, and topoisomerase IV from repression by ciprofloxacin.[21–23] Then four major groups of *qnr* genes (*qnr*A*qnr*B*qnr*C and *qnr*S) and two plasmid-mediated quinolones resistance (PMQR) genes *aac(6')-Ib-cr* and another *qep*A encoding various aminoglycoside transferases that modify ciprofloxacin to facilitate an efflux pump protein were identified.[20,24] Bifunctional acetyltransferase protein coded by aac(6')-bI-cr acetylate amino nitrogen in the piperazinyl group of fluoroquinolones such as norfloxacin, ciprofloxacin, and aminoglycoside such as amikacin and kanamycin.[25] This gene commonly exist in multi-resistance plasmids as a cassette in integrin.[26]

Bacteria can resist the hydrophilic fluoroquinolones such as norfloxacin and ciprofloxacin by plasmid mediated efflux pump QepA which is the major group of facilitator family, exist in plasmid encoding to aminoglycoside ribosomal methylase *rmt*B.[27–29] Increasing prevalence of PMQR have been reported worldwide in clinical isolates of *K. pneumoniae*, however, no detailed reports of the quinolone resistance and it mechanism have been described in Iraq. The aim of this study was to detect the resistance rate of *Klebsiella pneumoniae* Iraqi isolates toward quinolone antimicrobial agents, to determine genetic mutations in *gyr*A and *par*C, to screen for efflux-pump activity, and PMQR genes.

Materials and Methods

Ethics Statement

The research was done as per the ethical guidelines of the Declaration of Helsinki 1975 and was approved by the Mustansiriyah University ethics committee. Informed written consent was taken from all recruited subjects.

Isolation and Identification of *K. pneumoniae*

Forty-three bacterial isolates from *Klebsiella pneumoniae* were collected between June and December 2019. These isolates were obtained from patients attending medical care services in Baghdad hospitals, Medical city hospital, Ibn Balady hospital, Al-Zahra hospital and Al-Yarmok hospital in Baghdad city. The isolates were distributed among thirteen isolates from wound infection, eight isolate was from a blood stream infection, eight from a urinary tract infection, five isolates from burn, four isolates from ear, three from sputum, one from each of fluid, and bronchial infection. Bacterial identification was based on morphological and biochemical tests, Vitek 2 system, followed by PCR and sequencing for *rpo* gene (housekeeping gene), the oligonucleotide sequence of *rpo*B primers are listed in Table 1. PCR products of representative samples were sequenced and submitted to GenBank under accession numbers ATB17136.1, ATB17140.1 and ATB17139.1.

Antimicrobial Susceptibility Test and Minimum Inhibitory Concentration (MIC) for Ciprofloxacin

The susceptibility to antimicrobial agents was performed by the using disc diffusion method and Muller-Hinton agar (MHA) according to the CLSI guidelines.[30] Ten antimicrobial discs were used including: Ak, amikacin (30 µg); GN, gentamicin (30 µg); IMI, imipenem (10 µg); FOX, Cefoxitin (30 µg); CRO, Ceftriaxion (30 µg); NOR, Norfloxacin (10 µg); ATH, Azithromycin (15 µg); LEV, Levofloxacin (5 µg); AUG, Amoxicillin+Clavulanic acid (30 µg) and CIP, Ciprofloxacin (5 µg). MIC for ciprofloxacin was also performed by the standard agar dilution method for the 27-resistant isolates according to the CLSI criteria. According to CLSI guidelines, MIC values of ≥ 4 ug/mL were considered as resistant.[30] *Escherichia coli* ATCC 25,922 was used as quality stander strain (Central Public Health Laboratory, Baghdad). MIC was recorded as the lowest concentration that inhibited bacterial division as evidenced by the absence of bacterial growth on the media.

Phenotypic Detection of Efflux Pump-Mediated Resistance

The phenotypic detection of Efflux pump mechanism was performed using EtBr-agar cartwheel (EtBrCW) method in

Table 1 The Primers Used in the Current Study for PCR Amplification

Target Gene	Primers 5 — 3	Size Products	T_m	References
*gyr*A	F-AAATCTGCTCGTGTCGTTGG-3 R- GCCATACCTACAGCAATACC-3	349bp	52 C	[35]
*par*C	F-AAGCCCGTACAGCGCCGTATT-3′ R′ -AAAGTTATCTTGCCATTCGCT-3′	327bp	60 C	[35]
*qep*A	F- AACTGCTTGAGCCCGTAGAT –3′ R - GTCTACGCCATGGACCTCAC - 3′	596bp	54 C	[36]
*qnr*B	F-GATCGTGAAAGCCAGAAAGG –3′ R- ATGAGCAACGATGCCTGGTA - 3′	476bp	52 C	[36]
*qnr*S	F- GCAAGTTCATTGAACAGGGT- 3′ R- TCTAAACCGTCGAGTTCGGCG- 3′	428bp	60 C	[36]
aac(6′)-Ib-	F- TTG CGA TGC TCT ATG AGT GGCTA R- CTC GAA TGC CTG GCG TGT TT	482bp	56 C	[43]
*rpo*B	F- GGC GAA ATG GCW GAG AAC - 3′ R- GAG TCT TCG AAG TTG TAA - 3 ′	1056 bp	50 C	[35]

MHA plates by using Ciprofloxacin and EtBr stain.[31] EtBrCW was done by using EtBr stain at concentration (5, 10, 15, 20, 25 µL/mL) to MHA plates containing ciprofloxacin ranging from 16 to 1024 µg/mL which prepared on the same day for experiment and protected from light, then the plates were swabbed with the bacterial inoculum starting from the center of the plate toward the edges, and then were incubated in dark space at 37°C for 18 hrs. Cultures were placed on ultraviolet transilluminator and photographed using a gel documentation system. The minimum concentration of EB that produced fluorescence of the bacterial mass was recorded,[32] taking corresponding *Escherichia coli* ATCC 25,922 strains as negative controls.

Detection of Plasmid-Mediated Quinolone Resistance Genes and Sequencing of *gyrA* and *parC*

DNA was extracted according to Manufacture instructions (wizbio, Korea), genomic DNA was eluted by adding 50 EB buffer (10 mM Tris-HCL, pH 9, 0.5 Mm EDTA) and visualized by electrophoresis on horizontal gels containing 1% agarose and stained with ethidium bromide. Isolates with reduced susceptibility to ciprofloxacin were subjected to PCR screening to detect the PMQR genes *qep*A*qnr*B*qnr*S, and*aac(6)Ib*. In addition, *gyr*A and *par*C in these isolates were amplified by PCR followed by sequencing. PCR mixture was prepared according to manufacture instructions (Promega/USA) by adding 3 µL of DNA template, 1.5 µL (0.6 pmol) of each forward and reverse primers, 12.5 µL of 2X GO TaqGreen mastermix and finally completed the volume to 25 µL by adding nuclease free water. Primer sequences and annealing temperatures are listed in Table 1. The amplification process was done by using thermocycler (TechNet-500/USA), the conditions of the PCR reaction included initial denaturation at 95C°/5 min, repeated 35 cycles of denaturation 95C°/30 sec, annealing for 30 sec and extension 72C°/1 min. Then a final extension at 72C°/10 min. PCR products for *gyr*A and *par*C were subjected to sequencing analysis which was carried out by Macrogen DNA Sequencing (Seoul, Korea), and analyzed with NCBI database.

Results

Isolation and Identification

Forty three *Klebsiella pneumoniae* clinical isolates were collected from different sources in different hospitals in Baghdad. These isolates were collected from wounds (n=13, 30.2%), blood (n=8, 18.6%), UTI (n=8, 18.6%), burns (n=5,11.6%), ear infections (n=4, 9.3%), sputum (n=3, 6.9%), and fluid and bronchial secretions (n=1, 2.3%).

Antibiotic Susceptibility Test

The tested isolates showed different antibiogram resistance activities. Highly resistance was observed towards ceftriaxone 86.04% (37/43) and gentamicin 69.7% (30/43). Resistance for both Ciprofloxacin and Levofloxacin was observed in 62.8% (27/43). Moderate resistance was observed to Amoxicillin +Clavulanic acid, Norfloxacin and Cefoxitin (55.8% (24/43).

53.4% (23/43) of isolates were resistant to imipenem and 48.8% (21/43) to amikacin, while only 44.1% (19/43) were resistant to Azithromycin (Figure 1).

The MIC was evaluated for the isolates which were ciprofloxacin resistant by disc diffusion (n= 27). These isolates were resistant to 16, 32, 64, and 128 µg/mL of ciprofloxacin, while 25/27 (96.1%) were resistant to 256 µg/mL, and 23/27 (83.4%) were resistant to 512 µg/mL. Finally, 9/27 (34.6%) of isolates were resistant to 1024 µg/mL. While, when we mixed Ciprofloxacin with Ethidium bromide (EtBr) for detection of efflux pump by agar cartwheel method, the results showed a decrease in resistance rates. The isolates showed 100% resistance to (16, 32, 64 µg/mL with EtBr, while decrease to 92.3% (24/27) in 128 and 256 µg/mL with EtBr, while reached 69.2% (18/27) in 512 µg/mL with EtBr. In presence of 1024 µg/mL ciprofloxacin with EtBr, resistance reached 19.2% (5/27). Table 2 showed the distribution of efflux activity with various concentrations of EtBr. 10/27 isolates showed a presumptive overexpression of efflux pump activity. The minimum concentration of EtBr at which strains with efflux activity showed fluorescence was 512 µg/mL.

Importantly, sequencing results showed that all quinolone resistant isolates (27/27, 100%) had the Ser83Leu substitution in *gyr*A, among which 24/27 (88.8%) co-carried the Asp87Asn genetic alteration. *par*C sequencing showed a common mutation of Ser80Ile in 21/27 (77.7%) and no other mutations were observed in the same gene (Table 2).

Molecular Detection of Plasmid-Mediated Quinolone Resistance Genes

Presence of PMQR genes was analyzed by PCR. The most predominant genes were *aac (6)-Ib* (25/27, 92.5%), *qnr*B (14/27, 51.8%), *qep*A (11/27, 40.7%), and *qnr*S (10/27, 37%) (Figure 2).

Discussion

Gram-negative bacilli, particularly *K. pneumoniae*, are major problematic organisms that have the ability to resist different types of antibiotics, including fluoroquinolones.[33,34] These opportunistic multi-drug resistant bacteria represent a serious challenge to infectious disease specialists worldwide.[35–42] Mechanisms of resistance to fluoroquinolones include mutations in their target proteins; DNA gyrase and topoisomerase IV, presence of PMQR determinants, expression of efflux pumps, and changes in permeability of the cell membrane.[43–45]

In the current study, a high rate of quinolone resistance was reported among *K. pneumoniae* (62.8%), and most of the isolates had MICs ≥ 512 µg/mL. The rate of resistance to fluoroquinolones varies according to the geographical distribution, e.g., resistance rates are 71.4% in Iran, 46.9% in Egypt, and 89% in India.[46–48]

The resistance to fluoroquinolones is mostly caused by mutations targeting the QRDR of *gyr*A, and *par*C of DNA gyrase and topoisomerase IV.[49] Sequencing of *gyr*A and *par*C

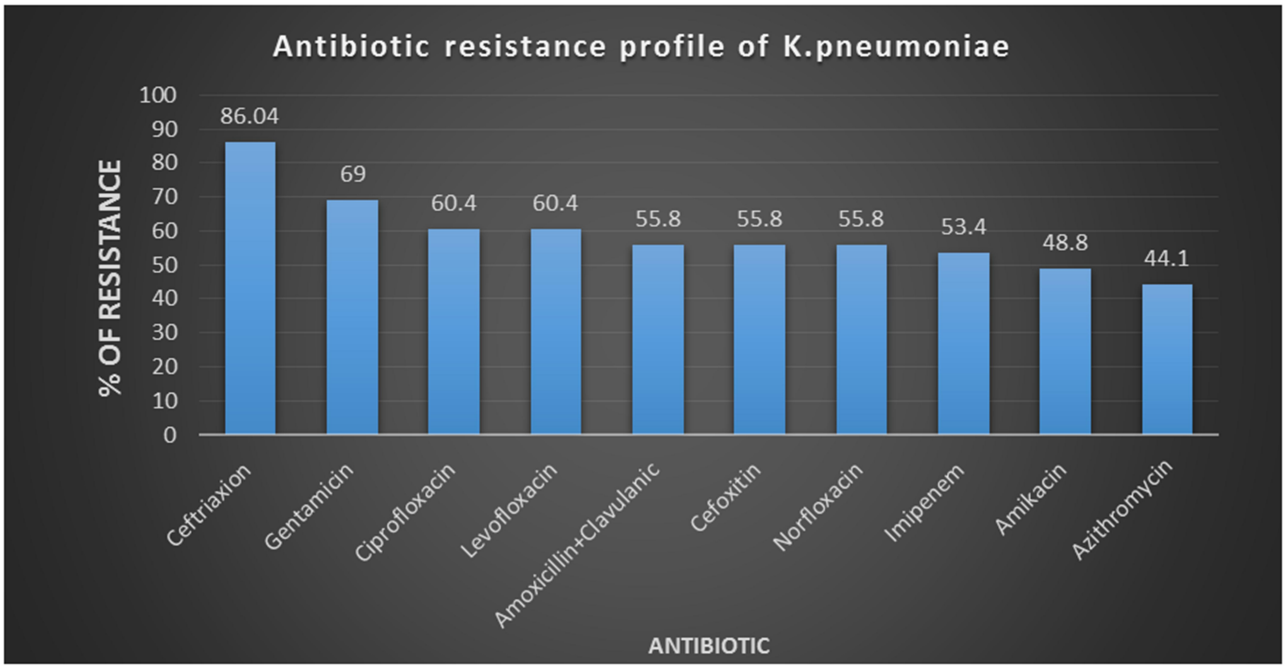

Figure 1 Antibiotic resistance profile of *K. pneumoniae*.

Table 2 Efflux Activity at Varying Concentrations of Ethidium Bromide and Corresponding *gyr*A and *par*C Gene Mutations

No	Isolate	Concentration of EtBr + Ciprofloxacin at Which Bacteria Started to Fluoresce (μg/mL)	*gyr*A	*par*C	Efflux Activity
1.	K1	1024	Ser83Leu, Asp87Asn	Ser80Ile	+
2.	K3	512	Ser83Leu, Asp87Asn	Ser80Ile	+
3.	K6	512	Ser83Leu, Asp87Asn	Ser80Ile	+
4.	K9	64	Ser83Leu, Asp87Asn	Ser80Ile	–
5.	K12	1024	Ser83Leu, Asp87Asn	Ser80Ile	+
6.	K13	512	Ser83Leu, Asp87Asn	Ser80Ile	–
7.	K15	512	Ser83Leu, Asp87Asn	Ser80Ile	+
8.	K17	512	Ser83Leu, Asp87Asn	Ser80Ile	+
9.	K18	1024	Ser83Leu, Asp87Asn	Ser80Ile	–
10.	K19	512	Ser83Leu, Asp87Asn	Ser80Ile	–
11.	K20	512	Ser83Leu, Asp87Asn	Ser80Ile	–
12.	K21	512	Ser83Leu, Asp87Asn	Ser80Ile	–
13.	K23	512	Ser83Leu, Asp87Asn	Ser80Ile	–
14.	K25	512	Ser83Leu, Asp87Asn	Ser80Ile	–
15.	K26	256	Ser83Leu, Asp87Asn	Ser80Ile	–
16.	K27	512	Ser83Leu, Asp87Asn	Ser80Ile	–
17.	K28	512	Ser83Leu, Asp87Asn	Ser80Ile	–
18.	K29	256	Ser83Leu, Asp87Asn	Ser80Ile	–
19.	K30	256	Ser83Leu, Asp87Asn	Ser80Ile	+
20.	K31	512	Ser83Leu, Asp87Asn	Ser80Ile	–
21.	K32	64	Ser83Leu, Asp87Asn	Ser80Ile	–
22.	K33	512	Ser83Leu, Asp87Asn	–	+
23.	K35	512	Ser83Leu, Asp87Asn	–	+
24.	K36	512	Ser83Leu, Asp87Asn	–	–
25.	K41	512	Ser83Leu	–	–
26.	K42	512	Ser83Leu	–	–
27.	K43	1024	Ser83Leu	–	+

showed that all tested *Klebsiella* isolates exhibited a Ser83Leu mutation in the *gyr*A, which is consistent with previous studies.[47,50] Moreover, most of the isolates had an additional Asp87Asn mutations in *gyr*A (88.9%) and Ser80Ile mutation in *par*C (77.8%), which is associated with high level of ciprofloxacin resistance.[51–53] Similar mutations were also reported by Al-Marzooq et al,[54] Ngoi et al,[55] Zeng et al,[56] and Guimarães et al.[57] On the other hand, the identified mutations are different from those observed by Azargun et al.[58] Resistance of *Enterobacteriaceae* to quinolones have been reported with alterations in genes other than the *gyr*A and *par*C. Lindbäck, Rahman, Jalal, Wretlind[59] and others have related mutation in *gyr*B and *par*E to resistance to quinolones.[60] No other mutations were observed in *par*C, however, other investigators have reported a Glu84Val substitution as the most common mutation in the *par*C gene.[61]

The presence of PMQR determinants on mobile genetic elements may leads to their dissemination among the *Enterobacteriaceae* family.[62] Most of our isolates carried the PMQR determinants. The fact that these genes are on mobile genetic elements constitutes a high risk of rapid dissemination to other Gram-negative bacteria.[63] The most common PMQR gene observed was the *aac(6)-Ib*, which is consistent with other reports.[63,64] It was reported that *aac(6)Ib* gene is common in Enterobacteriaceae and codes for resistance to gentamycin, tobramycin, kanamycin, and amikacin, and this explain the high percentage of this type of resistance in *Klebsiella pneumoniae*.[65,66] Among the qnr group of PMQR, qnrB was the most prevalent. Other investigators have also reported the predominance of qnrB type of PMQR in ciprofloxacin resistant isolates.[58,67] However the frequency of qnrB is higher than that

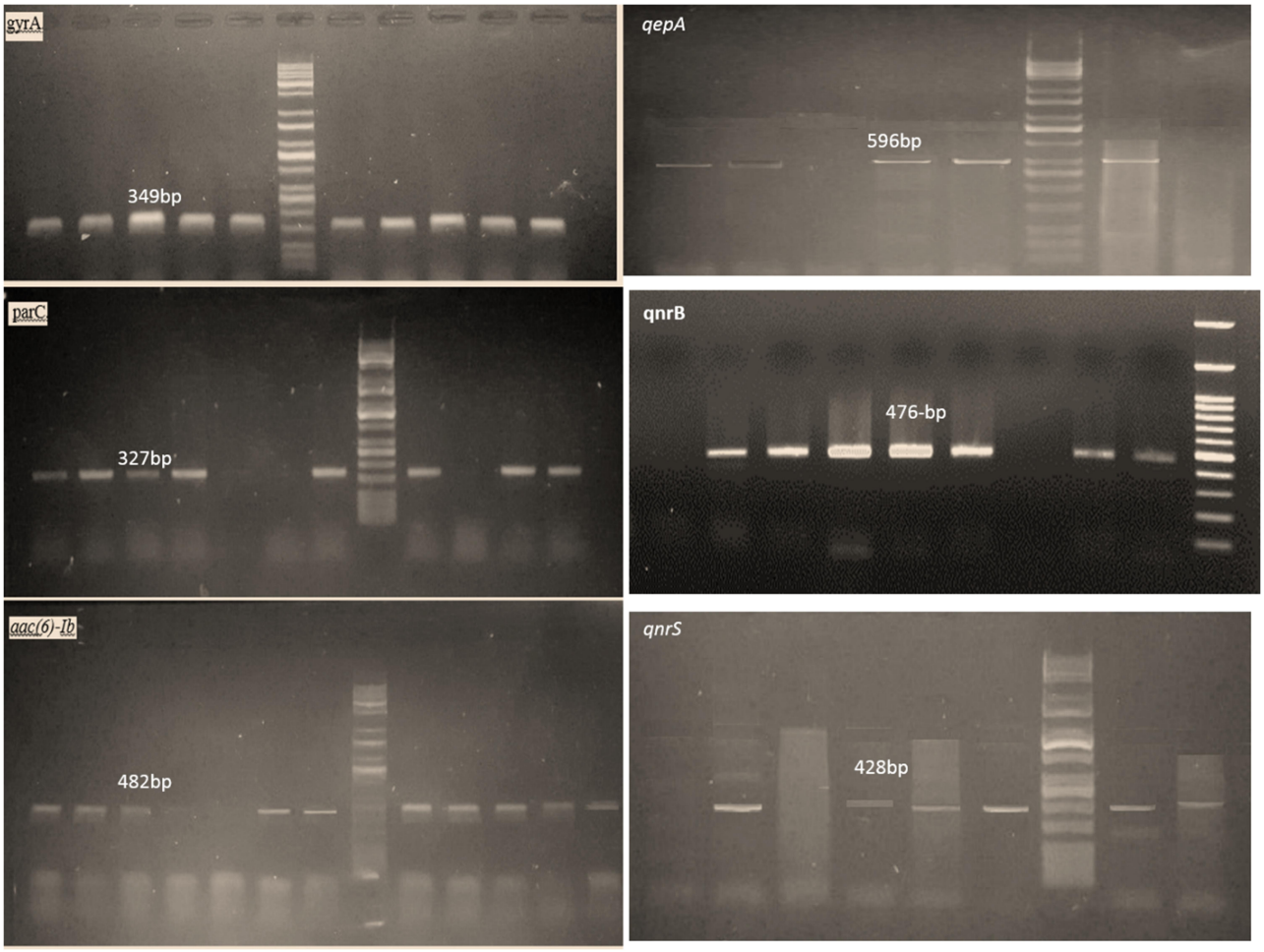

Figure 2 Gel electrophoresis for *gyr*A*par*C*aac(6)Ib, qep*A*qnr*B, and*qnr*S genes.

observed in other countries such Iran, Korea, and China (21.7%, 3.9% and 2.5%, respectively).[58,63,68] The differences in the incidence of mutations in DNA gyrase, and topoisomerase IV or prevalence of PMQR determinants could be related to the frequency of fluoroquinolones prescription, study area and period.

The EtBr-agar cartwheel screening method showed efflux activity in 27 strains. Efflux systems have a critical role in development mechanistic drug resistance in Gram-negative bacteria, these pump solutes out of the cell, permitting to microorganism to adjust their internal environment by getting out toxic substance like metabolite, antimicrobial agents, and quorum sensing signal molecules.[69] Our study reveals the emergence of efflux pump-mediated drug resistance in MDR Gram-negative bacteria in Iraq.

In fact, Gram-negative pathogens rely on tripartite protein assemblies that span their double membrane to pump antibiotics from the cell. The tripartite complex consists of an inner membrane protein (IMP) of the resistance nodulation cell division (RND) family, outer-membrane protein (OMP), and a periplas-mic membrane fusion protein (MFP) which connect the other two proteins. Efflux-mediated drug resistance is prominent in clinically significant MDR Gram-negative bacteria.[29,70]

Conclusions

Quinolone resistance is common in *K. pneumoniae* isolates in Baghdad. The frequent mutation in *gyr*A and *par*C, and the presence of PMQR genes is alarming.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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