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Research Article

Molecular detection of plasmid-mediated quinolone resistance genes among clinical isolates of *Klebsiella pneumoniae* during Covid-19 pandemic

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Abstract

Plasmid-mediated quinolone resistance (PMQR) genes confer low resistance to Fluoroquinolones (FQs). This study aims to detect five PMQR genes among FQs-resistant *Klebsiella pneumoniae* isolated from various clinical specimens. Out of 120 *K. pneumoniae* isolates, 68 FQs-resistance *K. pneumoniae* were included in a molecular study. Standard microbiological tests were used for identification and antimicrobial susceptibility. For the detection of PMQR genes, conventional polymerase chain reaction was used. A molecular study revealed that (73.5%) of samples harbored PMQR genes, and among them, 58% were co-carriages of PMQR gene variants. *Aac* (6')-*Ib-cr* gene was predominant (47.1%) among samples, and *qepA* had the lowest percentage (11.8%), *qnr* genes were (32.4%) (29.4%) (20.6%) *qnrS*, *qnrB*, and *qnrA* respectively. Overall, high percentages of PMQR genes were detected, and almost all of samples were phenotypically resistant to ciprofloxacin. As well, there was a significant statistical relationship between phenotypically ESBL-producers and *qnrB* and *qepA* genes.

Keywords

K. pneumoniae, Fluoroquinolones, PMQR, ESBLs, PCR

Introduction

Over the last three decades, resistance to fluoroquinolones (FQs) has been extensively studied in human and veterinary isolates of bacteria, with a growing trend toward resistance being associated with heavy usage (Pham et al. 2019). Previously was believed that FQs resistance could be acquired uniquely through chromosomal mutations (Hooper and Jacoby 2015). In 1998, Martínez-Martínez and Jacoby discovered that plasmid could transfer resistance genes to reduce susceptibility to FQs between bacteria; after that, plasmid-mediated quinolone resistance PMQR was first discovered in a clinical isolate of *K. pneumoniae*.

During the last two decades, three different mechanisms have been associated with the phenomenon of PMQR genes (Rodríguez-Martínez et al. 2016), first: *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnr VS*, and *qnrS* genes code for proteins of the pentapeptide repeat family that protects topoisomerase IV and DNA gyrase from FQ inhibition (Rahman et al. 2017; Acheampong et al. 2019;

Al-Rafyai et al. 2021). Second: *aac* (*6'*)-*Ib-cr* is a variant of *aac* (*6'*)-*Ib* conferring resistance to aminoglycosides (e.g., amikacin), the **-cr** include two mutations (Asp-179Tyr and Trp102Arg) this is a bifunctional variant able to acetylate (confer resistance) FQs with a piperazinyl substituent (ciprofloxacin and norfloxacin) while FQs that do not have this substitution (i.e., levofloxacin) are unaffected by this enzyme. The most common PMQR gene in FQ-susceptible and resistant clinical isolates is the *aac* (*6'*)-*Ib-cr* gene (Machuca et al. 2016). Third: *OqxAB* and *qepA* genes. *QepA* is a plasmid-mediated efflux pump gene that reduces susceptibility to hydrophilic FQs, particularly enrofloxacin, norfloxacin, and ciprofloxacin (Rahman et al. 2017).

In 2018 a novel mechanism of PMQR genes was identified on a pUM505 plasmid named the *CrpP* gene, which codes an enzyme-modifies ciprofloxacin (Chávez-Jacobo et al. 2018). Soon after, other studies defined that the *CrpP* gene is not always related to ciprofloxacin resistance (Hernández-García et al. 2021), and a recent study claimed that *CrpP* is not a fluoroquinolone-inactivating enzyme (Zubyk and Wright 2021). PMQR genes have changed the resistance pattern to FQs, especially among *Enterobacteriales* (Vieira et al. 2020). Although the PMQR genes confer low-level resistance that does not surpass the clinical susceptibility level, they accelerate the selection of higher-level resistance and make infection with pathogens carrying PMQR more challenging to treat (Rahman et al. 2017; Ayobola et al. 2021). Besides, a study in 2018 claimed that PMQR confirms sufficient resistance to ciprofloxacin in *Salmonella typhimurium* without any chromosomal mutations (Kuang et al. 2018).

Resistance to FQs is widespread in many Gram-negative bacteria that produce extended-spectrum beta-lactamase (ESBLs), including *K. pneumoniae* (Higashino et al. 2017). Moreover, PMQR genes are frequently co-located with ESBL genes on plasmids; resistance plasmids containing genes for ESBLs can be transferred, which facilitates the spread of PMQR determinants among *Enterobacteriaceae* species (Azargun et al. 2018; Xiong et al. 2021).

The importance of this study, according to our knowledge, is the first study in Iraqi Kurdistan to detect PMQR genes among ESBL-producers and non ESBL-producers FQs-resistant *K. pneumoniae* from clinical isolates, remarkably during covid-19 pandemic FQs extensively used among inpatient and outpatients. In addition, this study may provide information about the prevalence of PMQR genes among *K. pneumoniae* in the Duhok Province Kurdistan/Iraq region.

Materials and methods

Study setting and bacterial isolates

A cross-sectional study was conducted between September 2021 and January 2022. During this period, 120 isolates of *K. pneumoniae* were collected from different clinical samples, including urine (73.3%), sputum (9.2%), pus (6.7%), blood (3.3%), vaginal swab (2.5%), vaginal discharge (2.5%), bronchial lavage (0.8%), pleural fluid (0.8%), and wound (0.8%). Samples were taken from outpatient and inpatient from several public and private hospitals in different districts of Duhok Province. Ages 14 years and above and not took antibiotics in the past three days from both sexes were included. All isolates were identified using conventional biochemical tests (Mahon et al. 2019) then isolates were preserved in brain heart infusion and 15% Glycerol at -20 °C until use (Addgene 2021).

Ethical considerations

The ethics committee permitted the study proposal and informed consent of the Duhok Polytechnic University and Duhok General Health Directorate, Kurdistan Region, Iraq.

Antimicrobial susceptibility testing

The Kirby-Bauer disk diffusion method was used for the antimicrobial susceptibility test, using Mueller Hinton agar (NEOGEN, USA). The following disks were used: ciprofloxacin (CP, 10 µg), levofloxacin (LOM, 5 µg), nalidixic acid (NA, 30 µg), norfloxacin (NOR, 10 µg), amoxicillin/clavulanic acid (AMC, 20+10 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), imipenem (IPM, 10 µg), aztreonam (ATM, 30 µg), gentamicin (GM, 10 µg), amikacin (AK, 10 µg), neomycin (N-10 µg), and nitrofurantoin (NF, 300 µg) (Bioanalyses, Turkey). The results were interpreted according to CLSI 2021 and EUCAST 2021 breakpoints guidelines.

ESBL confirmatory test

The double-disc synergy test (DDST) was used to detection of phenotypically ESBL-producing *K. pneumoniae* using follow antibiotics: cefotaxime (30 µg), ceftazidime (30 µg), and (ATM, 30 µg) of amoxicillin/clavulanic acid (AMC, 20+10 µg) as described by (Iqbal et al. 2017).

Selecting isolates and DNA extraction

Among 120 isolates, only 68 FQs-resistance *K. pneumoniae* were selected for molecular study. DNA extraction was carried out with an optimized heat shock method, as described by (Higashino et al. 2017). The purity of samples was 1.75–1.9 (260/280), and concentrations were between 20–80 (ng/ul) by using the NanoDrop 2000 (Thermofisher, USA). The template DNA was stored at -20 °C for PCR applications.

PCR Assay and gel electrophoresis

PCR reaction mixtures (20 µl) contained 10 µl 1× Add taq-Master mix (Addbio, South Korean), 2 µl DNA template, 2 µl of forward and reverse primers 10 Pmol (Macrogen,

South Korean), and 6 µl Distilled water. A set of 5 primers of PMQR were used Table 1. Using the following program: **Initial denaturation** at 94 °C for 5 min 1 cycle. **Denaturation** at 94 °C for 1 min 30 cycles followed by **annealing temperature** (57 °C for *qnrA*, *qnrB*, and *qnrS*, 54 °C for *aac* (*6'*)-*Ib-cr* and 60 °C for *qepA* genes), **extension** 1 min at 72 °C. A **final extension** at 72 °C for 10 min 1 cycle. For *K. pneumoniae* 16S– 23S ITS (gene specific-species): **Initial denaturation** was 15 mins at 94 °C 1 cycle. **Denaturation** 30S at 72 °C 30 cycles followed by **annealing** 30 s 55 °C, **extensions** 50 s at 72 °C. A **final extension** at 72 °C for 5 mins. The amplicon was resolved by electrophoresis on 1.5% agarose gel (Simply, USA) and visualized after staining with a safe stain by using 1× TAE buffer (GENETBIO, South Korea) and 100 bp DNA ladder (GeneDireX, USA).

Table 1. Primers used in this study.

Genes	Primer sequences	Base pairs	References
QnrA	TTCTCACGCCAGGATTTGAG TGCCAGGCACAGATCTTGAC	571	Bouchakour et al. 2010
QnrB	TGGCGAAAAAATTGAACAGAA GAGCAACGATCGCCTGGTAG	594	Bouchakour et al. 2010
QnrS	GACGTGCTAACTTGCGTGAT AACACCTCGACTTAAGTCTGA	388	Bouchakour et al. 2010
Aac (6')-Ib-cr	TTGCGATGCTCTATGAGTGGCTA CTCGAATGCCTGGCGTGTTT	482	Eftekhar and Seyedpour 2015
QepA	CTGCAGGTACTGCGTCATG CGTGTTGCTGGAGTTCTTC	403	Cattoir et al. 2008
K. pneumoniae 16S–23S ITS	ATTTGAAGAGGTTGCAAACGAT TTCACTCTGAAGTTTTCTTGTGTTC	130	Liu et al. 2008

Statistical analysis

Frequencies, crosstab, P value, and percentages were used to describe the data analysis of the study sample. SPSS v23.0 (SPSSInc, Chicago, IL, USA).

Results

Sixty-eight FQs-resistance *K. pneumoniae* from clinical isolates were chosen for molecular study; 42 were urine and mainly from female patients, while pleural fluid and bronchial lavage contained the lowest percentage. Generally, outpatients were higher than inpatients Table 2.

Table 2. Distributions of clinical specimens related to sexes and hospital status.

Samples	Hospital status				Total
	Inpatient		Outpatient		
	Male	Female	Male	Female	
Urine	2	5	5	30	42
Pus	2	2	2	2	8
Sputum	5	2	0	1	8
Blood	3	0	0	0	3
Wound	2	1	0	0	3
HVS	0	0	0	2	2
Pleural fluid	0	1	0	0	1
Bronchial lavage	1	0	0	0	1
Total	15	11	7	35	**68**

Ciprofloxacin showed the highest rate of resistance, 92.6%, among FQs. At the same time, norfloxacin and ofloxacin showed a lesser resistance rate among FQs. In addition, 32 samples were ESBL-producers Table 3.

Table 3. Antibiotic susceptibility and ESBL tests of *K. pneumoniae*.

Antibiotics	Sensitive (%)	Intermediate (%)	Resistance (%)	Total (%)
Ciprofloxacin	4 (5.9)	1 (1.5)	63 (92.6)	
Levofloxacin	12 (17.6)	12 (17.6)	44 (64.7)	
Norfloxacin	25 (36.8)	3 (4.4)	40 (58.8)	68 (100.0)
Ofloxacin	23 (33.8)	5 (7.4)	40 (58.8)	
Nalidixic acid	9 (*21.4*)	*6* (*14.3*)	*27* (*64.3*)	*42* (*100.0*)*
Amoxicillin/ Clavulanic acid	13 (19.1)	10 (14.7)	45 (66.2)	
Cefotaxime	11 (16.2)	1 (1.5)	56 (82.4)	
Ceftazidime	11 (16.2)	7 (10.3)	50 (73.5)	
Aztreonam	16 (23.5)	10 (14.7)	42 (61.8)	68 (100.0)
Imipenem	32 (47.1)	3 (4.4)	33 (48.5)	
Amikacin	20 (29.4)	11 (16.2)	37 (54.4)	
Gentamicin	32 (47.1)	1 (1.5)	35 (51.5)	
ESBL -Producing		32 positives	36 negatives	**68 (100.0)**

*twenty-six samples were not urine samples therefore **NA** not applicable.

Among 32 carriages of *aac* (*6'*)-*Ib-cr*, 31 samples were resistant to ciprofloxacin, and among eight samples harboring *qepA*, all were resistant to ciprofloxacin. In addition, there was a significant relationship between ESBL-producers and harboring *QepA* and *QnrB* genes Table 4.

Table 4. Distributions of PMQR genes among ESBL-producers and FQs-resistance.

Target genes	Total of PMQR (%)	ESBL producer			Phenotypic pattern FQs resistant	FQs No.
		Yes	No	P. value		
QepA	8 (11.8)	7	1	0.01	CIP	8
					NAL, NOR, OFX and LVX	6
QnrS	22 (32.4)	11	11	0.46	CIP	21
					LVX	14
					NAL	8
					NOR and OFX	13
QnrB	20 (29.4)	13	7	0.05	CIP	19
					LVX	12
					NOR AND OFLO	11
					NAL	8
QnrA	14 (20.6)	9	5	0.12	CIP	13
					LVX	7
					NOR, OFLO and NA	6
Aac (6')-Ib-cr	32 (47.1)	17	15	0.24	CIP	31
					LVX	23
					NOR and OFX	22
					NAL	15

In a total of 50 *K. pneumoniae* harboring PMQR genes, 27 samples harbored more than one PMQR gene variants and only one sample was with all targeted PMQR genes Table 5.

Table 5. Frequency of PMQR genes harbored by each sample.

Harboring	Two genes of PMQR	Three genes of PMQR	Four genes of PMQR	Five genes of PMQR	Total of co-carriage (%)
Outpatients	9	8	3	1	21
Inpatients	5	1	None	None	6
Total of PMQR	14	9	3	1	**27 (54.0)**

Discussion

PMQR genes consist of three different mechanisms, and about nine genes are responsible for those mechanisms (Rahman et al. 2017). However, in the present study only following five genes (*qnrA*, *qnrB*, *qnrS*, *aac* (*6'*)-*Ib-cr* and *qepA*) included.

Plasmids carrying PMQR have been identified mainly in *Enterobacteriales*, including *Klebsiella*, *Enterobacter*, *Salmonella* species and *Escherichia coli* (Jacoby, 2018). Since *K. pneumoniae* is the major cause of hospital-acquired pneumonia, urinary tract infections, septicemia, and soft tissue infections are also considered risk factors for severe community-acquired infections (Wang et al. 2020). In addition, the first PMQR gene was detected in *K. pneumoniae*, and most studies indicate a greater frequency of PMQR genes in *K. pneumoniae* than in *E. coli* (Eftekhar and Seyedpour 2015; Ferjani et al. 2015). Therefore, it was worth studying *K. pneumoniae*. Notably, there are no studies on PMQR among *K. pneumoniae* or other bacteria in Iraqi Kurdistan.

Since most of the samples in this study came from urine, most of the *K. pneumoniae* isolates were found in urine. Our result shows that out of 68 samples, urine samples were the most common among other samples 42/68, which is near to the study done in Iran by (Eftekhar and Seyedpour 2015), and among those 42 urine samples, 35 were from females. These results are near to the study done in Iraq by (Mohammed et al. 2019; Ahmed Hasan et al. 2021).

A molecular study of this study found that 47.1% of samples harbored the *aac* (*6'*)-*Ib-cr* gene, which it appears that the *aac* (*6'*)-*Ib-cr* gene is more prevalent than any other *qnr* genes; this result agrees with similar studies conducted in the south of Iraq, in Baghdad, Kareem et al. (2018) found out of 27 *K. pneumoniae* 92.5% harbored *aac* (*6'*)-*Ib-cr* and in Najaf, in a total of 74 *K. pneumoniae* 52.7% has been reported (Al-Hilali et al. 2021). In Tehran, Iran was 53.2% (Eftekhar and Seyedpour 2015), Kashan-Iran (70.1%) (Shams et al. 2015), Saudi Arabia, 51.6% (Al-Agamy et al. 2018), in Jordan, 50% (Idris et al. 2017). Syria, out of 123 *E. coli* and *K. pneumoniae*, 75.6% had *aac* (*6'*)-*Ib-cr* (Alheib et al. 2015). High percentages of this gene could be back to the overuse of aminoglycosides or hydrophilic FQs in that region and time, as it is known bacteria, to survive could transfer antibiotic resistance genes among other bacteria (Jacoby et al. 2014). The *aac* (*6'*)-*Ib-cr* gene plays a crucial role in the emergence of clinical ciprofloxacin resistance. Hence, this is especially concerning at the nosocomial level, where strains carrying *aac* (*6'*)-*Ib-cr* should be rapidly identified and treated with non-hydrophilic FQs, such as ofloxacin or levofloxacin (Frasson et al. 2011).

Our results found that the percentage of *qnrS* was 32.4%. This result near to Nourozi et al. (2020) in Tehran, Iran, found that in a total of 80 *K. pneumoniae* isolates 34% had *qnrS*, and Kareem et al. (2018) found that in all 27 *K. pneumoniae*, 37% had *qnrS* in Baghdad, Iraq. In Turkey, the percentage was higher than in our study; out of 22 *K. pneumoniae*, 86.4% harbored *qnrS* (Unlu and Demirci 2020). On the other hand, some studies revealed fewer percentages; a study in Mashhad, Iran, found that among 130 *K. pneumoniae* (15.4%) of isolates harbored *qnrS* (Izadi et al. 2017), and another study in Tehran, Iran revealed that 79 isolates 2.5% harbored *qnrS* (Eftekhar and Seyedpour 2015). Azadpour et al. (2014) in Lorestan, Iran, found only one *qnrS* (5.5%) among 18 *K. pneumoniae*. Al-Hilali et al. (2021) detected only one *qnrS*. Similarly, Hadi et al. (2016) found that out of 20 *K. pneumoniae*, only one sample had the *qnrS* gene. While in Mosul, Mahmood and Abdullah (2015), among 57 isolates, did not find any *qnrS* gene. The reason behind these variations could be back to the sample size.

We found that 29.4% of isolates had *qnrB*. Our result is in accordance with the two studies done in Tehran, Iran, in which about 79 *K. pneumoniae*, 30.4% carried *qnrB* (Eftekhar and Seyedpour 2015), and out of 88 *K. pneumoniae*, the *qnrB* was present in 43% of isolates (Nourozi et al. 2020). In Togo, West Africa found out of 64 *Klebsiella* spp. 30.8% had *qnrB*. While in the south of Iraq, different percentages have been reported. Kareem et al. (2018) in Baghdad reported 51.8%, Mosul 15.8% (Mahmood and Abdullah 2015) Najaf 23% (Al-Hilali et al. 2021). Another study in Najaf by Alshammari and Al-Skhattat (2015) found that out of 89 *K. pneumoniae* isolates from clinical and environmental specimens, only one sample had *qnrB*. These variations in the frequency of *qnrS* and *qnrB* genes may be due to the geographic distribution of the PMQR genes or criteria of selection of FQs-resistance bacteria from clinical samples in that time and region.

A 20.6% of our samples harbored *qnrA*. This percentage has not previously been reported in Iraq among *K. pneumoniae* or other bacteria from clinical isolates. In their isolates, Hadi et al. (2016) and Al-Hilali et al. (2021) and found *qnrA* only in two samples. Alshammari and Al-Skhattat (2015) isolate one. Mahmood and Abdullah (2015) did not find *qnrA*. Similarly, some studies in Iran did not find *qnrA* (Azadpour et al. 2014; Eftekhar and Seyedpour 2015; Abbasi and Ranjbar 2018). In comparison, our result is near to Mahdane et al. (2022), which found that out of 80 *E. coli* and *K. pneumoniae* samples, 26.8% harbored *qnrA*. In general, *qnrB* seems somewhat more common than *qnrA* (Jacoby et al. 2014).

During the present study, *qepA* had the lowest percentage of other *PMQR* genes, which was 11.8% in a total of 68 isolates. However, a high percentage has been reported by Kareem et al. (2021) in Iraq, in a total of 27 *K. pneumoniae* (40.7%) harbored *qepA*, while Alshammari and Al-Skhattat (2015) found 4.5% in total of 89 *K. pneumoniae* had *qepA*. Some studies from other countries, such as Syria and Jordan, did not find *qepA* (Alheib et al. 2015; Idris et al. 2017). In Iran, out of 247 *K. pneumoniae*, 2% harbored *qepA* (Goudarzi et al. 2015).

In a total of 68 samples, 50 samples harbored PMQR genes, 46% had one gene, and 54% had more than one gene. Higher levels of FQs resistance have been noticed

when the plasmid carries two or more FQs gene resistance (Jacoby et al. 2014). High frequency of *qnrB* and *aac* (*6'*)-*Ib-cr* has been reported in *K. pneumoniae* (Eftekhar and Seyedpour 2015), but in our study, co-carriage of *qnrA* and *aac* (*6'*)-*Ib-cr* found in 20% of samples, and 14 isolates had *qnrB* and *aac* (*6'*)-*Ib-cr*. It is uncommon for a single isolate to possess more than two PMQR genes (Vinué et al. 2020). While in the current study, 26% of samples had three or more PMQR genes, which consider a high percentage. High co-carriage of PMQR genes in our study could be back to the over and misusing of FQs, improper hygiene and not strict guidelines for infection control leading to bacteria becoming resistant in the community and hospital settings.

Beta-lactam and FQs antibiotics are the most significant antibiotic agents used to treat *K. pneumoniae* infections (Goudarzi et al. 2015). Numerous studies have proved the link between PMQR determinants and the expression of ESBLs (Azargun et al. 2018). In the current study, there was a statically significant relationship among phonetically ESBL-producers and *qepA* and *QnrB*, while for other genes, statistically was not significant; this may be because the ESBL production was confirmed only by DDST, although this test considered the most accurate than other phenotypic confirmatory tests (Iqbal et al. 2017) however, the coexistence of *AmpC* gene may result in false negatives (Kaur and Chopra 2013). In comparison, some studies were confirmed genotypically. For example, a study in Azerbaijan and Iran revealed that genotypically confirmed *Enterobacteriales* that are ESBL-producing in UTI isolates had high FQs resistance and PMQR genes (Azargun et al. 2018). Another study in which DDST confirmed ESBL-producers found PMQR was more frequent in ESBL-producing *K. pneumoniae* (Izadi et al. 2017). However, FQ resistance in ESBL-producers is an urgent health concern in countries where ESBL-producing *K. pneumoniae* is prevalent (Higashino et al. 2017). Therefore, Monitoring and epidemiological studies are required to stop the spreading FQs resistance among ESBL-producers.

Conclusion

During this study, most samples were resistant to ciprofloxacin; nearly half of the samples were ESBL-producers. A molecular study detected a high percentage of PMQR genes. The *aac* (*6'*)-*Ib-cr* gene was more prevalent than any other PMQR gene. Among PMQR-harbored samples, 54% had more than one PMQR gene.

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