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Passer Journal

Passer 5 (Issue 1) (2023) 207-212

http://passer.garmian.edu.krd/

Prevalence of Silent Plasmid-Mediated Quinolone Resistance (PMQR) Genes among Clinical Isolates Fluoroquinolone-Sensitive *Klebsiella pneumoniae*

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Received 02 March 2023; revised 16 April 2023;
accepted 16 Apil 2023; available online 22 April 2023

DOI: 10.24271/PSR.2023.386813.1269

ABSTRACT

Plasmid-mediated quinolone resistance (PMQR) genes confer varying degrees of fluoroquinolone (FQ) resistance. Silent antibiotic resistance genes are DNA sequences typically not expressed, spread among strains through horizontal gene transfer, and could be active due to gene transfer. This study aimed to detect silent PMQR genes among FQs-sensitive *K. pneumoniae* isolated from clinical samples in Duhok province, Iraq. Standard microbiological methods were performed to identify *K. pneumoniae*, and then these bacteria were confirmed by species-specific genes. Samples were subjected to an antimicrobial susceptibility test by the Kirby-Bauer disc diffusion technique, then detection of targeted PMQR genes was done by the conventional polymerase chain reaction (PCR) method. Out of, 120 *K. pneumoniae*, 32 FQs-sensitive *K. pneumoniae* isolates were subjected for the molecular study; 53.1% of isolates (17/32) harbored silent PMQR genes, and among harbored PMQR genes, the frequency of genes was 12/17 (70.6%), 6/17 (35.3%), 5/7 (29.4%), 4/17 (23.5%) for, *qnrA*, *qnrB*, *qnrS*, and *aac (6')-Ib- cr* respectively, in addition, 9/17 (53%) of isolates harbored more than one gene of PMQR genes. Furthermore, the *qnrA* gene had the highest percentage of PMQR genes, while the *qepA* gene was not detected. The dissemination of silent PMQR genes could offer a future risk for public health under selective pressure of an antibiotic for a long time or transfer to other bacteria and then become active genes.

Keywords: ARGs, Silent genes, PMQR, K. pneumoniae, Fluoroquinolone.

1. Introduction

Klebsiella pneumoniae (K. pneumoniae) is a bacterium that commonly causes infections in both hospital and community settings[1,2]. It causes various infectious diseases and gained notorious for its near pan drug-resistant properties[1,2]. Several new antimicrobial resistance genes for the first time were found in *K. pneumoniae* such as *KPC, OXA-48, NDM-1* and *qnr* [1,2].

Fluoroquinolones (FQs) are one of the most valuable and widely used class of antibacterial drugs in the world and are extensively used for Enterobacterial infections. FQs resistance occurs by chromosomal mutations and plasmid-mediated quinolone resistance (PMQR) genes, which confer low-level FQ resistance and promote the selection of mutational high-level resistance[3,4]. Since 1998, studies have emphasized on the emerging of PMQR genes among the phenotypic FQs-resistant bacteria[5-7].

While a few studies concentrate on the silent antibiotic resistance genes among phenotypic sensitive bacteria[8]. The idea of an antibiotic **resistome** was advanced as a conceptual framework for understanding antibiotic resistance, all known and unknown regularly expressed, silent or proto-resistance genes in the world are grouped together in resistome[9-11].

Antibiotic resistance silent genes are DNA sequences that are typically not expressed[12]. However, it is an interesting fact that silent genes could be converted to be active after recombination or mutations like the rest of normal genes[13-15], as well as, studies suggested that due to a lack of expression, these genes do not confer resistance, but it will function and confer clinical resistance determinants if mobilized into the new host[8,12,14,16].

Several studies were achieved on the PMQR genes. The diagnosis of bacterial antibiotic resistance is usually based on the bacterial phenotypic resistance which may provide limited knowledge for understanding the possibility of bacterial evolution. Thus, the present study is designed to investigate and track the prevalence of silent PMQR genes among FQs-sensitive *K. pneumoniae* isolated from the clinical samples.

2. Methods and Materials

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Peer-reviewed under the responsibility of the University of Garmian.

2.1 Patients, samples, and sample collection

This cross-section study was conducted on patients aged from 14 years to 75 years, from both sexes who attended public and private hospitals in Duhok province, Iraq from September 2021 to January 2022. The samples were collected from urine, sputum, wound pus, blood, high vaginal swab, bronchial lavage, pleural fluid, and wound.

2.2 Ethical consideration

Prior to conducting the study, the ethics committee approved the proposal and informed consent was obtained from both the Duhok Polytechnic University and the Duhok General Health Directorate in the Kurdistan region of Iraq (18082021-8-18).

2.3 Identification and antimicrobial susceptibility test of *K. pneumoniae*

Bacterial cultures were obtained from patients attending various microbiology labs in the Duhok province. The isolates were sent to the microbiology lab at Azadi Teaching Hospital for final identification. Blood and MacConkey agar (NEOGEN, USA) were used for primary inoculation. To confirm the presence of *Klebsiella* species, morphological and biochemical tests such as IMViC tests (Alpha Chemika, India), motility test (by wet mount and culturing on SIM Media), and urease test were performed[17]. In addition, conventional PCR was used to identify and confirm *K. pneumoniae* using species-specific genes[18] (Table 1). The antimicrobial susceptibility profile of these bacterial isolates was determined using the Kirby-Bauer disc diffusion method depending on Clinical Laboratory Standards Institute (CLSI), 2021 and European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2021 recommendations[19,20]. Nalidixic acid (30 µg), norfloxacin (10 µg), ofloxacin (5 µg), ciprofloxacin (10 µg), and levofloxacin (5 µg) were selected and used as FQs.

2.4 Molecular characterization of PMQR genes

The phenotypically sensitive-FQs *K. pneumoniae* were selected for molecular detection of PMQR genes, using conventional PCR (Applied Bioscience, USA™) and gel electrophoresis. DNA extraction was carried out by boiling method[18] and PCR reactions were performed in 20 µl containing 2 µl DNA template, 10 µl Taq master mix, 2 µl primers 10 Pmol, and 6 µl DH_2O. PCR conditions and programs were depended on previous studies[6,21-23] (Table 1). The amplified PCR products were run on 1.5% agarose gel on 1x TAE buffer and read on a UV transilluminator (Wealtec, USA™).

Table 1: Primers used in this study.

PMQR genes	Primer sequences	Base pairs	References
QnrA	**F**-TTCTCACGCCAGGATTTGAG **R**-TGCCAGGCACAGATCTTGAC	571	[21]
QnrB	**F**-TGGCGAAAAAATTGAACAGAA **R**-GAGCAACGATCGCCTGGTAG	594	[6]
QnrS	**F**-GACGTGCTAACTTGCGTGAT **R**-AACACCTCGACTTAAGTCTGA	388	[21]
Aac (6')-Ib-cr	**F**-TTGCGATGCTCTATGAGTGGCTA **R**-CTCGAATGCCTGGCGTGTTT	482	[22]
QepA	**F**-CTGCAGGTACTGCGTCATG **R**-CGTGTTGCTGGAGTTCTTC	403	[22]
K. pneumoniae rRNA spacer	**F**-ATTTGAAGAGGTTGCAAACGAT **R**-TTCACTCTGAAGTTTTCTTGTGTTC	130	[23]

2.5 Statistical analysis

Statistical analysis was performed using, frequencies, crosstab, and percentages SPSS program v23.0 (SPSS Inc, Chicago, IL, USA).

3. Results and Discussion

3.1 Characterization and distribution of *K. pneumoniae* isolates

Out of 120 isolates of *K. pneumoniae* were obtained from patients residing in different places within Duhok province. Of these isolates, only 32 isolates of FQs-sensitive *K. pneumoniae* (25 isolates from female patients and 7 isolates from male patients) were included in the PCR study. Among these patients, only two patients were hospitalized, and the remaining 30 were outpatients. The 32 isolates were obtained from various sources, with 27 isolates collected from urine samples and 5 isolates collected from vaginal swabs and sputum samples. Of the remaining 88 isolates. 68 isolates were found to be resistant to FQs, and subjected to PCR study with results published[18]. The remaining 20 isolates were not included in the PCR study.

3.2 Antimicrobial susceptibility test

Table 2 shows the sensitivity of 32 isolates of *K. pneumoniae* to various FQs antibiotics. Of note, these antibiotics belong to different generations of FQs and nalidixic acid is only used for urine samples. The results showed that (93.7%), (100%), (100%), (96.9%), (93.7%), and (93.7%) of isolates were fully sensitive to nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin, and levofloxacin, respectively. The remaining five samples exhibited intermediate sensitivity to FQs.

Table 2: Antibiotic sensitivity of (32) *K. pneumoniae* isolates to FQs.

Fluroquinolones		Sensitive No. (%)	Intermediate No. (%)	Total No. (%)
Nalidixic acid	**1st generation**	25 (93.7)	2 (6.25)	27* (100.0)
Norfloxacin	**2nd generation**	32 (100.0)	0 (0.0)	32 (100.0)
Ofloxacin		32 (100.0)		
Ciprofloxacin		31 (96.9)	1 (3.1)	
Levofloxacin	**3rd generation**	30 (93.7)	2 (6.3)	

*Five samples were not urine samples. Therefore, nalidixic acid not applicable to non-urine samples.

K. pneumoniae naturally exhibits high levels of susceptibility to FQs. The five isolates showed intermediate sensitivity to FQs, which means that they were not fully sensitive but also not resistant. Therefore, the reduced sensitivity observed in these isolates may be attributed to the limitations of the disk diffusion testing method, which may not be as effective in detecting low-level resistance to certain antibiotics[24]. Additionally, the presence of silent PMQR genes could also contribute to the reduced sensitivity, as silent genes could be expressed at a very low level (typically silent genes not expressed)[12,25].

3.3 Percentages and distribution of PMQR genes

Among 32 FQs-sensitive *K. pneumoniae*, 17 of them harbored PMQR genes and the other 15 isolates were only phenotypiclly sensitive to FQs without harboring PMQR genes. The distribution of PMQR genes among isolates was as follows: For *qnrA* 12/17 (70.6%), *qnrB* 6/17 (35.3%), *qnrS* 5/7 (29.4%), and *aac (6)-Ib-cr* 4/17 (23.5%). Notably, no detection of the *qepA* gene was observed in any of the isolates studied. (Figure 1) and (Table 3).

Table 3: Distributions of PMQR genes among (17) isolates of FQs-sensitive *K. pneumoniae.*

No. of samples	PMQR genes	No. of Co-carriage samples
1	*QnrB*	-
2	*QnrB, qnrA*	**2**
3	*QnrA*	-
4	*QnrA, qnrS*	**2**
5	*QnrA*	-
6	*QnrA, qnrB*	**2**
7	*QnrA*	-
8	*Aac (6')-1b- cr*	-
9	*QnrA, aac (6')-1b- cr*	**2**
10	*QnrS*	-
11	*QnrA*	-
12	*QnrA, aac (6')-1b- cr*	**2**
13	*QnrB, qnrS*	**2**
14	*Aac (6')-1b- cr*	-
15	*QnrA, qnrB*	**2**
16	*QnrA, qnrB, qnrS*	**3**
17	*QnrA, qnrS*	**2**

The PMQR genes have changed the pattern of FQs resistance, particularly in Enterobacterales[26]. *K. pneumoniae* is a shelter for antimicrobial resistance genes and occasionally spreads genes horizontally[27]. Exposure to antimicrobial agents may convert silent antibiotic resistance genes (ARGs) into acquired ARGs and spread them[28]. Out of 32 FQs-sensitive *K. pneumoniae*, 53% harbored one or more PMQR genes. Based on the available literature, there is currently no evidence of similar percentages of FQs-sensitive *K. pneumoniae* harboring PMQR genes in Iraq or other countries, indicating that the results of this study are unique and have not been reported elsewhere[29,31] usually, studies focus on isolates that are already resistant to antibiotics, rather than those that are still sensitive. This could lead to an underestimation of the prevalence of PMQR genes in phenotypically sensitive bacteria. On the other hand, the *qepA* gene was not detected in the study (as shown in Figure 1), it is generally known that the prevalence of this gene is low worldwide, even in antibiotic-resistant bacteria[18]. However, it is important to note that the prevalence of the *qepA* gene can vary depending on several factors such as the study population, the type of bacteria, and the geographical location. Therefore, continued monitoring of the prevalence of this and other PMQR genes is crucial to guide appropriate antimicrobial therapy and infection control measures[32-34].

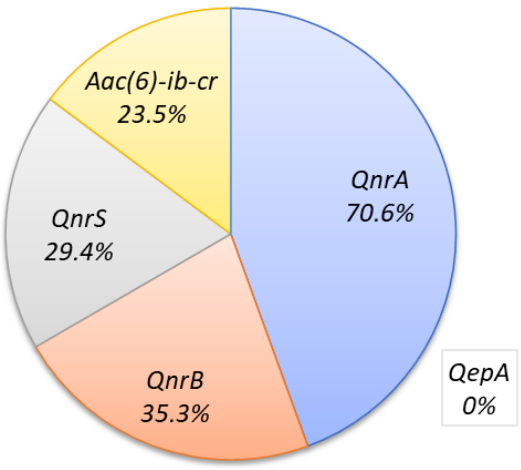

Figure 1: Prevalence and distribution of targeted PMQR genes qnrA, qnrB, qnrS, and aac (6)-Ib-cr in clinically isolated FQs-sensitive *K. pneumoniae.*

3.4 Co-carriage of silent PMQR genes

Figure 2 demonstrates that in a total of 17 PMQR-positive isolates, eight isolates contained two different types of PMQR genes, while one isolate contained three different types of PMQR genes. Each color in the figure represents a particular combination of PMQR genes that were present in the isolates. The green color indicates that three isolates contained both *qnrB* and *qnrA* genes. Table 3 details the distributions and co-carriage (combinations) of all 17 PMQR-positive isolates, providing a

more comprehensive representation of the relationships between PMQR genes in these isolates.

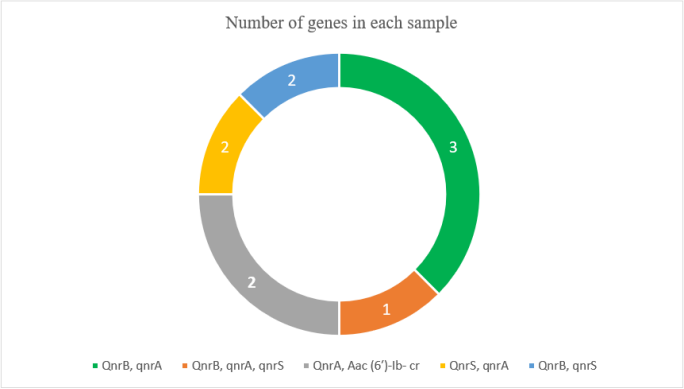

Figure 2: Distribution of co-carriage PMQR genes in (9) isolates of *K. pneumoniae.*

These findings suggest that multiple PMQR genes can coexist in the same bacterial isolate, which could potentially complicate efforts to control the spread of antimicrobial resistance. Even though these genes are silent, they can still pose a potential threat to public health. Silent genes can be activated under certain conditions[13-15]. It is known that plasmids or strains with two or more PMQR genes, such as *qnr* and *aac (6')-Ib-cr*, exhibit higher levels of phenotypically FQ-resistance[3]. Among 17 isolates of *K. pneumoniae* that harbored PMQR genes, 53% of isolates had more than one PMQR gene; nevertheless, they did not exhibit resistance to FQs. The *aac (6')-Ib-cr* gene is the most common PMQR mechanism found in FQ-resistant and sensitive clinical isolates [18], while in the current study, *qnrA* was most predominantly isolated. There could be several reasons that the *qnrA* gene was most predominantly isolated in the present study including antibiotic use, geographic location, and bacterial population dynamics[35,36]. Overall, the high co-carriage of PMQR genes in the present study could be related to over and misusing of FQs, improper hygiene, and the absence of clear and rigorous protocols for infection control measures. Therefore, continued monitoring and surveillance of PMQR genes are essential to understanding the evolution and spread of antibiotic resistance.

Numerous studies conducted worldwide have confirmed the existence of PMQR genes in clinical samples, these observations may help us to understand the phenomenon: A study in Syria, found out of 99 *Escherichia coli* (*E. coli*) and 23 *K. pneumoniae*, 42 isolates of both genera were sensitive to ciprofloxacin, they found that *qnrS and qnrB* genes in 28.5% and *aac (6')-Ib-cr* were among 64.28% of ciprofloxacin-sensitive and intermediate isolates; The study only detected genes without investigate silent genes or their effects on clinical isolates[32]. There are additional studies that emphasize the presence of different silent antibiotic-resistant genes among sensitive bacteria[12,37, 38]. These studies give us a great look at on the silent gene pool, which is not found by standard microbiological testing. In 2010, in Morocco, a study conducted on 39 extended-spectrum beta-lactamases-producers Enterobacterales, the *qnrA* and *qnrB* were identified among three ciprofloxacin-sensitive strains. They concluded that the presence of *qnr* genes among FQ-sensitive might lead to FQs-resistance during human infection[6]. Similarly, some researchers claim that these are normally expressed but are silent in lab conditions (in vitro) [39]. On the contrary, Stasiak *et al*., (2021) argued that silent genes could contribute new resistance features, and there is evidence that silent genes can be transmitted and activated, making the isolates phenotypically resistant[12,14]. A study in India found that 7 out of 43 *E. coli* were susceptible to FQs, and *qnr* genes were present in these FQs-sensitive *E. coli*. The researchers interpreted these genes as silent and suggested they could pose a threat by increasing the minimum inhibitory concentration of ciprofloxacin in future clinical isolates[40].

Moreover, in a study conducted on 40 *Salmonella* strains, 16 strains were sensitive to chloramphenicol and carried the *catA1* gene. The *catA1* gene found in sensitive strains was 99% similar to the gene found in resistant *Salmonella*. The researchers concluded that a deletion in the *catA1* gene's promoter was the reason for the absence of gene expression. Based on this finding, they hypothesized that the *catA1* gene could potentially function as a pool gene in the environment and be transferrable among bacteria[41]. In contrast, Enne et al. (2006) found that sensitive *E. coli* strain harbored silent resistance genes including *sul1*, *tetA*, *aadA1*, and *blaOXA*-2, and that their promoter regions were intact. When the plasmid from these silent isolates was transferred to another strain, expression was observed. They concluded that those genes are silenced because the chromosomal host is not plasmid by itself[25]. The same case has been experienced with Shiga toxin-producing *E. coli* the silent *aadA* gene in the Shiga toxin-producing *E. coli* was not fully expressed until it was moved to *Hafnia alvei*[42]. Similarly, D *et al*., (2019) investigated the expression of the *qnrB* gene in FQs-sensitive *E. coli* under gut conditions but found no changes despite intact gene and promoter region. Plasmid sequencing revealed the presence of a prophage (viral genome that has integrated into the DNA of a bacterial host cell)[15]. Prophages make up 20% of bacterial genomes and act as regulators by controlling gene expression through genome excision. However, their potential role in gene silencing remains unclear[43].

Our study highlights the potential threat of existing and co-carriage of silent PMQR genes in FQs-sensitive *K. pneumoniae* isolates, even if they are initially silent. This phenomenon can complicate efforts to control the spread of antimicrobial resistance. However, the mechanisms underlying gene silencing and activation remain unclear and may be influenced by various factors, such as the promoter region, chromosomal host, and prophages.

Conclusion

The current study found that 17/32 of FQs-sensitive *K. pneumoniae* isolates harbored silent PMQR genes, with *qnrA* being the most common gene detected. Additionally, 9/17 isolates carried more than one PMQR gene. This highlights the potential future risk for public health if these silent genes become active. However, research is needed to determine the specific factors contributing to the lack of expression of silent ARGs and their potential impact on the rising of antibiotic resistance.

Conflict of interests

There are no conflicts of interest associated with this publication.

Acknowledgements

We would like to thank all members of the Research Center of College of Science/ Duhok University and Azadi teaching hospital for their friendly collaboration in addition to all persons who helped us during conducting this work.

Author contribution

The sample collection, laboratory work, and manuscript writing were carried out by Mustafa M. Abdulkareem, while Najim A. Yassin provided supervision and contributed to reviewing the manuscript. Masood A. Abdulrahman contributed to the project by conducting data analysis and reviewing.

Funding

Author has not received any funds for this work.

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