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RESEARCH ARTICLE OPEN ACCESS

Study of Plasmid-Mediated Quinolone Resistance in *Klebsiella pneumoniae*: Relation to Extended-Spectrum Beta-Lactamases

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Abstract

***Klebsiella pneumoniae* (*K. pneumoniae*) is an important pathogen associated with various infections. The emergence of antibiotic resistances, such as quinolone resistance and those due to extended-spectrum beta-lactamases (ESBL), reduces the available choices for treatment. The objectives of the current study include the evaluation of the prevalence of the plasmid-mediated quinolone resistance genes *qepA, acrA, acrB,* and *aac(6')-Ib-cr* by polymerase chain reaction (PCR) in *K. pneumoniae* and the determination of the mechanism relating these genes to the ESBL phenotype and resistance to other groups of antibiotics. In total, 300 clinical isolates of *K. pneumoniae* were included in the study. Isolates were subjected to antibiotic sensitivity tests using the disc diffusion method. Quinolone resistance by the minimum inhibitory concentration method and detection of ESBL resistance by the double disc diffusion method were also determined. PCR analyses revealed the prevalence of *acrA, aac(6')-Ib-cr, acrB,* and *qepA* in 74.3%, 73.7%, 71%, and 6.7% of the isolates, respectively. Quinolone-resistant isolates positive for plasmid-encoded genes represented 82.7% of *K. pneumoniae* isolates positive for ESBL activity. The results also showed that the isolates of *K. pneumoniae* carrying plasmid-encoded quinolone resistance genes had significantly increased resistance to amikacin , amoxicillin/clavulanate, gentamicin, and cefoxitin than those isolates without quinolone resistance genes. Therefore, there was a high prevalence of *acrA, acrB,* and *aac(6')-Ib-cr* among *K. pneumoniae* and the prevalence of quinolone resistance was significantly associated with the ESBL resistance phenotype. Moreover, the presence of quinolone resistance genes was associated with resistance to aminoglycosides, namely amikacin and gentamicin.**

Keywords: Antibiotic resistance, *K. pneumoniae*, quinolone resistance genes, gram-negative bacilli

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INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) are non-motile, encapsulated gram-negative bacilli that may be found in the environment, such as soil and water, and on medical devices.[1,2] *K. pneumoniae* is associated with various human infections, such as urinary tract, respiratory tract, wound, and blood stream infections. Antibiotic therapies against *K. pneumoniae* include cephalosporins, aminoglycosides, and quinolones. However, resistance to commonly used antibiotics has emerged.[3,4]

Fluoroquinolone resistance is among the antibiotic resistances identified in *K. pneumoniae* in the last few years. Fluoroquinolone resistance can be mediated via two different mechanisms: through mutations in the chromosomally encoded genes for gyrase and topoisomerase IV enzymes or through plasmid-mediated quinolone resistance (PMQR) determinants, namely, Qnr determinants, QepA and AcrAB efflux pumps, and aminoglycoside-acetyltransferase Ib-cr enzymes.[5] QepA efflux pumps extrude antibiotics to the extracellular environment, leading to decreased antibiotic activity. The other efflux system, AcrAB, acts as a chemical transporter that transports various antibiotics to the extracellular environment, leading to antibiotic resistance.[6,7] AcrAB consists of an outer membrane component TolC, an inner membrane transporter AcrB, and a periplasmic protein AcrA that bridges the two integrated membrane components. *aac(6')-Ib-cr* encodes an acetyltransferase AAC(6')-Ib variant with substitutions in tryptophan 102 to arginine and in aspartic acid 179 to tyrosine. These substitutions lead to fluoroquinolone acetylation and to a reduction in susceptibility to fluoroquinolones and aminoglycosides, such as tobramycin, kanamycin, and amikacin.[8] Moreover, there are reports about the association between the presence of quinolone resistance and extended-spectrum beta-lactamases (ESBL) in *K. pneumoniae*.[9,10]

The objectives of the current study were to evaluate the prevalence of the PMQR genes *qepA, acrA, acrB,* and *aac(6')-Ib-cr* by polymerase chain reaction (PCR) in *K. pneumoniae* and to determine their relationship with the ESBL phenotype and with other antibiotic resistances.

MATERIALS AND METHODS

Bacterial Strains

The analysis included 300 isolates of *K. pneumoniae* obtained from different clinical samples (100 blood, 80 urine, 70 wound, and 50 sputum cultures) from King Fahd Hospital, Jazan, Saudi Arabia, from January 2018 to May 2020. *K. pneumoniae* isolates were identified using standard microbiological techniques, including Gram staining and manual biochemical identification methods (according to the Clinical and Laboratory Standards Institute [CLSI] guidelines).[11] Biochemical identification included the use of triple sugar iron agar, a motility test, the Simmons citrate test, and ornithine and lysine decarboxylation tests (Oxoid-Thermo Fisher Scientific, USA). *Escherichia coli* (*E. coli*) ATCC 25922 was used as the positive control for all biochemical reactions.

Antibiotic Susceptibility Tests by the Disc Diffusion Method

Antibiotic susceptibility tests were performed according to the CLSI guidelines using the disc diffusion method.[11] Briefly, pure colonies of *K. pneumoniae* were used for the preparation of cultures with a turbidity equivalent of 0.5 McFarland standards; cultures were spread over Mueller–Hinton agar. Discs were either impregnated with 5 µg of cefoxitin, 10 µg of imipenem, 20 or 10 µg of amoxicillin/clavulanate, or 30 µg of ceftazidime, tetracycline, cefotaxime, trimethoprim/sulfamethoxazole, gentamicin, amikacin, or cefepime (Oxoid-Thermo Fisher Scientific,). For antibiotic susceptibility testing, *Escherichia coli* strain ATCC 25922 was used as the quality control strain.

Minimum Inhibitory Concentrations (MICs) of the Quinolone Ciprofloxacin

The microdilution method (according to the CLSI guidelines) was used to determine the MIC of ciprofloxacin for the isolates.[11] The results were interpreted according to the CLSI guidelines; isolates were classified as resistant to ciprofloxacin at MICs ≥ 4.0 µg/mL, as ciprofloxacin sensitive at MICs ≤ 1.0 µg/mL, and as ciprofloxacin intermediates at MICs between 1 and 2.0 µg/mL. *E. coli* ATCC 25922 was used as the quality control strain.

Determination of Extended-Spectrum Beta-Lactamase (ESBL) activity in *K. pneumoniae*

The double disc diffusion method was used to determine the ESBL activity for isolates of *K. pneumoniae* resistant to ceftazidime and cefotaxime according to the CLSI guidelines. The double disc method was performed using discs impregnated with 30 µg of cefotaxime and 30 µg of ceftazidime alone or in combination with 10 µg of clavulanic acid. An increase of 5 mm or more in the inhibition zone around the discs after combining the beta-lactam antibiotic with clavulanic acid was considered positive for ESBL activity.[11] The ESBL-positive and negative control strains used in this study were *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603, respectively.

Determination of PMQR genes in *K. pneumoniae*

DNA Extraction

DNA from isolated colonies of *K. pneumoniae* was extracted using a Qiagen DNA extraction kit (Qiagen, Hampshire, United Kingdom). Extracted DNA was kept frozen at -20°C till amplification.

Molecular identification of *K. pneumoniae*

Table 1 shows the results from the molecular identification of *K. pneumoniae* isolates by PCR using universal 16S rRNA bacterial primers (16S forward and reverse primers). The PCR was carried out in a total volume of 50 µL using Dream Tag Green Master Mix (Thermo Fisher Scientific) containing 25 µL of master mix ready to use, 1 µL (10 pmol) of each reverse and forward primers, 5 µL of DNA template, and 18 µL of nuclease-free water. The mixture was subjected to an initial denaturation process at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C, and primer extension at 72°C for 1 min, and a final extension cycle at 72°C for 5 min. Negative and positive controls were included in each experiment. PCR products were analyzed by gel electrophoresis using 1.5% (wt/vol) agarose gels in Tris-Acetate EDTA buffer stained with ethidium bromide and visualized using a Gel Doc XR imaging system (Bio-Rad).

Standard Sequencing and Data Analysis

The PCR products were subjected to a high purification process. An ABI PRISM® 3730XL Analyzer with 96 capillary type using a BigDyeTM Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems) was used to perform sequencing reactions according to the manufacturer's protocols. Single-pass sequencing was performed on each template using 16S F primers. Gel elution was performed using the MG Gel Extraction SV (MD007) kit (MGmed), following the manufacturer's instructions.

The sequence similarity was examined and compared to the reference sequences through the Basic local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/BLAST/) and GenBank (www.ncbi.nlm.nih.gov/genbank/).[12]

An evolutionary tree was constructed for the sequenced samples using molecular evolutionary genetic analysis (MEGA 5).

PCR for PMQR gene detection in *K. pneumoniae*

The amplification of qepA, acrA, aac(6')-Ib-cr, and acrB was carried out using the

Table 1. Genes, primers and the amplified base pair (bp)

Gene	Sequence of the primer	bp
16S	5'- AGA GTT TGA TCC TGG CTC AG - 3'	
	5'- ACG GCT ACC TTG TTA CGA CTT - 3'	1500
QepA	5'- CTGCAGGTACTGCGTCATG -3'	
	5'- CGTGTTGCTGGAGTTCTTC -3'	403
acrA	5'- TCTGATCGACGGTGACATCC -3'	
	5'- TCGAGCAATGATTTCCTGCG -3'	157
acrB	5'- CAATACGGAAGAGTTTGGCA -3'	
	5'- CAGACGAACCTGGGAACC -3'	64
Aac(6')-Ib	5'- TTGCGATGCTCTATGAGTGGCTA -3'	
	5'- CTCGAATGCCTGGCGTGTTT -3'	611

Table 2. Similarity to some *K. pneumoniae* isolates from Gen Bank

Gen Bank sequence ID	Country
MH396759.1	Nigeria
MT415713.1	China
MH542253.1	India
MK156321.1	Turkey

primers listed in Table 1. A Qiagen (ready to use) amplification mixture (25 µL) was used for the entire process.

The amplification process was performed as follows: an initial denaturation step at 94°C for 5 min, 36 amplification cycles consisting of 45 s at 94°C, 45 s at 55°C, and 45 s at 72°C, and a final extension step at 72°C for 5 min.[8]

Gel electrophoresis of the amplification products was carried out on a 1% agarose gel stained with ethidium bromide.

Positive PCR products were purified, and direct sequencing was performed to confirm the positive results (Thermo Fisher Scientific).

Statistical Analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPPS) 22 and were stated as number and percentage. The results were compared using the chi-square test, P values < 0.05 were considered as statistically significant.

RESULTS

Molecular identification

To confirm the biochemical identification results, approximately 1500 bp of the 16S gene of each isolate were amplified (Fig. 1) and partial sequencing of the PCR products using the 16S forward primer was performed. BLAST search results showed that the 16S sequences from the isolates from the Jazan region aligned with many *K. pneumoniae* sequences published in GenBank (Table 2). Sequences were also used to determine the evolutionary relationship between *K. pneumoniae* isolates from the Jazan region and other isolates from GenBank (Fig. 2).

Table 3. Prevalence of ESBL and quinolone resistance among isolated *K. pneumoniae*

Phenotypic resistance	No. of isolates	%
ESBL	197	65.7
Quinolone resistance	240	80

Table 4. Frequency of *acA, acrB, qebA* and *aac (6')-Ib-cr* in *K. pneumoniae* (n=300)

Gene	No.	%
acrA	223	74.3
acrB	213	71
qebA	20	6.7
aac (6')-Ib-cr	221	73.7

Quinolone resistance, as determined by ciprofloxacin MICs ≥ 4.0 µg/mL, was found in 240 (80%) of the *K. pneumoniae* isolates, and ESBL was determined in 197 (65.7%) of the isolates as well (Table 3).

A PCR study searching for PMQR genes was positive in 233 of the isolates. acrA was found in 74.3% of the isolates, whereas *aac(6')-Ib-cr, acrB,* and *qepA* were found in 73.7%, 71%, and 6.7% of the *K. pneumoniae* isolates, respectively (Table 4).

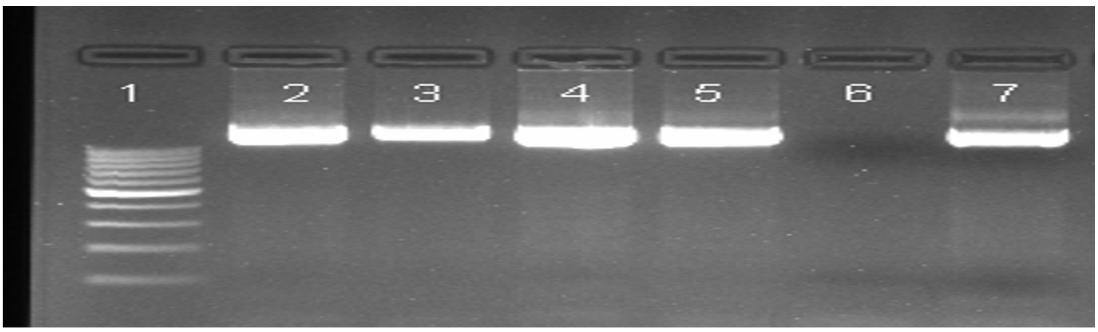

Fig. 1. Agarose gel electrophoresis image for 16S gene of *K. pneumoniae* using universal primers. Lane1; 100bp ladder, lane 2; positive control, lanes 3,4,5,7 isolate samples and lane 6 negative control.

K. pneumoniae positive for PMQR genes represented 82.7% of the isolates that were also positive for ESBL, as determined by the double disc method (Fig. 3).

Antibiotic resistance was compared between PMQR-positive and negative *K. pneumoniae* isolates (Table 5). The results showed that the isolates of *K. pneumoniae* positive for PMQR genes had significantly increased resistance to amikacin (P = 0.0001), amoxicillin/clavulanate (P = 0.0001), gentamicin (P = 0.001), and cefoxitin (P = 0.002).

DISCUSSION

Recently, there has been an increase in worldwide reports on PMQR genes in *K.*

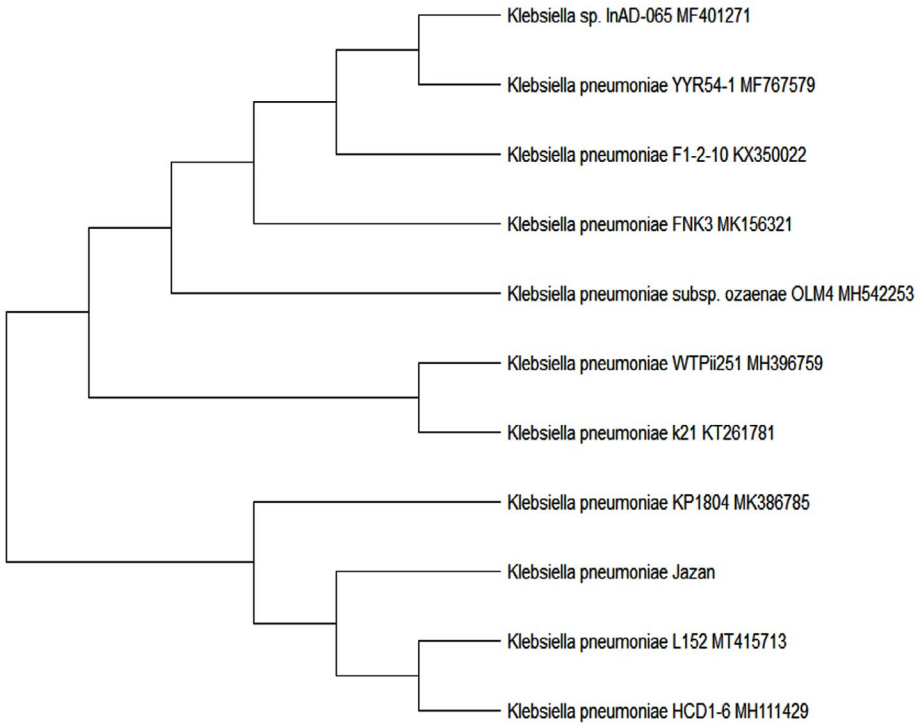

Fig. 2. Evolutionary relationship of taxa for *K. pneumoniae* isolates from Jazan region.

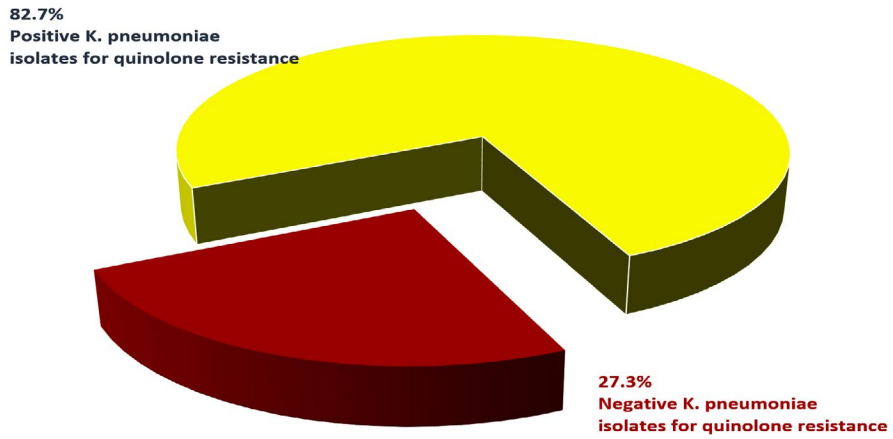

Fig. 3. Positive *K. pneumoniae* isolates for quinolone resistance genes among isolates positive for ESBL. P=0.01

Table 5. Comparison of antibiotics resistance between positive versus negative *K. pneumoniae* for the studied genes

Antibiotics	Positive *K.pneumoniae* isolates for genes (n=233) No. %	Negative *K.pneumoniae* isolates for genes (n=67) No. %	P
Amikacin	101	13	P=0.0001
Tetracycline	89	22	P=0.3
amoxicillin–clavulanate	156	26	P=0.0001
chloramphenicol	169	49	P=0.5
Cefotaxime	158	55	P=0.01
Ceftazidime	155	50	P=0.5
Gentamicin	103	15	P=0.001
Trimethoprim sulfamethoxazole	92	23	P=0.7
Cefepime	106	34	P=0.7
Cefoxitin	122	50	P=0.002
Imipenem	106	29	P=0.4

pneumoniae. Most of these studies report a higher prevalence of PMQR genes in *K. pneumoniae* compared than in *E. coli.*[13,14] However, the rates of prevalence vary considerably with the geographic area.[13,15]

In the present study, 80% of the *K. pneumoniae* isolates were found to be resistant to quinolones, according to the MIC study. A high frequency of resistance to quinolones has been reported previously, ranging from 41% to 89%.[16-18] Resistance to fluoroquinolone may be attributed to improper use of quinolone, especially in urinary tract infections, leading to the transference of resistance genes to other susceptible *Enterobacteriaceae* by horizontal plasmid transfer.

A significant association between ESBL production and the presence of PMQR genes was found (P = 0.01). This phenomenon has also been shown in previous studies, with differences in prevalence rates ranging from 5% to 48%.[19-22] Moreover, a recent study reported that all *K. pneumoniae* strains with quinolone resistance genes were also ESBL strains.[10] The co-resistance to fluoroquinolones and beta-lactams was explained by the presence of quinolone-resistant genes, including ESBL determinants, in identical mobile genetic elements.[10]

The molecular investigation done in the present study revealed that the *acrA* and *acrB* frequencies in *K. pneumoniae* were 74.3% and 71%, respectively. These results are in line with a previous study by Heidary et al.[8] in which it was determined that efflux systems mediated antibiotic resistance; the AcrAB efflux pump, which plays a vital role in the resistance of *K. pneumoniae* to quinolone was included.[23-25]

The second most prevalent gene was *aac(6')-Ib-cr.* Previous studies also reported a high frequency of *aac(6')-Ib-cr* associated with quinolone resistance.[8] *Aac(6')-Ib-cr* (ciprofloxacin resistance) is a variant of Aac(6')-Ib; it is responsible for the resistance to tobramycin, amikacin, and kanamycin due to two amino acid substitutions involved in acetylation and subsequent reduction reactions Thus, Aac(6')-Ib-cr may be responsible for diminishing the activity of several antibiotics, such as norfloxacin and ciprofloxacin subsequently reduced.[26-28] Therefore, in addition to quinolone resistance, PMQR determinants can play a significant role in resistance to other antibiotics, particularly β-lactams and aminoglycosides. This hypothesis supports the finding of increased resistance to amikacin and gentamicin among isolates positive for quinolone resistance genes.

The least frequently determined gene was *qepA.* Previous studies have determined a prevalence ranging from 0% to 12%.[29-31]

The current study highlights the emergence of the quinolone resistance plasmid-

encoded genes *acrA, acrB, qepA,* and *aac(6')-Ib-cr* in clinical isolates of *K. pneumoniae*. A high prevalence of all these genes was found, except for qepA. Moreover, the existence of quinolone resistance genes was associated with resistance to aminoglycosides, such as amikacin and gentamicin.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not Applicable.

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