Egyptian Journal of Medical Microbiology Volume 34 / No.3 / July 2025 173-182 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Study of *entB* Gene and Some Quinolone Resistance Genes (*qnrB* and *acc(6')-Ib-cr*) of *Klebsiella pneumoniae* in Community-Acquired Infections in Kirkuk City

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Key words:
Community-acquired Klebsiella pneumoniae, entB, qnrB, aac (6')-Ib-cr, PCR

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ABSTRACT

Background: *Community-acquired Klebsiella pneumoniae (CAKP) infections arise in people outside hospital environments and can affect various body parts. There is a growing problem with antibiotic resistance, particularly fluoroquinolone resistance, which limits treatment choices for CAKP infections.* ***Objective:*** *This study aimed to detect the entB gene and some fluoroquinolone resistance genes qnrB and aac(6')-Ib-cr of K. pneumoniae in Community-acquired infections.* ***Methodology:*** *Two hundred fifty-six different clinical samples were collected from outpatients of both sexes suffering from community-acquired infections aged less than one to eighty years. K. pneumoniae was isolated and identified using traditional microbiological methods, and subsequently confirmed with the VITEK 2 compact system. Antibiotic sensitivity was assessed for Gemifloxacin and Norfloxacin by disc diffusion method, and the minimum inhibitory concentration of Ciprofloxacin was evaluated using VITEK 2 AST-GN-419 cards. EntB, qnrB, and aac(6')-Ib-cr genes were detected using polymerase chain reaction (PCR).* ***Results:*** *Only thirty-eight non-duplicate K. pneumoniae isolates were identified. The antibiotic susceptibility test revealed 55.3% resistance of isolates to the ciprofloxacin, 50% to the gemifloxacin, and 42.1% to norfloxacin. PCR analysis showed that of all 38 K. pneumoniae isolates, 100% harbored the entB gene. Among 38 isolates, only 26 isolates were phenotypically resistant to fluoroquinolone and they were involved in the molecular detection of plasmid mediated-quinolone-resistant genes (PMQR), the acc(6')-Ib-cr gene was identified in 13 (50%) isolates. In contrast, qnrB was found in 9 (34.6%).* ***Conclusion:*** *A high prevalence of virulence factors can enhance the pathogenicity of K. pneumoniae in infections acquired in the community. Among the factors determining PMQR, the aac (6')-Ib-cr gene tends to be prevalent, followed by the qnrB gene.*

INTRODUCTION

The Gram-negative bacteria *Klebsiella pneumoniae* (*K. pneumoniae*) is known to cause various human diseases and is considered an opportunistic pathogen[1]. This bacteria is associated with several disseminated diseases, such as urinary tract infections (UTIs), pneumonia, surgical site infections, and septicemia in hospital and community settings[2].

The pathogenicity of *K. pneumoniae* mostly results from many virulence factors that enable it to bypass innate host immunity and sustain infection in a mammalian host. These virulence characteristics facilitate its survival in various circumstances and thus contribute to developing disease in the human host. The infectious ability of *K. pneumoniae* is affected by many virulence factors, including fimbrial adhesins, lipopolysaccharides, capsules, biofilm formation, and siderophores[3]. Enterobactin siderophores (*entB*) produced by *K. pneumoniae*, are small molecules that can extract iron from the ferrous-chelating proteins found in their host. This enables the bacterium to evade neutralization by the host[4].

K. pneumoniae is part of the ESKAPE pathogenic group that contains *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, all of which avoid antimicrobial agents through several mechanisms[5]; especially acquiring antibiotic resistance genes, to counteract frequently used antimicrobial agents[6]. Fluoroquinolones (FQs) are one of the last antibacterial options for treating infections induced by *K. pneumoniae*[7]. Among *Enterobacteriaceae*, it is the most prevalent fluoroquinolone-resistant bacterium. One of the most common fluoroquinolones used for treating bacterial infections is ciprofloxacin. Because of their wide range of effects and widespread use in treating infectious diseases, fluoroquinolones rapidly grow resistant to many bacteria[8].

Quinolone resistance can develop from two mechanisms: chromosomal mutation or PMQR. Chromosomal mutations alter target enzymes and the binding affinity of medications. Chromosomal alterations occur in genes located within the quinolone resistance-determining regions (QRDR). The *qnr* genes (A, B, C, D, and VC) protect DNA gyrase and topoisomerase IV against the effects of quinolone antibiotics, leading to plasmid-mediated quinolone resistance[9]. The *acc(6')-Ib-cr* gene, which codes for a specific kind of aminoglycoside transferase that acetylates certain fluoroquinolones, is another mechanism known to contribute to fluoroquinolone resistance[10]. Regarding the rise in antibiotic resistance among *K. pneumoniae* isolates and the crucial role of virulence genes on their pathogenicity, the current study aims to assess the prevalence of enterobactin gene and some fluoroquinolone resistance genes in Community-acquired *Klebsiella pneumoniae* infections in Kirkuk City.

METHODOLOGY

Patient and sample collection

The current study included two hundred and fifty-six (256) outpatients from four major hospitals in Kirkuk from September 2023 to December 2023. The 256 clinical samples were collected from various body locations (such as urine, wounds, sputum, burns, bronchial wash, diabetic foot ulcers, and vaginas), only 38 *K. pneumoniae* were isolated.

The outpatients were aged from <1 to 80 years of both sexes. The samples obtained from outpatients were categorized as infected according to the clinical symptoms demonstrated by each patient.

Sputum and urine specimens were collected in sterile, clean, properly labeled, leak-proof containers free of visible contamination and transported to the laboratory immediately. For the collection of burns, diabetic foot ulcers, high vaginal swabs, and wound exudate, sterile cotton swabs with transport media (Amie's) were used to collect the specimen from the infected sites with aspect precautions, they were subsequently transported to the microbiology laboratory for additional analysis.

Isolation and bacteriological identification of *K. pneumoniae* isolates

All clinical samples were cultured separately on MacConkey and Blood agars, incubating for 24 hours at 37°C. After that, Eosin methylene blue agar was used to subculture big, lactose-fermenting mucoid colonies to differentiate between *K. pneumoniae and E. coli.* Isolated colonies were purified via subculturing on MacConkey agar; isolates thought to belong to the *Klebsiella* genus were selected after incubation for identification based on conventional biochemical tests. All *K. pneumoniae* isolates were confirmed by Vitek2 gram-negative (GN) identification cards and the isolates were then maintained in Brain heart infusion broth with 20% glycerol at −20°C[11].

Antimicrobial susceptibility testing (AST) of *Klebsiella pneumoniae* isolates

The Disc diffusion method for testing antibiotic susceptibility

Antibiotic susceptibility testing for gemifloxacin (5 µg) and norfloxacin (10 µg) (Himedia, India); was performed on all identified *K. pneumoniae* isolates. The Kirby-Bauer disk diffusion on Muller Hinton agar was used to estimate the susceptibility following Clinical and Laboratory Standard Institute CLSI (2023) guidelines[12].

The Vitek2 system for AST

The sensitivity testing of all examined *K. pneumoniae* isolates was conducted by calculating the minimum inhibitory concentration (MIC) for ciprofloxacin (0.06, 0.12, 0.5, 1 ug/mL) with Vitek 2 GN AST-N419 cards.

Molecular detection of *K. pneumoniae entB, qnrB,* and *acc(6')-Ib-cr* genes

Extraction of genomic DNA

K. pneumoniae isolates genomic DNA (gDNA) was isolated utilizing the Blood/Cultured Cells genomic DNA extraction mini kit (FAVORGEN, Taiwan), this kit was developed to extract DNA from Gram-negative bacteria. The DNA fragment was preserved at -20ºC.

Quantitation of *K. pneumoniae* gDNA

A Quantus Fluorometer was employed to measure the purity and concentration of isolated DNA and evaluate the quality of samples for subsequent applications. For 1 µl of DNA, 200 µl of diluted Quantifluor Dye was added. The DNA concentration readings were recorded after five minutes of incubation at room temperature[13].

Selection and preparation of primers

The sequences of the oligonucleotide forward (F) and reverse (R) primers designed to detect *entB*, *aac(6')-Ib-cr*, and *qnrB* as listed in Table (1) below:

Table 1: The primers utilized in PCR amplification for the detection of *entB*, *qnrB*, and *acc(6')-Ib-cr* genes

Name of primer	Oligonucleotide sequence (5`→3`)	Annealing temp.	Amplicon size	References
entB	F: ATTTCCTCAACTTCTGGGGC	58°C	371bp	14, 15
	R: AGCATCGGTGGCGGTGGTCA			
aac(6')-Ib-cr	F: TTGCGATGCTCTATGAGTGGCTA	54°C	482bp	15
	R: CTCGAATGCCTGGCGTGTTT			
qnrB	F: GATCGTGAAAGCCAGAAAGG	52°C	469bp	15
	R: ACGATGCCTGGTAGTTGTCC			

Macrogen Company supplies these primers in a lyophilized form. A 100 pmol/µl stock solution was prepared by diluting lyophilized primers in 300-µl nuclease-free water. A working solution of these primers was prepared by mixing 10 µl of the primer stock solution (stored at -20°C) with 90 µl of nuclease-free water to attain a concentration of 10 pmol/µl, therefore preventing repetitive thawing and refreezing[16].

PCR reaction setup protocol and thermal cycling

PCR was used to identify *entB*, *aac(6)-Ib-cr*, and *qnrB* genes by amplifying these specific DNA targets using designated primers. The steps for DNA amplification for detection of *entB*, *aac (6)-Ib-cr*, and *qnrB* genes were performed in a final volume of 20 µl, including 10 µl of Go Taq Green Master Mix (Promega, USA), 2 µl of template DNA, 1 µl of forward primer, 1 µl of reverse primer, and 6 µl of nuclease-free water. The optimal conditions to identify these genes are listed in Table (2) below:

Table 2: Thermal cycling protocol for PCR assay

Target gene	Steps of amplification	Temperature (˚C)	Time (Min.: Sec.)	Cycles
entB	Initial denaturation (predenaturation)	95	05:00	1
	Denaturation	95	00:30	30 cycles of amplification
aac(6)-Ib-cr	Annealing	58,52,54	00:30	
	Extension	72	00:30	
qnrB	Final extension	72	07:00	1
	Hold	10	10:00	

Agarose gel electrophoresis and DNA loading

The resultant products of PCR were loaded on 1.5% agarose gel in 1X Tris-acetate-EDTA buffer (TAE)-electrophoresis buffer (Promega, USA). The pores in the gel were filled with 5 µl of PCR products. The power supply was switched on at 100 volts per milliampere for 60 minutes; the DNA migrated from the cathode to the anode. A gel imaging system (OWL Electrophoresis System, Thermo, USA) was used to view the 0.5 µg/ml Ethidium Bromide (Promega, USA) stained bands in the gel. The size of the PCR products was determined using a DNA ladder of 100 bp (Promega, USA).

Statistical analysis

The data were examined using SPSS software (version 28.0), and a p-value of less than 0.05 indicated statistical significance.

RESULTS

Patients and *K. pneumoniae* isolate characteristics

Two hundred fifty-six (256) clinical samples were obtained from outpatients in four major hospitals: Azadi Teaching Hospital, General Pediatrics Hospital, Kirkuk General Hospital, and AL-Naser Hospital in Kirkuk City. Based on the results of cultural and biochemical testing, thirty-eight (14.8%) isolates of *K. pneumoniae* were identified among the 256 samples.

The results in Figure (1) indicate that the highest distribution of *K. pneumoniae* was in 16 patients (42%) were aged 21-40 years, whereas nine patients (24%) were aged 1-20 years, 7 patients (18%) were aged 41-60 years, and 5 patients (13%) were aged 61-80 years. One patient (3%) belonged to the age group <1 year. The relationship between the age group patterns of *K. pneumoniae* infection was statistically highly significant (p-value = 0.003).

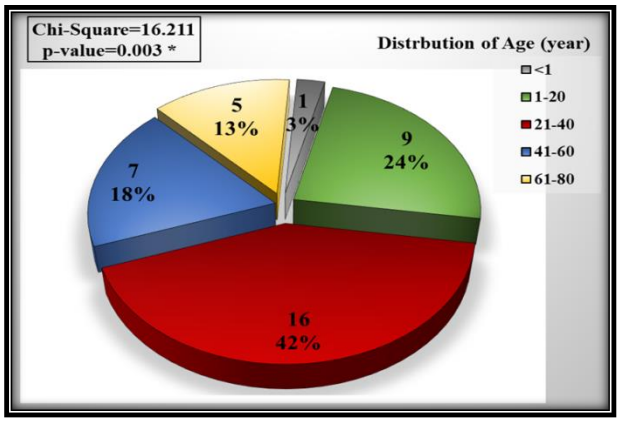

Fig. 1: Distribution of *K. pneumoniae* isolates by patient age

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Isolation and bacteriological identification of *Klebsiella pneumoniae* isolates

Based on the results of gram staining and macroscopic examination of the cultured plate as shown in Table (3), 110 (74.3%) of the bacterial isolates from patients with different infections were gram-negative. In comparison, 38 (25.7%) were gram-positive, with a p-value of 0.001, representing a highly significant distribution.

Table 3: Distribution of gram-negative and gram-positive bacterial isolates among different clinical samples

Bacteria strain	Number	%	p-value
Gram Positive isolates	38	25.7	Chi-Square= 35.027 P<0.001**
Gram Negative isolates	110	74.3	
Total	148	100%	

The morphological features of the colonies on MacConkey, blood agar, and EMB agar carried out preliminary identification of *K. pneumoniae* isolates (No. 38). On MacConkey agar, *K. pneumoniae* colonies were mucoid, big, and pink sometimes with centers that are cream-colored because of lactose fermentation. Furthermore, *K. pneumoniae* colonies on blood agar had a mucoid, round, greyish-white, translucent-opaque morphology and demonstrated gamma hemolysis (non-hemolytic).

To distinguish *K. pneumoniae* from *E. coli* colonies, all isolates were cultivated on EMB, resulting in large, mucoid colonies that were pink to purple, devoid of a green metallic sheen. In addition, an array of conventional biochemical assays were performed to identify *K. pneumoniae* isolates according to[11] and finally, all *K. pneumoniae* isolates were confirmed with the Vitek 2 compact system.

Antimicrobial susceptibility testing of *K. pneumoniae* isolates

All 38 *K. pneumoniae* isolates exhibited varying resistance levels to fluoroquinolones, with the highest resistance rates noted against Ciprofloxacin at 55.3%, 50% to Gemifloxacin, and 42.1% to Norfloxacin, out of 38 *K. pneumoniae* isolates 26 isolates that is resistant and intermediate to fluoroquinolones involved in the molecular study of FQS resistance genes as shown in Table (4).

Table 4: Antibiotic susceptibility tests of *K. pneumoniae*

Class of antibiotic	Antibiotics	R	%	I	%	S	%	T
Fluoroquinolones	Ciprofloxacin	21	55.3	2	5.2	15	39.5	38
	Gemifloxacin	19	50	3	7.9	16	42.1	38
	Norfloxacin	16	42.1	2	5.3	20	52.6	38

Molecular detection of *K. pneumoniae entB, qnrB,* and *acc(6')-Ib-cr* genes

The concentration and purity of extracted DNA from colonies identified as *K. pneumoniae* were assessed using the Quantus fluorometer. The concentration ranged from 2.1 to 84 ng/µl. Molecular testing showed that all 38 *Klebsiella pneumoniae* isolates (100%) were positive for the *entB* gene. Of 38 isolates, only 26 isolates were fluoroquinolone-resistant. Concerning these 26 isolates, 13 isolates (50%) were positive for *acc (6')-Ib-cr* gene while 9 isolates (34.6%) were positive for the *qnrB* gene, as shown in Figures (2, 3, and 4), respectively.

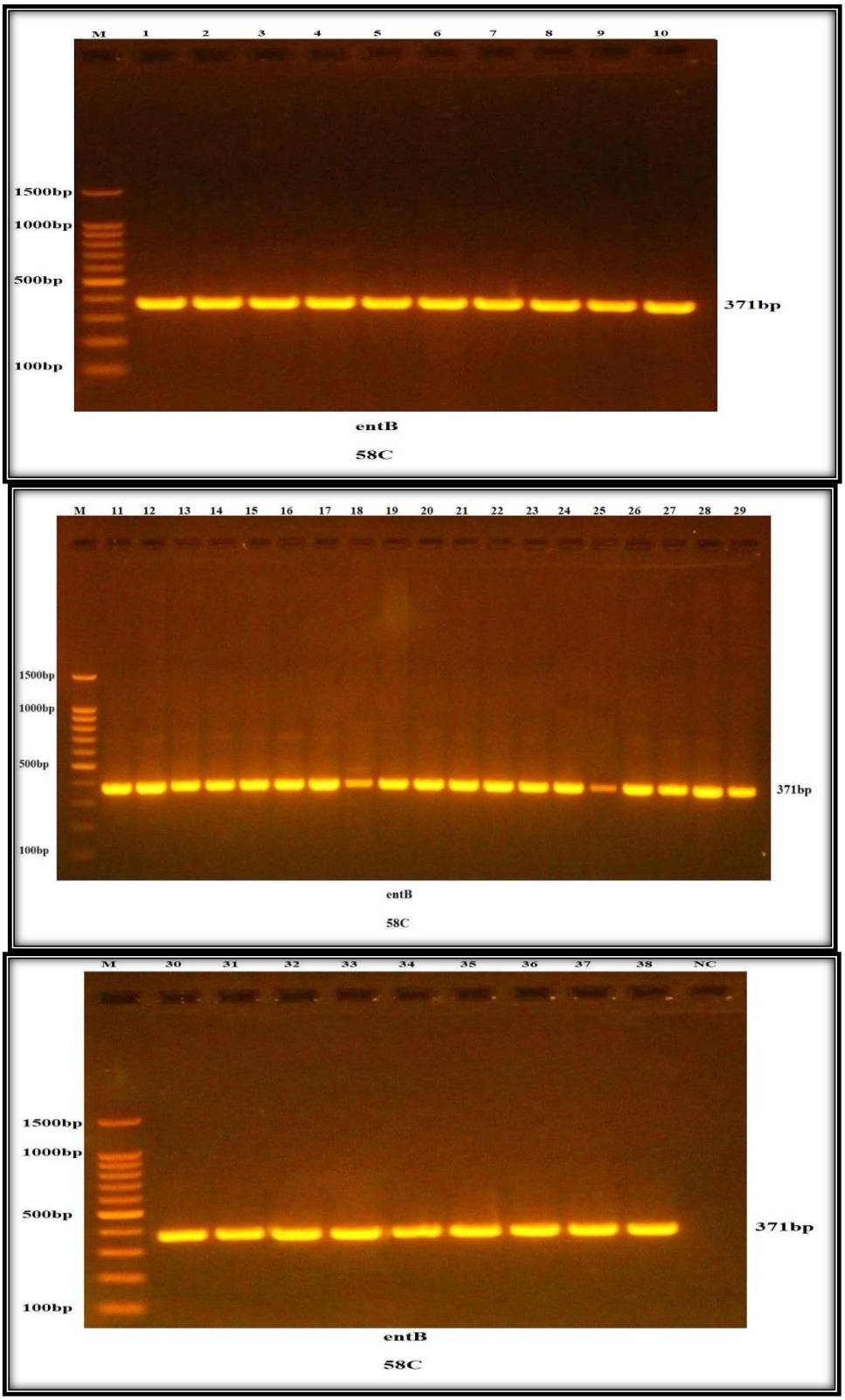

Fig. 2: The *entB* gene amplification results of *K. pneumoniae* were separated using 1.5% agarose gel electrophoresis and subsequently stained with ethidium bromide. M: 100 bp ladder marker. NC: negative control. Lanes 1-38 exhibit 371 bp PCR products

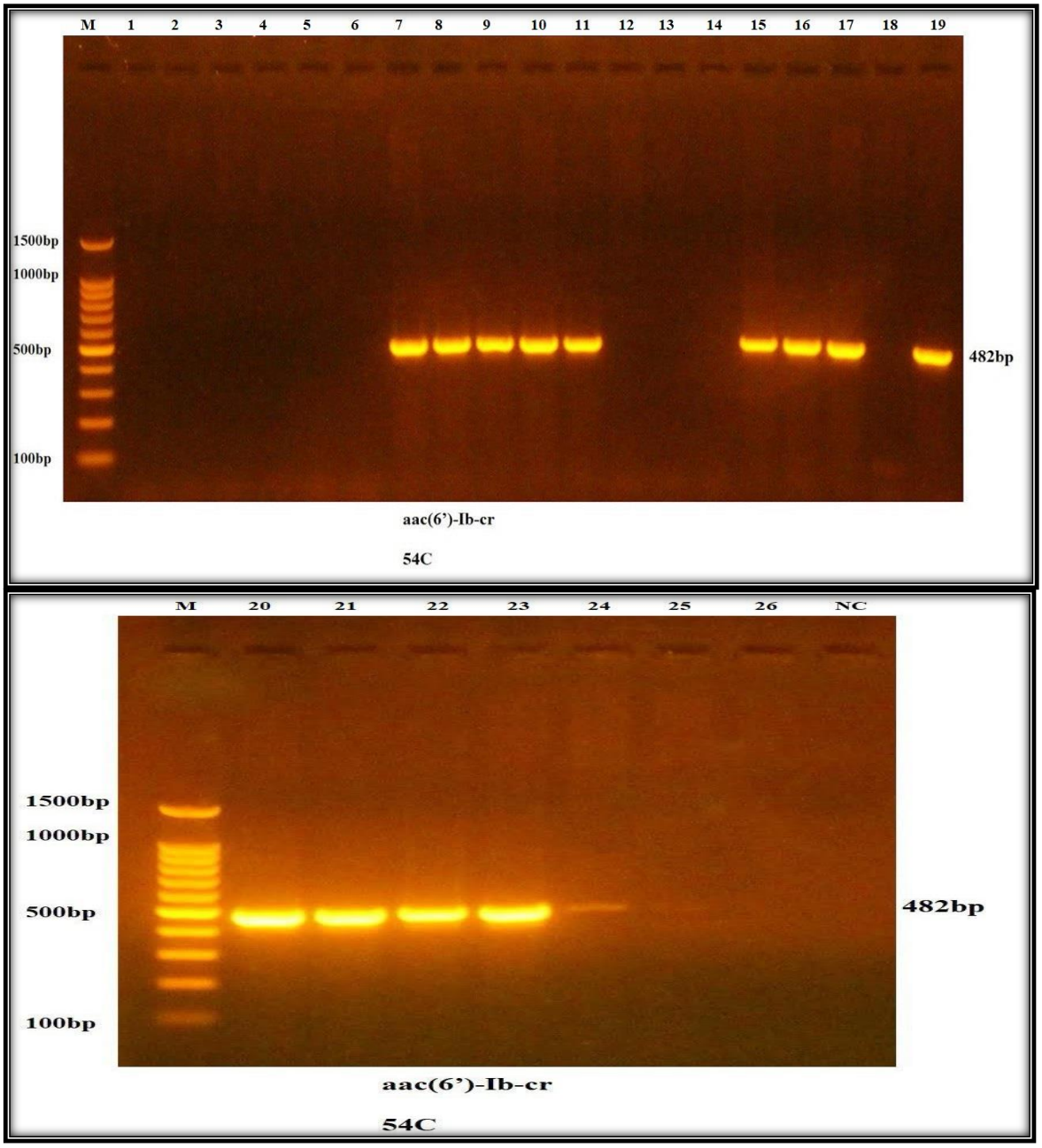

Fig. 3: The *aac(6')-Ib-cr* gene amplification results of *K. pneumoniae* were separated using 1.5% agarose gel electrophoresis, stained with ethidium bromide. M: 100 bp ladder marker. NC: negative control. Lanes 1-26 exhibit PCR products with 482 bp

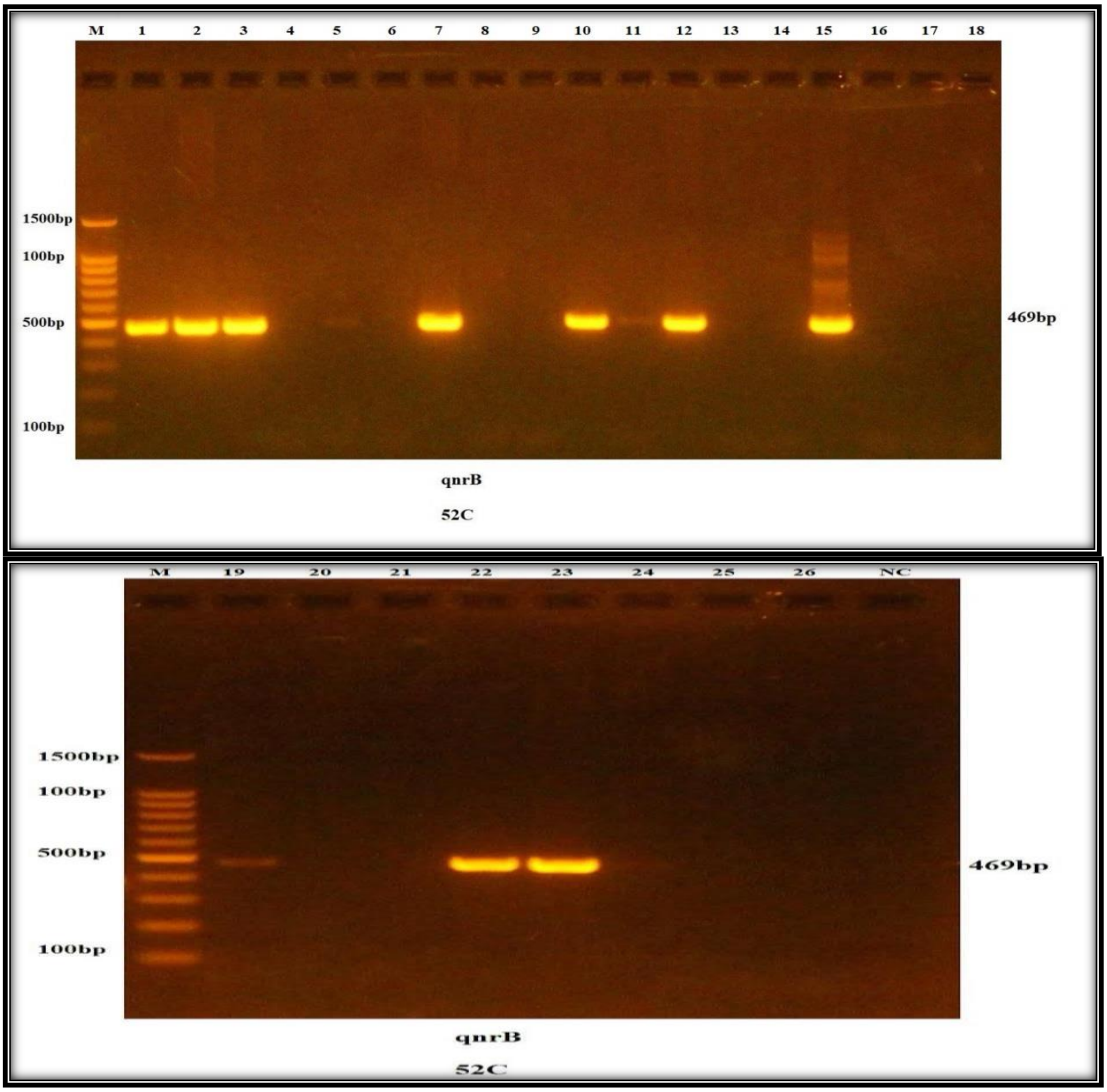

Fig. 4: The *qnrB* gene amplification results of *K. pneumoniae* separated using 1.5% agarose gel electrophoresis, stained with ethidium bromide. M:100 bp ladder marker. NC: negative control. Lanes 1-26 exhibit PCR products with 469 bp

DISCUSSION

The frequency of *K. pneumoniae* infection in this study was in line with the previous research results[17] that found that 19 of the infected individuals with *K. pneumoniae* were between the ages of (20 and 40 years) and three (10%) individuals were aged >60 years. In contrast, our results disagreed with the results of a study[18], which showed that the highest infection rate was among those aged 1–20 (18.1%), followed by those aged 41–60 (15.9%). The relationship between the age group profiles of *K. pneumoniae* infection was determined to be statistically insignificant. The increased infection rate in the age group 21-40 years may be attributed to the young age group participating in outdoor activities, making them the most active group[19]. In addition, the increased infection was also seen in the age group 40-60 years may be because increased age correlates with a heightened risk of *K. pneumoniae* infection due to a greater incidence of concurrent illnesses[20].

Of the isolates, 110 (74.3%) were gram-negative bacteria, whereas 38 (25.7%) were gram-positive bacteria, demonstrating the prevalence of gram-negative bacteria in Community-acquired infections. These results were comparable with Thapa *et al* [21], which demonstrated that (161/224) isolates 71.9% were gram-negative bacteria, whereas just (63/224) isolates 28.1% were gram-positive bacteria.

Gram-negative bacteria are responsible for various illnesses, including pneumonia, bloodstream infections, surgical site infections, and meningitis, among others. Gram-negative bacteria exhibit resistance to several medicines, indicating a potential for developing

resistance to most current antibiotics. This phenomenon may be ascribed to their inherent capabilities to discover alternate methods of developing resistance, hence resulting in considerable illness and mortality globally. The significant frequency of bacterial isolates identified in this study illustrates the necessity for efficient monitoring and surveillance of bacterial illnesses in resource-constrained healthcare settings[22].

Regarding the fluoroquinolone class, the resistance levels of *K. pneumoniae* to Ciprofloxacin and Norfloxacin (both of them are second-generation fluoroquinolones) in this investigation were 55.3% and 42.1%, aligning with the results of previous local study[23], which revealed a resistance rate at 57.1% and 42.8%, respectively. Furthermore, Gemifloxacin is a fourth-generation fluoroquinolone. The resistance rate of 50% reported in the current study, agreed with a study done in India in which resistance of *K. pneumoniae* isolated from wound infections toward Gemifloxacin was (51%) [24]. Furthermore, a local study done in Baghdad[25] reported the resistance levels of Ciprofloxacin and Norfloxacin were 60.4% and 55.8% respectively which nearly agrees with our study results regarding these antibiotics. In contrast, a study conducted in Pakistan in which *K. pneumoniae* isolated from outpatient UTIs exhibited the highest rate of resistance to Ciprofloxacin and Norfloxacin at 88.9% and 96.3%, which is confused with the current study result[26].

Antibiotic resistance mechanisms emerged because of the overuse of these drugs to treat antibiotic-associated infections. Of all antibacterial agents, Ciprofloxacin has the highest global consumption. Topoisomerase target change by mutation is one of the ways that might cause resistance to quinolones. The Qnr determinant resides on a plasmid-encoded integron, initially identified in *Klebsiella pneumoniae* and subsequently in *E. coli* as well as *Enterobacter cloacae*[27].

In our investigation, the *entB* gene encoding enterobactin was detected in 100% of all isolates, aligning with the result of a study carried out in Iran[6] which exhibited all isolates were positive for the *entB* gene. In addition, our results were inconsistent with a study, which also found that the *entB* gene was present in 90.5% of all isolates[28] but disagreed with another study, which reported a lower prevalence than our results of this gene (68%) [29]. The capacity to get iron during infection is essential for *K. pneumoniae* pathogenesis. The main strategy employed by numerous pathogens is the synthesis of siderophores, which are molecules that have a greater affinity for iron than host transport proteins[15].

The most important class of antibacterial drugs for treating bacterial infections is fluoroquinolones. Clinical isolates of bacteria resistant to fluoroquinolones have been rising recently[30].

This study investigated 26 fluoroquinolone-resistant isolates of *K. pneumoniae* to assess the frequency of PMQR genes: *aac(6')-Ib-cr* gene and *qnrB* gene. Out of 26 isolates half of them 13 isolates 50% harbored the *aac(6')-Ib-cr* gene while 9 isolates 34.6% harbored the *qnrB* gene. This finding was inconsistent with the research done in Iran[31], which found that MDR *K. pneumoniae* isolated from community-acquired infections had a frequency of 50% for the *aac(6')-Ib-cr* and 25% for the *qnrB* genes. In another study carried out in Baghdad[10] in which *K. pneumoniae* isolates were obtained from inpatients and outpatients' different clinical samples reported that 47.1% of isolates were positive for *aac(6')-Ib-cr* gene and only 29.4% of isolates positive for *qnrB.* PMQR genes are thought to significantly contribute to the development of quinolone-resistant strains due to their high horizontal transferability. The *aac(6')-Ib-cr* gene was frequently identified in *Enterobacteriaceae* isolates globally, indicating its significant spread to other Gram-negative species[10].

However, studies regarding the occurrence of PMQR genes in community isolates are somewhat limited. Therefore, we believe our study was carried out for the first time in Kirkuk City.

CONCLUSION

A high frequency of virulence factors such as gene-encoding siderophores (*entB*) enhances the pathogenicity of *K. pneumoniae* in infections acquired in the community.

There was a moderate frequency of Ciprofloxacin resistance in *Klebsiella pneumoniae* isolated from community-acquired infections.

Among the factors determining PMQR, the *aac(6')-Ib-cr* gene tends to be prevalent. These factors have a high degree of transferability. Concerning, this can lead to an increase in the transmission of fluoroquinolone resistance among clinical isolates by horizontal transfer of PMQR genes.

Ethical Approval

This cross-sectional study was carried out following the ethical principles originating in the Declaration of Helsinki. It was conducted with the patient's verbal and analytical consent before sample collection. The study protocol and the patient information and consent form obtained permission from a local ethics committee according to the document number and date on 2023-9-17.

Declaration

Consent for publication: Not applicable**.**

Availability of data and material: Data are available upon request.

Competing interests: The author(s) declare that the content of this article has not been published previously and will not be submitted for publication elsewhere while the manuscript is under review.
Funding: Authors did not receive any grants from funding agencies.

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