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Molecular Characterization of Carbapenemase (*Blakpc-2*) Gene From *Klebsiella Pneumoniae* Involved in Urinary Tract Infection in Al-Najaf City

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ABSTRACT

The human gastrointestinal tract, eyes, respiratory system, and genitourinary tract are all home to K. pneumonia. KPCs, or Klebsiella pneumonia carbapenemases, are β-lactamase enzyme types that have the ability to hydrolyze β-lactam drugs. Because of many co-morbid diseases, immunological suppression, and critical sickness, this KPC-producing strain infection can be fatal. In this study K. pneumonia was isolated from urinary tract infected patients, Where seven clinical urine specimen obtained from patients has UTI were gathered from different diagnostic Al-Sajjad Hospital in Najaf province the KPC gene was amplified by PCR. In this present study, a particular primer derived from the antibiotic sensitivity (blaKPC-2) gene is used to screen the urine samples collected for Klebsiella pneumoniae stains utilizing culture techniques then sequence analysis of the blaKPC-2 gene was conducted in order to gain a fresh understanding of the genetic variations. It's interesting to note that, while comparing our strains' blaKPC-2 open reading frame to previously released sequences, we discovered 6 mutations. Therefore, of the seven samples analyzed, three exhibited a positive band. The purpose of the primer was to amplify the bacteria's blaKPC-2 antibiotic sensitivity gene.

Keywords: *K. pneumoniae, Carbapenemases, KPC, Cloning, Sequencing.*

Article Information
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1. INTRODUCTION

The facultative anaerobic, rod-shaped, gram-negative, non-motile, encapsulated, lactose-fermenting bacteria Klebsiella pneumoniae [1]. It has been generally considered an opportunistic pathogen causing nosocomial infections and recently an increasing number of severe *K. pneumoniae* infections have been reported in Asian countries [2,3]. This species also produces bacteriocins, Given that K. pneumoniae is typically found in the host's microbiota, the synthesis of bacteriocins may actually be detrimental. Similar or closely related bacterial strains are typically inhibited in their growth by the proteinaceous toxins generated by these bacteria [4]. β-Lactams, also known as carbapenems, are used to treat infections brought on by Enterobacteriaceae that produce extended-spectrum beta-lactamases (ESBLs). K. pneumoniae is distinct due to a variety of metabolic characteristics, including the synthesis of the enzyme carbapenemase, which confers resistance to the medication carbapenem [5,6].

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The blaKPC genes are found usually in vast plasmids varied in lenth and composition and different K. pneumoniae clones may carry KPC β lactamases is proof of their ease of transmission [7]. *K. pneumoniae* is also capable of decarboxylation and sodium citrate use[5,8]. ACHN-490 a aminoglycoside is shown to significantly lower concentratdemonstrates that ACHN-490 is a potential replacement for aminoglycosides in the future for treating *K. pneumonia* strains that are resistant to drugs[9]. Human gastrointestinal tract, eyes, respiratory system, and genitourinary tract are all home to *K. pneumoniae*. Ions of *K. pneumoniae* more than all of the other aminoglycosides which and KPC-producing strain infection can cause mortality due to critical illness, immune-suppression, and multiple co-morbid conditions [6,7]. KPCs are float among the globe and go away to endemic parts in populations other than USA [9,10]. In this study *K .pneumonia* was set aside from urinary tract infected patients, and the KPC gene was amplified by PCR and sequenced.

2. MATERIALS AND METHODS

Urine sample Collection

Collected of seven clinical urine specimen obtained from patients has UTI were gathered from different diagnostic Al-Sajjad Hospital in Najaf province from October 2023 to May 2024. A carefully performed, clean-voided, midstream sample is usually satisfactory. The samples were gathered and carefully labeled in a sterile, spin-won conical tube. The urine sample tubes were kept in storage at 4°C after collection.

Isolation of bacteria

Klebsiella pneumoniae has emerged as the predominant urinary tract pathogen in SCI patients. There is lot of bacteria observed in the agar plates. From there based on the colony morphology seven bacteria were isolated and subcultured. The contents were thoroughly mixed in distilled water, if needed add enough Agar to the media. The prepared media was autoclaved at 121◦C for 15-20 minutes. After autoclaving, the media was left to cool at about 45-50oC. The Nutrient Agar was supplemented with antibiotics Nystatin (50mg/ml) to avoid fungus and mixed well. The clean sterile Petri plates were taken and labeled with date, media and sample no. Pour the media into sterilized Petri plates and allow the media to solidify.

Table 1: Composition of Nutrient Agar.

Contents	Weight
Beef extract	**10g**
Peptone	**40g**
Agar	**15g**
Distilled water	**1000 ml**
pH	**7.2**

Gram staining

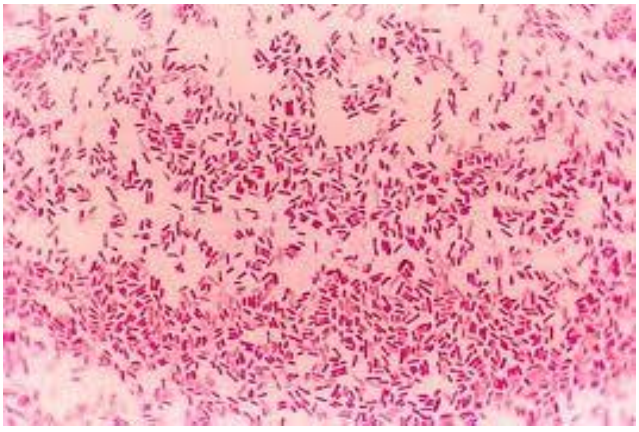

Figure 1: Gram negative bacilli was observed in 100x oil immersion objectives.

Genomic DNA isolation from bacterium

Through the use of the N-cetyl-N, N, N-trimethyl-ammonium bromide (CTAB) technique, the bacteria's whole genomic DNA was extracted.

Table 2: Composition of Extraction buffer

Stock Solution	Buffer composition
1 M Tris HCl	**100 mM Tris HCl**
1M EDTA	**100 mM EDTA**
4 M NaCl	**1.4 M NaCl**
	1% CTAB
	Proteinase K - 0.03µg\µl

3.4. Quantification of Isolated DNA

Utilizing a UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany), the amount of extracted DNA was measured. To obtain a 50-fold dilution, 1 µl of stock DNA was combined with 49 µl of sterile DW. The clarity of the DNA preparation was verified by recording the A260/A280 ratio.

3.5. Design and Synthesis of Primer

The 612 bp amplicon size of the *Klebsiella pneumonia* carbapenemase gene was used for the primer design.The Primer 3 Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) was utilized to design the appropriate primers, and Sigma Corporation USA manufactured the oligonucleotides that were designed.

2.1. DNA Isolation and PCR

From the samples, DNA was separated and the *KPC* gene was amplified by PCR using specific primers as described earlier [11]. Following protocol was followed for PCR amplification, 30 cycles of amplification with 60 seconds of denaturation at 94°C, 60 seconds of annealing at 56°C, and 60 seconds of extension at 72°C. A final extension was performed for two minutes at 72°C after this phase.

2.3. Sequence Analysis

The partial sequence of *blaKPC-2* gene was used to generate phylogenetic making use of Clustal W. The alignment did not include the variable or incomplete locations at the 5′ and 3′ ends of the gene sequences. All sites with alignment gaps were not included in the analysis. Using the sequences provided here and various K. pneumonia sequences previously deposited in the GenBank database, a rooted phylogenetic tree was built.

Table 3: Primer Details **.**

Primer	Sequences (5’- 3’)	GC %	Tm Value	Length	Product Size
FP	CTA CGA TAC GGG AGG GCT TAC	57.1	60.0^0C	21	612 bp
RP	TCT TAG ACG TCA GGT GGC ACT	52.4	59.9^0C	21	

Table 4: Reagents and the optimal PCR reaction mixture.

PCR components	Volume (µl)
Nuclease free water	10.75
10X reaction buffer with $MgCl_2$ (1.5mM)	2.00
dNTP mix (2.5mM)	2.00
Primer I (10picomoles/ µl)	2.00
Primer II (10picomoles/ µl)	2.00
Taq DNA polymerase (5U)	0.25
Template DNA (50ng/ µl)	1.00
Total volume	**20.0**

3. RESULTS

Bacterial isolation

Klebsiella pneumoniae has considered as the triumphant urinary tract pathogenic in SCI patients. There is a lot of bacteria observed in the agar plates. From there based on morphological characters of the colonies 7 bacteria were isolated and sub-cultured.

Genomic DNA isolation and quantification

After cultivating negative bacteria on LB broth medium, genomic DNA was extracted using a modified CTAB technique. In a 1% Agarose gel, the extracted DNA was electrophoresed (Figure 2). The findings of the study of the amount and quality of DNA using a UV visible spectrophotometer are shown in Table 5. The separated DNA's purity is indicated by the A260/280 value. The good quality DNA will show a value of 1.8.

Table 5: The optimum condition of detection *blaKPC1*

Phase	Temperature and time	No. of cycle
Initial denaturation	94°C for 2 min	
Denaturation	94°C for 1min	
Annealing	56°C for 30 s	30 cycles
Extension	72°C for 2min	
Final extension	72°C for 6 min	

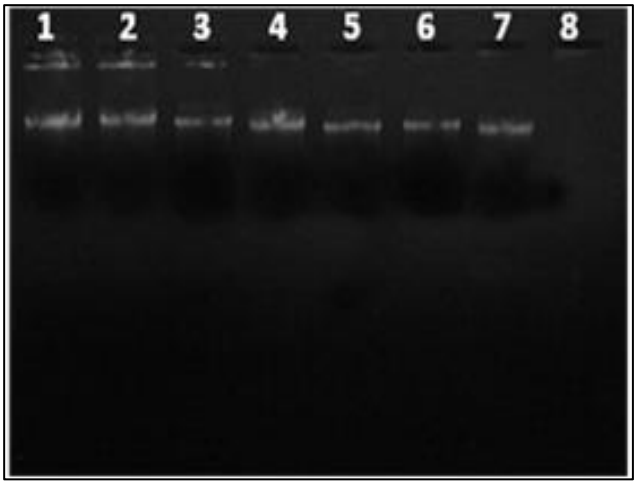

Figure 2: Genomic DNA isolated from the bacteria.

Table 6: Concentration of the isolated genomic DNA

DNA Sample	Concentration (ng/ µl)	A260/280
1	201	1.72
2	222	1.73
3	120	1.84
4	143	1.80
5	300	1.62

PCR amplification of the carbapenemase *KPC-2 (blaKPC-2)* gene

By using PCR to detect *blaKPC* genes molecularly, labs can swiftly determine whether this crucial resistance factor is present. Given that these genes have been shown to have the ability to spread quickly both horizontally and vertically, early detection is critical to limiting their dissemination. Using Primer 3 software, species-specific primers were created for *Klebsiella pneumoniae* based on drug-resistant *blaKPC-2* gene sequences found in NCBI GenBank. When treating serious infections brought on by Klebsiella pneumoniae that produces extended-spectrum β-lactamase (ESBL), carbapenems are usually the preferred medication, and the prevalence of carbapenem resistance in Klebsiella has been steadily rising. The anticipated primers were first verified in silico and then in a wet laboratory. An amplicon unique to the *blaKPC-2* gene of the anticipated size may be produced by the primers. The starting points were in the figure bellow.

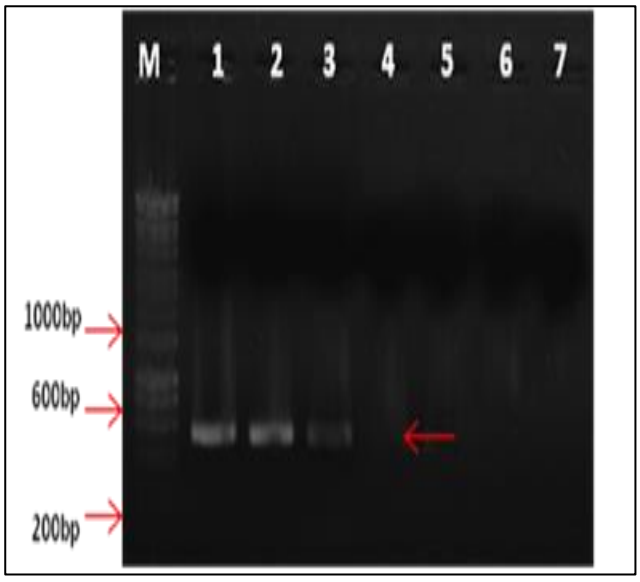

Figure 3. PCR amplification of ***blaKPC-2*** partial gene by specific primers (M- 200bp ladder, Line 1, 2, 3- *blaKPC-2* gene product, 4, 5, 6, 7- Negative samples).

The released gene product of *blaKPC-2* Gene was sequenced after being eluted from the gel. To ensure the accuracy of the results, we matched the sequences to their corresponding GenBank sequences. The sequence was found to have a "perfect" match (similarity, 99%) with Gen Bank sequences of the relevant gene using BLAST. Employ Clustal W sequence alignment (http://align.genome.jp/), the N-J tree with branch length was shown to illustrate the link between the closest K. pneumoniae strains in the NCBI database and the blaKPC-2 gene (Figure 4).

Sequence alignment by clustal W

The GenBank database also contains the *blaKPC-2* gene sequence of *Klebsiella pneumoniae*, which is 99% identical to this sequence. Using ClustalW sequence alignment (http://align.genome.jp/), the N-J tree with branch length was shown, illustrating the relationship between the closest *Klebsiella pneumoniae* strains in the NCBI database and the *blaKPC-2* gene (Fig. 7). The partial blaKPC-2 gene amplified by the particular primer has a sequence that closely matches (99%) that of *Klebsiella pneumoniae*. Additional work was done to get greater understanding of the genetic distinctions between the gastritis and peptic ulcer groups by sequencing the *blaKPC-2* genes from 11 strains. Interestingly, we discovered 6 alterations within the blaKPC-2 open reading frame of our strains rapprochement with formerly published sequences *blaKPC-2*. However, recently published epidemiological data suggest that *blaKPC-2* gene condition be inverted geographic differences in *Klebsiella pneumoniae* strains rather than a virulence-associated trait.

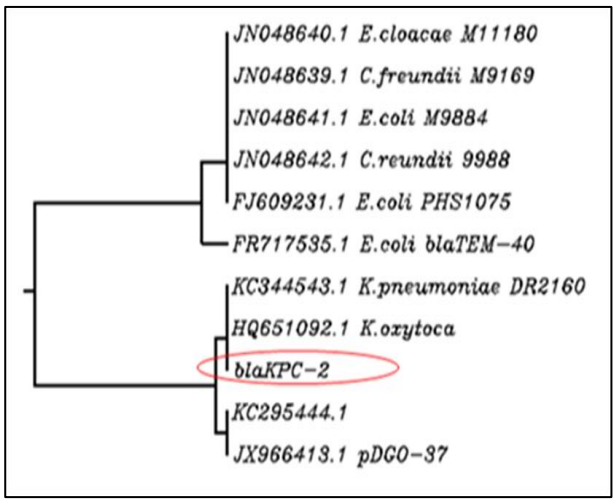

Figure 4. Tree plot was constructed with the NJ method using ~612bp fragment of the *blaKPC-2* gene partial sequences showing the relationship of *Klebsiella pneumoniae*.

4. DISCUSSION

This study was conducted on 7 cases presenting with UTI caused by CRKP from October 2023 to May 2024 at the First Affiliated Hospital of Furat Al-Awsat Technical University in Al-Najaf Province, Iraq. Human infections are caused by the *K. pneumoniae* bacteria, and in high-frequency nosocomial *K. pneumoniae* outbreaks, the patient population serves as the primary reservoir [12]. There was a serious public health emergency when *K. pneumoniae* harboring carbapenemases first appeared and spread throughout the world[10]. Greece and Italy had fatality rates of 37% and 41.6%, respectively, according to numerous publications on KPC-producing *K. pneumoniae* epidemics that were documented in Canada, Korea, Greece, Kenya, and Italy[13–15]. In numerous locations across the globe, there has been a sharp rise in the recurrence of producers among clinical isolates, which is associated with the prevalence of resistance [16,17]. The global community continues to document the steady rise in carbapenem-resistant strains, which is still regarded as a serious global problem [13,14]. Although high-level resistance to carbapenems has been shown in recent investigations, carbapenems remain the most effective treatment against multiple antibiotic-resistant *K. pneumonia* [18,19]. Despite the fact that this kind of resistance has been extensively researched worldwide, studies on the subject are still scare and don't fully capture the severity of the issue as of yet, particularly with regard to the carbapenem antibiotic, which is severely limited in its ability to treat bacterial infections. Consequently, we examined the prevalence of pathogenic *K. pneumoniae* that is resistant to carbapenem in hospital to better understand its danger in UTI. In addition to, previous study survey findings show that more infected of *K. pneumoniae* isolates were found in urine. Our findings coincide with a study from Al-Azhar University in Egypt[8,20]. Furthermore, additional research conducted in Uganda found that blood, pus, and urine were the primary sources of *K. pneumoniae* isolates [21]. However, it was discovered by Lopes et al. and Hussein et al. [22,23] that isolates of *K. pneumoniae* resistant to carbapenem had increased expression of *blaKPC*. Finding out if the K. pneumoniae isolate generates carbapenemase is necessary to perform epidemiological studies and establish the most effective way to treat illnesses [24]. Parrott et al.'s investigation from New York, however, [25] verified that the majority of K. pneumoniae isolates were obtained from blood cultures, then from wound swabs. Furthermore, it was discovered by Palmeiro et al.[26] that blood sample produced the greatest number of isolates.

Consequently, Variations in sample type and case count, conditions, timings, locations, and countries, as well as overall patient health, could account for this difference in results. Last but not least, over the past ten years, molecular investigation of bacterial resistance

has produced a plethora of knowledge. Much progress has been achieved in our understanding of the distribution and transmission of resistance markers among the species with the use of molecular amplification techniques. It hasn't yet materialized, nevertheless, the early prediction that molecular methods will outperform phenotypic susceptibility testing in standard diagnostic susceptibility testing. The range of point mutations or resistance-causing genes, as well as the labor-intensive nature of the amplification techniques used today, are obstacles that still need to be overcome. Automated amplification techniques in conjunction with DNA chip technology could potentially.

5. CONCLUSION

In this work, we verified a PCR technique for *blaKPC-2* gene identification that is quick, sensitive, and specific. Out of the seven samples analyzed, three exhibited a positive band. The purpose of the primer was to amplify the bacteria's *blaKPC-2* antibiotic sensitivity gene.

6. Ethical approval

The research was conducted by the ethical principles specified in the Helsinki Declaration. Before collecting the sample, a local ethics committee reviewed and authorized the research protocol, subject information, and consent form. This approval was granted by document number 53847, which includes the date of December 27, 2023.

7. Acknowledgment

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8. Conflicts of Interest

The authors state they have no conflicting financial objectives.

9. Funding

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10. Authors' contributions

Najat M. Flyyih, Alaa H. Abd Ali and Ameer A. Karim: Literature review and collection of referenced data, data analysis, and manuscript drafting. ***Najat M. Flyyih***: Analysis of referenced data, editing of final manuscript. ***Alaa H. Abd Ali*****:** Review design, Analysis of referenced data, editing of final manuscript.

Abbreviations

CTAB: *Cetyltrimethylammonium bromide*

GN: *Gram-negative*

KPC: *Klebsiella pneumoniae carbapenemase*

MDR: *Multidrug-resistance*

ESBL: *Extended-Spectrum Beta-Lactamase*

PCR: *Polymerase chain reaction*

UV-VIS: *Ultraviolet-visible (UV–VIS) spectrophotometry*

SCI: *Spinal Cord Injury*

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