AL-KUFA JOURNAL FOR BIOLOGY
ISSN (Print): 2073-8854 **ISSN (online):** 2311-6544

Original Research Paper

Study the genetic variations of Extended Spectrum Beta-Lactamase genes (*bla*SHV and *bla*CTX-M) in *Klebsiella pneumoniae* isolated from clinical samples.

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Article history
Received: 16/5/2025
Revised: 18/6/2025
Accepted: 22/7/2025

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Abstract: *Klebsiella pneumoniae* exhibits notable multidrug resistance in burn patients; genomic characterization of resistance determinants is pivotal for elucidating resistance mechanisms and guiding precise therapeutic interventions. The aim of this study to investigate genetic variation among *Klebsiella pneumonia* isolates obtained exclusively from burn wound infections by analyzing *bla*SHV, *bla*CTX-M genes. Between November 2023 and March 2024, 95 burn swabs were collected from male and female patients in five Baghdad hospitals. After culturing and DNA extraction, PCR was performed, and three amplicons (*bla*SHV, *bla*CTX-M) were Sanger-sequenced by Macrogen- Korea. Of the 95 burn swab samples, 44% were identified as *Klebsiella pneumoniae* using the VITEK 2 Compact System, with isolates obtained from 43% male and 57% female patients. A high level of resistance was observed against Ceftriaxone, Ceftazidime, Cefepime, and Cefotaxime, whereas Imipenem and Meropenem exhibited complete efficacy, with no detected resistance. Molecular analysis of the *bla*SHV and *bla*CTX-M genes was conducted on ten multidrug-resistant isolates. The *bla*SHV gene was detected in 100% of isolates, while *bla*CTX-M was present in 30%. Gene sequences for (JH85, JH86, JH93) submitted to GenBank showed high similarity (99–100%). One mutation (G→A at position 78) was noted in the *bla*CTX-M sequence from isolate JH93. Phylogenetic analysis linked this variant to CTX-M-14. The study highlights a high prevalence of ESBL genes, especially *bla*SHV and *bla*CTX-M genes, underscoring the need for continuous surveillance and proper treatment of resistant *K. pneumoniae* in burn patients.

Keywords*: K. pneumonia*, ESBL, *bla*SHV, *bla*CTX-M, burns.

1. Introduction

Klebsiella pneumoniae is a common Gram-negative, nonmotile opportunistic pathogen. *K. pneumoniae* frequently causes urinary, respiratory, and bloodstream infections in people with underlying illnesses [1]. With the increased use of antibiotics, multidrug-resistant (MDR) *K. pneumoniae* has grown more widespread, offering additional challenges and impediments in clinical treatment. At the onset, burn injury prevention should be given top priority, as it is a global public health concern, particularly in poor and underdeveloped countries. Burn patients are more vulnerable to pathogens such as MDR bacteria, fungi, and viruses, especially during hospitalization. Burn victims experience decreased immune system responses, abnormal vascular organization, and increased oxidative stress. Burn patients are more susceptible to MDR-

DOI: https://doi.org/10.36320/ajb/v17.i3.20310

bacterial and deadly viral infections due to immunosuppression, prolonged hospitalisation, and geographic considerations (Microbial infections in burn patients). Common burn wound infections include *Klebsiella* spp., *Pseudomonas aeruginosa*, and Staphylococcus aureus. These bacteria release virulence factors that contribute to invasive infection [2]. The World Health Organization has identified extended-spectrum β-lactam (ESBL) production as a significant public health issue [3]. ESBLs are enzymes that hydrolyze the beta-lactam core structure of penicillins, cephalosporins, monobactams, and carbapenem, there by neutralizing their antibacterial activity [4]. Strains of *K. pneumoniae* that can manufacture ESBLs are extremely active against a wide range of beta-lactam drugs. Furthermore, these virulent strains are potential of becoming resistant to several types of non-beta-lactams, making it difficult to treat infections [5]. The genes producing ESBL enzymes have been shown from both bacterial chromosomes and plasmid DNA, plasmid-mediated transfer of β-lactamase genes among bacteria is a crucial contributor in dissemination of drug resistance [6,7]. There are approximately 350 different ESBL variation, which are grouped into nine evolutionary and structural families based on their amino acid sequence, such as *bla*SHV, *bla*CTXM, *bla*TEM, *bla*OXA, *bla*PER, *bla*TLA, *bla*VEB, *bla*GES, and *bla*BES5, commonly *bla*CTX-M, *bla*TEM, and *bla*SHV, which are harboured by self-transmissible conjugative plasmids that are horizontally transferred between the same and other species of bacteria [8,9]. Molecular methods can detect major genes like *bla*TEM, *bla*CTX−M, and *bla*SHV, as well as their antibiotic resistance pattern, providing valuable information about their epidemiology and aiding in rational antimicrobial therapy formulation [10]. The aim of this study to investigate genetic variation among *Klebsiella pneumonia* isolates obtained exclusively from burn wound infections by analyzing *bla*SHV, *bla*CTXM genes, molecular characterization will be used to identical any potential mutations or genetics differences within these isolates.

2.Methodology

Samples collection

From five hospitals in Baghdad, Al- Shahid Chazi, Al-Yarmouk teaching hospital, Al- Karama teaching hospital, Al- Karkh general hospital and teaching laboratories / Medical city, about ninety-five burns swabs were collected from patients, males and females, from period spanned over six months from November 2023 to end of Mars 2024.

Bacterial Isolation and Identification

Burn wound swabs were first plated on Blood agar (Himedia, India), and Chrom agar orientation (Chrom agar, France), then incubated aerobically at 37˚C for 24 hrs. Pure colonies isolated and subsequently inoculated on MacConkey agar (Oxide, England), and bacterial identification was performed by using VITEKS2 Compact System (Biomericux, France).

Antibacterial Susceptibility Testing

By using Kirby-Bauer disc diffusion method [11, 12], antibiotics susceptibility was performed, and the Zone of inhibition was measured. A 0.5 McFarland standard inoculum was prepared by suspending bacterial colonies in saline within a test tube. A sterile cotton swab was then used to evenly spread the suspension on to Mueller-Hinton agar plates for antibiotic susceptibility testing against the following antibiotics Ceftriaxone (CRO), Kanamycin (K), Ceftazidime (CAZ), Cefixime (CFM), Levofloxacin (LEV), and Gentamicin (CN) by (Bioanalyse, Turkey), and Cefepime (CPM) Ciprofloxacin (CIP), Cefotaxime (CTX), by (Himedia, India). After overnight incubation at 37˚C, the diameters of the inhibition zones surrounding the antibiotic discs were measured in millimeters (mm) using a metric ruler.

Molecular detection of blaSHV, and blaCTX-M

Genomic DNA was extracted from *K. pneumoniae* colonies that overnight grew on Nutrient broth according to the protocol of ABIOpureTM Total DNA (ABIOpure USA). DNA concentration and purity were assessed using the Quantus Fluorometer (Promega, USA) [13]. The extracted DNA was screened for *bla*SHV, and *bla*CTX-M genes using the primers from Macrogen Company (Macrogen, Korea) that are reported in table 1. These primers [14,15] were supplied in a lyophilized form. Lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100pmol/µl as a stock solution. A working solution of these primers was prepared by adding 10µl of primer stock solution (stored at freezer -20 C) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl.

DOI: https://doi.org/10.36320/ajb/v17.i3.20310

Table 1: Sequences of primers

Primer Name	Sequence 5`-3`	Product Size (bp)	Ref.
*bla*SHV-F	AGCCGCTTGAGCAAATTAAAC	713	[14]
*bla*SHV-R	ATCCCGCAGATAAATCACCAC		
*bla*CTX-M-F	ATGGTGACAAAGAGAGTGCA	869	[15]
*bla*CTX-M-R	CCCTTCGGCGATGATTCTC		

The PCR was performed using Thermal Cycler (Thermo Fisher Scientific, USA). The PCR reaction mixture (25 µl) consisted of 12.5 µl Go Taq Green Master Mix (Promega, USA), 2 µl template DNA, 1 µl of both forward and reverse primers, and 8.5 µl Nuclease Free Water [13]. The optimal conditions for PCR thermal cycler were programmed for our gens interesting that are reported in tables (2 and 3), respectively. The PCR amplification was verified by electrophoresis at 100v/mAmp for 60min on 1.5% agarose gel stained with 1µl(10mg/ml) ethidium bromide in 1X TAE buffer (Promega, USA) using a DNA ladder (100-1500 bp), supplied by Promega (USA), as a molecular weight marker. The UV- Transilluminator (Major Science, Taiwan) was used for the observation of PCR products under 320nm UV light [16].

Table 2: The optimal conditions for amplifying *bla*SHV gene

PCR Steps	Temperature (°C)	Time (min)	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	55,60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

Table 3: The optimal conditions for amplifying *bla*CTX-M gene

PCR Steps	Temperature (°C)	Time (min)	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	55,60	00:30	
Extension	72	00:30	
Final extension	72	07:00	1
Hold	10	10:00	

DNA sequencing

Three PCR products that referred to the blaSHV, and blaCTX-M genes from forward ends were sent for Sanger sequencing using ABI-12730XL capillary automated sequencer by Macrogen corporation-Korea, results were received by email then analyzed using Geneious software.

3. Results and discussion

Our study was conducted on ninety- fifty (95) burn swabs collected from hospitalized patients. With more accuracy and reliability, forty-four (44%) isolates were identical as *K. pneumonia* by using VITEKS2 Compact System (Figure 1), 19(43%) were male and 25(57%) were female patients. The antibiotics susceptibility profile against 11 different antibiotics that reported in tables - (4) according to Clinical and Laboratory Standards Institute (CLSI) guidelines 2022 [17], revealed that varying resistance patterns among the bacterial isolates. A significant percentage of isolates exhibited resistance to multiple antibiotics indicating potential multidrug resistance, the highest resistance (less effective) was observed against Ceftriaxone, Ceftazidime, Cefepime and Cefotaxime. Conversely Imipenem, and Meropenem demonstrated the highest efficient with only (0.00%) of isolate being resistant.

Fig.1.Colony morphology and lactose fermentation phenotypic identification characteristics of K. pneumoniae on MacConkey agar for

Table 4: Antimicrobial susceptibility of ESBL -producing *K. pneumoniae* isolated from burn wound infection.

No.	Antibiotic agent	Code and conc.	Dimension of inhabitation zone (mm) (ClSI 2022)		
			Susceptible (S)	**Intermediate (I)**	**Resistant (R)**
1.	Ceftriaxone	CRO (10µg)	**0(0%)**	**5(9.6%)**	**38(90.4%)**
2.	Kanamycin	K (30µg)	**13(30.9%)**	**4(9.5%)**	**25(59.5%)**
3.	Ceftazidime	CAZ (30µg)	**10(23.8%)**	**0(0%)**	**32(76.1%)**
4.	Cefixime	CFM (5µg)	**7(16.6%)**	**0(0%)**	**36(85.4%)**
5.	Levofloxacin	LEV (5µg)	**10(23.8%)**	**8(19.0%)**	**24(57.1%)**
6.	Gentamycin	CN (10µg)	**24(57.1%)**	**2(4.7%)**	**26(61.9%)**
7.	Cefepime	CPM (30µg)	**6(16.6%)**	**0(0%)**	**36(85.4%)**
8.	Ciprofloxacin	CIP (5µg)	**29(69.0%)**	**0(0%)**	**13(30%)**
9.	Cefotaxime	CTX (30µg)	**3(7.1%)**	**0(0%)**	**39(92.8%)**
10.	Imipenem	IPM (10 µg)	**42(0%)**	**0(0%)**	**0(0%)**
11.	Meromem	MEM (10µg)	**42(0%)**	**0(0%)**	**0(0%)**

Following a sensitivity test, the most resistant *Klebsiella* isolates were selected, PCR analysis was conducted on 10 samples to detect the prevalence of ESBL (*bla*SHV, and *bla*CTX-M) genes. The prevalence of the *bla*SHV gene in *K. pneumoniae* isolates were 100% of the isolates, while the prevalence of *bla*CTX-M genes was (30%) of total *k.pneumoniae* isolates (Figure 2, 3).

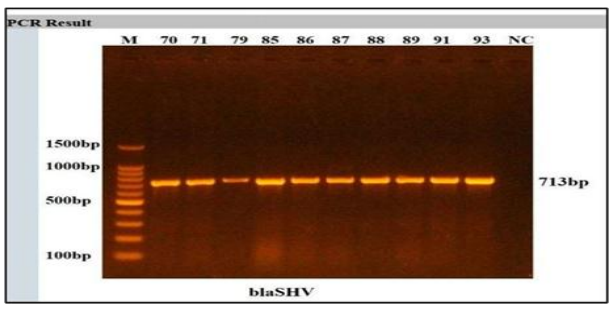

Fig.2. PCR products of *bla*SHV genes of bacterial species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 70-93 resemble 713bp PCR

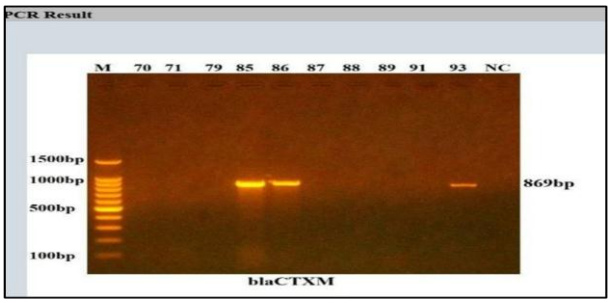

Fig.3. Results of the amplification of *bla*CTXM genes of bacterial species were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker.Lanes 70- 93 resemble 869bp PCR products

DOI: https://doi.org/10.36320/ajb/v17.i3.20310

Subsequently, three isolates (MDR), were selected for sequencing analysis, the nucleotide sequences of *bla*SHV and *bla*CTX-M genes of our local isolates (JH85, JH86, JH93) reported in this study have been submitted at the National Center for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov, under accession numbers as showed in table -5. Isolates were chosen to perform the nucleotide sequencing of the *bla*SHV and *bla*CTX-M genes. PCR product was sent for Sanger sequencing using ABI3730XL, after which sequencing was conducted by Macrogen DNA Sequencing in Seoul, Korea. The nucleotide sequences for the *bla*SHV and *bla*CTX-M genes from *K. pneumoniae* reference strains sourced from various regions globally (accessible in the public database: Gen Bank) were downloaded and aligned using Geneious software.

Table 5: The accession numbers for the nucleotide sequences of the *bla*SHV and *bla*CTX-M genes of *K. pneumoniae* local isolates

Isolate code	*bla*SHV	*bla*CTX-M
JH1		LC835495
JH85	LC835667	
JH86	LC835668	LC835496
JH93	LC835669 .	LC835497.

Note: the isolated code JH1 are the same isolate JH85 but in different code.

Aligning of the obtained sequence with the reference strains in GenBank confirmed the correct identification of blaSHV and blaCTX-M genes by PCR. The alignment results of the amplified blaSHV sequences of K. pneumoniae revealed the presence of no nucleic acid variation in the analyzed (JH85, JH86, JH93) samples in comparison with the most similar referring reference nucleic acid sequences. Meanwhile, our the local amplified blaCTX-M sequences (JH85, JH86) in comparison with the reference sequence, also exhibited similarity (99-100) % with no mutation in the genes, whereas the alignment result of the local amplified isolate blaCTX-M (JH93) sequences with the reference strain with accession number LR596807.1, revealed 800 out of 802 nucleotide identities, a single nucleotide variation at position 78with a substitution of adenine for guanine (G>A), and a single gap observed (Figure 4).

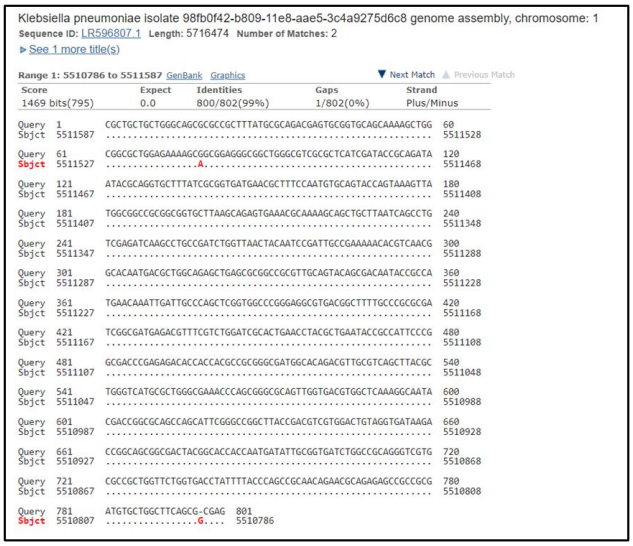

Fig 4: DNA Sequences Multiple Alignment of the blactx-m HJ93 isolate with their corresponding reference sequences of the 801bp amplicon of the *bla*CTX-M gene (GenBank accession number LR596807.1). The observed substitution was highlighted according to the position in the PCR products

Based on these analyses, a phylogenetic tree (Figure 5) was constructed to illustrate the genetic relationships. We can observe that some plasmids cluster together in the tree. This indicates that these plasmids are more closely related in terms of gene sequence than plasmids in other clusters or chromosomal regions. The position of the K. pneumoniae chromosome in the tree relative to the plasmids is important. If the chromosome branch is far from all plasmid branches, this indicates that the plasmids have different evolutionary histories. In contrast, a closer relationship may indicate some kind of gene exchange or a recent common ancestor between certain plasmid lineages and chromosomes of certain genes. And the present results from phylogenetic tree revealed that the variant was closed to blaCTX-M-14 gene. The presence of the gene blaCTX-M (encoding extended-spectrum beta-lactamase CTX-M) in several of the listed plasmids is a key point. The phylogenetic tree can help to visualize whether plasmids carrying this resistance gene form different clusters. If plasmids carrying blaCTX-M are close to each other, this may indicate a common origin or horizontal transfer of this resistance gene between related plasmids. The overall structure of the tree illustrates the genetic diversity of the analyzed plasmids. The longer the branches between clusters, the greater the divergence.

DOI: https://doi.org/10.36320/ajb/v17.i3.20310

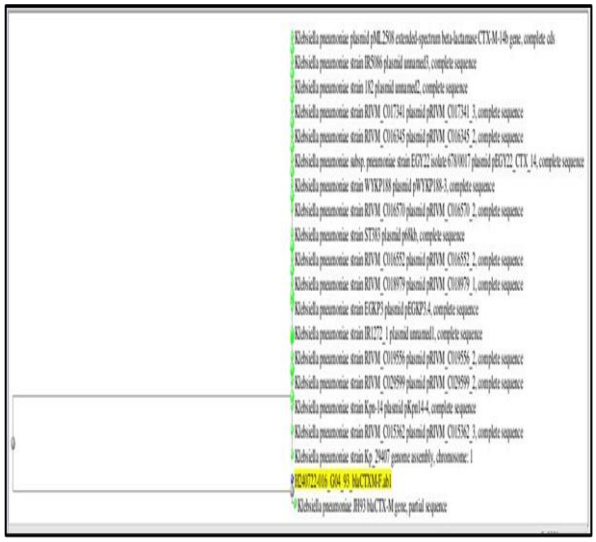

Fig 5: The phylogenetic tree of *bla*CTX-M gene from HJ93 isolate with the reference genes from GENE BANK.

Genetic variation in ESBL genes, have been studied in several researches for its role in bacterial resistance, adaptability and altering target sites or increasing antibiotics degradation. This study investigates the sequence diversity in *bla*SHV, and *bla*CTX-M gene *in K. pneumonia* isolates to better understand their contribution to resistance.

The isolates were obtained exclusively from burn wound infections we examined using a total of ninety- fifty (95) burn swabs, including forty-four (44%) isolates were identical as *K. pneumonia*. These confirmed isolates were distributed between 19(43%) male patients and 25(57%) female patients. When comparing these findings with similar studies conducted in different regions have reported varying rates of isolation and sex-based distribution. For instance, some investigations have shown a higher prevalence among male patients, while others indicated no significant difference between sexes. These discrepancies may be attributed to differences in sample size, hospital settings, demographic of patients, or methodology of diagnostic. Nonetheless, the consistent identification of this pathogen across studies highlights its clinical relevance and the importance of continuous surveillance.

The antimicrobial susceptibility analysis demonstrated diverse resistance level among K. pneumonia isolates growth, notably, high rates of MDR resistance against Ceftriaxone, Ceftazidime, Cefepime and Cefotaxime than other types of antibiotics. These outcomes align with findings consistent with previous studies conducted in [18, 19, 20, 21], which also reported high resistance rates among burns infections. This widespread resistance has been indicating frequent use as a first line treatment in severe infections, particularly in burns units or excessive use of antimicrobial agent in recent years, along with the lack of strict health regulation and surveillance. In contrast, Imipenem, and Meropenem antibiotics were demonstrated full effectiveness against all bacterial isolates. When compared with studies of [22, 23, 24], similar patterns of full susceptibility were observed. This consistent efficacy may due to broad spectrum activity and attributed to act through binding affinity to mechanisms.

With regard to molecular analysis, our study was consistent with those reported in previous investigations [25,26,27], a study conducted that all samples harbored blaSHVgene, while three of them (85,86,93) combinate the resistant blaCTX-M with blaSHV genes. Also, other results demonstrated a high prevalence in blaSHV gene, with variable distribution rates, such as study by [28], in Iraq, and, [29], [30] in Iran, conducted the highest frequency of genes was blaSHV gene than blaCTX-M genes. In contrast, studies conducted by [31, 32] reported that the blaCTX-M genes were the most prevalent among the detected genes. This variability in genes prevalence, which fluctuates across different regions and countries, may be attributed to several factors, including local antimicrobial usage patterns and environmental condition.

Regarding The BLAST analysis of the genes sequence of blaSHV gene (JH85, JH86, JH93), and blaCTX-M (JH85, JH86), which demonstrated a high similarity (99-100) % with no observed mutations in the genes, when compare with reference sequence in database, although it exhibited increased resistance to a broader spectrum of antibiotics evaluated in this study, this may be explained that our isolates have another MDR encoded genes. otherwise, in the alignment of the local isolate blaCTX-M (JH93) showed a single nucleotide variation at position 78with a substitution of adenine for guanine (G>A).

Phylogenetic analysis revealed that this variant closely clusters with the blaCTX-M-14 allele, a well-known ESBL gene associated with clinical resistance. This close genetic relatedness may reflect local evolutionary divergence or selective pressure occurring within the burn unit, an environment known for high antibiotic use and limited external microbial influx. The detection of blaCTX-M on multiple plasmids supports the critical role of horizontal gene transfer in the dissemination of ESBL genes [33, 34], particularly in high-risk

environments such as burn units, where antibiotic pressure and patient-to-patient transmission promote genetic fixation. Recent studies [35, 36] have highlighted the genetic stability of blaSHV and blaCTX-M genes in K. pneumoniae isolated from high-risk clinical environments, suggesting that restricted environmental exposure and clonal spread contribute to low genetic variation. The limited or absence genomic diversity observed among our isolates supports this hypothesis, emphasizing the importance of continuous molecular surveillance and infection control practices to curb the persistence of resistant strains in such settings.

Conclusion

99-100% sequence similarity observed in blaSHV and blaCTX-M genes among K. pneumoniae isolates from burn wounds reflects a high degree of genetic conservation. Exactly the blaCTX-M gene in isolate JH93 showed a single nucleotide variation (G>A at position 78). Prevalence of blaSHVgenes, harbored in all samples, while three of them (85,86,93) combinate the resistant blaCTX-M with blaSHV genes Imipenem and Meropenem demonstrated the highest effectiveness against Klebsiella pneumoniae isolates.

Acknowledgement

Special thanks are extended to the Institute of Genetic Engineering and Biotechnology for Postgraduate Studies for providing the necessary facilities, research environment, and technical support that enabled the execution of the experimental part of the study.

Funding Information

No external funding was received for this study

Author's Contributions

All authors equally contributed to sample collection and manuscript writing. All authors read and approved the final version of the manuscript.

Ethics

Ethical approval was obtained from the authorized ethics committee, and a formal task facilitation letter from the Institute of Genetic Engineering to the Iraqi Ministry of Health authorized sample collection and research procedures.

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