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Antibiotic Resistance Patterns and Molecular Typing of *Klebsiella pneumoniae* Isolates from Clinical and Water Samples in Diyala Governorate, Iraq, Using ERIC-PCR and BOX-PCR

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Abstract **Objectives:** *Klebsiella pneumoniae* bacteria are one of the most prominent causes of healthcare-associated infections globally, especially after the widespread proliferation of antibiotic-resistant strains, which are the cause of serious complications and increased mortality rates. Therefore, BOX-PCR and ERIC-PCR techniques were used to detect the genetic relationship between bacterial isolates, which is a necessary step to understand the epidemiological spread and limit their transmission. **Methodology:** The study involved the collection of 200 samaaaples: (150) clinical samples from various sources and (50) water samples from the Public Health Laboratory in Diyala Governorate. 26 isolates of *Klebsiella pneumoniae* bacteria were isolated. In this study, twelve antibiotics from different classes were tested to determine their effectiveness against *Klebsiella pneumoniae* bacteria using the Kirby-Bauer disk diffusion method. DNA was extracted from the 26 isolates using the boiling method. ERIC-PCR and BOX-PCR techniques were used to determine the genetic relatedness of these bacteria. **Results: A**ccording to the source of the samples, they were distributed as follows: 5 out of 45 urine samples (11.1%), 4 out of 40 wound samples (10%), 4 out of 30 burn samples (13.3%), 3 out of 35 sputum samples (8.6%) and 10 out of 50 water samples (20%). Molecular typing using ERIC and BOX repetitive sequences showed clear diversity among *Klebsiella pneumoniae* isolates, with an absence of a direct correlation between the genotype and the source of the isolate. Dendrogram analysis showed the genetic relationship between antibiotic-resistant *Klebsiella pneumoniae* isolates. **Conclusion:** All *Klebsiella pneumoniae* bacteria were resistant to antibiotics and the genetic profiling techniques BOX-PCR and ERIC-PCR were used to detect the presence of genetic diversity among the studied isolates, with no correlation found between the environmental source and the genotype. It was also shown that these two techniques are essential for studying the genetic diversity of *Klebsiella pneumoniae* and they are simple, reproducible, rapid and inexpensive tools.

Key Words *Klebsiella pneumoniae*, Clinical Samples, ERIC-PCR, Dendrogram Analysis, BOX-PCR, Molecular Typing, Environmental Samples

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative bacterium belonging to the Enterobacteriaceae family, the members of which are rod-shaped. It is considered part of the normal gastrointestinal flora and is an opportunistic pathogen, especially among people suffering from immunodeficiency [1].

Klebsiella pneumoniae bacteria are among the causes of nosocomial infections, as they cause a wide range of infections such as urinary tract infections, liver abscesses and bacteremia. These bacteria are able to survive in hospitals, persist on environmental surfaces and colonise human skin, the intestines and the respiratory tract and they have the ability to spread between contaminated environments and medical equipment [2]. The treatment of infections caused by *Klebsiella pneumoniae* is very difficult due to the resistance it shows against antibiotics, thus representing a major treatment challenge because of the bacteria's inherent and acquired resistance. *Klebsiella pneumoniae* is considered the second most common gram-negative pathogen, after Escherichia coli, as is known in healthcare environments. The spread of Multidrug-Resistant (MDR) organisms or 'superbugs' has escalated and this raises concerns about increased anxiety and more severe consequences [3]. Genotyping is a key tool for combating pathogens and for monitoring the spread or dominance of certain strains within healthcare institutions, or for determining whether certain infections are linked to disease outbreaks [2].

One of the important points for preventing the spread of hospital-associated infections and healthcare settings is to know the relatedness between bacterial species. Polymerase chain reaction based on repetitive sequence elements (ERIC-PCR) is a molecular investigation method for repetitive nucleotide sequences within the bacterial genome, grouping bacterial strains into categories. These repetitive elements (Enterobacterial Repetitive Intergenic Consensus-ERIC) vary in pattern and number within the bacterial genome. ERIC-PCR is a reliable and low-cost method for molecular typing of the Enterobacteriaceae family, which distinguishes between the genetic diversity of strains [1]

Another technique that is a useful molecular marker in the analysis of genetic diversity is the BOX-PCR technique. BOX-PCR sequences are conserved, short, repetitive DNA sequences found in the genomes of most bacterial species. When amplifying BOX elements, a distinctive banding pattern appears, which helps in differentiating between different bacterial strains [4]. Both of these techniques, ERIC-PCR and BOX-PCR, are among the elements frequently used in molecular typing. BOX elements are mosaic repetitive elements consisting of three subunits: boxA, boxB and boxC, with lengths of 59, 45 and 50 nucleotides respectively. Similarly, for ERIC-PCR sequences, they are 126bp long with a conserved central inverted repeat, located between non-coding transcription regions in the chromosome. The BOX primer anneals to the boxA subunit of the BOX elements, while the ERIC primer synthesizes DNA sequences outwards from the inverted repeats [5].

This study aims to conduct molecular genotyping on clinical and environmental isolates to understand the epidemiological relationship and the extent of the spread of closely related genotypes among the different sources of the isolates.

METHODS

About 200 samples were collected from various sources. They included 150 clinical samples (urine, wound infections, burn infections and sputum) from Baqubah Teaching Hospital and 50 environmental samples (water) sent to the Public Health Laboratory for examination in the Food Department in Diyala Governorate, during the period from August 2025 to November 2025. For the purpose of initial isolation and diagnosis, the collected samples were cultured on MacConkey agar and the environmental samples were cultured on nutrient broth. *Klebsiella pneumoniae* bacterial isolates were identified on differential EMB agar based on phenotypic characteristics on culture and differential media and HiCrom agar, which is a selective medium for *Klebsiella pneumoniae*, was used to confirm the diagnosis.

Antibiotic Susceptibility Test (AST)

The Kirby-Bauer method [6] was used to test the sensitivity of *Klebsiella pneumoniae* bacteria using twelve antibiotic discs.

Table 1: Primers Oligonucleotide Sequences Used in This Study

Primer	Oligo sequence (5'-3')	Product size bp
BOX	CTACGGCAAGGCGACGCTGACG	Variable
ERIC	ATGTAAGCTCCTGGGGATTCA AGTAAGTGACTGGGGTGAGCG	Variable

Table 2: Amplification Program of Primers

Amplified gene	Initial denaturation	No. of cycle	Denaturation	Annealing	Elongation	Final extension
BOX	94°C/ 5min	35	94°C/ 1min	40°C/1min	72°C/2 min	72°C/10min
ERIC	94°C/ 16min	35	94°C/ 30 sec	48°C/2min	72°C/4min	72°C/16min

Genotyping Detection

DNA Template Preparation by Boiling Method: The DNA template was prepared using the boiling method as described in the study by Ali and Khudhari [7]. Five colonies isolated from an overnight bacterial culture were suspended in 2 mL of distilled water and then placed in a water bath for ten minutes. Subsequently, centrifugation was performed and the resulting supernatant was used as the DNA template for the PCR reaction.

PCR Amplification

The PCR amplification procedure for the genetic level to detection *Klebsiella pneumoniae* local isolates Table 1 by follow step.

A total volume of PCR mixture was 25 µL (12.5 of Master Mix 2x, 5µL of template DNA, 1µL of each of the forward and reverse primer and 5.5 µL nuclease free water) in Uniplex PCR Eppendorf tubes but amount changed in multiplex PCR, mixed briefly via vortex then been placed in thermocycler polymerase chain reaction. The program used for each multiplex PCR mixture was illustrated in the Table 1.

Primer Dilution

The oligonucleotide primers in a lyophilised state were dissolved and first diluted with Nuclease-free D.D.W, according to the manufacturer's recommendations, to obtain a concentration of 100 pmol/µL. This stock solution was then diluted using Nuclease-free D.D.W to reach a concentration of approximately 10 pmol/µL. This method included all primers as shown in Table 2. The specifications for all gene primers were obtained from Alpha DNA Company, USA.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to detect PCR amplification products, which were visualised using ethidium bromide staining and a UV transilluminator in 1× TBE buffer [8].

ERIC and BOX Results Analysis

ERIC and BOX patterns were analysed via MEGA X online data analysis.

RESULTS

Isolation and Identification of *Klebsiella pneumoniae*

The total bacterial growth appeared in 147 samples as positive bacterial growth, which represents (73.5%), where sixteen clinical isolates were isolated at a rate of (10.7%) and ten environmental isolates at a rate of (20%) of *Klebsiella pneumoniae* bacteria, while other bacterial species accounted for 121 at a rate of (60.5%) (Table 3).

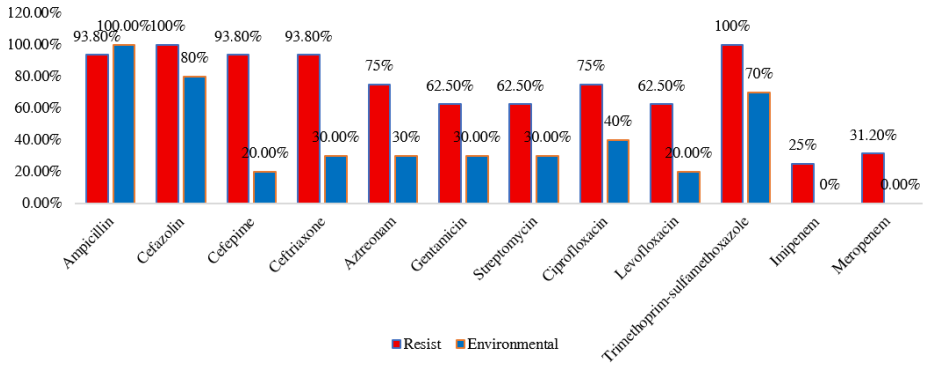


Figure 1: Antibiogram of *Klebsiella pneumoniae*

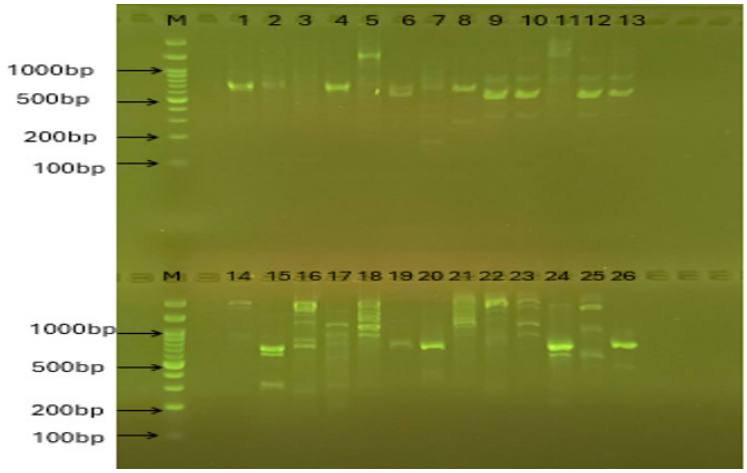


Figure 2: Gel Electrophoresis for ERIC-PCR of 26 Isolates *Klebsiella pneumoniae* using 1.5%

Table 3: Source of *Klebsiella pneumoniae* Isolates

Source	*Klebsiella pneumoniae* isolates
Urine (U)	5
Wounds (Sw)	4
Burns (Sb)	4
Sputum (S)	3
Water (W)	10
Total	26

Antibiogram

About 26 isolates of *Klebsiella pneumoniae* bacteria were tested against 12 antibiotics as shown in Figure 1. Clinical isolates showed varying degrees of antibiotic resistance; the highest resistance rate for clinical isolates was to Trimethoprim-sulfamethoxazole and Cefazolin at 100%, followed by Ampicillin, Cefepime and Ceftriaxone at 93.8%. Meropenem showed 31.2% resistance and the lowest resistance rate was for the antibiotic Imipenem at 25%. As for the environmental isolates, they showed a less intense resistance pattern than the clinical isolates; the highest resistance recorded in water isolates was 100% for the antibiotic Ampicillin, while Trimethoprim-sulfamethoxazole showed 80 and 70% respectively. Streptomycin, Ceftriaxone, Aztreonam and Gentamicin showed 30% resistance. As for Cefepime and Levofloxacin, resistance was recorded at 20%. All environmental isolates showed sensitivity against Meropenem and Imipenem.

Result of ERIC PCR

Figure 2 shows the gel electrophoresis image of the ERIC-PCR results. The number of bands ranged from 1 to 6 bands, while the size ranged between 100bp and 3kbp. The degree of similarity between strains was determined using banding pattern analysis via the MEGA X software. According to the electrophoresis results and based on the Dice method for statistical analysis and the UPGMA method for clustering and in light of the electrophoresis and software analysis results, the *Klebsiella pneumoniae* isolates showed genetic diversity.

Figure 2 shows the gel electrophoresis image of the ERIC-PCR results. The number of bands ranged from 1 to 6, while the size ranged between 100bp and 3000bp. To determine the degree of similarity between the strains, band pattern analysis was used.

ERIC-PCR analysis using heat mapping and hierarchical clustering showed a clear variation in band patterns among the studied isolates, reflecting significant genetic diversity. The heat map showed the distribution of the presence and absence of bands across a wide range of genetic sizes, where red cells represent the presence of the band, while light cells indicate its absence.

The cluster analysis in Figure 3 showed the grouping of isolates into several distinct groups based on the degree of similarity in banding patterns. It was observed that some isolates, such as (SW)4, (W)19 and (U)3, clustered in the same group, indicating a potential genetic proximity.

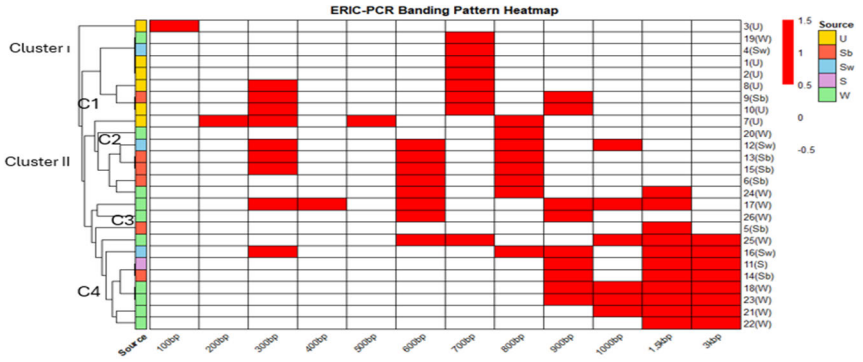


Figure 3: Dendrogram Generated from ERIC-PCR Fingerprinting of 26 Isolates *Klebsiella pneumoniae*

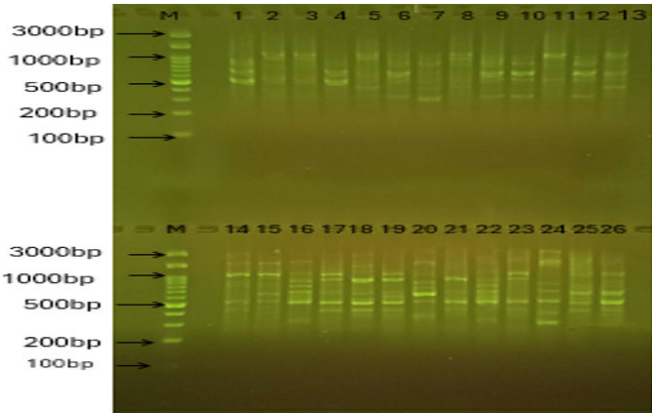


Figure 4: Gel Electrophoresis for BOX-PCR of 26 Isolates *Klebsiella pneumoniae* using 1.5% Concentration

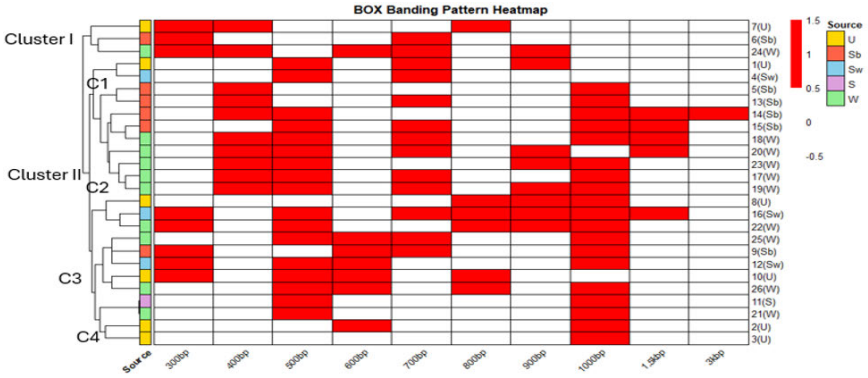


Figure 5: Dendrogram Generated from BOX-PCR Fingerprinting of 26 Isolates *Klebsiella pneumoniae*

In contrast, a group appeared at the bottom of the diagram, such as (W)23, (W)22 and (W)21, with a distinct pattern represented by the clear presence of bands with large sizes (3kbp, 1.5kbp), suggesting a genetic specificity that might reflect a different strain or a specific environmental adaptation. It is notable that some isolates from different sources clustered in the same group, which implies the absence of a direct correlation between the environmental source of the isolate and the genetic pattern.

These results indicate the presence of genetic polymorphism among the isolates, which may be due to factors such as horizontal gene transfer or differences in environmental pressures.

Result of BOX-PCR

Figure 4 shows the gel electrophoresis image of the BOX-PCR results. The number of bands ranged from 1 to 7, while the size ranged between 300bp to 3kbp. To determine the degree of similarity between the strains, banding pattern analysis was used using the MEGA software. Figure 5 shows the presence of distinct clusters of isolates based on the similarity of genetic band patterns, where red cells indicate the presence of a band, while light cells represent their absence. The clusters were divided into Cluster I and Cluster II, with C1, C2, C3 and C4 branching from Cluster II. Some isolates, such as (W)24, (Sb)6 and (U)7, clustered together, indicating high genetic similarity and the possibility of a

common origin or evolutionary proximity between them. In some groups, isolates taken from different sources (U, W, Sb, SW) appeared together in the same group. This indicates that genetic similarity is not directly related to the source of the isolate. In other cases, it was observed that some isolates from the same source were distributed into different groups, reflecting the existence of genetic variation within the same environment. These results highlight the importance of using BOX-PCR technology as an effective tool in studying genetic diversity and epidemiological relationships between bacterial isolates.

DISCUSSION

Klebsiella pneumoniae is highly virulent and can cause severe community-acquired infections with a high mortality rate. Recently, antibiotic-resistant strains have spread widely across the world [8]. *Klebsiella pneumoniae* is one of the most common Multi-Drug Resistant (MDR) pathogens and is a significant source of healthcare-associated pathogens affecting humans [9].

In the current study, the isolation rate of *Klebsiella pneumoniae* bacteria from clinical samples was recorded at (10.7%), which is a lower rate than in some studies [10,11]. For the study by Abed and Mnif [12], where the rate was (8%), the isolation rate was comparable to the current study. The isolation rate of *Klebsiella pneumoniae* in water was recorded at (10%), which is similar to the results of the study by Khalefa *et al.* [13].

High levels of antibiotic resistance were observed, particularly against Cefazolin at 100%, consistent with Iraqi studies [14]. In a study by Masalane *et al.* [15], bacterial resistance to the antibiotic Trimethoprim-sulfamethoxazole was also 100%, which aligns with this study. Imipenem and Meropenem showed the highest efficacy, recording the lowest resistance rates, consistent with the study by Li *et al.* [16], where resistance to both antibiotics was (20.1%). In water isolates, ampicillin showed the highest resistance at 100.0%, as all isolates were resistant to this antibiotic [17].

Given the important role played by *Klebsiella pneumoniae* bacteria in hospital-acquired infections, genotyping plays a useful role in identifying sources of infection and consequently, in preventing hospital-acquired infections [18].

The results of the current study using the ERIC-PCR technique showed that some isolates of different origins clustered within the same group and this result agrees with Mahmud *et al.* [19], where the researchers observed that isolates from environmental sources clustered in the same group with clinical isolates. Therefore, the clustering of antibiotic-resistant *Klebsiella pneumoniae* isolates of different origins within the same group may indicate genetic similarity.

The BOX-PCR results, as shown in Figure 5, were divided into two clusters and a unique clone called Cluster one. Four groups branched from Cluster two. Despite the difference in the sources and locations of the isolates, a phylogenetic similarity was observed, indicating an epidemiological relationship between these isolates. These results are consistent with the findings of Al-Qaysi *et al.* [3] regarding the genetic similarity among antibiotic-resistant *Klebsiella pneumoniae* isolates collected from different sources.

CONCLUSION

The results of the current study revealed that all *Klebsiella pneumoniae* isolates showed a Multi-Drug Resistant (MDR) pattern through antibiotic susceptibility testing. Using molecular genotyping techniques BOX-PCR and ERIC-PCR, a clear genetic diversity was evident among the studied isolates, in addition to the presence of genetically similar isolates despite their different sources. These results indicate that there is a possibility that some strains were transmitted or spread between environments.

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