Egyptian Journal of Medical Microbiology Volume 34 / No.4 / October 2025 431-436 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Molecular Screening of Clinical *Klebsiella pneumoniae* Isolated from Ramadi Hospitals

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Key words:
Klebsiella pneumoniae, MDR, MLST, ST. Allelic profile

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ABSTRACT

Background: *Molecular characterization of bacterial strains is essential for understanding the epidemiology of healthcare-associated infections. Strain-level typing enables the differentiation between epidemic strains, which spread between individuals, and endemic or sporadic strains.* ***Aim:*** *This study aimed to identify clinical Klebsiella pneumoniae strains using Multilocus Sequence Typing (MLST) to enhance epidemiological surveillance.* ***Methodology:*** *Multidrug-resistant (MDR) K. pneumoniae isolates were subjected to molecular characterization using MLST. Sequence analysis was performed in accordance to Pasteur Institute's MLST database protocols.* ***Results:*** *Sequencing of seven housekeeping genes for 10 MDR K. pneumoniae isolates revealed that three distinct sequence types (STs): ST16, ST218, and ST283. These STs were recorded for the first time in Iraq according to the Pasteur MLST database.* ***Conclusion:*** *Phylogenetic analysis by MLST scheme of K.pneumoniae strains with ST283 indicated that they are related to strains which discovered in Brazil with same Sequence Type (STs). While the relatedness of ST16 strains close to strains discover in UK . So, the capability of genetic relatedness of different strains of infectious pathogen is very important for the study of epidemiological and surveillance which essential for developing public health control approaches.*

INTRODUCTION

Bacterial typing is crucial for epidemiological studies of bacterial infections. Recent advancements in molecular methods have significantly improved diagnostic methods, leading to molecular techniques largely replacing older phenotypic typing methods[1,2]. Therefore, typing based on nucleic acids sequencing can be used to pathogenesis typing, which can help us understand how they develop, spread, and how to prevent them. This is because conserved genes are found in all organisms and remain stable over time. The most accurate and reliable way to identify and classify organisms is to sequence specific regions of their DNA that are known to be conserved across different strains such: Multilocus sequence typing (MLST)[3]. Multilocus Sequence Typing (MLST) is a molecular method for bacterial identification and classification, it analyzes the DNA sequences of seven "housekeeping genes," which are essential genes highly conserved across bacteria. These gene sequences are amplified using PCR, then sequenced and compared to a database of known MLST types. This comparison assigns the bacteria to a specific MLST type, revealing its genetic lineage and potential for causing disease [4].

METHODOLOGY

Isolation and Identification of *K. pmneumonae* strains:

In this study, *K. pneumoniae* strains were isolated from UTI and sputum samples. Each sample was cultured on MacConkey agar and blood agar and incubated at 37°C for 24 hours. *K. pneumoniae* colonies were identified by morphological characteristics, Gram staining, and standard biochemical tests such as lactose fermentation, indole, methyl red, Voges-Proskauer, and citrate utilization tests. The study was conducted between February and October 2023 in (Ramadi city/Iraq) hospitals and some private clinics. 140 patients of both sexes and varying ages. Subsequently, the Vitek 2 Compact system with a GN (Gram-Negative) card was utilized for definitive species identification according to the manufacturer's instructions (bioMérieux, France).

Antibiotics susceptibility test:

Antimicrobial susceptibility testing for each pure isolate was performed according to **Kirby-Bauer disc diffusion method**, as outlined by CLSI Institute 5 guidelines, and also utilized the automated **Vitek-2 compact system** with AST cards[5]. Ten antimicrobial agents were tested, including Amox-clav, Ceftriaxon,

Cefotaxime, Ceftazidime, Meropenem, Amikacin, Levofoxaccin, Colistin, Ciprofloxacin, and Piperacillin-Tazobactam.

Phenotypic confirmation test for extended- spectrum β-lactamases (ESBLs).

- **Modified hodge test (MHT) :**In order to identification of **ESBLs** and Carbapenems producing *K. pneumoniae*, all β-lactam-resistant strains susceptible isolates were identify then examined by phenotypic (MHT) according to [6]. Clover leaf result gave indicator for positive for this test.
- **Double disk synergy test (DDST):** The test steps were done according to [7];[8].
- **Modified cephalosporine inactivation methods (mCIM) and eCIM:** The methods were done according to [5].

Molecular Screening:

Bacterial DNA was extracted using a Promega DNA mini Kit (Promega, USA). All PCR reactions were carried out in Applied Bio-system 2720 thermal cyclers (USA) using primers for MLST housekeeping genes (details in Table 1). *Escherichia coli 25922* genomic DNA served as a negative control. PCR programs were adapted from the Pasteur Institute, with minor adjustments, and also followed methods described in reference [8]. PCR products were purified using a Qiagen-Germany PCR purification kit. The purified MLST gene DNA fragments were then sent to Macrogen Company in Korea for Sanger sequencing using an ABI3730XL automated DNA sequencer, employing both forward and reverse primers. For data processing, allelic profiles and sequence types (STs) were determined using the Pasteur Institute database website.

Table 1: Primers used for the determination of Multilocus Sequence type (MLST).

Gene		Sequence of forward and reverse Primer 5´- 3´	PCR Product Size bp	Annealing Temp.	Reference
gapA gene	F	TGA AAT ATG ACT CCA CTC ACG G	**662bp**	**60**	Pasteur Institute/Online
	R	CTT CAG AAG CGG CTT TGA TGG CTT			
rpoB gene	F	GGC GAA ATG GCW GAG AAC CA	**1075 bp**	**50**	
	R	GAG TCT TCG AAG TTG TAA CC			
mdh gene	F	CCC AAC TCG CTT CAG GTT CAG	**756 bp**	**50**	
	R	CCG TTT TTC CCC AGC AGC AG			
pgi gene	F	GAG AAA AAC CTG CCT GTA CTG CTG GC	**556 bp**	**50**	
	R	CGC GCC ACG CTT TAT AGC GGT TAA T			
phoE gene	F	ACC TAC CGC AAC ACC GAC TTC TTC GG	**602 bp**	**50**	
	R	TGA TCA GAA CTG GTA GGT GAT			
infB gene	F	CTC GCT GCT GGA CTA TAT TCG	**462 bp**	**50**	
	R	CGC TTT CAG CTC AAG AAC TTC			
tonB gene	F	CTT TAT ACC TCG GTA CAT CAG GTT	**539 bp**	**45**	
	R	ATT CGC CGG CTG RGC RGA GAG			

RESULTS

Identification of *Klebsiella pneumoniae*

This study examined 140 sputum and urine samples obtained from patients with various ages and both sexes, and collected from Al-Ramadi Teaching General Hospital and Al-Ramadi Teaching for Children and Maternity Hospital. These patients were presented with urinary tract infections (UTIs) and pneumonia. Of the total specimens collected, 90 (64.2%) yielded positive bacterial cultures, while 50 (35.0%) were culture-negative likely due to prior antibiotic therapy or suboptimal sampling conditions. Bacterial isolates were initially identified based on colonial morphology and standard biochemical tests (Table 2). Final confirmation was achieved using the Vitek 2 Compact system with GN (Gram-Negative) identification cards (bioMérieux, France).

Among the 90 culture-positive samples, 70 isolates were identified as *Klebsiella pneumoniae*. Further analysis of the source of the positive cultures revealed that 45 isolates (64.2%) originated from urine samples and 25 isolates (35.7%) from sputum. The remaining 20 isolates, although culture-positive, were attributed to bacterial species other than *K. pneumoniae*, indicating the presence of a variety of pathogens in the clinical specimens.

Table 2: Biochemical tests for *K. pneumoniae* isolates

Test	*K.pneumoniae*
MacConkey agar medium	Lactose ferment (+)
Gram-stain	G- rod
"Catalase test"	+
"Oxidase test"	-
"Indole Test"	-
"Methyl Red Test"	-
VP Test	+
"Citrate Utilization Test"	+
Urease	+

Antimicrobial Susceptibility

Our study investigating the antibiotic resistance profiles of 70 *K. pneumoniae* isolates employed Kirby-Bauer disk diffusion and the Vitek-2 compact system [5]. The results revealed a high degree of antibiotic resistance diversity among the *K. pneumoniae* isolates (Figure1). Notably, over 65% of the isolates exhibited resistance to at least one agent from three or more antimicrobial categories, classifying them as multidrug-resistant (MDR). Furthermore, 35% of the isolates were categorized as XDR.

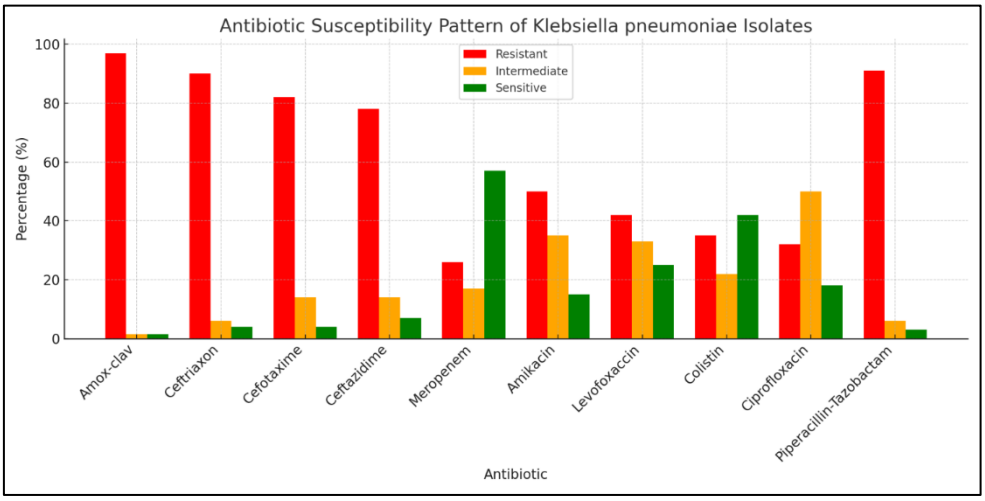

Fig. 1: Antimicrobial Susceptibility Results

We examined thirty multidrug-resistant (MDR) isolates for the production of carbapenemases and ESBLs. The **modified Hodge test**, a phenotypic method, confirmed the production of both serine and metallo-β-lactamases in 28 out of 30 isolates (93%). For ESBL production in cephalosporin-resistant *K. pneumoniae*, all isolates tested positive using the **MCIM method**. Finally, the **imipenem-EDTA disk method** was employed to phenotypically detect metallo-β-lactamases, revealing that 20 out of 30 isolates (66.6%) were positive for DDST as ESBL.

Molecular Screening of MLST Analysis

The PCR electrophoresis results confirmed that all target genes were successfully amplified and fully identified through MLST screening (Figure 2).

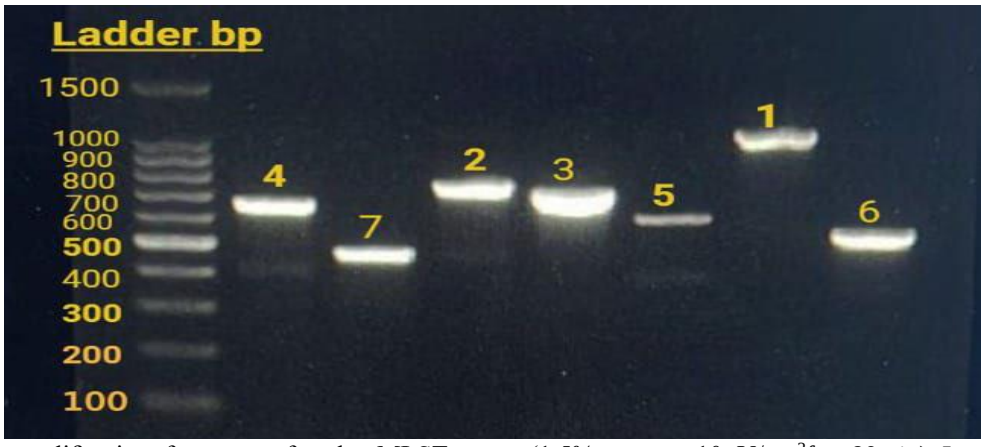

Fig. 2: PCR amplification fragments for the MLSTgenes, (1.5%agarose, 10 V/cm^{2}for 90min) Lane1(*rpoB* gene 1075bp), Lane 2(*mdh* gene 756), Lane 3(*phoE* gene 602bp), Lane 4(*gap* gene 662bp), Lane 5 (*pgi* gene 556bp), Lane 6(*tonB* gene 539bp), Lane 7(*inf*B gene462bp).

Ten bacterial isolates, previously identified as highly resistant to multiple antimicrobial agents, underwent molecular typing. Sequencing of housekeeping genes and subsequent analysis using the MLST databases at the Pasteur Institute revealed the presence of three sequence types (STs): ST16, ST218, and ST283. Notably, these specific STs represent newly identified strains within Iraq, as per the Pasteur Institute's MLST database records (Table 3).

Phylogenetic analysis of *K.pneumoniae* strains using MLST scheme of ST283 indicated that the registered strains under study is more close to strains which discovered in Brazil with same STs (fig.3). While the relatedness of ST16 strains under study were to strains discover in UK (fig.4).

Table 3: The sequence type (STs) and other details for strain that demonstrated according to Institute Pasteur-databases.

ID	Strain Name	ST	Date of isolation	Origin	Type of Infection	Sample	Antibiotic Resistance
1	HSAN11-IQ	16	2022	colonization	UTI	Urine	MDR
2	HSAN22-IQ	16	2022	infection	Pneumonia	Sputum	XDR
3	HSAN45-IQ	218	2023	infection	UTI	Urine	MDR
4	HSAN55-IQ	283	2022	infection	Pneumonia	Sputum	MDR
5	HSAN60-IQ	283	2022	infection	Pneumonia	Sputum	MDR
6	HSAN63-IQ	283	2022	infection	Pneumonia	Sputum	XDR

ST: sequence type, XDR: Extensively Drug resistance , MDR: Multidrug resistance.
UTI: Unary Tract Infection.

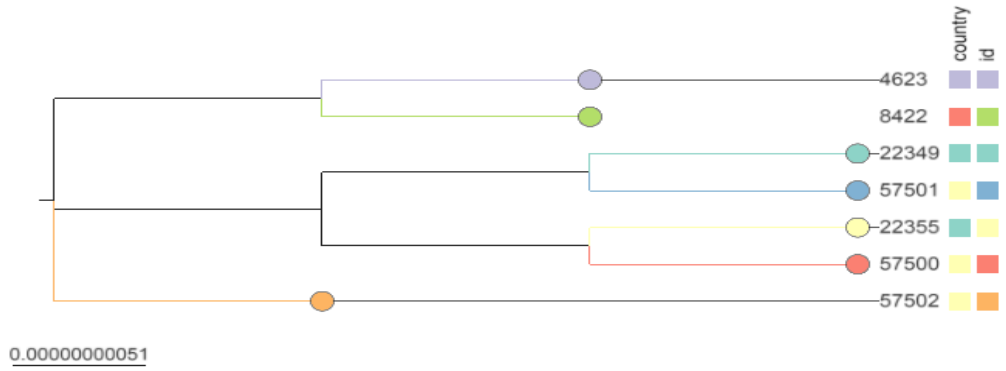

Fig. 3: Phylogenetic Newick Tree Analysis by MLST Scheme, indicate the relatedness of strains under study with ST283; ID: 57500,57501 with strains ID: 22349,22355 in Brazil.

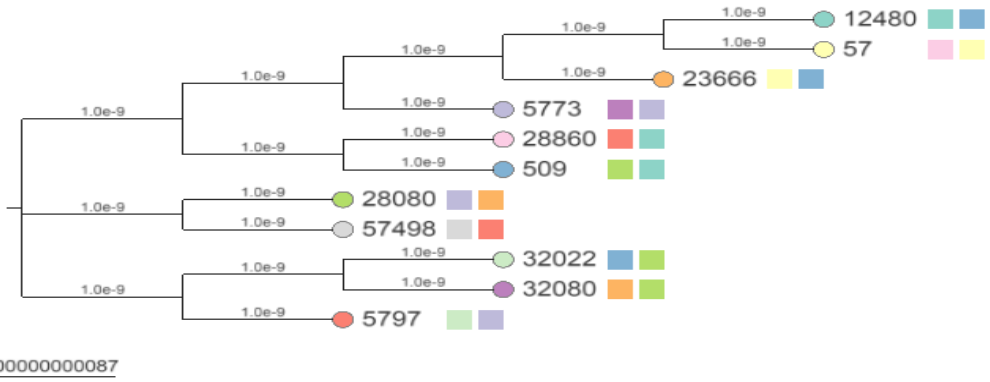

Fig. 4: Phylogenetic Newick Tree Analysis by MLST Scheme, indicate the relatedness of strains under study with ST16; ID: 57498 with strains ID: 28080 in United Kingdom.

DISCUSSION

Infections with carbapenemase-producing *K. pneumoniae* are tough to treat, leading to high death rates. This is because plasmids in *K. pneumoniae* are key to how these bacteria spread and pick up genes for antibiotic resistance and virulence. These extrachromosomal DNA molecules are responsible for many key bacterial traits, leading some researchers to consider them as independent organisms [9]. Scientists discovered some bacteria that are almost impossible to treat and others that are completely untouchable by current antibiotics, this is a worrying development as it could make even common bacterial infections extremely difficult to control in the future [2,].

Carbapenem-resistant bacteria are a growing public health concern, and *K. pneumoniae* is a prime example. While carbapenems are typically the last line of defense against multi-drug resistant infections, carbapenem-resistant *K. pneumoniae* (CRKP) strains are emerging, leading to high mortality rates in infected patients[10]. Resistance to carbapenems in *K. pneumoniae* is due to either production of carbapenemase enzymes, alteration of porin or upregulation of efflux pump [11].

Out of thirty multidrug-resistant (MDR) *K. pneumoniae* isolates examined, the study aimed to screen for the production of carbapenemases and extended-spectrum β-lactamases (ESBLs) using various phenotypic methods. The MHT serves as a general screening tool for carbapenemase activity, including both serine and metallo-β-lactamases. A 93% positivity rate indicates a high prevalence of carbapenemase production among the isolates. This is a concerning finding as carbapenemases confer resistance to last-resort β-lactam antibiotics, significantly limiting treatment options. Although MCIM is generally used for carbapenemase detection, the statement implies it was applied to cephalosporin-resistant isolates to suggest ESBL activity. A universal positive result suggests widespread ESBL production among cephalosporin-resistant *K. pneumoniae*. This supports the notion that these isolates are producing enzymes that hydrolyze broad-spectrum cephalosporins, contributing to therapeutic challenges. These findings underscore a critical resistance threat posed by K. pneumoniae in clinical settings, particularly in the context of limited therapeutic options. The simultaneous presence of ESBLs and carbapenemases especially MBLs-highlights the need for stringent infection control measures, antibiotic stewardship, and further molecular studies to identify the specific resistance genes involved.

To investigate the genetic relationships among *K. pneumoniae* strains, we performed Multilocus Sequence Typing (MLST). This involved screening the genomic DNA of multidrug-resistant (MDR) isolates for the presence of seven housekeeping genes: *tonB, infB, phoE, pgi, mdh, gapA*, and *rpoB*, using PCR. The amplified genes were then sequenced to identify unique alleles. These alleles were subsequently assigned numbers, creating allelic profiles that defined the sequence types (STs) of each strain. Finally, all the data was analyzed using the Pasteur Institute MLST database and scheme to determine the relatedness of the strains. The accession gene numbers for each gene were assigned by the Pasteur Institute after comparing sequence of housekeeping genes under the study with the genes of the same bacteria in the Pasteur Center's gene database, so the **(ST)** is the result of the combination of the alleles at the different loci. Ten bacterial isolates, previously identified as highly resistant to multiple antimicrobial agents, underwent molecular typing. In the Pasteur database, only four isolates of *K. pneumoniae* belonging to ST283 have been recorded, all classified as KpII. Our recent study identified an additional three ST283 isolates from sputum samples of patients with nosocomial infections at Ramadi Teaching Hospital. These new isolates were also classified as KpII and are molecularly very similar, suggesting they belong to the same strain.

It's important to note that the classification of K. *pneumoniae* phylogroups has evolved. While it was traditionally divided into KpI, KpII, and KpIII, recent research proposes reclassifying KpII as a new species, *K. quasipneumoniae*. This new species would encompass two subspecies: *K. quasipneumoniae* subsp. *quasipneumoniae* (corresponding to KpII-A) and *K. quasipneumoniae* subsp. *similipneumoniae* (corresponding to KpII-B)[12]. In 2016, the Institut Pasteur of Madagascar identified the first ST283 strain in Africa. Through MLST typing, they determined this strain to be *K. quasipneumoniae* subsp. *quasipneumoniae*. In 1997, scientists in Paris identified the first strain of *K. pneumoniae* with a specific genetic profile known as ST16. This particular strain was found in a sample taken from a person's respiratory system. Fast forward to today, and the Pasteur Institute has logged over 824 different *K. pneumoniae* strains with ST16 in their public database. Interestingly, while ST16 is known to be very common in Asia, it has now been identified for the first time in Iraq. When analyzed the genetic relationships of strains with ST283, they found that the Iraqi strains they were studying were genetically very similar to strains previously found in Brazil that also had the ST283 profile. However, when they looked at the ST16 strains from Iraq, they discovered that these were more closely related to strains that had been found in the UK.

This ability to accurately tell the difference between various strains of infectious microbes is incredibly important. It allows experts to conduct thorough epidemiological and surveillance studies, which in turn helps in designing effective public health strategies to control disease outbreaks. Multilocus sequence typing (MLST), the method used in this analysis, has become a widely accepted technique for this kind of detailed genetic fingerprinting. Its use has significantly enhanced

our understanding of how bacteria evolve and has provided valuable insights into how bacterial diseases spread.

CONCLUSION

Phylogenetic analysis by MLST scheme of *K.pneumoniae* strains with ST283 indicated that is more close to strains which discovered in Brazil with same STs. While the relatedness of ST16 strains close to strains discover in UK. The capability of correctly differentiate between different strains of infectious pathogen is very important for epidemiological and surveillance, and for developing public health control approaches.

Ethical Approval

This study received approval from the **Ethics Committee of Al Anbar Medical Research University** on December 21, 2023, under **approval number 342**. All participants provided **informed consent,** and no individuals under the age of 16 were included in the study. Conflict of interest.: The authors declare no conflict of interest.

Declarations:

Consent for publication: Not applicable

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

Competing interests: The author(s) declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

This manuscript has not been previously published and is not under consideration in another journal.

Funding: Authors did not receive any grants from funding agencies.

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