

**Republic of Iraq
Ministry of Higher Education
& Scientific Research
University of Al-Qadisiyah
College of Sciences
Department of Biology**



**Identification and Molecular Characterization of Drug Resistant
Klebsiella pneumoniae Isolates and Multilocus Sequence Typing
of A Hypermucoviscous Strain**

A Thesis

**Submitted to the College of Sciences
University of Al-Qadisiyah in partial
Fulfillments of the requirements for the Master
degree of Science in Biology**

By

Hayder Ali Hussein Al-jammali

B.Sc. (University of Al-Qadisiyah,.Biology, 2010)

Supervised By

Assist. Prof. Dr. Dhuha Mahdi Jabir

2025 AD

1446 AH

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

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Supervisor: Assist. Prof. Dr.

Dhuha Mahdi Jabir

College of Science, University of Al-Qadisiyah

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I certify that this thesis was carried out in the Department of Biology, and I nominate it to be presented for discussion.

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Signature :

Name : **Najla Abdullah Dawoud**

Scientific degree: **Assist. Prof. Dr.**

Address: Department of Microbiology, College of Biotechnology, University of Al-Qadisiyah..

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Signature :

Name : **Lama Fouad Manhar**

Scientific degree: **Assist. Prof. Dr.**

Address: College of Environmental Sciences, University of Al-Qasim Green.

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Name : **Sahar Abdul Ameer Haraj**

Scientific degree: **Prof. Dr.**

Address: Department of English, College of Education, University of AL-Qadisyiah

Date : / / 2025

Committee Certificate

We are examining committee, certify that we have read this thesis **(Identification and Molecular Characterization of Drug Resistant *Klebsiella pneumoniae* Isolates and Multilocus Sequence Typing of A Hypermucoviscous Strain)** and have examined the student " **Hayder Ali Hussein** " in its contents, we find it adequate as a thesis for the award of the Degree of Master in Biology

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Address: Dean collage of sciences

Date: / / 2025

Dedication

- *To my Lord ...the night is not sweet except with your remembrance, the day is not sweet with your obedience, and the moments are not good except for remembering you.*
- *To the one who delivered the message and led the trust ... Muhammad is the messenger of Allah.*
- *To my master, the Imam of the Age and Time*
- *To the one who gave me everything he had ... My Father -To whose supplication was the secret of my success ... My Mother*
- *To the seed of my heart and hope for tomorrow ... My wife and My children -To everyone who supported me, even with a smile.*

Hayder

Acknowledgements

Praise be to Allah and peace and blessings be upon the Prophet Muhammed may Allah bless him and his family.

I would like to express my sincere gratitude to my supervisor, **Assist Prof. Dr. Dhuha Mahdi Jabir**, for her invaluable assistance in solving the challenges I faced in this work. She always supports me in every step of my research with her valuable ideas and information.

I am also pleased to extend my thanks to the Deanship of the College of Sciences, represented by **Professor Dr. Salwan Ali Obaid**, and to the Head of the Department of Biology, represented by **Professor Dr. Layth Sareea Mutar**.

I extend my thanks and gratitude to all the staff of the Microbiology Unit, Laboratory Division, at Al-Diwaniyah Teaching Hospital, for their assistance and facilitation during one of the stages of my dissertation.

I extend my sincere gratitude to my family for their support in completing my thesis. Finally, I also extend my thanks to everyone who helped me complete this work.

Hayder

Summary

Multidrug-resistant *Klebsiella pneumoniae* is a global public health problem. Over the last decades, several hypermucoid strains of *K. pneumoniae* have emerged with a distinctive feature from classical strains. Infections due to hypermucoviscous strains have increased with significant mortality and morbidity.

This study aimed to investigate most common virulence genes associated with the multiple antimicrobial resistance property in *K. pneumoniae*, as so as investigate the incidence of hypermucoviscous strains among hospital acquired infections with *K. pneumoniae*. For this purpose 316 clinical samples have been collected from Al-Diwaniyah Teaching Hospital during the period from 20th of October 2024 to the 20th of January 2025, distributed as the following 113 from urine, 98 from septum, 70 from wound and 35 from blood. All samples have been collected in tubes equipped with transport media and immediately transferred to the laboratory in the laboratory the samples streaked on both blood and MacConkey agar and incubated at 37 °C for 24 hours. The growing bacteria were diagnosed depending on Gram stain and several biochemical tests, then indol negative, positive, Voges-Proskauer test, positive Simmon's citrate test, Catalase production and urease positive gram-negative rods were diagnosed as *Klebsiella pneumoniae*. For more identification GN/ID cards Vitek2 system (BioMérieux\ France) has been used. The results showed that *K. pneumoniae* were isolated from 71(30%) of 316 clinical sample most of them were from septum samples with percentage 33.7%. indicating that bacteria are responsible for most cases of pneumonia. Vitek2 compact system was also used to utilized to determine susceptibility of the isolated *K. pneumoniae* to different antimicrobial agents, according to National Committee for Clinical Laboratory Standards (CLSI 2024) and the results demonstrated a moderate frequency of

multidrug-resistant (MDR) isolates at 20 (28.2%) and a relatively lower frequency of extensively drug-resistant (XDR) isolates at 11 (15.5%).

Notably, no pan-drug resistant (PDR) isolates were detected. In order to evaluate the incidence of hypermucoviscous (hmvKp) strains string test was used, the results showed that 3 of 71 (4.2%) isolates were positive for string test. All of them were from sputum samples, indicating an increased prevalence of hypervirulent strains among pneumonia patients. The study also include investigation of some virulence genes associated with multiple resistance to antimicrobial agents. Those genes were (*mrkD*, *rmpA*, *blaKPC*, *blaVIM*). the results showed that *rmpA* gene was isolated from the hypermucoviscous strains only.

Multilocus Sequence Typing (MLST) was conducted for the hmvKp strain. PCR for seven housekeeping genes ((*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) was used the results showed that the hypermucoviscous *K. pneumoniae* strain was belong to the clonal group CG147 and sequence type ST 392. Which consider the first report for this Strain Iraq. The strain has recorded in pasture institute with serial number (ID: 76145).

The public health implications of clonal ST392 expansion are profound. CRKP strains are classified as "urgent threats" by the CDC due to limited treatment options and high mortality rates. The genetic stability observed in HAJ39201 isolates suggests that existing infection control measures, such as contact precautions and antimicrobial stewardship, may be insufficient to curb transmission. Enhanced surveillance leveraging real-time genomic databases like BIGSdb-Pasteur and Pathogen watch.

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List of Abbreviations

Abbreviations	Description
ABC	ATP Binding Cassette
<i>AcrAB</i>	Acriflavine resistance protein AB
blaKPC	<i>Klebsiella pneumoniae</i> carbapenemase gene
blaVIM	Verona Integron-encoded Metallo- β -lactamase
CDC	Centers for Disease Control
CG	Clonal Group
CLSI	Clinical Laboratory Standards Institute
CPS	Capsular Polysaccharide
CRKP	Carbapenem-Resistant <i>Klebsiella pneumoniae</i>
ClustalW	Cluster Alignment Weights
DMSO	Dimethyl sulfoxide
EefABC	Enterobacterial efflux System A,B, and C
EDTA	Ethylene diamine tetra acetic acid
ESBLs	extended-spectrum β -lactamases
<i>luxS</i>	S-ribosyl homocysteine lyase

<i>gapA</i>	Glyceraldehyde 3-phosphate dehydrogenase
GN	Gram Negative
GT buffer	Guanidine Thiocyanate buffer
Kdo	Keto deoxyoctulosonate
KPF	<i>Klebsiella pneumoniae</i> Fimbyiae
ICU	Intensive Care Unit
<i>infB</i>	Translation initiation factor 2
<i>iutA</i>	Iron Transport uptake system gene A
LPS	lipopolysccharide
MBL	Metallo- β -lactamase
<i>Mdh</i>	Malate dehydrogenase
MDR	Multidrug-Resistant
MEGA	Molecular Evolutionary Genetics Analysis
MLST	Multilocus Sequence Typing
<i>mrkD</i>	Mannose Resistance <i>Klebsiella</i> fimbriae
MSbA	Multidrug resistance Subunit BA
NEB Tm	New England Biolab Melting Temperature
<i>uge</i>	Uridine Diphosphate
OD	Optical Density
PDR	Pan-Drug Resistant
<i>Pgi</i>	Phosphoglucose isomerase
<i>phoE</i>	Phosphoporine E
<i>rmpA</i>	Regulator of mucoid phenotype A
<i>rpoB</i>	Beta-subunit of RNA polymerase B
ST	Sequence Type
TBE	(Tris – Borate – EDTA) Buffer
<i>tonB</i>	Periplasmic energy transducer
TSI	Triple Sugar Iron
XDR	Extensively Drug-Resistant

1.1. Introduction

Klebsiella pneumoniae, a Gram-negative bacterium belonging to the Enterobacteriaceae family, is a major opportunistic pathogen implicated in both community-acquired and nosocomial infections (Muraya *et al.*, 2022).

It is known to cause a wide spectrum of infections including pneumonia, meningitis, liver abscess, urinary tract infections, bacteremia, and wound infections (Martin & Bachman, 2018). The frequent misuse of antibiotics has led to the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, significantly increasing mortality and morbidity (Ding *et al.*, 2022).

The pathogenicity of *K. pneumoniae* is mediated by a combination of core and accessory virulence genes. Among the most studied virulence genes is *mrkD*, which encodes the adhesion subunit of type 3 fimbriae, enabling biofilm formation by binding to extracellular matrix proteins like collagen (Murphy *et al.*, 2013).

Another critical gene is *rmpA* (regulator of mucoid phenotype A), which enhances capsule production and is closely associated with the hypermucoviscous phenotype and severe infections. Additionally, antimicrobial resistance is largely attributed to *blaKPC* and *blaVIM* genes, which encode carbapenemases—enzymes that degrade carbapenems, the last-resort antibiotics for MDR infections (Ramadan *et al.*, 2022).

In Iraq, several studies have reported the presence of the carbapenems genes *blaVIM* (up to ~28.8%) and *blaKPC* (~20–80%) in *K. pneumoniae* clinical isolates from Baghdad between 2020–2022 (Hamad, 2022; Hasan *et al.*, 2022). However, none have concurrently examined the presence of *rmpA* or *mrkD*, representing a significant research gap in linking virulence and resistance.

Case reports and small-scale studies have documented the emergence of hypermucoviscous *K. pneumoniae* (hmKp) in Iraq as early as 2016, with confirmed string-test positive isolates and virulence-associated genes such as *rmpA* and *iutA*.

A particularly concerning phenotype is hypermucoviscous *K. pneumoniae* (hmKp), characterized by enhanced capsule production and the ability to cause metastatic, life-threatening infections even in healthy individuals (Thapa *et al.*, 2025).

Strains exhibiting this phenotype are frequently linked to sequence types such as ST23, ST65, and ST86, commonly found in East Asia and now increasingly reported worldwide (Choby *et al.*, 2020).

Genotyping methods, especially multilocus sequence typing (MLST), have provided robust frameworks for understanding the clonal structure and transmission dynamics of *K. pneumoniae*. MLST analyzes seven housekeeping genes to assign sequence types (STs), allowing cross-study and international comparisons (Liu & Ji, 2020; Ruppitsch *et al.*, 2015). The lack of published MLST-based surveillance data in Iraq represents another critical gap, particularly concerning hypervirulent clones and their resistance profiles.

Although hypervirulent *Klebsiella pneumoniae* clones-particularly those belonging to sequence types ST23, ST65, and ST86 have been frequently reported in East Asia and have increasingly emerged in other regions, no published studies from Iraq have confirmed the detection of ST23 or similar hypervirulent clones through molecular typing. While several Iraqi reports have described resistance genes such as *blaKPC*, *blaVIM*, and virulence genes including *rmpA* and *mrkD*, limited attention has been given to genotypic profiling using techniques like multilocus sequence typing (MLST), (Choby *et al.*, 2020).

1.2. Aims of the Study

The current study aims to the investigation of some virulence factors in *K. pneumoniae* and their association with antimicrobial resistant through the following objectives:

1. Isolation and identification of *Klebsiella pneumoniae* by traditional biochemical tests and by the Vitek2 system.
2. Investigation of hyperviscosity phenotypically in the isolated bacteria by string test.
3. Evaluate the antimicrobial resistance of the isolates using the Vitek2 system.
4. Investigation of the ability of isolated bacteria to form biofilm by microtiter plate.
5. Detection of some virulence genes and resistance genes of *K. pneumoniae* isolates by PCR.
6. Genotyping analysis of hypermucoviscous *Klebsiella pneumoniae* isolates by multilocus sequence typing (MLST).

2. Literatures Review

2.1. History and Taxonomy of *Klebsiella pneumoniae*

In 1882, Carl Friedlander described *Klebsiella pneumoniae* for the first time. He described an encapsulated bacillus found in the lungs of a patient who died from pneumonia. Originally named. Friedlander's bacillus, it was not until 1886 that the bacterium received the name is *Klebsiella*.

Klebsiella belongs to the Enterobacteriaceae family. *Klebsiella pneumoniae* is an opportunistic pathogen. Some natural flora may be endemic to the intestine and other areas of the body, taking advantage of the body's frailty during infection with other diseases. Therefore, it can be separated from a variety of infections (Kelly L Wyres et al., 2020). *Klebsiella* is so-called after the German microbiologist Edwin Klebs (1834–1913) (Righini, 2020). According to Bergey's Manual of Systematic Bacteriology, Second Edition (Grimont and Grimont, 2009). It is classified to the following:

Domain: Eubacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Klebsiella*

Species: *pneumoniae*

However, the truth that *Klebsiella* genus have been re-classified according to the medical importance as two former species: *K. oxytoca* and *K. pneumoniae* subspecies *aerogenes*, *ozaenae* and *rhinoscleromatis* (Barer *et al.*, 2019).

Klebsiella pneumoniae is classified into three phylogroups (KpI, KpII, and KpIII) based on gene sequences. These phylogroups have been re-designated as three species: *K. pneumoniae* (KpI), *K. quasipneumoniae* (KpII), and *K. variicola* (KpIII), all of which can infect humans (Elliott *et al.*, 2016; Wyres & Holt, 2016).

2.2. Characteristics of *Klebsiella pneumoniae*

Klebsiella pneumoniae is an opportunistic, Gram-negative, facultative anaerobic rod-shaped bacteria measuring 0.3-1 µm in diameter and 0.6-6 µm in length. It can be found single, in pairs or in short chains. *K. pneumoniae* is lactose-fermenting, non-motile, non-spore-forming, and oxidase-negative. Colonies on agar plates seem mucoid due of a thick polysaccharide capsule. *K. pneumoniae* colonies on MacConkey agar are big, pink, spherical, and mucoid, indicating lactose fermentation and acid generation (Sharma *et al.*, 2015).

Klebsiella pneumoniae is a Gram-negative, encapsulated Non-motile bacterium discovered in the environment and can colonize in animals, plants and humans (Jondle *et al.*, 2018).

Klebsiella pneumoniae is the causal agent for a variety of illnesses, including pneumonia. Urine tract infections, in addition to pneumonia, are a result of *Klebsiella* invasion. It also leads to bloodstream infection (BSI), surgical site infection, Especially in elderly, neonates and

immunocompromised, they are at risk (Hossain, 2023). Strains of *K. pneumoniae* is increasingly found to be resistant to multiple classes of antibiotics which makes it difficult to treat infections caused by this bacterium (Paczosa & Mecsas, 2016).

2.3. Pathogenesis of *Klebsiella pneumoniae*

Klebsiella pneumoniae is an opportunistic pathogen bound with both community-acquired and nosocomial infections, such as pneumonia, urinary tract infections, septicemia and wound infections, with the increasingly multidrug-resistant (MDR) *K. pneumoniae* being a major serious public health problem. The virulence of bacteria is provided by a wide range of factors that can progress to infection and antibiotic resistance. The organism's polysaccharide capsule is the most important virulence component, allowing the bacterium to prevent the host organism's destruction and serum death. *Klebsiella* species lacking a capsule tend to be less virulent, and 77 distinct kinds of capsules have been identified. Gram-negative bacteria's outer surface is coated in lipopolysaccharides, which constitute a second virulence factor (Tsereteli *et al.*, 2018).

It has been established that the detection of lipopolysaccharides triggers an inflammatory response in the host organism and is a primary cause of the sequel in septic shock and sepsis. Fimbriae, the other virulence component, enables the pathogen to attach itself to host cells. Another virulence component that the bacterium needs in order to infect its host is siderophores. Siderophores acquire iron from the host to allow the infectious bacterium to spread. *Klebsiella pneumoniae* is among the few

bacteria with substantial antibiotic resistance due to genomic modifications (Rønning, 2019; Tsereteli *et al.*, 2018).

2.4. Clinical Epidemiology

Klebsiella species are numerous in nature and may be found in soil, water, and on other surfaces. In humans, *K. pneumoniae* frequently colonizes at numerous mucosal surfaces, including the upper respiratory tract and the gut, where colonization rates vary greatly across people depending on their environment and exposures (Martin & Bachman, 2018).

According to previous research, *Klebsiella* colonization prevalence varies between 18.8% and 87.7% in Asia and between 5% and 35% in Western nations (Russo & Marr, 2019). *Klebsiella* carrier rates in fecal samples in non-hospital settings vary from 5 to 38%, but nasopharyngeal carrier rates range from 1 to 6%. Skin-related *Klebsiella* infections are uncommon and are regarded as temporary rather than chronic.

Colonization rates in the gastrointestinal system can reach 77% in hospitalized patients, whereas they can reach 19% in the nasopharynx (Podschun & Ullmann, 1998). Gastrointestinal colonization is an important reservoir for infection and transfer to other locations because of its widespread occurrence in people (Dorman & Short, 2017). The association between colonizing strains of *K. pneumoniae* and strains isolated from the infection sites has been validated by more recent research (Dorman & Short, 2017; Martin *et al.*, 2016).

In a similar way (Gorrie *et al.*, 2017), investigated the association between colonization and susceptibility to *K. pneumoniae* infection in 498

ICU patients. They discovered that 16% of the patients colonized with *K. pneumoniae* became infected, compared to only 3% in the non-carriers. Whole-genome sequencing indicated that the patients had been colonized with the same strain. From a genetic viewpoint, these investigations indicated that gastrointestinal microbiota is a key source of nosocomial *K. pneumoniae* infections, with self-colonizing strains accounting for 80% of the cases. The primary cause of the shift from colonization to infection is the host's defenses being compromised by underlying illnesses or immunomodulatory treatments (Meatherall *et al.*, 2009).

According to study by (Mohamed & Al-Taai, 2023), the biodiversity of *K. pneumoniae* has widely been reported to be associated with different pathological progress community-acquired and nosocomial infections. About 250 clinical samples included urine, sputum and swabs from wound and burns samples were collected from Al-Batool Teaching Hospital (38.4%), Baqubah Teaching Hospital (61.6%) and private laboratories in Baqubah and Diyala, Iraq. Positive rates of nosocomial acquired infection were sputum 98%, urine 96%, and swabs from wound and burns 94%, while positive rates of community acquired infection were sputum 60%, urine 60%, and swabs wound and burns 30%.

2.5. Virulence Factors

There are several virulence factors for *Klebsiella pneumoniae* that act in association with pathogen and encompass capsule antigens, adhesive factors, endotoxin producer such as lipopolysaccharide and the serum killer effect resistance and system for the acquisition an iron (Siderophore) and the multi-resistance for the antibiotics that were the leading cause in hospital propagation acquiring infections,

since the infections percentage 80% that resulted in the current alternative treatment (Jasim *et al.*, 2020).

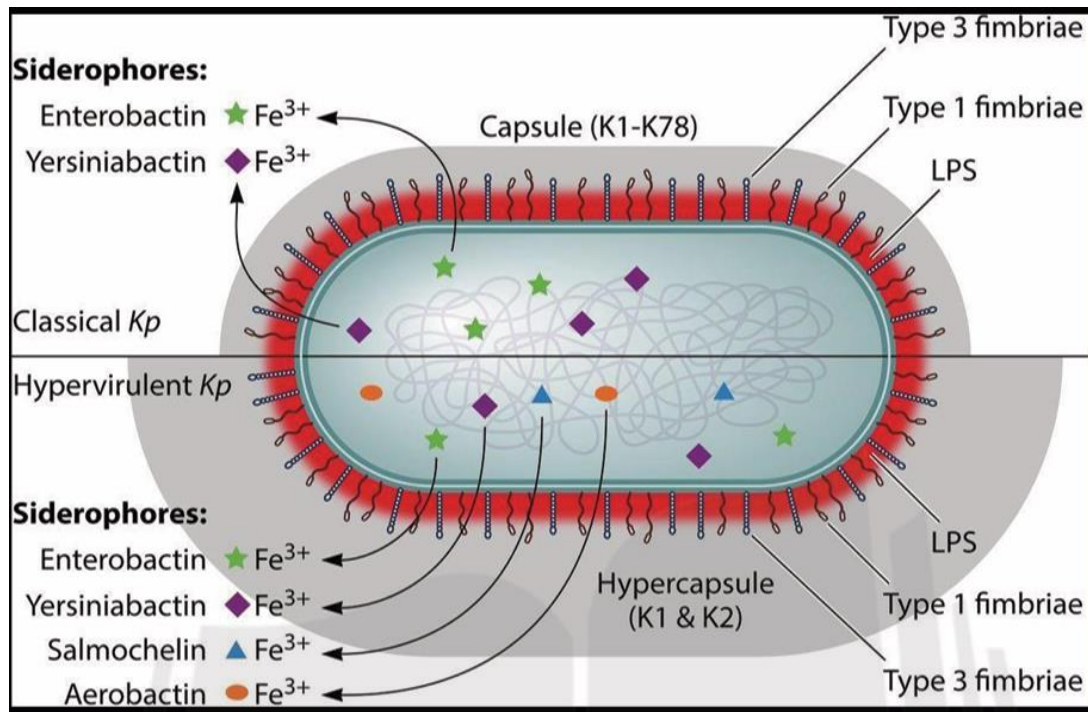


Figure (2-1): Schematic diagram of the virulence factors of classical and hypervirulent *Klebsiella pneumoniae* (Paczosa & Mecsas, 2016).

2.5.1. Capsule Polysaccharide

One of the most significant virulence factors of *Klebsiella pneumoniae* is capsule polysaccharide. Four to six sugars (glucose, galactose, mannose, fructose, and rhamnose) make up the capsular repeating subunits. Uronic acids are frequently negatively charged substances (Brisse *et al.*, 2006; Podschun & Ullmann, 1998). The bacterial surface is covered in thick layers of fibrillous structures called capsular material, which shields the bacteria from macrophage phagocytosis and stops bactericidal serum factors from killing them (Lin *et al.*, 2010). According to study by

(Sikarwar & Batra, 2011), there are around 80 distinct capsular (K) serotypes and subtypes with comparable antigenicity but distinct polysaccharide backbones.

K. pneumoniae usually produce both smooth lipopolysaccharide (LPS) with O antigen molecules and capsule polysaccharide (K antigen) on the surface. A single mutation in a gene that encodes for a UDP galacturonate 4- epimerase (*uge*) makes a strain with the O⁻:K⁻ phenotype (lack of capsule and LPS without O antigen molecules and outer core oligosaccharide) (Regué *et al.*, 2004). According to study of (Regué *et al.*, 2004) the *uge* gene was present in all the *K. pneumoniae* strains tested, the *K. pneumoniae uge* mutants were unable to produce experimental urinary tract infections in rats and were completely avirulent in two different animal models (septicemia and pneumonia).

2.5.2. Lipopolysaccharide

Lipopolysaccharide is made up of three components: the core polysaccharide that connects lipid A and O-antigen; the extremely variable O-antigen, which is the outermost component of LPS; and the very conserved and hydrophobic lipid A, which is anchored in the outer membrane.

2.5.2.1. Lipid A

Lipid A is produced through a multi-step biosynthesis enzymes in the cytoplasm and inner membrane which then flipped out flipase into outer membrane, which is then transported by the ABC transporter MsbA and ultimately anchored in the outer membrane. The virulence of some Enterobacteriaceae pathogens alternates due to covalent changes of lipid A

that occur through lipid A transporter and are catalyzed by various modification enzymes upon environmental inducers.(Raetz *et al.*, 2007). The modification of *K. pneumonia* lipid A participates to resistance to host natural responses, particularly including resistance to antibacterial peptides, and the mutation of alteration of the enzyme genes cause the attenuation of *K. pneumoniae* virulence as tested in various animal models (Llobet *et al.*, 2011). Lipid A and core polysaccharide , but not O-antigen are needed for phagocytosis resistance by mouse alveolar macrophages, that has a significant function in the defense of the host against *K. pneumoniae* (March *et al.*, 2013).

2.5.2.2. O-antigen

K. pneumoniae has been found to have at least nine O-antigen groups (O1, O2, O2ac, O3, O4, O5, O7, O8, and O12) (Bei Li *et al.*, 2014). The enzymes encoded by a six-gene wb cluster are responsible for the production of O-antigen. comprise wzm, wzt, wbbM, glf, wbbN, and wbbO with relation to their transcriptional orientation. Given the substantial chemical variety in different O-antigen groups, the wb cluster exhibits high genetic variation in matching coding sequences while maintaining the gene's structure (Hsieh *et al.*, 2012). O1 is the most prevalent serotype among clinical isolates of *K. pneumoniae*, and hyper-mucoviscous (invasive) strains are more likely to have it than non-tissue invasive ones (Hsieh *et al.*, 2012). *K. pneumoniae* O-antigen helps bacteria resist complement-mediated killing by blocking the arrival of complement components to activators (like porins and rough LPS) (Merino *et al.*, 2000). Serum resistance is higher in O1-serotype isolates than in non-O1-serotype isolates (Hsieh *et al.*, 2012).

2.5.2.3. Core Polysaccharide

For *K. pneumoniae*, only two types of core polysaccharide—type 1 and type 2—have been identified. These were produced by the products of two different 13-gene *wa* gene clusters (Fresno *et al.*, 2007; Regué *et al.*, 2005). *HldD*, *waaF*, *waaC*, *wabK*, *waaL*, *wabM*, *waaQ*, *wabG*, *wabH*, *orf10*, *waaA*, *waaE*, and *coaD* make up the type 2 *wa* gene cluster (Fresno *et al.*, 2007). Only two genes separate the two *wa* gene clusters: The last two outer core residues, Kdo and 1,d-HepI, are integrated by type 1's *wabI* and *wabJ*, which encode 3-deoxy-d-manno-octulosonic acid (Kdo) transferase and heptosyltransferase, respectively; type 2 has the two corresponding genes, *wabK* and *wabM*, which are involved in the transport of the final two outer core Glc residues (Regué *et al.*, 2005).

2.5.3. Fimbriae

At least four types of fimbriae, namely type 1 fimbriae, type 3 fimbriae (Struve *et al.*, 2009), KPF-28 adhesin and Kpc fimbriae (Wu *et al.*, 2010) have been experimentally reported for *K. pneumoniae*. The previous three, as opposed to KPF-28, are synthesized by hypermucoviscous O1:K1 strain NTUH-K2044 with detected complete genome sequences and NTUH_K2044 still harbors six uncharacterized fimbrial gene loci :kpa, kpb, kpd, kpe, kpf and kpe (Wu *et al.*, 2010).

2.5.3.1. Type 1 fimbriae

Type 1 fimbriae are thin, rigid, adhesive thread like surface appendages on the outer membrane, and the appendages are primarily consist of repeating FimA subunits with an adhesion molecule FimH at the end (Kline *et al.*, 2010). According to study (Rosen *et al.*, 2008) *K. pneumonia*

type 1 fimbriae extend beyond the capsule and mediate bacterial adhesion to mannose-containing structures on host cells or on extracellular matrices by the adhesion FimH. *K. pneumoniae* type 1 fimbriae are essential for the initial establishment of urinary tract infection, but have no impact on the capability of *K. pneumoniae* to inhabit the intestine or infect the lung (Struve *et al.*, 2008).

2.5.3.2. Type 3 fimbriae

Type 3 fimbriae of *K. pneumoniae* are described as 2-4nm wide and 0.5- 2 μ m long appendages. *mrkA* encodes the fimbrial subunit, that is polymerized to compose the helical fimbrial shaft. The adhesion subunit, with the capability to connect to collagen molecules, is encoded by *mrkD* and set at the tip of the fimbriae. *mrkB*, *mrkC* and *mrkF* encode the chaperone, usher and scaffolding proteins, respectively which are responsible for fimbrial synthesis / stabilization (Murphy *et al.*, 2013). Type 3 fimbriae mediate in vitro adhesion to epithelial cells, kidney and lung tissues, most probably in a mannose resistant manner (Stahlhut, Struve, & Krogfelt, 2012). Type 3 fimbriae is a major participator to *K. pneumoniae* biofilm formation, but have no role in the intestine and pulmonary infections (Struve *et al.*, 2009). *K. pneumoniae* induced urinary tract infections are frequently associated with the formation of *K. pneumoniae* biofilms on indwelling urinary catheters. Both type 1 and type 3 fimbriae, functioning in a compensating pattern, induce *K. pneumoniae* biofilm formation on urinary catheters (Stahlhut, Struve, Krogfelt, *et al.*, 2012). In addition to type 1 fimbriae, type 3 fimbriae act as another significant colonization factor for *K. pneumoniae* biofilm associated to

urinary infections belong to the indwelling of urinary catheters (*Murphy et al.*, 2013).

The chromosomes of *K. pneumoniae* possess a conserved fimbriae-encoding region comprising the regulatory gene locus mrkHIJ, the type 3 fimbriae gene cluster mrkABCDEF, and a four-gene interspacing region and the type 1 fimbriae gene locus fimBEAICDFGHK (*Struve et al.*, 2009).

2.5.4. Efflux Pumps

K. pneumoniae produces the efflux pump *AcrAB*, which exports both antibiotics (e.g., quinolones and β -lactams) and host-derived antimicrobial factors (e.g., antimicrobial compounds in human bronchoalveolar lavage fluid and antimicrobial peptides). *AcrAB* also works as a resistance determinant of *K. pneumoniae* to host innate immune responses (*Padilla et al.*, 2010). The inhibition of *AcrAB* not only leads to a multidrug resistance phenotype, but also to a reduced ability to cause pneumonia in a murine model (*Padilla et al.*, 2010). The production of other *K. pneumoniae* efflux pump, namely EefABC, is not associated to any antibiotic resistance phenotype, but it gives acid tolerance in vitro and high competition possibility in the host GI tract (*Coudeyras et al.*, 2008).

2.5.5. Outer Membrane Proteins (OmpA)

Research suggests that CPS is necessary but insufficient to reduce airway epithelial cell-mediated inflammation (*Regueiro et al.*, 2011). OmpA, a key outer membrane of Gram-negative bacteria, it is extremely preserved among Enterobacteriaceae. *K. pneumoniae* OmpA, independent of CPS, is important for preventing the activation of airway epithelial cells

and so participate to the attenuation of the airway epithelial cell-mediated inflammatory response (March *et al.*, 2011). Absence of OmpA makes *K. pneumoniae* is more sensitive to antimicrobial peptides, but has no impact on CPS production, that is known to be responsible for resistance to antimicrobial peptides (Llobet *et al.*, 2009). It is believed that *OmpA* is included in the activation of as yet- unknown systems consecrated to improve the cytotoxicity of antimicrobial peptides. *OmpA* also participants to resistance to phagocytosis by alveolar macrophages (March *et al.*, 2011).

K.pneumoniae produces two major outer membrane porins-OmpK35 _OmpK36- through which hydrophilic molecules (e.g., nutrients and cephalosporins/carbapenems) spread into the bacteria (Tsai *et al.*, 2011). In addition, *K. pneumoniae* expresses alternative porins, such as KpnO (García-Sureda *et al.*, 2011) and OmpK26 (Srinivasan *et al.*, 2012), in order to compensate for the absence of OmpK35/36. Loss of any of OmpK36, KpnO or Ompk26 leads to increase resistance to cephalosporins/carbapenems and decreased virulence in mouse models of acute systemic infections, whereas absence of OmpK35 has no effect on antibiotics resistance and virulence(March *et al.*, 2013; Srinivasan *et al.*, 2012; Tsai *et al.*, 2011). Absence of OmpK36 remodels the surface structure of *K.pneumoniae* and thereby modifies the binding of phagocytes, leading to increased sensitivity to phagocytosis and so an attenuation in virulence (March *et al.*, 2013; Tsai *et al.*, 2011).

2.5.6. Siderophore Production

Siderophores (from the Greek: "iron carriers") is relatively low-molecular-weight, ferric ion-specific chelating compounds released by

bacteria in response to iron deficiency for acquisition. Iron is extracted from insoluble forms by mineralization and sequestration. Siderophores are proteins that bind iron and have a molecular weight between 400 and 1500 Da. The function of these molecules is to scavenge iron from the environment and make the practically necessary mineral accessible to microbial cells (Kannahi & Senbagam, 2014). Siderophores are tiny, iron-chelating molecules with high affinity that are essential to the pathogenicity of several Gram-negative bacteria. *K. pneumoniae* secretes siderophores that are necessary for bacterial growth and reproduction (Khasheii *et al.*, 2021). Notably, hypervirulent *K. pneumoniae* excrete quantitatively further and biologically more efficient siderophore molecules than classical *K. pneumoniae*, delineating an additional mechanism by which hypervirulent *K. pneumoniae* increases its pathogenic potential (Russo *et al.*, 2011).

2.5.7. Urease Production

Many pathogens of gut, including *K. pneumoniae*, can produce cytoplasmic urease so as to hydrolyze urea to ammonia and CO₂ as a source of nitrogen for growth. The ureDABCEFG operon encodes structural subunits (UerA, UreB and UreC) of the metalloenzyme urease and accessory nickel-binding proteins (UreD, UreE, UreF and UreG) which are responsible for the integration of nickel ions into the active site of the urease enzyme (W. Li *et al.*, 2014). The inhibition of *K. pneumoniae* urease based metabolism of urea will impair the pathogen growth in the host GI tract, where urea is abundant (Maroncle *et al.*, 2006).

2.6. Mechanisms of Antibiotic Resistance in *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen responsible for a wide range of hospital- and community-acquired infections. Its ability to acquire and disseminate antibiotic resistance determinants has made it one of the most critical priority pathogens according to the World Health Organization. Multiple mechanisms contribute to its resistance phenotype, often acting synergistically to confer high-level resistance to various antibiotic classes.

The first and most important mechanism is the production of hydrolyzing enzymes, particularly β -lactamases, which include extended-spectrum β -lactamases (ESBLs), AmpC β -lactamases, and carbapenemases such as *KPC*, *NDM*, *OXA-48*-like enzymes. These enzymes inactivate β -lactam antibiotics by hydrolyzing the β -lactam ring, rendering the drug ineffective.

A second mechanism involves reduced permeability of the outer membrane, often due to the loss or mutation of porin proteins (e.g., *OmpK35*, *OmpK36*). This structural change limits the entry of hydrophilic antibiotics, especially carbapenems, into the bacterial cell.

Third, *K. pneumoniae* employs efflux pumps, such as the AcrAB–TolC system, to actively expel a wide range of antibiotics from the periplasmic space and cytoplasm, decreasing their intracellular concentration below therapeutic levels.

A fourth important mechanism is target site modification, which involves mutations or enzymatic alterations of antibiotic binding sites. Examples include modifications in DNA gyrase and topoisomerase IV leading to fluoroquinolone resistance, changes in ribosomal subunits conferring resistance to aminoglycosides

and macrolides, and alterations in lipid A (e.g., through *mgrB* or *pmrB* mutations) that result in colistin resistance.

Finally, biofilm formation plays a crucial role in resistance and persistence. Within biofilms, bacterial cells are embedded in an extracellular polymeric substance (EPS) matrix that acts as a physical and chemical barrier, impeding antibiotic penetration and enhancing tolerance to hostile environmental conditions, including host immune responses.

These mechanisms can coexist in the same isolate, making *K. pneumoniae* extremely difficult to treat and underscoring the urgent need for novel therapeutic strategies and robust infection control measures (Braun *et al.*, 2024).

2.7. Hypervirulent (hvKp) and Classical *K. pneumoniae* (cKp)

Klebsiella pneumoniae mainly has two types that pose a danger to our health: classical (cKp) and hypervirulent (hvKp). Classical *K. pneumoniae* is universally recognizable by clinicians and microbiologists worldwide. The classical *K. pneumoniae* has their ability to cause nosocomial and community-acquired infection and develop resistance to antimicrobial (Shon *et al.*, 2013). In 1986, it first described the emergence of hypervirulent *K. pneumoniae* by Taiwanese doctors in a patient with liver abscess. The hypervirulent strains have shown unique features compared to classical *K. pneumoniae* (Russo *et al.*, 2014). The hypervirulent strains show a striking capacity to cause serious infections in young healthy individuals and immunocompetent hosts in addition to infected patients have pyogenic liver abscess (Bulger *et al.*, 2017). Unlike classical *K. pneumoniae* and other enteric Gram-negative bacilli, hypervirulent *K.*

pneumoniae strains can cause metastatic infections (Liu *et al.*, 2014). The hypervirulent strains characterized by hypermucoviscosity colonies appear when the growth of the bacteria on blood agar. This phenotype defined as a "string test" was done as an official lab test to recognize hypervirulent from classical *K. pneumoniae* where hypervirulent isolates yield a positive result for the test (Russo *et al.*, 2018). While most hvKp strains exhibit hypermucoviscosity, not all hypermucoviscous strains are truly hypervirulent. Thus, the string test is helpful but not definitive for identifying hvKp. First in Taiwan, and then in Korea, Vietnam, Japan, Australia, South America, and Europe, a rising number of infection caused by this bacteria was being reported (Mei *et al.*, 2017).

2.8. Multidrug Resistance (MDR) (multi-resistance to Antibacterial Agent's)

This topic describes acquired resistance to at least one antimicrobial agent from three or more antimicrobial classes (Sweeney *et al.*, 2018). The spread of carbapenem-resistant *K. pneumoniae* isolates, both locally and internationally, creates a therapy problem due to less effective antibiotics for therapy, leading to an increase in the evolution of extensively drug-resistant (XDR) and pan drug-resistant (PDR) Gram-negative bacteria (Alkofide *et al.*, 2020). Extensively drug-resistant (XDR) is defined as an isolate that is resistant to at least one agent in all but one or two available classes and pan drug-resistant (PDR) is defined as an isolate that is resistant to all agents in all available classes (Sweeney *et al.*, 2018). XDR for acquired nonsusceptibility to at least one antimicrobial agent in all antimicrobial classes, with the exception of two or fewer antimicrobial

classes, and PDR for acquired nonsusceptibility to all antimicrobial agents from all classes (Bedos *et al.*, 2021).

2.9. Tendency in Molecular Epidemiology

The first Metallo- β -lactamase (MBL) found in a Carbapenem-Resistant *Klebsiella pneumoniae* (CRKP) isolate was IMP-1 in 1996 in Singapore (Koh *et al.*, 1999). Since then, (CRKP) isolates generating IMP have been identified internationally, mostly in South and Southeast Asia (Bin Li *et al.*, 2014). VIM MBLs are the most common on the world scale. In 2004, an epidemic caused by VIM-1 generating CRKP strains occurred in France following the hospitalization of a patient in Greece (Kassis-Chikhani *et al.*, 2006). Since then, numerous VIM subtypes have been found, including VIM-12, VIM-19, VIM-4, VIM-27, VIM-26, and VIM-39, particularly in CRKP-endemic nations. These VIM variations are genetically connected, and they can originate from one another as a result of small genetic events like point mutations. The blaVIM genes are often incorporated in a class 1 integron (Karampatakis *et al.*, 2016).

ST147, according to the Institute Pasteur method, has been the most commonly found among VIM-producing *K. pneumoniae* isolates (Arca-Suárez *et al.*, 2022; Hasan *et al.*, 2014). KPC is the most common of the carbapenemases. KPC-1 was first discovered in the United States of America in 1996 and has since spread fast to the east coast. It is thought to be endemic in most of New York (Landman *et al.*, 2007). However, the significant dissemination of KPC-producing CRKP occurred in 2007 with an epidemic of CRKP isolates expressing KPC-2 in Crete, Greece. These isolates showed clonal proliferation and were shown to be clonally linked

to the previously characterized New York clone (Maltezou *et al.*, 2009; Pournaras *et al.*, 2009).

Since then, this clone has predominated and been dubbed the 'hyperepidemic Greek clone' (Giakkoupi *et al.*, 2009). According to the Centers for Disease Control and Prevention (CDC), around 70% of KPC-2 generating CRKP isolates are classified to ST258 (Kitchel *et al.*, 2009). Antimicrobial multidrug resistance has been linked to ST258 (Giakkoupi *et al.*, 2010). However, because only a small percentage of individuals colonized with ST258 KPC-2-producing CRKP isolates progress on to become infected, these isolates are regarded as opportunistic and low-virulent pathogens. With comparatively low virulence and death rates, these CRKP isolates form long-lived reservoirs (Tzouvelekis *et al.*, 2013). Individuals with chronic illnesses and co-morbidities are more susceptible to infection (Borer *et al.*, 2012). KPC-2 CRKP, which belongs to ST39, has just surfaced (Räsänen *et al.*, 2019).

CRKP isolates harboring concurrently VIM-1 and KPC-2 are usually assigned in ST147, meaning that they are commonly related with VIM-1 (Papagiannitsis *et al.*, 2013). However, CRKP isolates producing concurrently VIM-1 and KPC-2 have recently been assigned to ST39, implying some kind of relatedness with KPC-producers (Karampatakis *et al.*, 2022). The type 3 fimbriae of *Klebsiella pneumoniae* are primarily composed of the structural subunit *MrkA*, while the *MrkD* adhesin plays a crucial role in mediating adhesion to host extracellular matrix components, particularly collagen, under in vitro conditions. This adhesive function facilitates bacterial colonization and contributes significantly to biofilm formation, enhancing persistence and resistance in clinical settings

(Schroll *et al.*, 2010). The type 3 fimbriae are expressed by several species of opportunistic pathogens and are characterized by their ability to mediate agglutination, in vitro, of treated erythrocytes (Clegg *et al.*, 2020).

The *MrkA* polypeptide comprises the major structural component of type 3 fimbriae and is polymerized to form the fimbrial shaft. The *MrkD* protein is believed to function as the type 3 fimbrial adhesin and mediates binding to extracellular matrix (ECM) proteins such as collagen molecules. The collagen-binding *MrkD* molecule appears to serve a minor impact in biofilm formation on these flexible surfaces, but the *MrkA* shaft protein is a significant contributor.

As a result, type 3 fimbriate bacteria may be more effective in bacterial binding, colonization, and biofilm development on plastic devices such as endotracheal tubes. In contrast, in-dwelling devices such as catheters and tubes are coated with host-derived material over time and in place. As a result, particular receptor-ligand binding, such as the MrkD-collagen interaction, may potentially play a role in biofilm formation during infection. *MrkD*-mediated binding to extracellular matrix may also play a role in epithelial surface colonization (Donlan, 2001). The presence of *MrkD* adhesin was required for growth on collagen-coated surfaces. Fimbriate strains missing the *MrkD* adhesin have difficulty adhering to and growing on these surfaces.

Similarly, pure human extracellular matrix and extracellular matrix produced by human bronchial epithelial cells cultured in vitro provided an appropriate substrate for *MrkD*-mediated biofilm formation, but no direct attachment to respiratory cells was found. Type 3 fimbriae may thus play

two functions in the early phases of adhesion and development on indwelling devices like endotracheal tubes. The *MrkA* polypeptide may enhance adsorption to abiotic polymers of newly implanted devices, but the *MrkD* adhesin may allow bacteria to cling to and thrive on polymers coated with host-derived proteins (Jagnow & Clegg, 2003).

The *rmpA* (Regulator of Muroid Phenotype A) gene is a plasmid-mediated give a highly muco-viscous phenotype encouraged and regulate the synthesis of the capsular polysaccharide (Rivero et al., 2010). It was initially described by (Yu et al., 2006) explained that *rmpA*-carrying strains were correlated with the hyper-mucoviscosity phenotype, in addition to the invasive clinical syndrome, mentioned that deletion of *rmpA* gene can reduce in mouse lethality tests by 1000-fold.

Plasmid borne *rmpA* and its isoform *rmpA2* encode the activator of *cps* gene transcription, CPS synthesis and hyper-mucoviscosity in *K. pneumoniae* K1/ K2 (Cheng et al., 2010; Hsu et al., 2011; Lai et al., 2003). In the K2 strain CG43, both *rmpA* and *rmpA2* are activators of biosynthesis of CPS and are important for virulence in mice (Cheng et al., 2010; Lai et al., 2003). In the K1 strain NTUH-K2044, besides plasmid-borne *rmpA* and *rmpA2*, there is yet *rmpA* paralog in the chromosome, and only plasmid carry *rmpA* encourages the transcription of *cps* gene (Hsu et al., 2011).

2.10. Genotyping(MLST: A Molecular Approach for Bacterial Typing and Surveillance)

The distinctions among infectious pathogen strains need to be made with accuracy for efficient analysis in epidemiology and surveillance, analysis of microbial population structure and dynamics, and ultimately, the design of enhanced public

health control methods. Various molecular typing methods capable of typing isolates globally (global epidemiology) and in localized epidemics (local epidemiology) have been suggested for advancing such broad objectives (Foley *et al.*, 2009). Since 1998, multilocus sequence typing has been the accepted method for molecular typing (MLST). MLST is founded on the now-established concepts and methods in the multilocus enzyme electrophoresis (MLEE) method. The varying sequences in a particular species are designated as different alleles for each gene, and the alleles at each of the seven housekeeping loci determine the allelic profile or type for any isolate (ST).

Consequently, each isolate is uniquely characterized by a set of seven numbers reflecting the alleles at the seven housekeeping loci. Most bacterial genera possess sufficient variation in housekeeping genes for provision of high numbers of alleles for each locus, such that billions of different allelic profiles may be differentiated with merely seven loci. Alternatively, isolate identity and tracking can be achieved directly through the use of nucleotide data, though it is less frequently applied in isolate tracking (Pérez-Losada *et al.*, 2018). For molecular typing, however, the method of choice is widely accepted for being MLST.

There are many examples of their use in summarizing pathogen population structure, in vaccine research, in tracking epidemic strain transmission, and in detection of the associated species and virulent strains for causing disease. Three developments in molecular microbiology allowed for this: an understanding in bacterial evolution and population biology, high-throughput nucleotide sequencing, and internet-based databases. Bacterial population analyses performed starting in the 1980s were pivotal for the development of MLST (Maiden *et al.*, 2013). As the number of schemes in use has increased, the most universally accepted pathogen typing method today is MLST.

Compared to earlier methods (serotyping; MLEE), the application of the variation in the DNA allows the generation of variable data (increased resolution) that is universally comparable (within schemes), readily validated, and readily shared between laboratories. MLST is an applicable method for wide-ranging application that can be fully automated and scalable from the single isolate level through thousands of specimens owing to the application of generic sequencing technology. Since the substances necessary for the analysis of MLST, such as DNA or dead bacteria, can be transported between laboratories easily without the issues surrounding infectious material, both the biological specimens and the generated data prove very portable.

In addition, through the storage and curating in electronic online schemes, the schemes for MLST become an internationally available resource (Pérez-Losada *et al.*, 2018). Multilocus Sequence Typing (MLST) is an approach based on nucleotide sequences for describing the genealogical relationships between bacterial species. It generates clear-cut results and is readily portable laboratory to laboratory owing to its nucleotide sequence-based nature. Housekeeping loci, rather than virulence, antigen, or other functional elements, are selected for investigation because, in all organisms, they occur (i.e., their product plays an essential function), and changes in them are widely believed to be neutral, subjected as they are to the same selective forces as the other parts of the genome.

3. Materials and methods**3.1. Materials****3.1.1. The laboratory devices and tools used in this study**

Table (3-1): The laboratory devices and tools used in bacterial isolations.

No.	laboratory device	Manufacture / Country
1	Autoclave	Prestige medical / England
2	Burner	Amal / Turkey
3	Centrifuge	PLC Series / Taiwan
4	Distiller	Lab Tech / India
5	Electric incubator	Memmert / Germany
6	Freezer -20 °C	Mettler / Switzerland
7	Hot Plate	Newal / China
8	Laminar air flow cabinet	Lab-Tech / Korea
9	Light Microscope	Olympus / Japan
10	Millipore Nalgene filters	Sigma / USA
11	Refrigerator	Arcelik / Turkey
12	Sensitive balance	Sartorius / Germany
13	Slides and cover slides	Schott / Germany
14	Standard wire loop	Hi media / India
15	Sterile cotton swabs	Almalak / China
16	Sterile cotton swabs with Amies medium	Almalak / China
17	Sterile syringe	Py-med / China
18	Test tube (sterile)	Superestar / India
19	Urine Cup	Almalak / China
20	Vitek 2 compact system	Biomerieux / France

Table (3-2): The laboratory devices and tools used in molecular study.

No.	laboratory device	Manufacture / Country
1	PCR thermocycler T100	BioRad/USA
2	Vortex mixer	Talboys/USA
3	Cell Disruptor Genie vortex	Scientific Industries/USA
4	High Speed Cold centrifuge	Hettich/Germany
5	High Speed centrifuge	Hettich /Germany
6	Sensitive Balance	Ohaus /USA
7	Water Bath	Polyscience/ USA
8	Micropipettes (1-10 μ l, 1-20 μ l, 10-100 μ l, 100-1000 μ l)	Gibson/ France
9	1.5 Microcentrifuge tubes	BioBasic/ Canada
10	Electrophoresis	Bioneer/ Korea
11	PowerPac HC Electrophoresis Power Supply	BioRad/USA
12	U.V transilluminator	Wised/Korea
13	Camera	Nikon/Japan
14	Tube Rack Double Panel Microcentrifuge PCR Centrifuge Tube Holder	P-ABC/ USA

3.1.2. The chemical materials used in this study:

Table (3-3): The chemical materials used in bacterial isolations

No.	chemical materials	Manufacture / Country
1.	Absolute ethanol	Bioneer/ Korea
2.	Glycerol	BDH/England

3.	Crystal violet	RBIO/ Switzerland
4.	Iodine	RBIO/ Switzerland
5.	Saffranin	RBIO/ Switzerland
6.	Glycerol(C ₃ H ₈ O ₃)	Scharlau/ Espine
7.	Methyl red	Biolife/ Italy
8.	Ethylene Diamine Tetra Acetic Acid (EDTA)	Hi Media/ India
9.	Sodium chloride (NaCl)	BDH/ England
10.	Urea solution	Mast Diagnostic/ England

Table (3-4): The chemical materials used in molecular study.

No.	chemical materials	Manufacture / Country
1.	Absolute ethanol	CHEM-LAB/ Belgium
2.	DNA Marker ladder 100-2000bp	Bioneer/ Korea
3.	Ethidium bromide	BioBasic/ Canada
4.	TBE (Tris – Borate – EDTA) buffer	iNtRON / Korea
5.	Agarose	iNtRON / Korea
6.	Free nuclease water	BioLabs/ UK

3.1.3 Culture Media

The following table showed the culture media that used in this study.

Table (3-5): Culture media used in this study

NO.	Media	Manufacture / Country	Purpose
1	Blood agar	Accumix/India	Cultivation of bacteria and detection of hemolysis
2	MacConkey Agar	Accumix/India	Utilized as a selective medium to differentiate lactose

			fermented gram negative bacteria
3	Brain heart infusion broth	Oxoid/ England	Used as a carrier medium for the purpose of growth and activation of isolates in experiments
4	Nutrient Agar (N.A)	Accumix/India	Growth ,activation and maintenance of bacteria
5	Nutrient Broth (N.B)	Accumix/India	Activation and maintenance of bacteria
6	Methyl red vogas–Proskaouragar	Hi-Media/ India	Used to differential bacteria based on their glucose metabolism, complete fermentation (MR test) or partial fermentation and production of acetoin (VP test)
7	Simon citrate agar	Accumix/India	Used to detect utilizing of citrate by bacteria as a single source of carbon
8	Triple Sugar Iron Agar	Hi-Media/ India	Used for identification Enterobacteriaceae, based on sugar fermentation and hydrogen sulphide production
9	Luria-Bertani (LB) broth	HiMedia/India	Used for general purpose bacterial growth
10	Trypticase soy broth	HiMedia/India	Supports good bacterial growth and stimulates biofilm formation.

3.1.4. Reagents and Dyes Used in the Study

Table (3-6) clarified the reagent and dyes that are used in this study.

Table (3-6): Reagents and dyes used in this study

NO.	Reagents and dyes	Purpose
1	Activated charcoal	Used to purify electrophoresis solutions from impurities, including Ethidium Bromide,

		improving the quality of results and reducing potential interferences during DNA analysis.
2	Loading dye	Gives the colour indication for migration of DNA. Also, to make the sample more dense so the sample will fall into the wells
3	Kovac's reagent	Used to detect bacterial ability to consume Tryptophan by producing the enzyme tryptophanase
4	Methyl red reagent	This reagent was used to detect the ability of bacteria to produce acid as a final product of the complete decomposition of sugars
5	Voges-Proskauer reagent	Use this detector to investigate the ability of the bacteria to partially decompose sugars.

3.1.5. Kits

Table shows all the kits used during study period (3-7).

Table (3-7): The kits used during current study

No.	Kit	Manufacture / Country
1	Presto™ Mini gDNA Bacteria Kit	Geneaid/ Taiwan
2	GoTaq® Green PCR master Mix	Promega/ USA
3	GN TEST KIT VTK2 20 CARDS	BioMérieux/ France
4	AST-N419 TEST KIT 20 CARDS	BioMérieux/ France
5	AST-NG76 TEST KIT 20 CARDS	BioMérieux/ France

3.1.6. Primers:

Table (3-8): The PCR oligonucleotides used in this study:

Oligo.	*Sequence (5'-3')	Amplicon	NCBI-Reference code
<i>RmpA-F</i>	ACTGGGCTACCTCTGCTTCA	367bp	X17518-1
<i>RmpA-R</i>	TCAGTAGGCATTGCAGCACT		
<i>MrkD-F</i>	GGAACCCACATCGACATTCA	515bp	MZ380316.1
<i>MrkD-R</i>	CAACGGTGATGCTGGACATG		
<i>BlaKPC-F</i>	CTGTAAGTTACCGCGCTGAG	544bp	NC_014312.1
<i>BlaKPC-R</i>	CAGGTTCCGGTTTTGTCTCC		
<i>BlaVIM-F</i>	AAGTCCGTTAGCCCATTCCG	548bp	CP034084.1
<i>BlaVIM-R</i>	ACTCATGAACGGCACAACCA		

*Note1: These oligonucleotides were designed by current study.

Note2: These oligonucleotides were designed by using NCBI-GenBank and Primer3 plus server.

Note3: These oligonucleotides were manufactured by Scientific Resercher.co. Ltd. Iraq.

3.2. Methods:

3.2.1. Study Design:

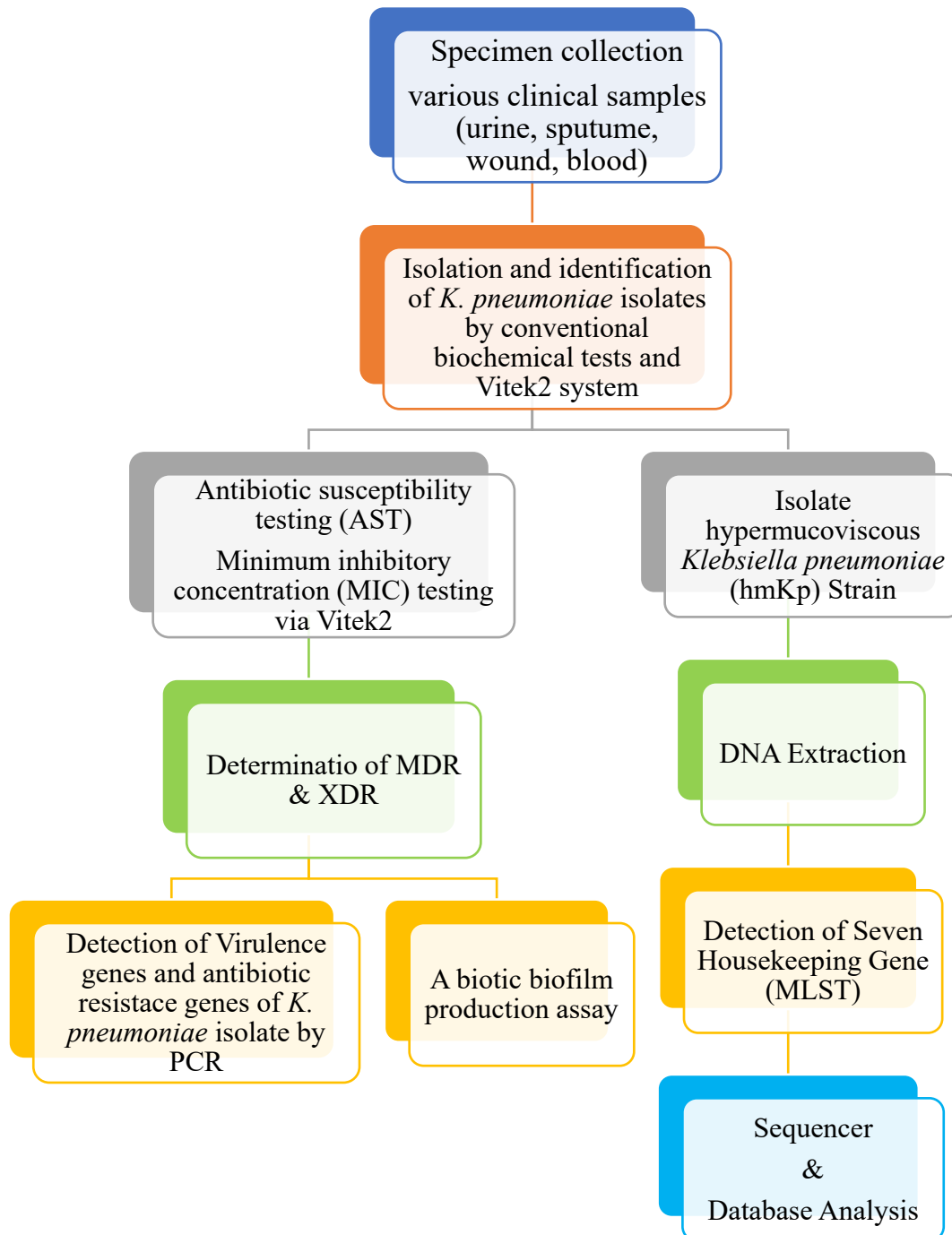


Figure (3-1): Study design.

3.2.2. Sample Collection

During the period from 20th of October 2024 to the 20th of January 2025, three hundred and sixteen (316) specimens (urine, sputum, wound, and blood) were collected from different clinical sources.

The specimens were collected from (Al-Diwaniyah Teaching Hospital). All samples transferred to laboratory and culture onto MacConkey agar medium incubated at 37° C for 24 hr. Isolates purified several times until pure isolates were obtained, exposed to microscopic and special biochemical tests then transferred to identification via Vitek 2 compact system.

3.2.3. Preparation of Culture Media

MacConkey agar, Brian Heart infusion broth, Blood agar, Trypticase soy broth, Luria-Bertani (LB) broth, Triple sugar iron agar, base was prepared as according to manufacturing company and autoclaved at 121° C and 15 PSI (lbs) pressure for 15 minutes.

3.2.4. Preparation of Reagents Used in Biochemical Tests**3.2.4.1 Methyl Red Reagent**

It was prepared according to the instructions of the company.

3.2.4.2. Voges-Proskauer Reagent

This reagent was used as indicator for Voges – Proskauer test in the set of sugar. It was prepared as :

Reagent A : 6g α -naphthol dissolved in 100 ml of 95% ethanol.

Reagent B: 16g KOH dissolved in 100 ml distilled water.

3.2.4.3 Kovac's Reagent

It was prepared according to the instructions of the company.

3.2.4.4 Gram stain

It has been done according to (Goldman & Green, 2009).

3.2.5. Bacterial Isolation

All collected specimens were grown separately at McConkey agar and Blood agar and then incubated for 24 hours at 37 ° C. After incubation and characterize the colonies depend on morphology (shape, color and texture). A single colony was taken from positive culture and it was identified according to the methods of (MacFaddin, 2000; Patel et al., 2017). Blood samples were also collected and cultured in blood culture bottles designated for the BACT/ALERT device, pending the device's results. After confirming a positive result in the blood samples, a small amount of bacterial culture was withdrawn using a medical syringe after sterilizing the bottle cap with alcohol (pathology vial). After the appropriate incubation period and the appearance of growth in the medium, the bacteria were diagnosed.

3.2.6. Identification Methods of *Klebsiella pneumoniae* Isolates

A single colony was taken from positive culture and identified According to methods of (Benson, 2002; Goldman & Green, 2009).

3.2.6.1. Morphological Tests

Colonial characteristics were tested such as the shape, mucoid, color, boundaries, and textures of the colonies.

3.2.6.2. Microscopic characteristics

Bacteria were examined microscopically after Gram staining. A small amount of the bacterial colony was spread with a drop of normal saline solution on a clean slide, heat-fixed, stained with crystal violet, fixed with alcohol, stained with safranin, and examined under oil immersion with a 100x magnification lens.

3.2.6.3. Biochemical Tests**3.2.6.3.1 IMVC test****1- Indole Test**

Tryptophan broth medium was inoculated with bacteria and incubated at 37 ° C for 24 hours. The few drops of Kovak's reagent were added to the tube formation of a pink-liquefied ring, indicating positive results.

2- Methyl Red Test

The MR-VP aircraft tube was designed according to the manufacturer instructions and inoculated with the culture of bacteria, which was incubated at 37 ° C for 24 hours, adding five drops of the methyl red reagent. The formation of a bright red color indicated positive results; the result was read immediately after the mixture.

3- Voges-Proskauer Test

The MR-VP broth medium was inoculated with the culture of bacteria and incubated for 24h at 37 ° C. A few drops of alpha-naphthol solution and KOH solution are pairing and mixed.

4- Simmon Citrate Test

The Simone Citrate was inoculated with a culture of bacteria at 37 ° C for 24 hours. A blue color indicated positive results.

3.2.6.3.2 Triple-Sugar-iron Test

This test was used to determine of a bacterium to ferment the glucose, lactose and sucrose, and to detect the ability of bacteria to produce the H₂S and gas. It was carried out by inoculating the slant of triple-sugar-iron agar slant with bacterial growth and incubated at 37 ° C for 24 hours the color changes as following:

Slant	Button	Result
Red	Red	Alkaline/ Alkaline
Red	Yellow	Alkaline/Acid
Yellow	Yellow	Acid/Acid

3.2.6.4. The Vitek2 Operating Procedure was as Follows:**1- General Setup:**

- a) The VITEK® 2 system was used according to the manufacturer's instructions (bioMérieux, 2021).
- b) It performs automated microbial identification using colorimetric reagent cards. It enables rapid identification of aerobic and anaerobic bacteria, yeasts, and other microorganisms.

2- Sample Collection and Preparation:

- a) Patient samples were collected and cultured on appropriate media to obtain isolated colonies.
- b) On the next day, the sample type (Gram-positive or Gram-negative) was determined to select the corresponding ID card.

3- Bacterial Suspension Preparation:

- a) A Kan tube was prepared with 3 mL of 0.45% NaCl saline (bioMérieux, France).
- b) Using a sterile swab, a sample was taken from the isolated colonies and transferred into tube number one (ID tube), then mixed thoroughly.

4- Densitometer Reading:

- a) The Densichek device was turned on and zeroed.
- b) The density of the suspension in tube one was measured using the device according to standard guidelines.
- c) Target concentration: 0.50 – 0.63 for Gram-negative bacteria.

5- Sample Transfer to ID and AST Cards:

- a) The sample was transferred from tube one to tube two using:
 - Red pipette 145 for Gram-negative bacteria.
- b) The ID card was inserted into tube one, and the AST card into tube two using the cassette.
- c) Each cassette had a unique number that must not be reused on the same day.

6- Software and Data Entry:

- a) The computer was turned on and the VITEK® 2 software was launched.

- b) AST cards were defined by selecting "Maintain AST Card Definitions" from the main menu.
- c) The AST code was entered, and the card numbers were scanned using the handheld barcode reader.
- d) All data were saved into the system.

3.2.7. Biofilm Formation Test: (Colorimetric Microtiter Method)

- A 96-well microtiter plate was used to assess biofilm formation.

1- Bacterial culture preparation:

- a) Each bacterial isolate was cultured in Luria broth for 18 hours at 37°C.
- b) Plate inoculation:
- c) 200 µL of trypticase soy broth (TSB) was added to each well of the microtiter plate.
- d) A 10 µL of a microbial suspension (prepared from a 24-hour culture adjusted to 0.5 McFarland turbidity) was added to each well.
- e) The plate was incubated at 37°C for 24 hours.

2- Washing step:

- a) After incubation, each well was emptied.
- b) Wells were washed three times with normal saline to remove non-adherent cells.

3- Staining:

- a) A 200 µL of 10% crystal violet was added to each well.
- b) The plate was incubated at room temperature for 20 minutes.

4- Washing and drying:

- a) Wells were washed again three times with normal saline.
- b) Plates were left to dry.

5- Solubilization:

- A 200 μ L of dimethyl sulfoxide (DMSO) was added to each well to solubilize the bound dye.

6- Measurement:

- The optical density (OD) was measured at 630 nm using an ELISA reader.

7- Interpretation of results according to (Bakhtiari *et al.*, 2021):

- $OD > 0.65 \rightarrow$ Strong biofilm formation
- $0.5 \geq OD > 0.3 \rightarrow$ Moderate biofilm formation
- $0.3 \geq OD > 0.15 \rightarrow$ Weak biofilm formation
- $OD \leq 0.15 \rightarrow$ No biofilm formation

3.2.8. Conventional PCR

The polymerase chain reaction (PCR) was done to detect some important virulence factor genes, a biofilm formation genes and antibiotic resistance genes. All the PCR primers used in this study were designed, and displayed as of table (3-8).

3.2.8.1. Isolation of DNA from Bacterial Cells

Bacterial genomic DNA was extracted from MDR and XDR *Klebsiella pneumoniae* isolates by using (Presto™ Mini gDNA Bacteria Kit) as and done according to company instructions as following steps:

1- Sample Preparation

A 1 ml of (18 hours) incubated cultured bacterial cells (up to 1×10^8) was transferred to 1.5 ml microcentrifuge tube then centrifuged at 10000 rpm for 1 minute then the supernatant was discarded.

2- Cell Lysis Steps

a- A 180 μ l GT buffer was added to the tube and the cell pellet suspended by vortex, then 20 μ l of Proteinase K was added and the mixtures were incubated at 60°C for 10 minutes. During incubation periods the mixtures tubes were inverted every 3 minutes.

b- A 200 μ l GB buffer were added to each tube and mixed by vortex for 10 seconds. Then the tubes incubated at 60°C for 10 minutes with inverted the tubes every 3 minutes through incubation periods.

3- DNA Binding steps

a- A 200 μ l absolute ethanol were added and immediately mixed by vortex, then precipitates if happen was broken by pipetting.

b- A GD column was placed in a 2 ml collection tube and all mixtures were transferred (including any precipitate) to the GD column. Then centrifuged at 10000 rpm for 1 minute. The 2 ml collection tubes that contains the flow-through were discarded and placed the GD column in a new 2 ml collection tube.

4-Washing Steps

a- A 400 μ l W1 buffer were added to the GD column, then centrifuged at 10000rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube.

b- 600 μ l Wash Buffer were added to the GD column. Then centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube. and the tubes were centrifuged again for 2 minutes at 12000 rpm to dry the column matrix.

5- Elution Steps

a- The dried GD column was transferred to a clean 1.5ml microcentrifuge tube and 100 μ l of pre-heated elution buffer were added to the center of the column matrix.

b- The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 1 minutes to eluted the purified DNA.

3.2.8.2. Estimation of Extracted Total DNA

The extracted total DNA was checked by using Nanodrop (Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer. USA) that measured DNA concentration (ng/ μ L) and checked the DNA purity at absorbance (260/280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, DNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipetted 2 μ l of free nuclease water and placed onto the surface of the lower measurement pedestals for blank the system.
3. The Nanodrop sampling arm was lowered and 1 μ l DNA sample measured.

3.2.8.3. Preparation of Primers Solution

The lyophilized primer was dissolved using deionizer distal water ddH₂O or Nuclease Free water to obtain 100 pmol/μl in the master tube, then 10 pmol/μl was prepared as a working solution by taking 10 μl from master tube and completed the volume to 100 μl by adding ddH₂O or Nuclease free water (90 μl).

3.2.8.4. PCR Master Mix Preparation

PCR master mix preparation for all genes were prepared by using (GoTaq® Green PCR master Mix) and this master mix done according to company instructions as following table (3.10):

Table (3-10): Standard PCR master mix protocol:

PCR Master Mix	Volume
DNA template (10ng)	5 μl
Forward primer (10pmol)	1.5 μl
Reveres primer (10pmol)	1.5 μl
G2 green Master mix	12.5 μl
PCR Water	4.5 μl
Total volume	25 μl

3.2.8.5. PCR Thermocycler Conditions

PCR thermocycler conditions protocol for each gene was calculated by using NEB Tm Calculator (version 1.16.7) online application and done by using conventional PCR thermocycler, as the following table (3-11):

Table (3-11): PCR thermocycler conditions protocol:

No.	Gene name	PCR Amplicon	Initial denaturation Temp./time	Denaturation Temp./time	Annealing Temp./time	Extension Temp./time	Cycle	Final extension Temp./time	Hold Temp./time
1	<i>rmpA</i>	367bp	95°C/ 3 min	95°C/ 30 sec	58°C/ 30 sec	72°C/ 60sec	30	72°C/ 5min	4°C/Cooling
2	<i>mrkD</i>	515bp	95°C/ 3 min	95°C/ 30 sec	57°C/ 30 sec	72°C/ 60sec	30	72°C/ 5min	4°C/Cooling
3	<i>blaKPC</i>	544bp	95°C/ 3 min	95°C/ 30 sec	58°C/ 30 sec	72°C/ 60sec	30	72°C/ 5min	4°C/Cooling
4	<i>blaVIM</i>	548bp	95°C/ 3 min	95°C/ 30 sec	58°C/ 30 sec	72°C/ 60sec	30	72°C/ 5min	4°C/Cooling

3.2.8.6. Preparation of Tris-Borate-EDTA (TBE) Buffer

Tris-Borate-EDTA (TBE) is the most commonly utilized buffer for RNA and DNA gel electrophoresis. The 10x solution was prepared by dissolving (10⁸) g of Tris base, 55 gm of boric acid, and 40 ml of 0.5 EDTA in 1000 ml of distilled water. However, the final concentration of working stock solution is prepared by adding 100 ml of 10× TBE buffer to 900 ml of sterile distilled water and stored at room temperature (Chen *et al.*, 2011).

3.2.8.7. PCR Product Analysis

The PCR products were analyzed by agarose gel electrophoresis method (Green & Sambrook, 2012) as following steps:

- 1- 1.5% Agarose gel was prepared in using 0.5X TBE and dissolving in microwave for 5 minutes, and left to cool for 50°C.
- 2- Then 3ul ethidium bromide stain were added into agarose gel solution.
- 3- Agarose gel solution was poured in tray after fixed the comb in proper position and left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray.
- 4- The gel tray was fixed in electrophoresis chamber and filled by 0.5X TBE buffer.
- 5- 10µl PCR product were loaded in to each well with added 5µl (DNA marker Ladder) in first well. Then electric current was performed at 100 volt and 80 AM for 1hour.
- 6- PCR products were visualized by using UV Transilluminator.

3.2.9. Multilocus Sequence Typing (MLST)

Multilocus Sequence Typing (MLST) Protocol for *Klebsiella pneumoniae* (Hypermucoviscous Isolate) .

3.2.9.1. Principle of MLST

Multilocus sequence typing (MLST) approach was employed in this study to genotype *Klebsiella pneumoniae* isolate at the strain level. MLST is a direct access database. The principal of MLST is monitoring the variations in the sequence of seven loci encoding for seven housekeeping genes. The variations in the sequence are the nucleotide level. Thus, each locus would have several alleles differing in their nucleotide sequences at the nucleotide level. The genomic DNA of the Hypermucoviscous strain to be genotyped is used as DNA template in seven separate PCR reaction to partially amplify 7 loci of seven housekeeping genes. The principal of this approach is amplification of 7 fragments encoding for 7 loci of housekeeping genes in *Klebsiella pneumoniae*. Normally, the PCR amplified DNA fragments for each locus do range from 450-500 bp the amplified DNA fragments are sequenced. The nucleotide sequence of each DNA fragment for each locus is aligned with the DNA sequences of the locus alleles that are deposited in the MLST database. Then the allele of each locus in the *Klebsiella pneumoniae* strain is defined. The combination of all 7 loci alleles is then compared to the ST deposited in the BIGSdb-Pasteur MLST database to define the ST of the *Klebsiella pneumoniae* strain.

3.2.9.2. Primers for Seven Loci Housekeeping Genes

Seven specific primer sets for seven loci housekeeping genes in *Klebsiella pneumoniae* from the reference sequence of each housekeeping gene locus was retrieved from the BIGSdb-Pasteur MLST database. The 7 housekeeping genes according to the BIGSdb-Pasteur MLST database for *Klebsiella pneumoniae* were

(*gapA*, *mdh*, *rpoB*, *pgi*, *phoE*, *infB*, and *tonB*). The designed primers were checked for melting temperature, 3' self complementarity, primer length, 3' complementarity, and specificity through the online program Primer-Blast.

3.2.9.3. PCR Amplification

Protocol follows the original conditions described by (Diancourt et al., 2005).

Table (3-12): Gene Specific Primer Sequence and PCR length of 7 Loci (housekeeping genes) for *K. pneumoniae*.

Locus	Putative function of gene	Primer sequence (5'-3')	Size (bp)
<i>rpoB</i>	Beta-subunit of RNA polymerase B	Vic3: GGCGAAATGGCWGAGAACCA	501
		Vic2: GAGTCTTCGAAGTTGTAACC	
<i>gapA</i>	Glyceraldehyde 3-phosphate dehydrogenase	gapA173: TGAAATATGACTCCACTCACGG	450
		gapA181: CTTCAGAAGCGGCTTTGATGGCTT	
<i>mdh</i>	Malate dehydrogenase	mdh130: CCCAATCTCGCTTCAGGTTTCAG	477
		mdh867: CCGTTTTTCCCCAGCAGCAG	
<i>Pgi</i>	Phosphoglucose isomerase	pgi1F: GAGAAAAACCTGCCTGTACTGCTGGC	432
		pgi1R: CGCGCCACGCTTTATAGCGGTTAAT	
<i>phoE</i>	Phosphoporphine E	phoE604.1: ACCTACCGCAACACCGACTTCTTCGG	420
		phoE604.2: TGATCAGAACTGGTAGGTGAT	
<i>infB</i>	Translation initiation factor 2	infB1F: CTCGCTGCTGGACTATATTCG	318
		infB1R: CGCTTTCAGCTCAAGAACTTC	
<i>tonB</i>	Periplasmic energy transducer	tonB1F: CTTTATACCTCGGTACATCAGGTT	414
		tonB2R: ATTCGCCGGCTGRGCRGAGAG	

Note: For *tonB*, MgCl₂ must be used at a concentration of 50 mM, while for all other genes, 25 mM MgCl₂ is required.

3.2.9.4. PCR Conditions

PCR amplification is performed under the following conditions:

An annealing temperature of 50°C is used for all genes, except for *gapA* (60°C) and *tonB* (45°C).

PCR Cycle:

1. Initial Denaturation: 94°C for 2 minutes
2. 35 Cycles of Amplification:
 - Denaturation: 94°C for 20 seconds
 - Annealing: 50°C (or 60°C for *gapA*, 45°C for *tonB*) for 30 seconds
 - Extension: 72°C for 30 seconds
3. Final Extension: 72°C for 5 minutes

3.2.9.5. PCR product purification

All amplified PCR products were purified using Gene JET® PCR purification kit prior to subjecting to DNA sequencing. The purification of PCR products was performed according to the instructions of the manufacturer. The protocol of PCR products purification using Gene JET®PCR purification kit was established in the following steps:

- 1- Binding buffer in a ratio of 1:1 was added to a PCR product mixture (100 µl of PCR product mixture: 100µl of binding buffer). The developed yellow color does indicate the optimal pH favoring DNA binding. (Note: orange or violet color development does necessitate the addition of 10 µl of 3 M sodium acetate, pH 5.2 solution to help turn the mixture color to yellow).
- 2- The mixture of step 1(800 µl) was transferred to the Gene JET purification column. Then the mixture was centrifuged at 10, 000 rpm for one minute. The flow-thorough was discarded.
- 3- Repeated step 2 until the entire solution was added to the column membrane.

- 4- Washing buffer (700 μ l) was added to the Gene JET purification column. Then the mixture was subjected to centrifugation at 10, 000 rpm for 30-60 seconds. The flow-through was discarded and the purification column was re-backed to the collection tube.
- 5- The empty Gene JET purification column was subjected to centrifugation for an additional one minute to guarantee complete removal any residual of ethanol included in the washing buffer. (Note. This step is mandatory to help minimize the possible inhibitory effects of the ethanol in the DNA sample in the subsequent steps.)
- 6- The GeneJET purification column was transferred to a clean 1.5 ml microcentrifuge tube. Then the elution buffer (50 μ l) was added to the center of the GeneJET purification column membrane. The DNA was eluted by centrifugation at 10, 000 rpm for 1 minute at room temperature.

3.2.9.6. DNA sequencing

All purified PCR products were subjected to DNA sequencing using Automated Fluorescent DNA Sequencing on the ABI Prism 377. Each DNA sample was sequenced in two directions using forward primer and reverse primer.

3.2.9.7. Analysis of DNA sequences for seven housekeeping genes

The obtained DNA sequences were analyzed using Bioedit software version 7.5. The sequences were cleaned to trim the overlapped, uncertain nucleotides from the entire sequence. The trimmed, clean DNA sequences were analyzed using the BIGSdb-Pasteur MLST database online server where each DNA sequence encoding for a given locus housekeeping gene was aligned against the BIGSdb-Pasteur MLST deposited DNA sequences of this locus. After conducting the multiple sequence alignment on the online server of BIGSdb-Pasteur MLST database, each locus housekeeping gene will be denoted an allele number.

3.2.9.8. Determination of ST profile for *K. pneumoniae* strains

After determination of the allele for each locus, the combination of seven loci alleles was subjected to analysis by the online server of BIGSdb-Pasteur MLST database. Where the combined allelic profile for a given strain is normally matched with the BIGSdb-Pasteur MLST deposited ST profiles. Finally, the ST profile for *Klebsiella pneumoniae* strain was submitted to the BIGSdb-Pasteur MLST database.

3.2.10. Statistical Data Analysis

Data were collected, summarized, analyzed and presented using statistical package for social sciences (SPSS) version 27 and Microsoft Office Excel 2016. Qualitative (categorical) variables were expressed as number and percentage.

The following statistical tests were used: Chi-square test was use to evaluate association between any two categorical variables provided that less than 20 % of cells have expected count of less than 5. However, Yates correction of Fischer exact test were used instead when chi-square test was not valid (in case that more than 20 % of cells have expected count of less than 5). The level of significance was considered at P-value of equal or less than 0.05.

4. Results and Discussion

4.1. Results of Isolation and Identification of *Klebsiella pneumoniae*

The present study includes collecting 316 samples from different clinical cases from Al-Diwaniyah Teaching Hospital during the period from 20th of October 2024 to the 20th of January 2025. For the purpose of *K. pneumoniae* isolation. The results showed that 71(22.5%) isolated *K. pneumoniae* specimens were obtained from a total of 316 patients specimens, distributed as the following including 21/71 (29.6%) urine samples, 33/71 (46.5%) sputum samples, 11/71 (15.5%) wound samples, and 6/71 (8.5%) blood samples, as shown in Figure 4-1.

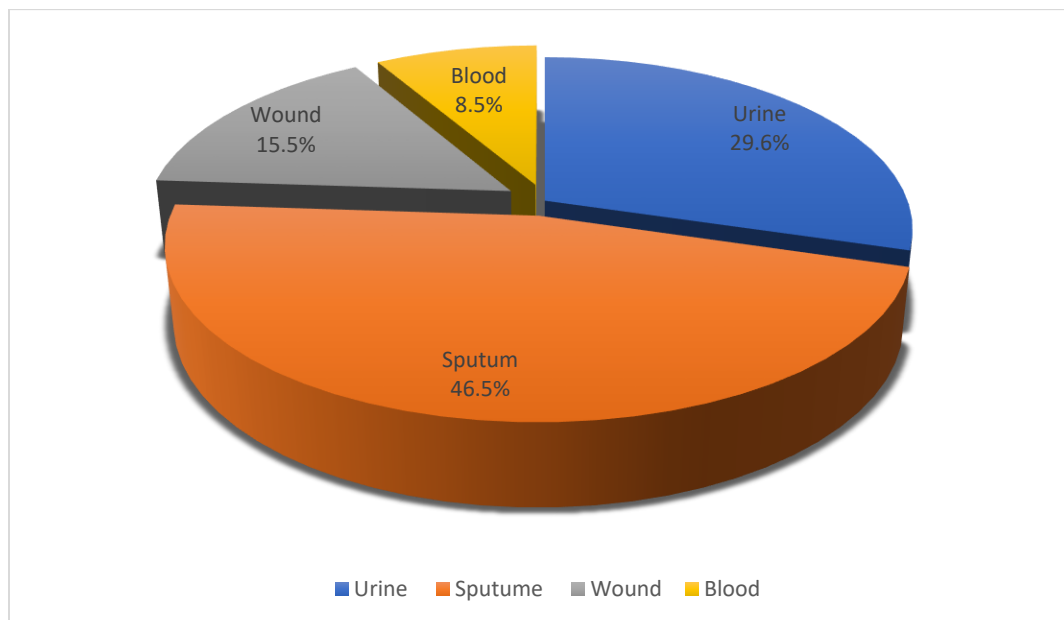


Figure 4-1: Distribution of *Klebsiella pneumoniae* isolates among different clinical specimens.

Figure 4-1 shows that a higher distributional analysis of *K. pneumoniae* strains, 46.5% of isolates were obtained from sputum, followed by urine 29.6%. The ratio conforms to the findings of (Al-Timimi, 2023; Pyakurel et al., 2021). As per (Ashurst & Dawson, 2018), *K. pneumoniae* colonizes mucosal human surfaces and

is the most common cause of hospital-acquired pneumonia. (Ranjbar et al., 2016) had documented that *K. pneumoniae* is the second most common cause of UTIs in hospital environments, supporting the finding of the present study.

In the research of (Raheem & Mohammed, 2021; Ssekatawa et al., 2021), wound, burn, sputum and urine specimens were employed in the analysis of the prevalence of *K. pneumoniae* in a hospital-based research and they were able to recover the micro-organisms from all of these specimens in supporting the current observation that *K. pneumoniae* can cause a wide range of clinical illness and can be isolated from a range of clinical specimens.

Aside from the standard biochemical tests including (negative Indole test, positive Voges-Proskauer test, positive Simmon's citrate test, Catalase production, Urease production, and Triple Sugar Iron (TSI) test (acid/acid, gas production) employed for the identification of *K. pneumoniae*, the isolates were furthermore diagnosed through the Vitek2 system by the GN/ID identification card of the diagnosis, wherein the isolates were confirmed as being returned to *K. pneumoniae* (Appendix 1). This method proved speed as well as accuracy in the identification of isolates with a probability ratio (99%–95%).

4.2. Distribution of *K. pneumoniae* Isolates from Different Clinical Specimens

Table (4-1) shows the frequency distribution of different clinical samples according to bacterial culture results. The current results showed a high frequency distribution of *K. pneumoniae* isolates from urine, sputum, wound, and blood samples (22.5%) based on diagnosis using conventional biochemical tests and confirmatory testing with the Vitek 2 compact system. This is consistent with a study of (Al-Timimi, 2023; Raheem & Mohammed, 2021).

Table(4-1): Distribution of *K. pneumoniae* isolates from different clinical specimens.

Clinical specimens	Total number	<i>K. pneumoniae</i> <i>n</i> =71	<i>P</i>
Urine	113	21(18.6%)	$\chi^2 = 10.445$ HS $P = 0.015$
Sputum	98	33(33.7%)	
Wound	70	11(15.7%)	
Blood	35	6(17.1%)	
Total	316	71(22.5%)	

n: number of cases; HS: High significant differences at $P < 0.05$

The distribution of *Klebsiella pneumoniae* isolates among different clinical specimens was analyzed using the Pearson Chi-Square test. The results revealed a statistically significant difference in the isolation rates across the clinical sources ($\chi^2 = 10.445$, $P = 0.015$). This indicates that the prevalence of *K. pneumoniae* is significantly associated with the type of clinical specimen. Specifically, sputum samples showed the highest frequency of *K. pneumoniae* isolates (33 out of 98; 33.7%), followed by urine (21 out of 113; 18.6%), blood samples (6 out of 35; 17.1%), and wound (11 out of 70; 15.7%). The observed frequencies notably deviated from the expected frequencies under the assumption of equal distribution.

These findings suggest that sputum is a major source of *K. pneumoniae* infections in the studied population, possibly reflecting the organism's affinity for respiratory tract colonization or infection in this clinical context. The statistically significant association underscores the need for targeted infection control strategies, especially in respiratory care settings.

4.3. String Test Results

One of the most important tests that used for phenotypical identification of hypermucoviscous *K. pneumoniae* is string test. the results showed that 3/71(4.23%) isolates were positive for the test (>5 mm string; Figure 4-2):



Figure (4- 2): Positive “string test” for a hypervirulent strain of *K. pneumoniae*.

It has been established for a long time that hypermucoviscosity is strongly correlated with hypervirulence in hmKp strains (Ballén *et al.*, 2021). ‘Hypermucoviscous’ (hmKp) and ‘hypervirulent’ (hypervirulent *K. pneumoniae*: hvKp) have thus typically been synonymous (Hu *et al.*, 2024). However, recent studies have established that hypermucoviscosity and hypervirulence are distinct phenotypes and cannot be synonymous with each other (Li *et al.*, 2022). *K. pneumoniae* hypervirulence is a product of multiple Hypervirulence cannot be conferred by a single phenotypic attribute or genetic variables (Morreale *et al.*, 2023). It is insufficient to declare one strain hypervirulent based solely on hypermucoviscosity ascertained by the string test (Makhrmash *et al.*, 2022).

4.4. Antibiotic Sensitivity Tests for Clinical Isolates

Besides the bacterial identity of the isolated *K. pneumoniae*, the Vitek2 compact system was utilized to determine susceptibility to the isolated *K. pneumoniae* to

different antibiotic agents, according to National Committee for Clinical Laboratory Standards Institute (CLSI 2024) and the results demonstrated a moderate frequency of multidrug-resistant (MDR) isolates at 20 (28.2%) and a relatively lower frequency of extensively drug-resistant (XDR) isolates at 11 (15.5%). Notably, no pan-drug resistant (PDR) isolates were detected.

Among the clinical sources, sputum and blood samples showed the highest number of MDR isolates (33.3%), followed by urine (23.8%), and wound isolates (18.2%). Regarding XDR, the highest frequency was observed in urine isolates (19.0%), followed by wound (18.2%) and sputum (15.2%), whereas no XDR isolates were detected in blood samples. These differences in susceptibility profiles across clinical sources emphasize the varying resistance burden depending on the infection site. Although no statistically significant association was found, this result suggests that resistance patterns are not strongly influenced by sample type in this dataset. This finding is important as it indicates a potentially uniform distribution of resistance and calls for further investigation using larger sample sizes or broader geographic representation, as showed in Table (4-2) and Figure (4-3).

Table (4-2): Antibiotic susceptibility patterns in *Klebsiella pneumoniae* isolates from various clinical sources:

Clinical source	No. of isolate	Susceptible (%)	MDR (%)	XDR (%)	PDR (%)
Urine	21	12(57.1)	5(23.8)	4(19.0)	0.00
Sputum	33	17(51.5)	11(33.3)	5(15.2)	0.00
Blood	6	4(66.7)	2(33.3)	0(0.0)	0.00
Wound	11	7(63.6)	2(18.2)	2(18.2)	0.00
Total	71	40(56.3)	20(28.2)	11(15.5)	0.00
χ^2		2.411 (NS)			
P value		0.878			

NS: Non-Significant differences $P > 0.05$

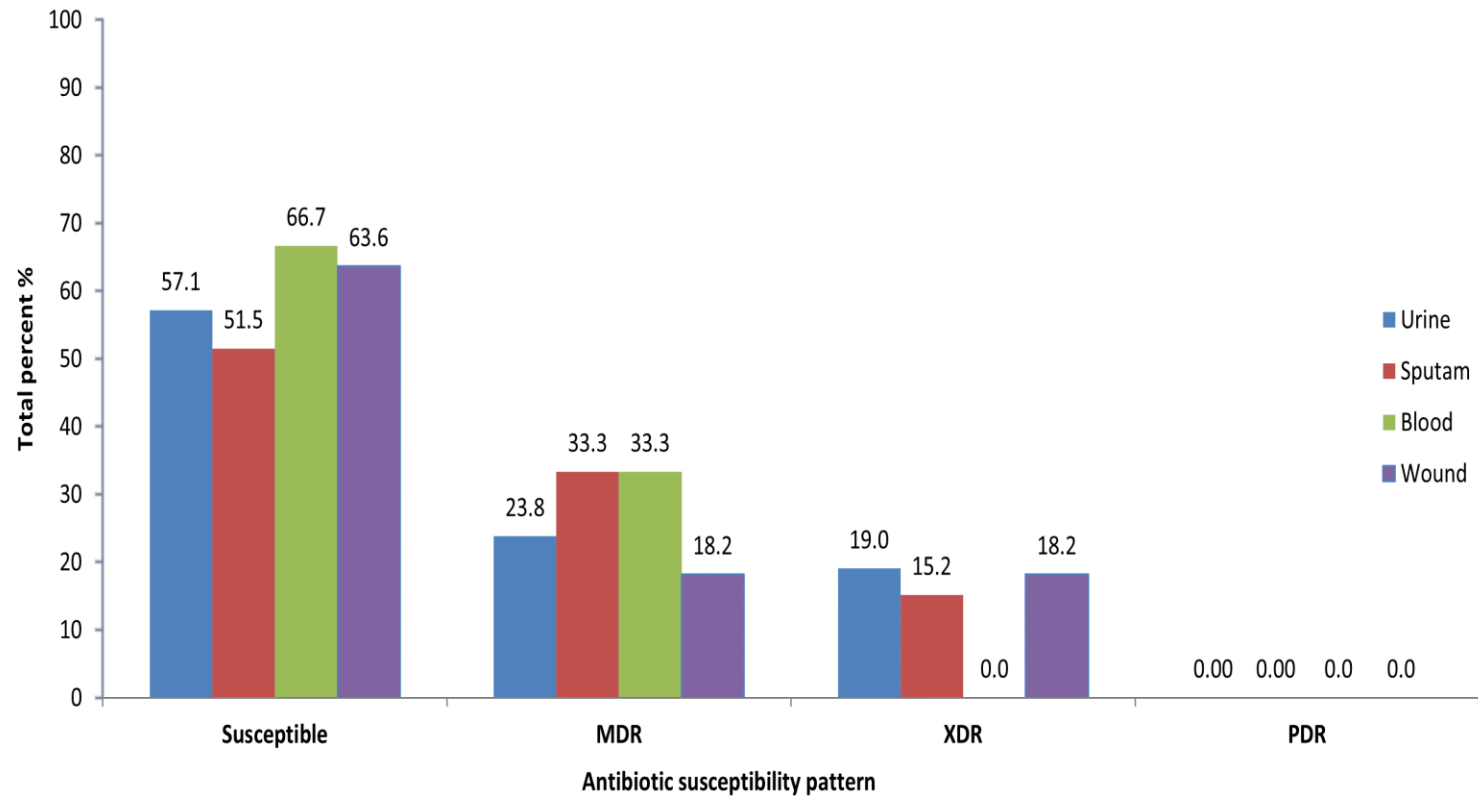


Figure (4-3): The prevalence (%) of antibiotic susceptibility pattern in *Klebsiella pneumoniae* isolates from different clinical infection patients' samples

The statistical analysis revealed a highly significant association ($\chi^2 = 354.6$, $P < 0.001$) between the types of antibiotics tested and the resistance profiles of *Klebsiella pneumoniae* isolates. This suggests that the bacterial response (sensitive, intermediate, resistant) is strongly dependent on the specific antibiotic used. This aligns with the observed variability in resistance across different antibiotic classes, as showed in Table (4-3) and Figure (4-4):

Table (4-3): Rates of sensitivity and resistance to antibiotics by *Klebsiella pneumoniae* isolates from clinical specimens

Antimicrobial Categories	Antimicrobial agents	Sensitive	Intermediate	Resistance
PENICILLINS	Ampicillin	1(1.4%)	0(0%)	70(98.6%)
	Piperacillin	41(57.7%)	0(0%)	30(42.3%)
CEPHALOSPORINS	Cefazolin	40(56.3%)	0(0%)	31(43.7%)
	Cefoxitin	53(74.6%)	0(0%)	18(25.4%)
	Ceftazidime	46(64.8%)	2(2.8%)	23(32.4%)
	Ceftriaxone	45(63.4%)	0(0%)	26(36.6%)
	Cefepime	46(64.8%)	0(0%)	25(35.2%)
B-LACTAM COMBINATION AGENTS	Piperacillin-tazobactam	52(73.2%)	0(0%)	19(26.8%)
	Ticarcillin-clavulanate	55(77.5%)	0(0%)	16(22.5%)
MONOBACTAMS	Aztreonam	48(67.6%)	0(0%)	23(32.4%)
CARBAPENEAMS	Imipenem	45(63.4%)	5(7.0%)	21(29.6%)
	Meropenem	47(66.2%)	2(2.8%)	22(31.0%)
	Ertapenem	59(83.1%)	0(0%)	12(16.9%)
LIPOPEPTIDES	Colistin	71(100%)	0(0%)	0(0%)
AMINOGLYCOSIDE	Amikacin	47(66.2%)	10(14.1%)	14(19.7%)
	Gentamicin	48(67.6%)	9(12.7%)	14(19.7%)
	Tobramycin	57(80.3%)	0(0%)	14(19.7%)
FLUOROQUINOLONES	Ciprofloxacin	46(64.8%)	9(12.7%)	16(22.5%)
	Levofloxacin	42(59.2%)	13(18.3%)	16(22.5%)
FOLATE PATHWAY ANTIAGONISTS	Trimethoprim-sulfamethoxazole	39(54.9%)	2(2.8%)	30(42.3%)
χ^2	354.6 (HS)			
P value	P < 0.001			

HS: High significant association $p < 0.05$

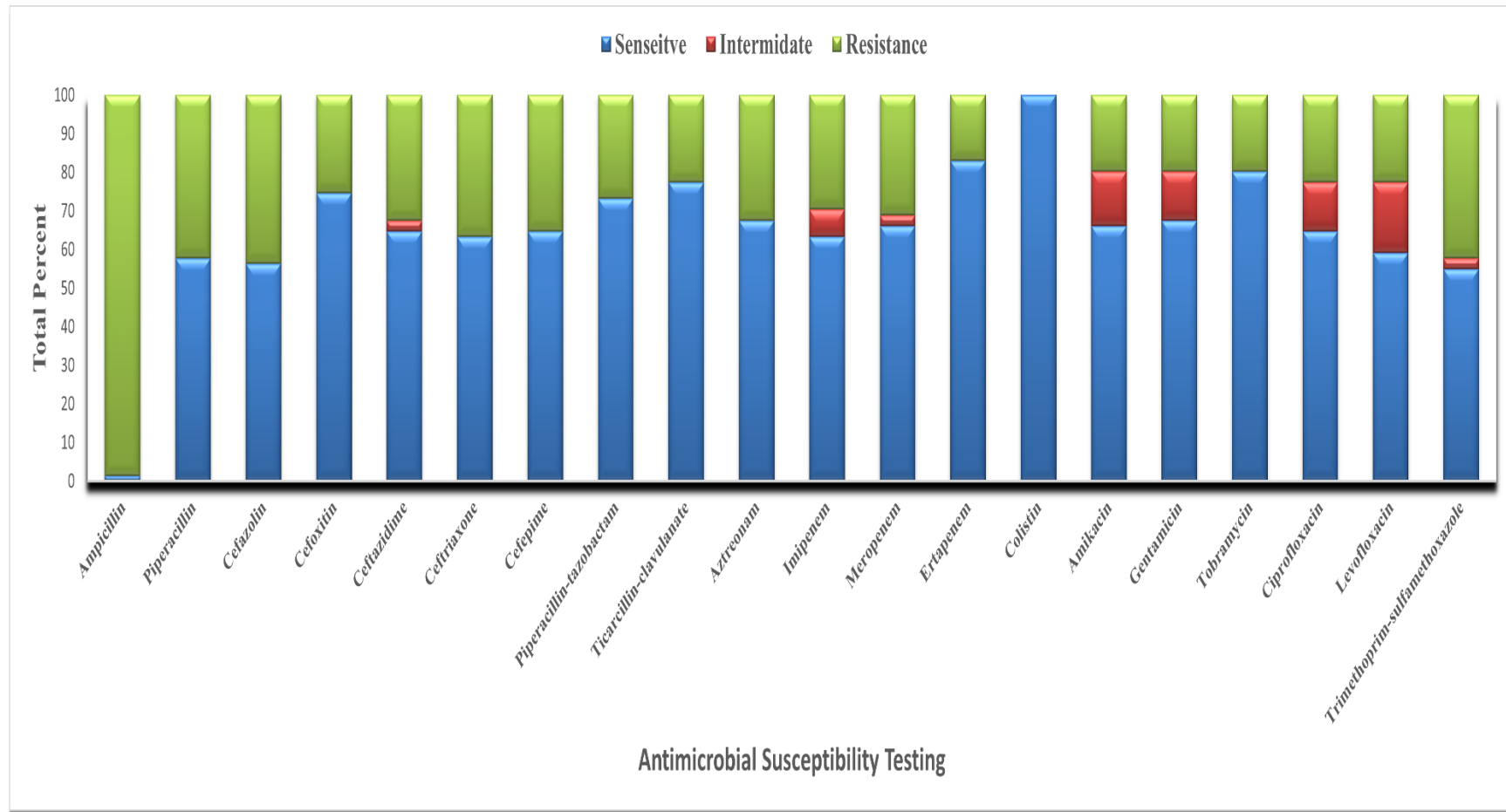


Figure (4-4): Antibiotic resistance profile in *Klebsiella pneumoniae* isolates

This finding suggests that resistance patterns are not evenly distributed among antibiotics. Certain antibiotics, such as target Colistin, exhibited complete sensitivity (100%), making it a last-resort but reliable option. However, caution must be taken due to its nephrotoxic effects and potential for resistance development with overuse.

While others, such as Ampicillin showed extremely high resistance (98.6%), rendering it largely ineffective for treating *K. pneumoniae* infections. This is consistent with several previous studies, including (87.1%) according to (Raheem & Mohammed, 2021) and (95.8%) according to (Srinivasan et al., 2012), reinforcing the conclusion that ampicillin is not a suitable choice for empirical therapy.

Piperacillin exhibited a resistance rate of 42.3%, higher than figures reported in previous studies, (17.5%) according to (Srinivasan et al., 2012) and (32.0%) according to (Aziz, 2021) indicating a regional rise in resistance, possibly due to local overuse or clonal expansion of resistant strains.

The resistance rate for Cefazolin was (43.7%) based on the current study, regarding Cefoxitin the resistance rate was (28.0%) according to (Hamid, 2022), This is consistent with the present study where the resistance ratio was (25.4%). With respect to Ceftazidime , the resistance rate was (35.0%) according to (Hamid, 2022), This is consistent with the current study where the resistance rate was (32.4%). With respect to Ceftriaxone the resistance rate was (31.0%) according to (Hamid, 2022), This is consistent with the present study where the resistance rate was (36.6%). The resistance rate to cefepime was (74.2%) according to (Raheem & Mohammed, 2021), (43.0%) according to (Aziz, 2021), and (35.2%) in this study, indicating that cefepime (CPM) is not a significantly bad option. Cephalosporins such as Cefazolin, Cefoxitin, Ceftazidime, Ceftriaxone, and Cefepime showed moderate resistance rates, generally ranging from 25% to 44%. These levels suggest

that while some cephalosporins may still be used in treatment, their effectiveness is diminishing and should be closely monitored.

On the other hand, For Piperacillin-tazobactam, the resistance rate was (37.0%) according to (Al-Timimi, 2023), and (26.8%) in this study. The resistant rate to aztreonam was 31.4 % according to of (Srinivasan *et al.*, 2012) and (41.7%) according to (Sakkas *et al.*, 2019) and in our study was (32.4%).

Combination beta-lactam agents such as Piperacillin–tazobactam (26.8%) and Ticarcillin–clavulanate (22.5%) retained good activity, reflecting their utility as part of empirical regimens.

Regarding Meropenem, the resistance rate was (79.2%) according to (Sakkas *et al.*, 2019) and (29.2%) according to (Raheem & Mohammed, 2021) and (31.0%) according to this study. Furthermore, the resistant rate for Imipenem was (88.8%) in a study by (Sakkas *et al.*, 2019) and (22.6%) according to (Raheem & Mohammed, 2021) and (29.6%) in the current study, indicating that genetic strains of *K. pneumoniae* in our community is different from that in other regions of world and Imipenem and Meropenem are still a good antibiotic choice. Ertapenem is also a good choice because the sensitivity rate was high (83.1%).

Resistance to carbapenems like Imipenem (29.6%), Meropenem (31.0%), and especially Ertapenem (16.9%) maintained relatively high sensitivity. This supports their continued role in treating resistant *K. pneumoniae*, although emerging resistance trends call for cautious usage.

On the other hand, For Amikacin, the resistance rate was (62.5%) according to (Sakkas *et al.*, 2019) and (22.6%) according to (Raheem & Mohammed, 2021) and (19.7%) in this study. Regarding gentamycin, the resistance rate was (45.8%) according to (Sakkas *et al.*, 2019) and (75.6%) according to (Srinivasan *et al.*, 2012)

and (19.4%) according to (Raheem & Mohammed, 2021) whereas in the current study, it was (19.7%). With respect to Tobramycin the resistance rate was (25.0%) according to (Hamid, 2022), and (19.7%) in this study.

Aminoglycosides including Amikacin, Gentamicin, and Tobramycin showed favorable sensitivity patterns (resistance ~19.7%), suggesting they are still effective options for treating multidrug-resistant isolates.

Regarding Ciprofloxacin, the resistance rate was (88.8%) according to (Sakkas *et al.*, 2019) and (38.7%) according to (Raheem & Mohammed, 2021) and (22.5%) in the present study. Regarding Levofloxacin, the resistance rate was (60.6%) according to (Srinivasan *et al.*, 2012) and 77.4% according to (Raheem & Mohammed, 2021) and (22.5%) in this study.

Fluoroquinolones like Ciprofloxacin and Levofloxacin demonstrated moderate resistance rates (22.5%), significantly lower than those observed in some global studies. These discrepancies may be due to local prescribing practices or specific genetic backgrounds of circulating strains.

Regarding Trimethoprim-sulfamethoxazole, the resistance rate was (71.8%) according to (Al-Saady, 2023) and (28.0%) according to (Hamid, 2022) and (42.3%) according to this study.

The findings emphasize the need for region-specific antibiotics stewardship programs. The variability in resistance profiles underscores the danger of relying on global or outdated susceptibility patterns when treating infections.

Empirical treatment protocols should be adjusted based on local antibiogram data, with periodic updates to reflect ongoing changes in resistance trends.

The high resistance to commonly used antibiotics such as ampicillin and cefazolin suggests that misuse or overuse of these agents may have contributed to the resistance burden.

Preserving the efficacy of last-line agents like colistin and carbapenems requires judicious use, restricted indications, and possibly enhanced infection control measures to prevent clonal dissemination of resistant strains.

From a clinical perspective, these results highlight the urgent need for ongoing surveillance of antibiotic resistance and reinforce the importance of evidence-based antibiotic selection. Empirical use of antibiotics should be guided by local resistance profiles to minimize the risk of therapeutic failure and reduce the spread of resistant strains.

4.5. Biofilm Formation Results

The biofilm formation in MDR and XDR *Klebsiella pneumoniae* isolates were measured by using colorimetric microtiter plate method-based crystal violet stain. The results showed that (13%) were strong for biofilms formation while (29%) moderate and (42%) weak biofilm formation and (16%) as non-biofilm producer as showed in Figure (4-5):

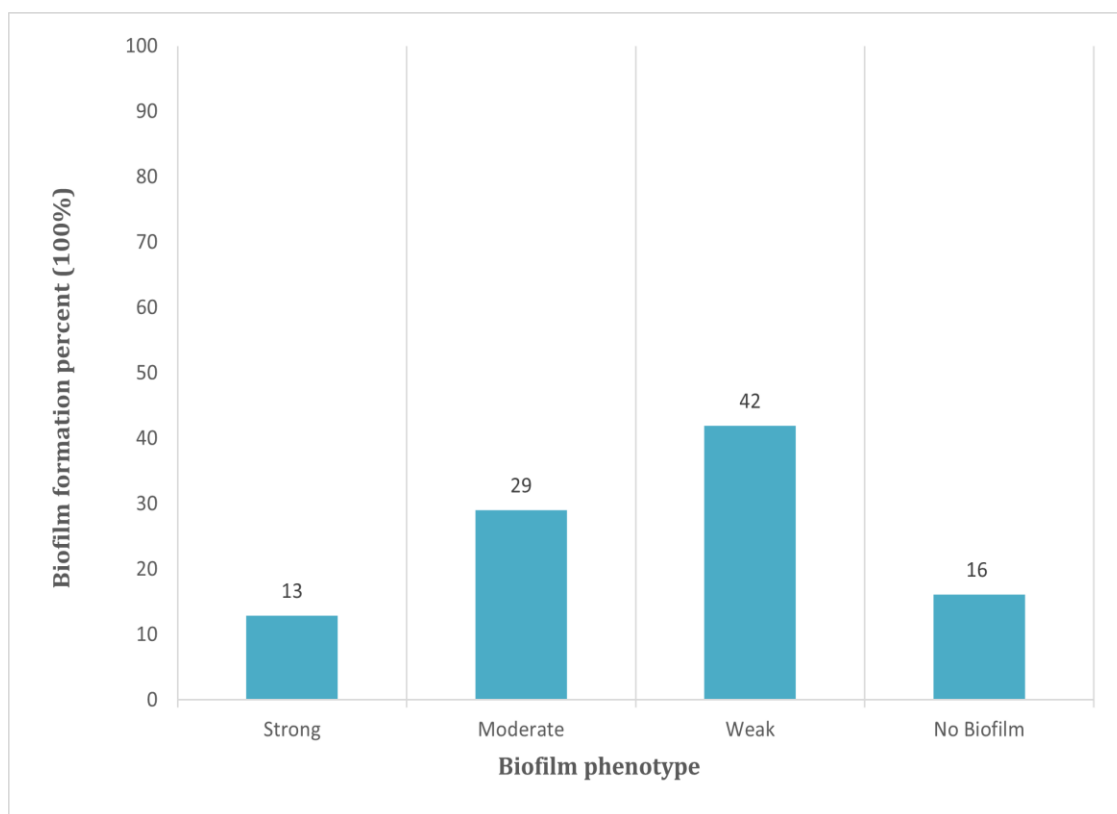


Figure (4-5): Phenotypic percentage of biofilm formation in MDR & XDR *Klebsiella pneumoniae* isolates.

In the process of biofilm formation, structural and regulatory genes play a crucial role by influencing colony aggregation through various mechanisms. These include modifications in the synthesis of transcriptional factors and the regulation of extracellular polysaccharide production (Alzubaidy *et al.*, 2019).

Biofilm formation is a major virulence factor for many pathogenic bacteria, particularly nosocomial pathogens such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The ability to form biofilms enhances bacterial survival by protecting cells from host immune responses and increasing their resistance to antibiotic agents (Donlan & Costerton, 2002).

In the current study, biofilm-forming ability was evaluated and categorized into four phenotypic groups: strong (13%), moderate (29%), weak (42%), and non-biofilm formers (16%). These findings suggest that the majority of isolates (84%) possess the capability to form biofilms, though predominantly in weak and moderate levels.

The predominance of weak biofilm formers (42%) may be attributed to variations in regulatory gene expression (such as *mrkA*, *rmpA*, *luxS*) or environmental adaptations rather than complete genetic inability. As previous studies have shown, biofilm formation is not a binary trait but rather a spectrum influenced by multiple factors including nutrient availability, oxygen levels, and surface type (Vuotto et al., 2014).

Strong biofilm producers (13%) are of particular clinical concern as they often exhibit enhanced tolerance to antibiotics like carbapenems and aminoglycosides due to the dense extracellular matrix that impedes drug penetration (Al-Timimi, 2023). Their low proportion in the current dataset may reflect a natural distribution or could indicate selective pressure in the clinical environment favoring less robust biofilm phenotypes.

Moderate biofilm producers (29%) also pose significant clinical risks, especially in device-associated infections. The presence of 16% non-biofilm producers indicates genetic or environmental suppression of biofilm-related gene pathways,

which may reduce pathogenicity but increase susceptibility to host defenses and antibiotics.

Overall, these results are consistent with other regional and international studies. For example, (Al-Saady, 2023) reported that 100% of *K. pneumoniae* clinical isolates in Iraq could form biofilms, with varying intensities. Similarly, (Al-Timimi, 2023) observed a high prevalence of weak and moderate biofilm producers among Iraqi isolates.

The predominance of weak and moderate biofilm producers highlights the importance of early detection and tailored antimicrobial strategies. Surveillance of biofilm phenotypes in clinical isolates remains crucial in understanding infection persistence and resistance development.

4.6. Genotypic Detection of (*mrkD*, *rmpA*, *blaKPC*, *blaVIM*) genes

In the current study, DNA from 31 isolates (20MDR and 11XDR) was isolated and amplified by polymerase chain reaction (PCR) to detect the presence of *the mrkD*, *rmpA*, *blaKPC* and *blaVIM* genes, as shown in Figure (4-6). The results of Agarose gel electrophoresis clarified in Figures (4-7) to Figure (4-14).

Klebsiella pneumoniae has emerged as a critical nosocomial pathogen, particularly due to its increasing capacity for multidrug resistance (MDR) and extensive drug resistance (XDR). The current study investigated the presence of key genetic determinants related to adhesion (*mrkD*)(100%), hypervirulence (*rmpA*)(9.7%), and carbapenem resistance (*blaKPC*)(9.7%) and the absence of (*blaVIM*) among MDR and XDR clinical isolates, as shown in Figure (4-6). These findings are compared with recent studies to contextualize their clinical and epidemiological relevance.

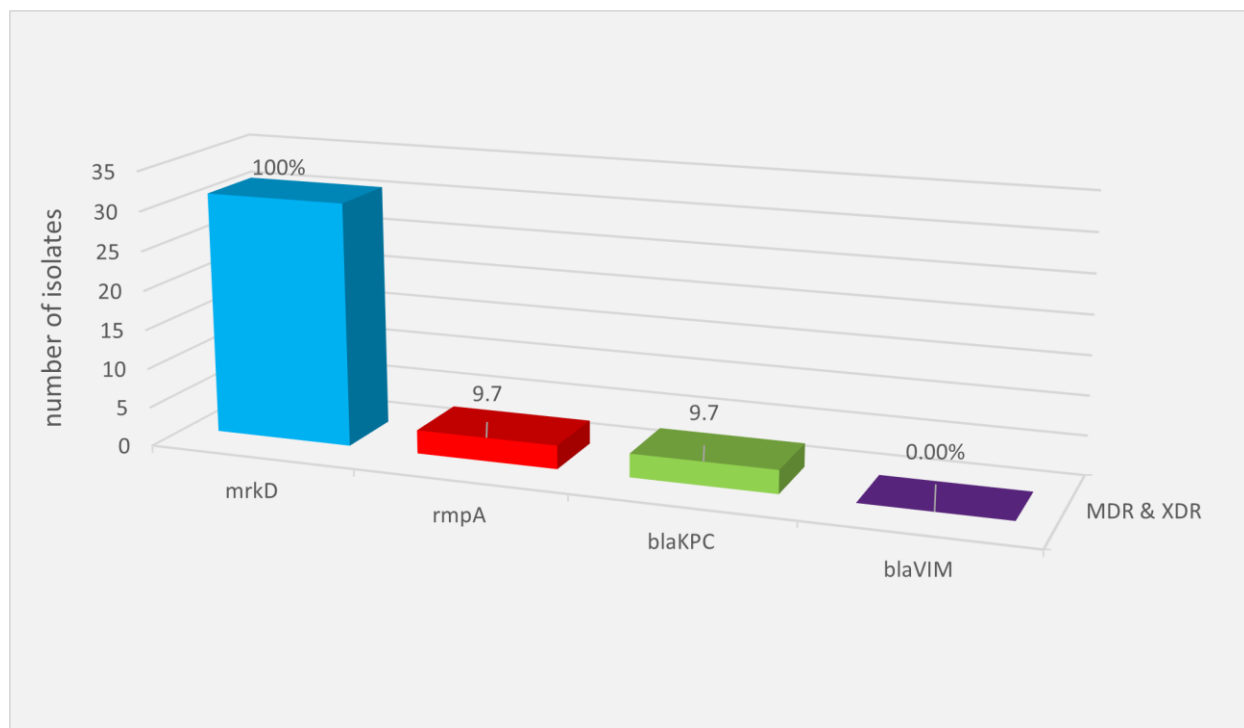


Figure (4-6): Distribution of Selected Genes in MDR and XDR *K. pneumoniae* Isolates.

4.6.1. Results of *mrkD* Gene

The high prevalence of the *mrkD* gene among both MDR and XDR isolates in this study aligns with its recognized role in biofilm formation and adhesion, key factors contributing to antimicrobial resistance and persistence in hospital environments. These results were close to what was reached by the (Bakhtiari *et al.*, 2021) study, which recorded the appearance of a gene at a rate of (69.09%) in the studied isolates. This supports the hypothesis that *mrkD*-mediated biofilm formation significantly contributes to resistance phenotypes and chronic infections (Li *et al.*, 2024).



Figure (4-7): Agarose gel electrophoresis image that showed PCR product analysis of *mrkD* gene in *Klebsiella pneumoniae* isolates. M (Marker ladder 2000-100bp). Lane (K1-K11) showed *Klebsiella pneumoniae* XDR isolates with positive *mrkD* gene at 515bp product size.

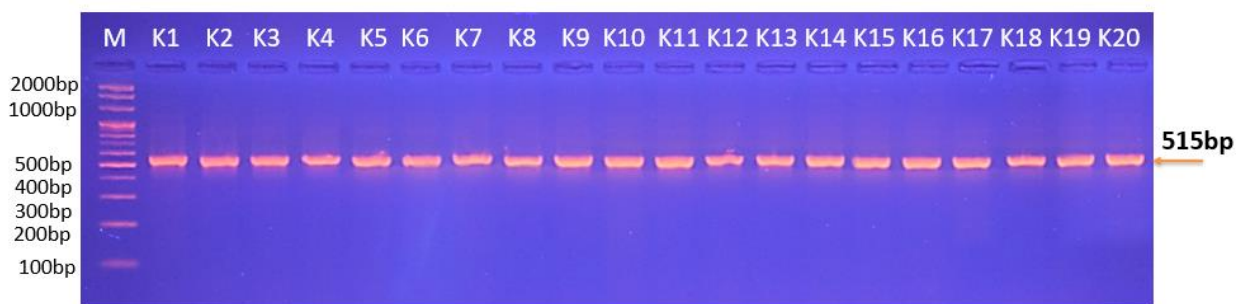


Figure (4-8): Agarose gel electrophoresis image that showed PCR product analysis of *mrkD* gene in *Klebsiella pneumoniae* isolates. M (Marker ladder 2000-100bp). Lane (K1-K20) showed *Klebsiella pneumoniae* MDR isolates with positive *mrkD* gene at 515bp product size.

4.6.2. Results of *rmpA* Gene

The results showed that only hypermucoviscous isolates produced *rmpA* (3 isolates) rate was (9.7%) as shown in Figure (4-6). The partial detection of the *rmpA* gene suggests the coexistence of hypervirulent strains among the MDR and XDR isolates. These results were close to what was reached by the study (Shakib *et al.*,

2018) in Iran, as it recorded the appearance of the gene at a rate of (5.7%) in the studied isolates, while the results were contrary to what was reached by the study of (Raheem & Mohammed, 2021), The proportion of the *rmpA* virulence factor was (38.7%). (Taha *et al.*, 2024) found that the presence of *rmpA* and *rmpA2* genes is associated with hypermucoviscous phenotypes and increased virulence in *K. pneumoniae* strains.

Although the presence of *rmpA* was lower in this study, its detection still poses a clinical threat due to its association with increased capsule production and evasion of host immune responses.

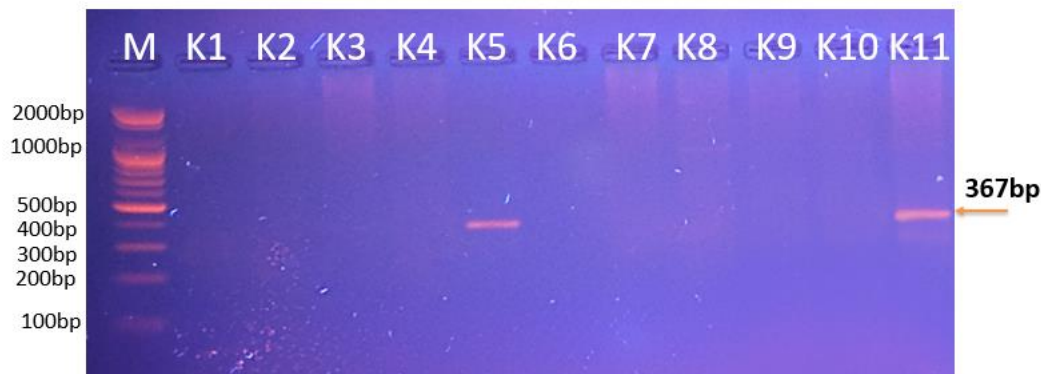


Figure (4-9): Agarose gel electrophoresis image that showed PCR product analysis of *rmpA* gene in *Klebsiella pneumoniae* isolates. M (Marker ladder 2000-100bp). Lane (K1-K11) showed some *Klebsiella pneumoniae* XDR isolates (K5&K11) with positive *rmpA* gene at 367bp product size.

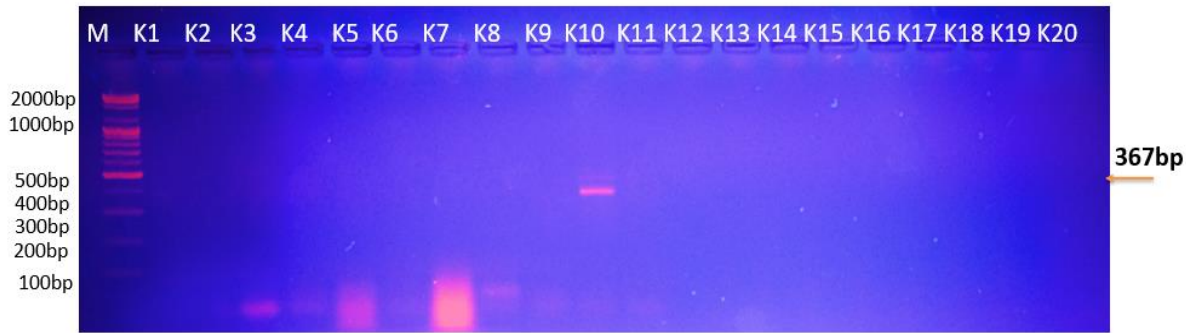


Figure (4-10): Agarose gel electrophoresis image that showed PCR product analysis of *rmpA* gene in *Klebsiella pneumoniae* isolates. M (Marker ladder 2000-100bp). Lane (K1-K20) showed some *Klebsiella pneumoniae* MDR isolates (K10) with positive *rmpA* gene at 367bp product size.

4.6.3. Results of *blaKPC* Gene

The identification of *blaKPC* rate was (9.7%), in some MDR and XDR isolates corroborates global trends of carbapenem resistance dissemination. The Centers for Disease Control and Prevention (CDC) reported that carbapenem-resistant Enterobacteriaceae, including *K. pneumoniae*, are a significant public health threat, with *blaKPC* being a common resistance mechanism. These results were close to what was reached by the study of (Al-Timimi, 2023), as it recorded the appearance of the gene at a rate of (2.5%) in the studied isolates, while the results were contrary to what was reached by the study of (Hamid, 2022), The proportion of the *blaKPC* virulence factor was (50.0%).



Figure (4-11): Agarose gel electrophoresis image that showed PCR product analysis of *blaKPC* gene in *Klebsiella pneumoniae* isolates. M (Marker ladder 2000-100bp). Lane (K1-K11) showed some *Klebsiella pneumoniae* XDR isolates (K5&K11) with positive *blaKPC* gene at 544bp product size.



Figure (4-12): Agarose gel electrophoresis image that showed PCR product analysis of *blaKPC* gene in *Klebsiella pneumoniae* isolates. M (Marker ladder 2000-100bp). Lane (K1-K20) Showed some *Klebsiella pneumoniae* MDR isolates (K10) with positive *blaKPC* gene at 544bp product size.

4.6.4. Results of *blaVIM* Gene

Interestingly, the absence of the *blaVIM* gene in all isolates contrasts with reports from other geographical regions. A study by (Al-Timimi, 2023; Hamid, 2022) documented a prevalence of *blaVIM* among *Klebsiella* spp. isolates in certain

regions, indicating geographical variability in the distribution of metallo- β -lactamase genes.

This suggests that regional differences in antibiotic usage and infection control practices may influence the distribution of resistance genes like *blaVIM*.

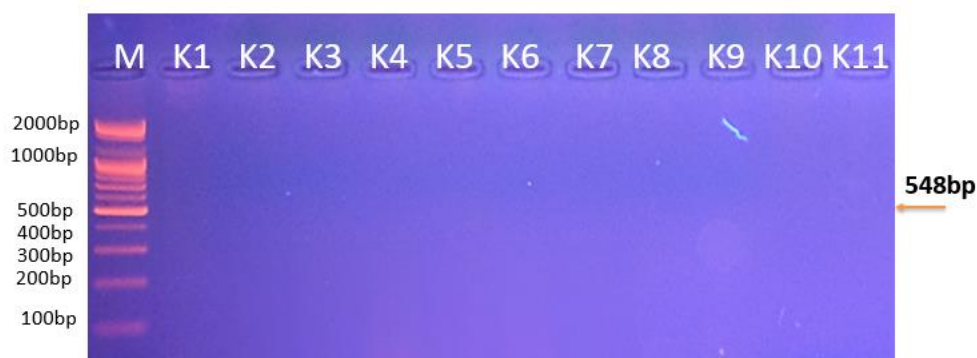


Figure (4-13): Agarose gel electrophoresis image that showed PCR product analysis of *blaVIM* gene in *Klebsiella pneumoniae* isolates. M (Marker ladder 2000-100bp). Lane (K1-K) Showed *Klebsiella pneumoniae* XDR isolates with negative *blaVIM* gene at 548bp product size.



Figure (4-14): Agarose gel electrophoresis image that showed PCR product analysis of *blaVIM* gene in *Klebsiella pneumoniae* isolates. M (Marker ladder 2000-100bp). Lane (K1-K20) Showed *Klebsiella pneumoniae* MDR isolates with negative *blaVIM* gene at 548bp product size.

4.7. Multilogues Sequence Typing of *K. pneumoniae*

4.7.1. Molecular Detection of Housekeeping Genes of *K. pneumoniae*

The results showed that the presence of seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) in *Klebsiella pneumoniae* isolated from clinical samples, as shown in Figure (4-15):

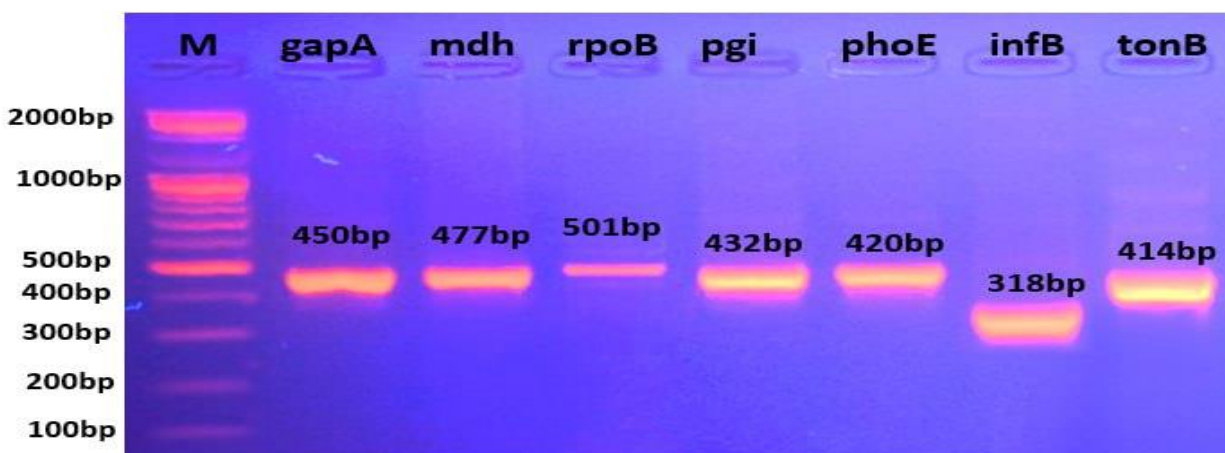


Figure (4-15): Agarose gel electrophoresis image showing the PCR analysis of seven housekeeping genes in a *Klebsiella pneumoniae* isolate. The ladder marker (100-2000 base pairs) and Lane (*gapA*, *mdh*, *rpoB*, *pgi*, *phoE*, *infB*, and *tonB*) showed positive isolates at a PCR product size of 450 bp, 477 bp, 501 bp, 432 bp, 420 bp, 318 bp and 414 bp respectively, and an electric current of 100 V and 80 mA was applied for 1 hour.

4.7.2. Housekeeping Gene Sequence Analysis

Polymerase chain reaction (PCR) for seven housekeeping genes were conducted for a multidrug-resistant *K. pneumoniae* isolate from a patient admitted to the respiratory care unit and partially sequenced. This isolate (hyperviscosity) positive for string test and carried the genes (*mrkD*, *rmpA* and *blaKPC*).

The differences between *K. pneumoniae* strain with respect to their alleles in the housekeeping genes were studied. A different allele number was assigned to each distinct sequence within the gene locus, and a distinct sequence type (ST) number was added to alleles group, according to the protocol described on the *K. pneumoniae* MLST website (www.pasteur.fr/mlst), as shown in Figure (4-16).

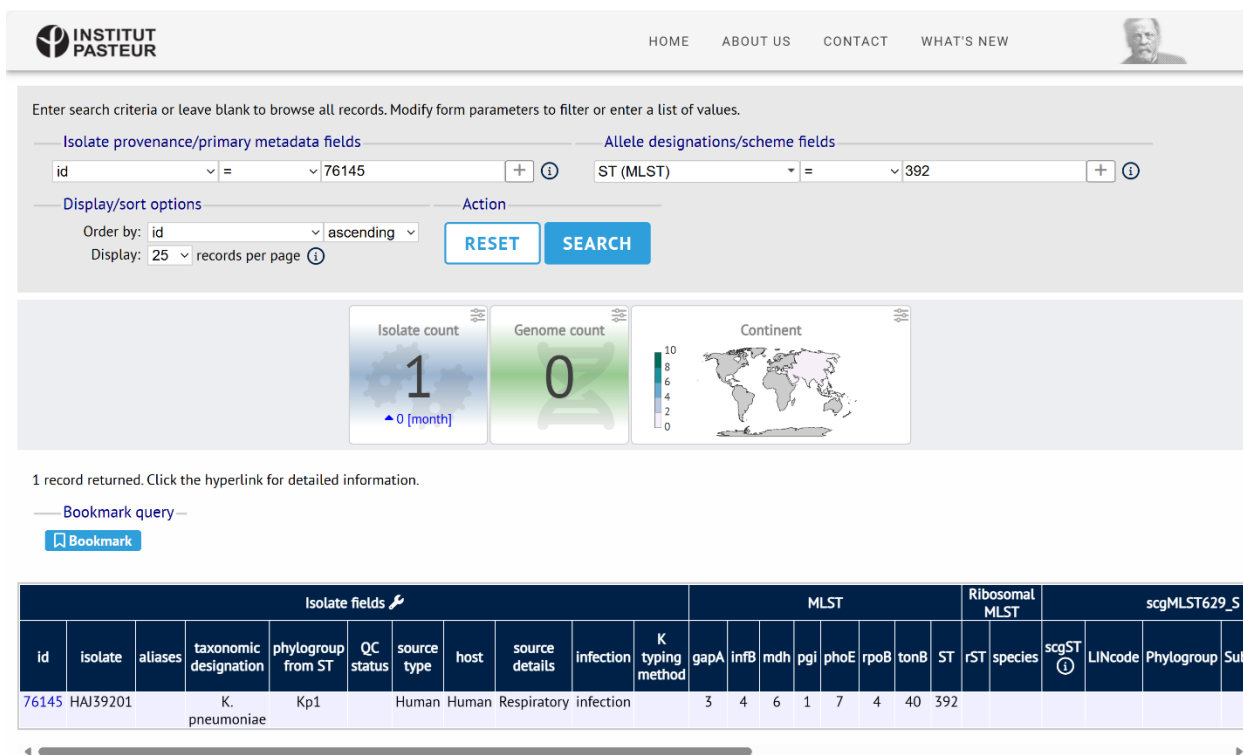
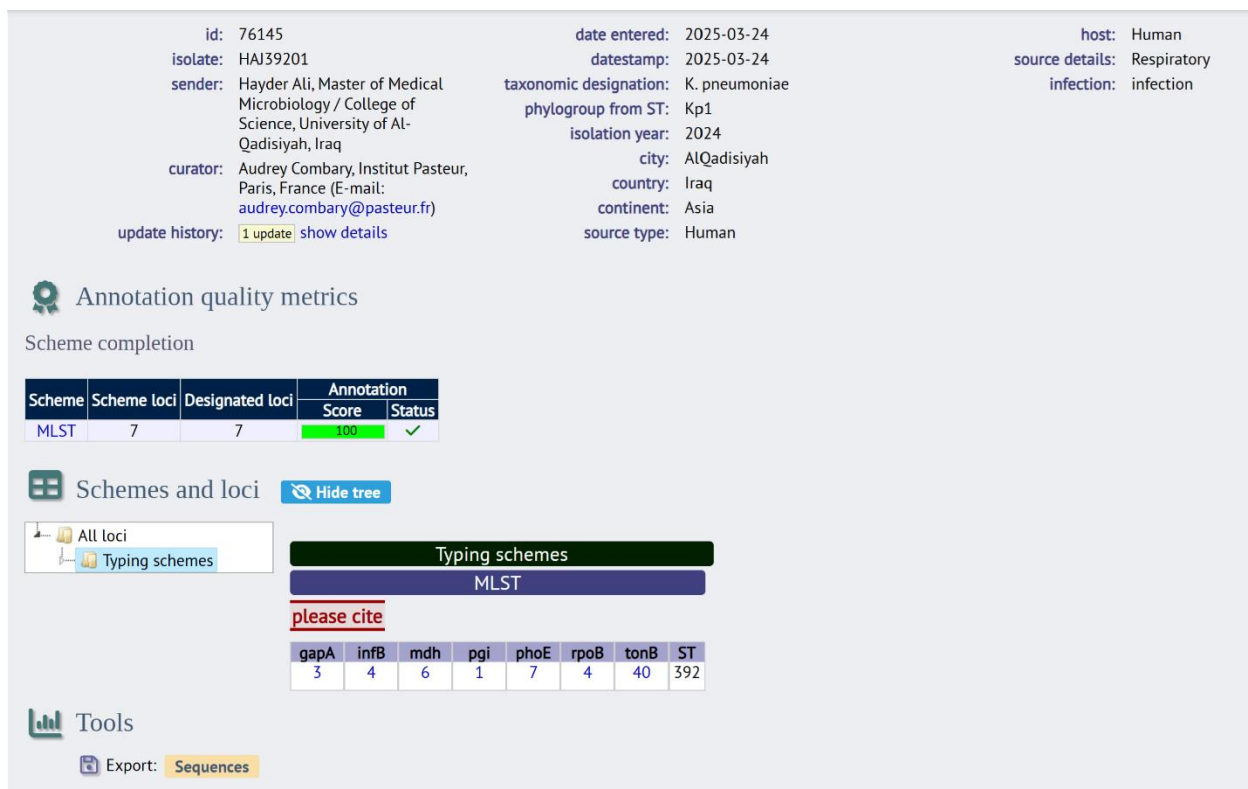


Figure (4-16): Multilocus sequence typing (MLST) allelic Profile identification of a distinct *K. pneumoniae* isolate of type ST392 in the BIGSdb-Pasteur database.

In this study, clinically relevant isolates of *K. pneumoniae* were analyzed using multi-locus sequencing (MLS) using seven key genes to clarify the relationship between local and global isolates. Multiple sequence alignment analysis of the seven gene sequences of the one isolate showed similarity (*) and divergence in the nucleotide sequences of the seven key genes. As shown in Figures (4-18 to 4-31).

The results of this study revealed the identification of *K. pneumoniae* isolate, which was registered in the BIGSdb-Pasteur database (ID:76145) and was the first in Iraq to have the ST392 sequence type, as shown in Figure (4-17):



Figure(4-17): The global database record for *Klebsiella pneumoniae* type (ID:76145) (ST392) shows a unique presence in Iraq.

DNA Sequences	Translated Protein Sequences																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
Species/Abbrv		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

Figure (4-18): Multiple sequence alignment analysis of MLST housekeeping *gapA* gene in local *Klebsiella pneumoniae* (HAJ39201) isolates and related [BIGSdb-Pasteur](#) data base *K. pneumoniae* MLST typing. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in MLST housekeeping *gapA* gene between isolates.

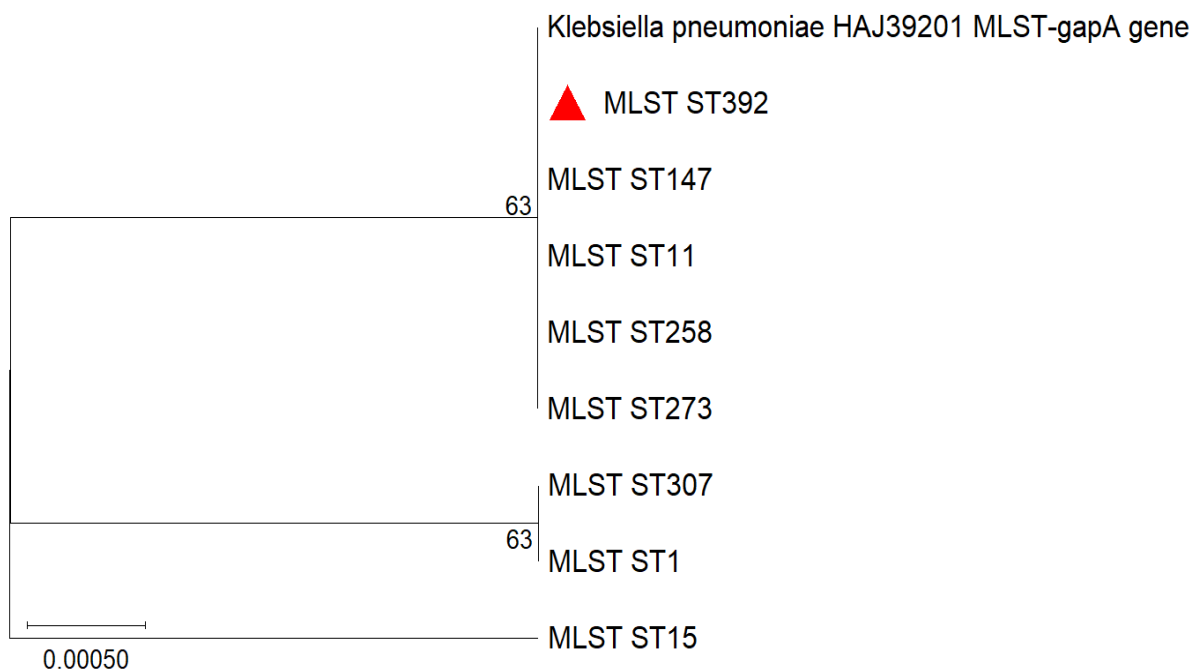


Figure (4-19): Phylogenetic tree analysis based MLST housekeeping *gapA* gene partial sequence in local *Klebsiella pneumoniae* (HAJ39201) isolate that used for confirmative genetic identification of MLST typing analysis. The phylogenetic tree was constructed using Neighbor-Joining tree method in (MEGA 12.0 version). The local *K. pneumoniae* (HAJ39201) Isolates were showed closed related to [BIGSdb-Pasteur](#) database *Klebsiella pneumoniae* MLST-ST392 at total genetic changes (0.00050%).

DNA Sequences	Translated Protein Sequences
Species/Abbrev	** *
1. <i>Klebsiella pneumoniae</i> HAJ39201 MLST- <i>infB</i> gene	GCACAGGCGACGGATATTGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCAC
2. MLST ST392	GCACAGGCGACGGATATTGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCAC
3. MLST ST147	GCACAGGCGACGGATATTGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCAC
4. MLST ST307	GCGCAGGCGACGGATATCGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCGC
5. MLST ST11	GCGCAGGCGACGGATATCGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCGC
6. MLAT ST15	GCGCAGGCGACGGATATCGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCGC
7. MLST ST258	GCGCAGGCGACGGATATCGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCGC
8. MLST ST273	GCACAGGCGACGGATATTGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCAC
9. MLST ST1	GCACAGGCGACGGATATTGTGGTTCTGGTGGTGGCGGCAGACGACGGCGTGATGCCAC

Figure (4-20): Multiple sequence alignment analysis of MLST housekeeping *infB* gene in local *Klebsiella pneumoniae* (HAJ39201) isolates and related [BIGSdb-Pasteur](#) data base *K. pneumoniae* MLST typing. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in MLST housekeeping *infB* gene between isolates.

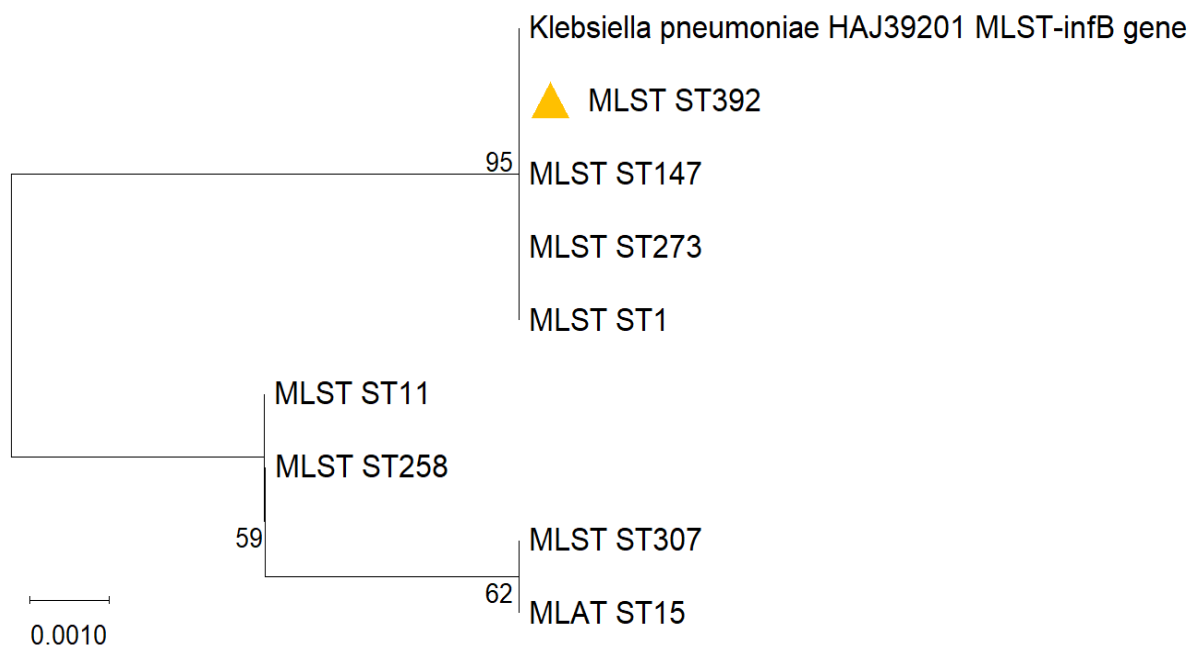


Figure (4-21): Phylogenetic tree analysis based MLST housekeeping *infB* gene partial sequence in local *Klebsiella pneumoniae* (HAJ39201) isolate that used for confirmative genetic identification of MLST typing analysis. The phylogenetic tree was constructed using Neighbor-Joining tree method in (MEGA 12.0 version). The local *K. pneumoniae* (HAJ39201) Isolates were showed closed related to [BIGSdb-Pasteur](#) data base *Klebsiella pneumoniae* MLST-ST392 at total genetic changes (0.0010%).

DNA Sequences	Translated Protein Sequences	
Species/Abbrv		* * * * *
1. <i>Klebsiella pneumoniae</i> HAJ39201 MLST-mdh gene	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCTGAGCTGAAA	
2. MLST-ST392	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCTGAGCTGAAA	
3. MLST-ST147	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCTGAGCTGAAA	
4. MLST-ST307	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCGGAGCTGAAA	
5. MLST-ST11	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCGGAGCTGAAA	
6. MLST-ST15	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCGGAGCTGAAA	
7. MLST-ST258	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCGGAGCTGAAA	
8. MLST-ST273	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCTGAGCTGAAA	
9. MLST-ST1	ACGCTGGACATCATCCGTTCCAATACCTTTGTGGCGGAGCTGAAA	

Figure (4-22): Multiple sequence alignment analysis of MLST housekeeping *mdh* gene in local *Klebsiella pneumoniae* (HAJ39201) isolates and related [BIGSdb-Pasteur](#) data base *K. pneumoniae* MLST typing. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in MLST housekeeping *mdh* gene between isolates.

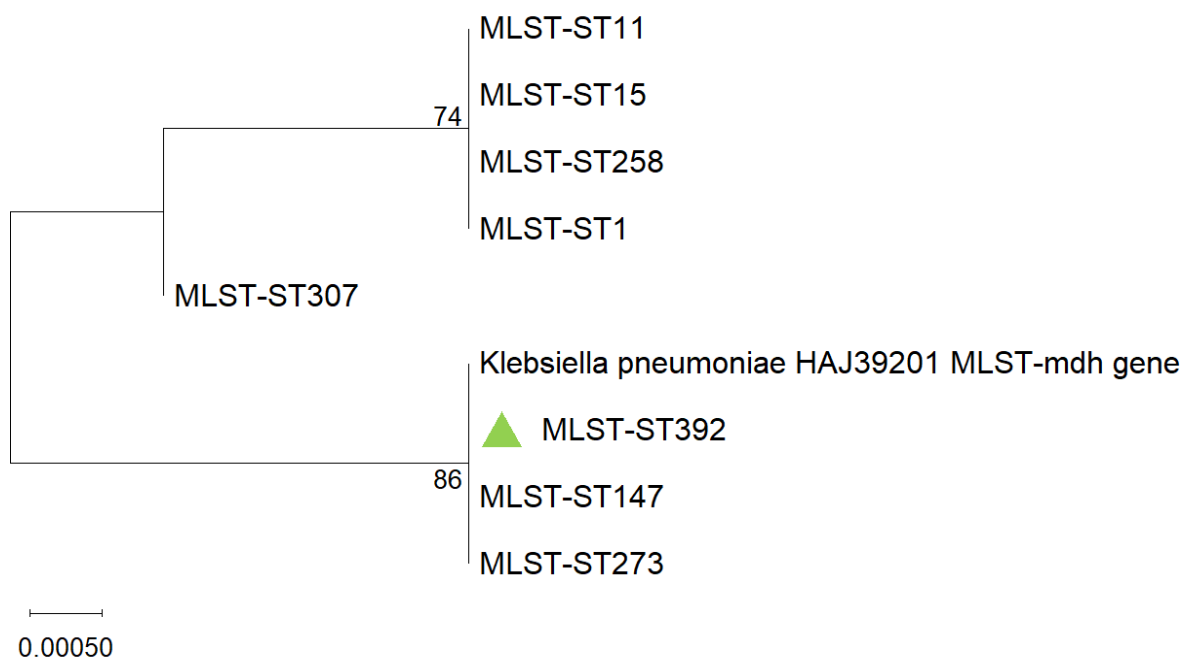


Figure (4-23): Phylogenetic tree analysis based MLST housekeeping *mdh* gene partial sequence in local *Klebsiella pneumoniae* (HAJ39201) isolate that used for confirmative genetic identification of MLST typing analysis. The phylogenetic tree was constructed using Neighbor-Joining tree method in (MEGA 12.0 version). The local *K. pneumoniae* (HAJ39201) Isolates were showed closed related to [BIGSdb-Pasteur](#) database *Klebsiella pneumoniae* MLST-ST392 at total genetic changes (0.00050%).

DNA Sequences	Translated Protein Sequences
Species/Abbrev	* * * * *
1. <i>Klebsiella pneumoniae</i> HAJ39201 MLST-pgi gene	G A G T C C A A C G G T A A G T A T G T T G A C C G T A A C G G C C A C G C G G T A G
2. MLST ST392	G A G T C C A A C G G T A A G T A T G T T G A C C G T A A C G G C C A C G C G G T A G
3. MLST-ST147	G A G T C C A A C G G T A A G T A T G T T G A C C G T A A C G G C C A C G C G G T A G
4. MLST-ST307	G A G T C C A A C G G T A A G T A T G T T G A T C G T A A C G G C C A C G C G G T A G
5. MLST-ST11	G A G T C C A A C G G T A A G T A T G T T G A C C G T A A C G G C C A C G C G G T A G
6. MLST-ST15	G A G T C C A A C G G T A A G T A T G T T G A C C G T A A C G G C C A C G C G G T A G
7. MLST-ST258	G A G T C C A A C G G T A A G T A T G T T G A C C G T A A C G G C C A C G C G G T A G
8. MLST-ST273	G A G T C C A A C G G T A A G T A T G T T G A C C G T A A C G G C C A C G C G G T A G
9. MLST-ST1	G A G T C C A A C G G T A A G T A T G T T G A C C G T A A C G G C C A C G C G G T A G

Figure (4-24): Multiple sequence alignment analysis of MLST housekeeping *pgi* gene in local *Klebsiella pneumoniae* (HAJ39201) isolates and related [BIGSdb-Pasteur](#) database *K. pneumoniae* MLST typing. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in MLST housekeeping *pgi* gene between isolates.

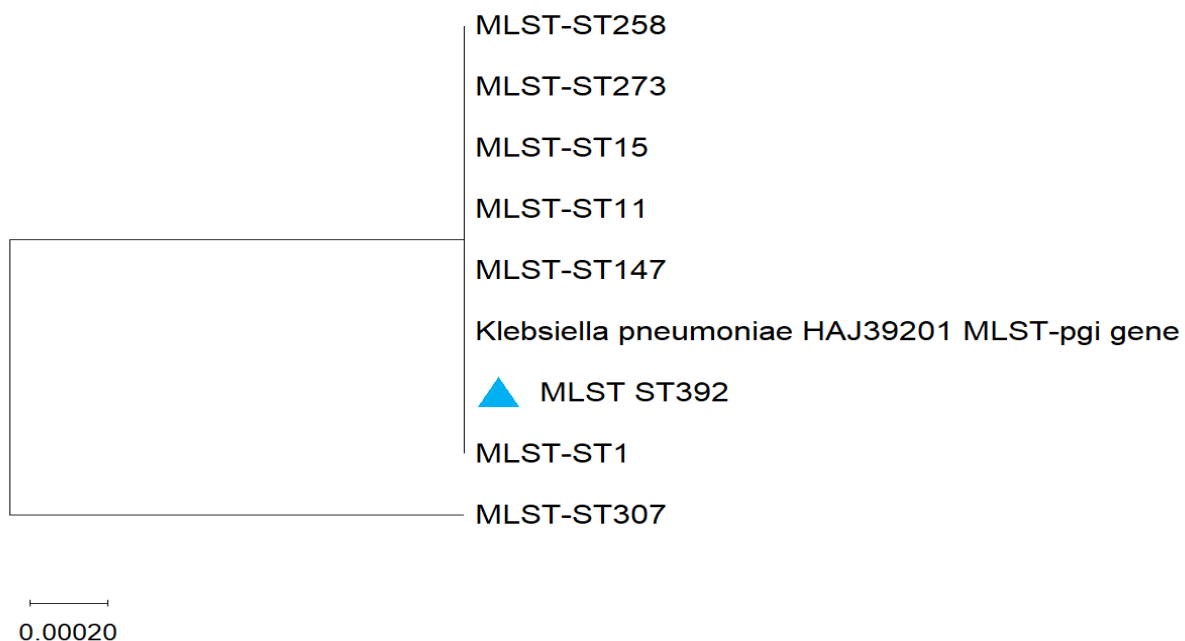


Figure (4-25): Phylogenetic tree analysis based MLST housekeeping *pgi* gene partial sequence in local *Klebsiella pneumoniae* (HAJ39201) isolate that used for confirmative genetic identification of MLST typing analysis. The phylogenetic tree was constructed using Neighbor-Joining tree method in (MEGA 12.0 version). The local *K. pneumoniae* (HAJ39201) Isolates were showed closed related to [BIGSdb-Pasteur](#) data base *Klebsiella pneumoniae* MLST-ST392 at total genetic changes (0.00020%).

DNA Sequences	Translated Protein Sequences
Species/Abbrv	* * * * *
1. <i>Klebsiella pneumoniae</i> HAJ39201 MLST-phoE gene	G A A G G G G T G G G G A G T G A G G A T C T G G T T A A C T A C A T T G A C G T G G G
2. MLST-ST392	G A A G G G G T G G G G A G T G A G G A T C T G G T T A A C T A C A T T G A C G T G G G
3. MLST-ST147	G A A G G G G T G G G G A G T G A G G A T C T G G T T A A C T A C A T T G A C G T G G G
4. MLST-ST307	G A A G G G G T G G G G A G T G A G A T C T G G T T A A C T A C A T C G A C G T G G G
5. MLST-ST11	G A A G G G G T G G G G A G T G A A G A T C T G G T T A A C T A C A T C G A C G T G G G
6. MLST-ST15	G A A G G G G T G G G G A G T G A A G A T C T G G T T A A C T A C A T C G A C G T G G G
7. MLST-ST258	G A A G G G G T G G G G A G T G A A G A T C T G G T T A A C T A C A T C G A C G T G G G
8. MLST-ST273	G A A G G G G T G G G G A G T G A G G A T C T G G T T A A C T A C A T T G A C G T G G G
9. MLST-ST1	G A A G G G G T G G G G A G T G A G G A T C T G G T T A A C T A C A T T G A C G T G G G

Figure (4-26): Multiple sequence alignment analysis of MLST housekeeping *phoE* gene in local *Klebsiella pneumoniae* (HAJ39201) isolates and related [BIGSdb-Pasteur](#) database *K. pneumoniae* MLST typing. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in MLST housekeeping *phoE* gene between isolates.

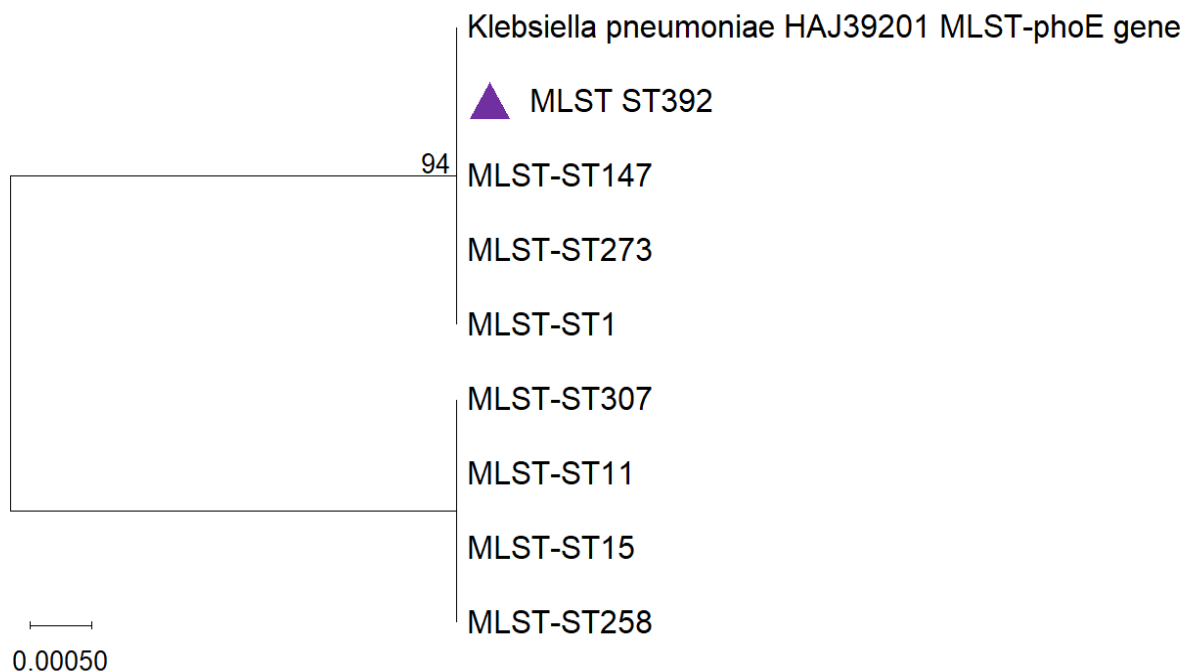


Figure (4-27): Phylogenetic tree analysis based MLST housekeeping *phoE* gene partial sequence in local *Klebsiella pneumoniae* (HAJ39201) isolate that used for confirmative genetic identification of MLST typing analysis. The phylogenetic tree was constructed using Neighbor-Joining tree method in (MEGA 12.0 version). The local *K. pneumoniae* (HAJ39201) *pneumoniae* MLST-ST392 at total genetic changes (0.0050%).

DNA Sequences	Translated Protein Sequences	
Species/Abbrev		* * * * *
1. <i>Klebsiella pneumoniae</i> HAJ39201 MLST-rpoB gene	GTGACCGACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	
2. MLST-ST392	GTGACCGACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	
3. MLST-ST147	GTGACCGACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	
4. MLST-ST307	GTGACCAACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	
5. MLST-ST11	GTGACCAACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	
6. MLST-ST15	GTGACCAACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	
7. MLST-ST258	GTGACCAACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	
8. MLST-ST273	GTGACCGACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	
9. MLST-ST1	GTGACCGACGGTGTGGTTACTGACGAAATTCACTACCTGTCT	

Figure (4-28): Multiple sequence alignment analysis of MLST housekeeping *rpoB* gene in local *Klebsiella pneumoniae* (HAJ39201) isolates and related [BIGSdb-Pasteur](#) database *K. pneumoniae* MLST typing. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in MLST housekeeping *rpoB* gene between isolates.

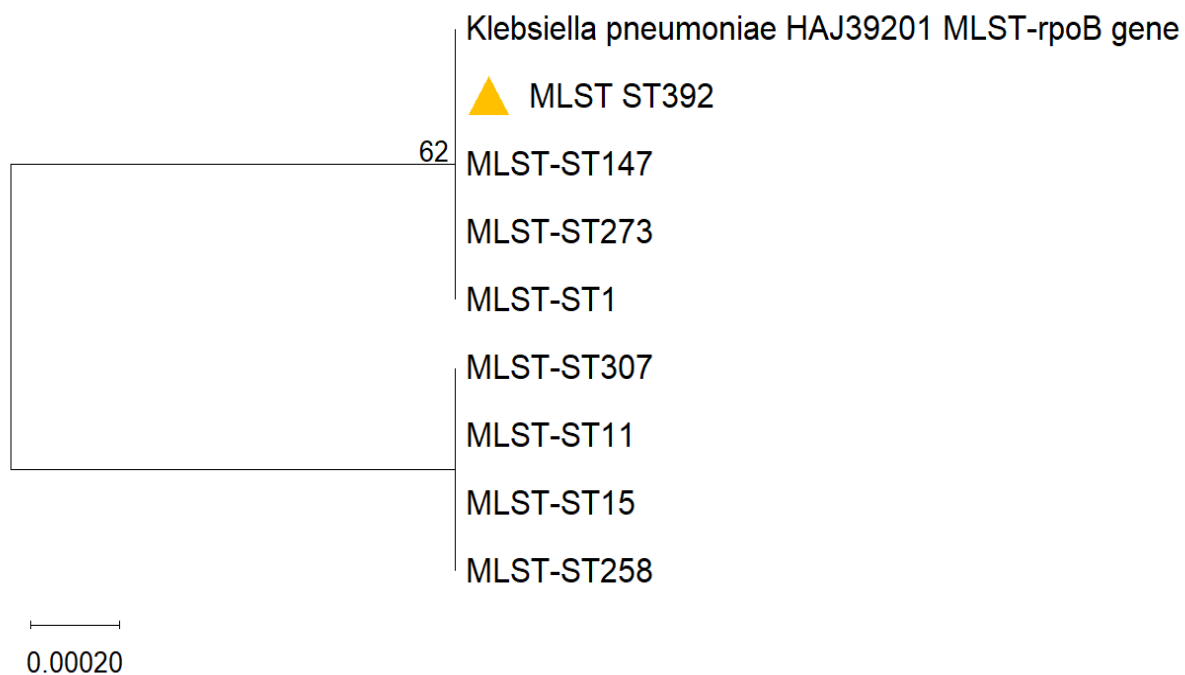


Figure (4-29): Phylogenetic tree analysis based MLST housekeeping *rpoB* gene partial sequence in local *Klebsiella pneumoniae* (HAJ39201) isolate that used for confirmative genetic identification of MLST typing analysis. The phylogenetic tree was constructed using Neighbor-Joining tree method in (MEGA 12.0 version). The local *K. pneumoniae* (HAJ39201) Isolates were showed closed related to [BIGSdb-Pasteur](#) database *Klebsiella pneumoniae* MLST-ST392 at total genetic changes (0.00020%).

DNA Sequences	Translated Protein Sequences
Species/Abbrev	
1. <i>Klebsiella pneumoniae</i> HAJ39201 MLST-tonB gene	AGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCGC
2. MLST-ST392	AGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCGC
3. MLST-ST147	AGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCGC
4. MLST-ST307	AGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCGC
5. MLST-ST11	CGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCGC
6. MLST-ST15	AGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCAG
7. MLST-ST258	CGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCGC
8. MLST-ST273	CGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCGC
9. MLST-ST1	CGCAGCGGCTAAACCCACCGTTACTGCTCCAAGCGGCCCGCGGGCGATCAGCCGCGTTTACGCCGTCTATCCAG

Figure (4-30): Multiple sequence alignment analysis of MLST housekeeping *tonB* gene in local *Klebsiella pneumoniae* (HAJ39201) isolates and related [BIGSdb-Pasteur](#) database *K. pneumoniae* MLST typing. The multiple alignment analysis was constructed using (ClustalW alignment tool. Online). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in MLST housekeeping *tonB* gene between isolates.

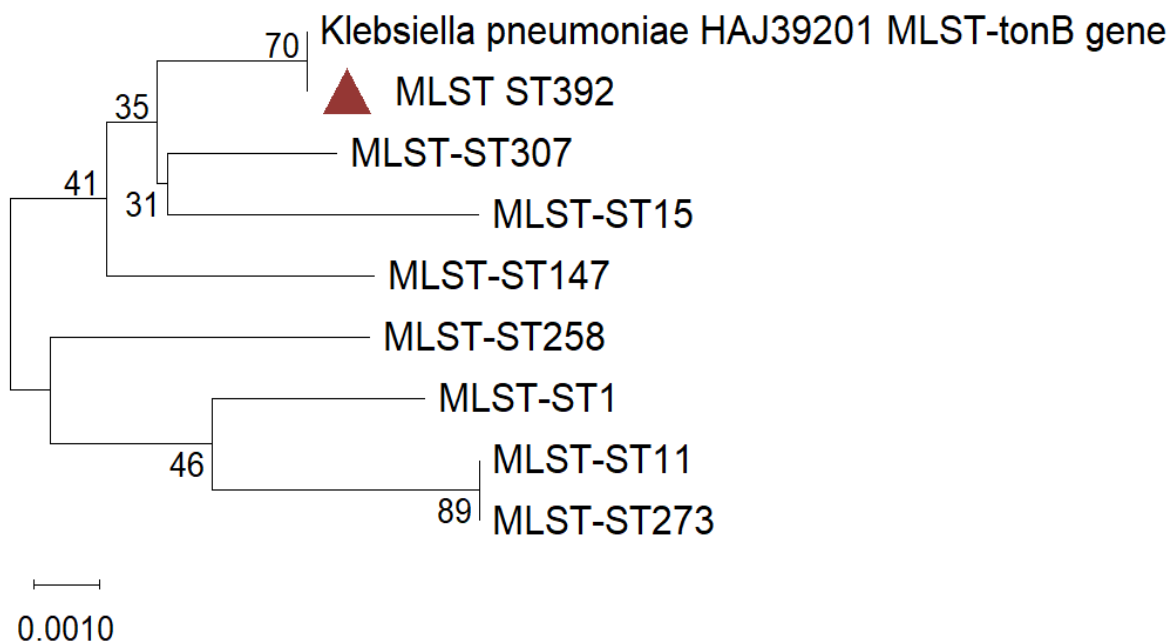


Figure (4-31): Phylogenetic tree analysis based MLST housekeeping *tonB* gene partial sequence in local *Klebsiella pneumoniae* (HAJ39201) isolate that used for confirmative genetic identification of MLST typing analysis. The phylogenetic tree was constructed using Neighbor-Joining tree method in (MEGA 12.0 version). The local *K. pneumoniae* (HAJ39201) Isolates were showed closed related to [BIGSdb-Pasteur](#) database *Klebsiella pneumoniae* MLST-ST392 at total genetic changes (0.00010%).

The advent of Multi-Locus Sequence Typing (MLST) has revolutionized microbial epidemiology by enabling high-resolution tracking of bacterial population dynamics, particularly in clinically significant pathogens such as *Klebsiella pneumoniae*. This opportunistic pathogen, notorious for its role in nosocomial infections and escalating antimicrobial resistance (AMR), necessitates robust genomic surveillance to mitigate outbreaks and inform therapeutic strategies (K. L. Wyres *et al.*, 2020). In the present study, MLST analysis of seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB* in local *K. pneumoniae* (HAJ39201) isolates revealed a striking genetic congruence with the globally disseminated sequence type ST392. This alignment was demonstrated through both multiple sequence alignments (ClustalW) and phylogenetic reconstructions (Neighbor-Joining method in MEGA 12.0), with genetic divergence as low as 0.0010%–0.00050% compared to ST392 reference strains in the BIGSdb-Pasteur database. These findings underscore the clonal expansion of ST392 associated lineages, a phenomenon increasingly linked to carbapenem resistance and hospital outbreaks across diverse geographical regions (Garcia-Gonzalez *et al.*, 2023; Liu *et al.*, 2022).

The alignment analyses, generated using ClustalW (Thompson *et al.*, 1994), consistently highlighted conserved nucleotide regions (denoted by asterisks) and sporadic substitution mutations across all seven loci. Such minimal genetic divergence suggests evolutionary stasis in these housekeeping genes, likely due to their critical roles in core metabolic functions (Lan & Reeves, 2001). For instance, *gapA* (glyceraldehyde-3-phosphate dehydrogenase) and *mdh* (malate dehydrogenase) are integral to glycolysis and the citric acid cycle, respectively, and their conservation aligns with studies emphasizing purifying selection in essential metabolic pathways (Palmieri *et al.*, 2019). Conversely, marginally higher variation

in *phoE* (0.00050%), a gene encoding an outer membrane pore protein, may reflect localized adaptive pressures, such as immune evasion or environmental stress responses. The *tonB* gene, which exhibited the lowest divergence (0.0010%), is pivotal for iron acquisition—a virulence determinant in *K. pneumoniae* (Hsieh *et al.*, 2008). Its conservation underscores the evolutionary constraint imposed by its role in pathogenicity, as iron limitation in host environments necessitates efficient uptake systems (Miethke & Marahiel, 2007).

Phylogenetic reconstructions using the Neighbor-Joining method (Saitou & Nei, 1987) clustered the HAJ39201 isolates within the ST392 clade with high bootstrap support. While this method is computationally efficient, recent critiques highlight its limitations in resolving complex evolutionary relationships, particularly in recombinogenic species (Didelot, 2021). Maximum likelihood or Bayesian approaches, which account for site-specific substitution rates, may offer finer resolution (Minh *et al.*, 2020). Nevertheless, the concordance between alignment and tree-based analyses here validates MLST's utility for preliminary strain characterization, as advocated by (Maiden *et al.*, 2013).

The predominance of ST392 in the HAJ39201 isolates raises critical epidemiological questions. ST392 has been implicated in carbapenem-resistant *K. pneumoniae* (CRKP) outbreaks in intensive care units across Asia and Europe, often harboring *blaKPC* and *blaNDM* resistance genes (Guo *et al.*, 2022; Lee *et al.*, 2021; Yang *et al.*, 2013). The genetic homogeneity observed in this study suggests either recent clonal introduction into the local population or persistent selection pressures favoring ST392, such as antibiotic overuse or horizontal gene transfer (HGT) of resistance determinants (Partridge *et al.*, 2018). Notably, ST392's association with mobile genetic elements (MGEs), including plasmids and transposons, facilitates the dissemination of AMR genes (Jiang *et al.*, 2025).

Future whole-genome sequencing (WGS) of HAJ39201 isolates could elucidate the presence of such MGEs and their role in resistance gene acquisition (Taylor *et al.*, 2021).

While MLST remains a gold standard for bacterial typing, its reliance on partial sequences (~500 bp) of housekeeping genes limits its resolution compared to WGS (Hilt & Ferrieri, 2022). For example, WGS can detect single-nucleotide polymorphisms (SNPs) in regulatory regions or accessory genes linked to virulence (e.g., *rmpA*, aerobactin) (Russo *et al.*, 2021). A study by (García-Cobos *et al.*, 2025) revealed that hypervirulent *K. pneumoniae* (hvKP) strains, though phylogenetically related to classical strains, possess distinct virulence plasmids undetectable by MLST. Thus, integrating WGS with MLST data could enhance our understanding of HAJ39201 pathogenic potential.

The public health implications of clonal ST392 expansion are profound. CRKP strains are classified as "urgent threats" by the CDC due to limited treatment options and high mortality rates (CDC, 2019). The genetic stability observed in HAJ39201 isolates suggests that existing infection control measures, such as contact precautions and antimicrobial stewardship, may be insufficient to curb transmission. Enhanced surveillance leveraging real-time genomic databases like BIGSdb-Pasteur and Pathogen watch (Argimón *et al.*, 2016) is critical for tracking emerging lineages. For instance, a 2023 report from the European Centre for Disease Prevention and Control (ECDC) identified ST392 as a high-risk clone in neonatal wards, emphasizing the need for targeted screening in high-risk populations (ASSESSMENT, 2025).

Regionally, the persistence of ST392 may reflect gaps in infection control protocols or environmental reservoirs, such as contaminated water systems or medical devices (Gorrie *et al.*, 2022).

Future research should prioritize multi-omics approaches to unravel the interplay between genomic stability and phenotypic plasticity in ST392. Proteomic and transcriptomic analyses could identify stress response pathways underpinning antibiotic tolerance. Additionally, *in vivo* models assessing virulence and immune evasion mechanisms in ST392 isolates would provide mechanistic insights (Cabrera *et al.*, 2025). Collaborative efforts between clinicians, microbiologists, and public health agencies are essential to design interventions tailored to ST392's transmission dynamics (Yang *et al.*, 2023).

Conclusions

According to the results of the current study, the study concluded the following

- 1- The incidence of multi drug resistant *Klebsiella pneumoniae* among hospital acquired infections is on the rise, in addition to the presence of (XDR) isolates.
- 2- The *mrkD* gene, which is implicated in bacterial adhesion and biofilm formation, was detected in all MDR and XDR isolates. Which clarifies its critical role in the persistence and pathogenicity of resistant *K. pneumoniae* strains, indicating its potential as a conserved virulence marker.
- 3- The *rmpA* gene, has found in hypermucoviscous isolates only. Therefore, it can be considering a key virulence factor associated with this phenotype, indicating a limited distribution but potential clinical significance due to its association with increased virulence.
- 4- The *blaKPC* gene, which encodes a carbapenemase enzyme conferring resistance to carbapenem antibiotics, was identified in two MDR and one XDR isolate, confirming the presence of carbapenem-resistant strains within the studied population.
- 5- The *blaVIM* gene, another important carbapenemase-encoding gene, was not detected in any of the isolates, suggesting its absence among the clinical samples examined in this study.
- 6- Multi locus sequence typing for hypermucoviscous strain revealed that the strain belongs to clonal group (CG) 147, sequence type ST (392) which is high-risk clone with enhanced pathogenic potential.
- 7- This the first recorded of ST392 in Iraq. Integrating MLST-based phylogenetics with metadata (e.g., geographical origin, source, year, and genetic markers) provides valuable insights into the epidemiology of *Klebsiella pneumoniae* and supports proactive infection control measures.

Recommendations

- 1- Conduct whole genome sequencing (WGS) of multidrug resistant and hypermucoviscous *klebsiella pneumoniae* isolates to provide deeper insights into resistance mechanisms, virulence factors and mobile genetic elements contributing to gene dissemination.
- 2- Perform longitudinal surveillance studies across multiple hospitals and regions to monitor the spread and evolution of high risk clones such as ST392 and CG147 especially in ICU and burn units.
- 3- Investigate the expression levels and regulatory mechanisms of the *mrkD*, *rmpA*, *blaKPC*, and other virulence/resistance genes under different environmental and antibiotic stress conditions using transcriptomic approaches (e-g-RT-qPCR, RNA-seq).
- 4- Investigate alternative therapies, such as phage therapy or anti-biofilm agents, targeting *mrkD*-mediated adhesion and biofilm formation to combat persistent infections.
- 5- Implement a national MLST database for *K. pneumoniae* in Iraq to facilitate molecular tracking of emerging clones and to integrate with international databases for global epidemiological comparison.

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Appendix

bioMérieux Customer:		Microbiology Chart Report		Printed December 8, 2024 8:55:54 AM CST													
Patient Name:				Patient ID:													
Location:				Physician:													
Lab ID: 4/12/2024/9				Isolate Number: 1													
Organism Quantity:																	
Selected Organism : <i>Klebsiella pneumoniae</i> ssp <i>pneumoniae</i>																	
Source:			Collected:														
Comments:																	
Identification Information		Analysis Time: 4.02 hours		Status: Final													
Selected Organism		99% Probability		<i>Klebsiella pneumoniae</i> ssp <i>pneumoniae</i>													
		Bionumber:		6607734753565010													
ID Analysis Messages																	
Biochemical Details																	
2	APPA	-	3	ADO	+	4	PyrA	+	5	IARL	-	7	dCEL	+	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	+
17	BGLU	+	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	+	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	+	29	TyrA	+	31	URE	+	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	+	37	MNT	+	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	+
46	GlyA	+	47	ODC	-	48	LDC	+	53	IHISa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Appendix 1: Biochemical reaction profile of *Klebsiella pneumoniae* by identified Vitek2 system.

bioMérieux Customer:

Microbiology Chart Report

Printed October 23, 2024 11:31:39 AM CDT

Patient Name: Nada , Ali

Patient ID: 22102024 5/21

Location:

Physician:

Lab ID: 22102024 5/21

Isolate Number: 1

Organism Quantity:

Selected Organism : *Klebsiella pneumoniae*

Source: urine

Collected:

Comments:	

Susceptibility Information			Analysis Time: 12.17 hours			Status: Final		
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation			
ESBL	POS	+	Ertapenem	<= 0.5	S			
Ampicillin	>= 32	R	Imipenem	2	I			
Piperacillin/Tazobactam	<= 4	S	Amikacin	32	I			
Cefazolin	>= 64	R	Gentamicin	8	I			
Cefoxitin	<= 4	S	Ciprofloxacin	2	I			
Ceftazidime	>= 64	R	Levofloxacin	4	I			
Ceftriaxone	8	R	Nitrofurantoin	128	R			
Cefepime	2	*R	Trimethoprim/ Sulfamethoxazole	>= 320	R			

*= AES modified **= User modified

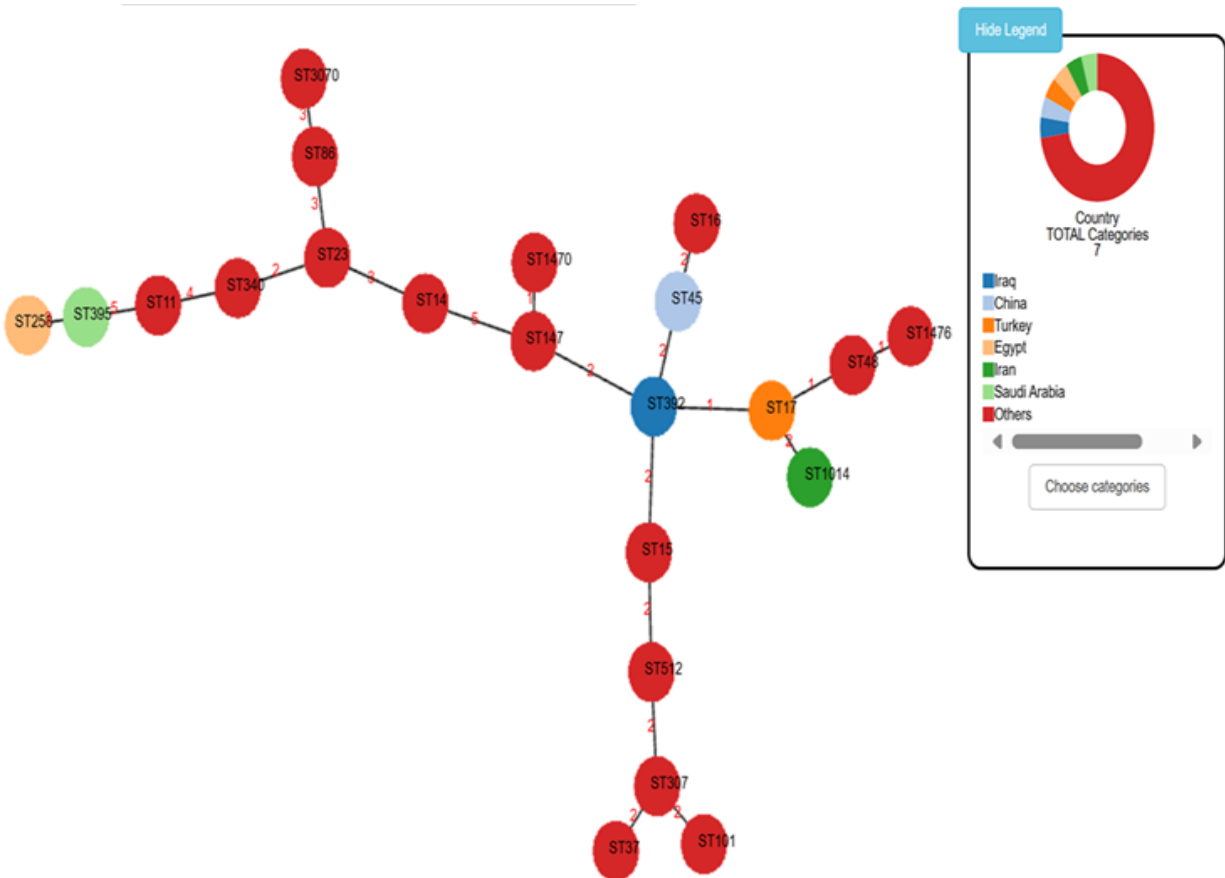
AES Findings	
Confidence:	Consistent

Appendix 2: Antimicrobial Susceptibility Test (AST) Results of *Klebsiella pneumoniae* Isolate by using Vitek2 system.

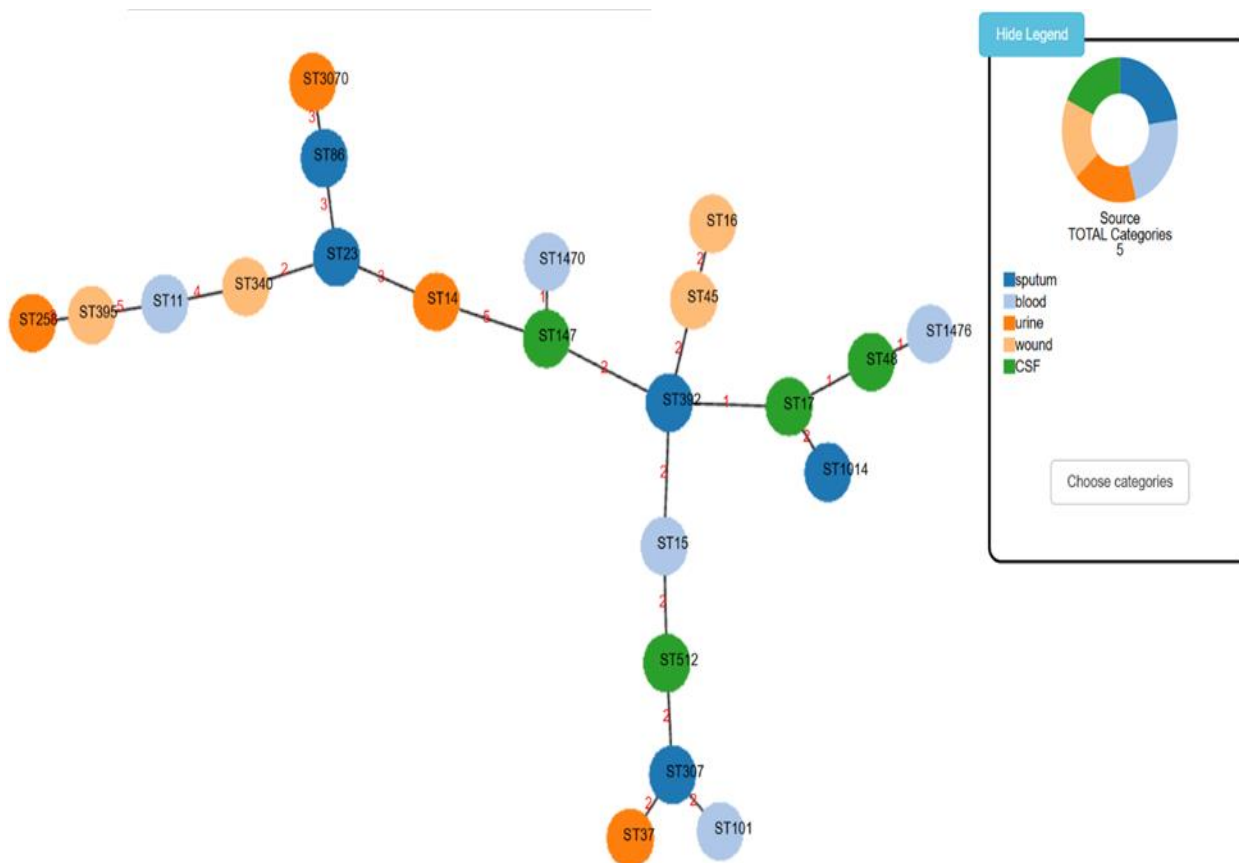
Appendix 3 Minimum Spanning Tree (MST) colored by Country.

The phylogenetic relationships of ST392 and related STs based on geographical origin.

(for more information sci.bio.mas.23.3@qu.edu.iq)



Appendix 4 Minimum Spanning Tree (MST) colored by Source of Isolation
 Visualization of ST392 and associated STs categorized by clinical sample type.
 (for more information sci.bio.mas.23.3@qu.edu.iq)



الخلاصة

تُعدّ الكلبسييلة الرئوية المقاومة للأدوية المتعددة مشكلة صحية عامة عالمية، حيث يتجاوز معدل الوفيات بها 60%. على مدى العقود الماضية، ظهرت عدة سلالات مفرطة اللزوجة من الكلبسييلة الرئوية، تتميز بسمة مميزة عن السلالات التقليدية. وقد ازدادت حالات العدوى الناجمة عن السلالات مفرطة اللزوجة، مع معدلات وفيات واعتلال مرتفعة.

هدفت هذه الدراسة إلى دراسة جينات الضراوة الأكثر شيوعاً المرتبطة بخاصية مقاومة مضادات الميكروبات المتعددة في الكلبسييلة الرئوية، كما هدفت الدراسة إلى معرفة مدى انتشار السلالات مفرطة اللزوجة بين عدوى المستشفيات التي تسببها الكلبسيلا الرئوية. ولهذا الغرض، جُمعت 316 عينة سريرية من مستشفى الديوانية التعليمي خلال الفترة من 20 أكتوبر 2024 إلى 20 يناير 2025. وزعت العينات على النحو التالي: 113 عينة من البول، و98 عينة من البلغم، و70 عينة من الجروح، و35 عينة من الدم. جُمعت جميع العينات في أنابيب مُجهزة بوسائط نقل، ونُقلت مباشرةً إلى المختبر. في المختبر، وُضعت العينات على كَلٍّ من أكار الدم وأكار ماکونكي، وحُضنت عند درجة حرارة 37 مئوية لمدة 24 ساعة. شُخِّصت البكتيريا النامية باستخدام صبغة غرام والعديد من الاختبارات الكيميائية الحيوية، ثم شُخِّصت العصيات سالبة الجرام (سلبية الإندول)، وموجبة لاختبار فوجيس-بروسكاور، واختبار سترات سيمون، وإنتاج الكاتالاز، والعصيات سالبة الجرام (إيجابية اليوريز) على أنها كليبسيلا رئوية. لمزيد من التشخيص، استُخدم بطاقة GN/ID في نظام فيتيك (BioMérieux/France). أظهرت النتائج أن كليبسيلا رئوية عُزلت من 71 (30%) من أصل 316 عينة سريرية، معظمها من عينات البلغم بنسبة 33.7%، مما يُشير إلى أن البكتيريا مسؤولة عن معظم حالات الالتهاب الرئوي. استُخدم نظام Vitek2 compact لتحديد حساسية بكتيريا *K. pneumoniae* المعزولة لمختلف العوامل المضادة للميكروبات، وفقاً للجنة الوطنية لمعايير المختبرات السريرية (CLSI) (2024). وأظهرت النتائج وجود تكرار معتدل للعزلات المقاومة للأدوية المتعددة (MDR) بلغ 20 عزلة (28.2%)، وتواتر منخفض نسبياً للعزلات المقاومة للأدوية على نطاق واسع (XDR) بلغ 11 عزلة (15.5%). والجدير بالذكر أنه لم يتم رصد أي عزلات مقاومة للأدوية المتعددة (PDR).

ولتقييم نسبة حدوث سلالات فرط اللزوجة المخاطية (hmvKp) استُخدم اختبار الخيط. أظهرت النتائج أن 3 من أصل 71 (4.2%) عزلة كانت إيجابية لاختبار الخيط. جميعها من عينات البلغم، مما يشير إلى زيادة انتشار السلالات شديدة الضراوة بين مرضى الالتهاب الرئوي.

تضمنت الدراسة أيضاً دراسة العديد من جينات الضراوة المرتبطة بمقاومة متعددة للعوامل المضادة للميكروبات. هذه الجينات هي (*blaVIM* ، *blaKPC* ، *rmrA* ، *mrkD*) أظهرت النتائج أن جين *rmrA* عُزل من سلالات فرط اللزوجة المخاطية فقط.

أُجريت تقنية التنميط التسلسلي متعدد المواضع (MLST) لسلالة hmvKp تم استخدام تفاعل البوليميراز المتسلسل (PCR) لسبعة جينات منزلية (*tonB* ، *rpoB* ، *phoE* ، *pgi* ، *mdh* ، *infB* ، *gapA*) أظهرت النتائج أن سلالة *K. pneumoniae* شديدة اللزوجة تنتمي إلى المجموعة المستنسخة CG147 ونوع التسلسل ST293 وهذا يتبر أول تسجيل عن هذا النوع في العراق. سُجلت السلالة في معهد باستر بالرقم التسلسلي (ID:76145).

إن الآثار الصحية العامة لانتشار النمط ST392 كبيرة للغاية، تُصنف سلالات CRKP على أنها "تهديدات عاجلة" من قبل مراكز السيطرة على الأمراض والوقاية منها (CDC) بسبب محدودية خيارات العلاج وارتفاع معدلات الوفيات. ان الاستقرار الجيني الملحوظ في عزلات (ID76145) يشير إلى تدابير السيطرة على العدوى الحالية، مثل احتياطات التلامس وإدارة مضادات الميكروبات، قد لا تكون كافية للحد من انتقال العدوى. لذا فإن يجب تعزيز أنظمة المراقبة بالاستفادة من قواعد البيانات الجينومية الفورية مثل BIGSdb-Pasteur و Pathogen watch وبالتالي، يُعد هذا أمراً بالغ الأهمية لتتبع السلالات الناشئة. في حين أن ST392 وقد تم التعرف عليها في العديد من المراكز السريرية باعتبارها سلالة عالية الخطورة في الأقسام حديثي الولادة، مما يبرز الحاجة إلى اجراء الفحوصات المستهدف في الفئات السكانية ذات الخطورة العالية.



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التشخيص والتوصيف الجزيئي لعزلات الكليبسيلا الرئوية المقاومة للأدوية
والتنميط التسلسلي متعدد المواقع لسلالة ذات لزوجة مخاطية مفرطة

رسالة

مقدمه الى مجلس كلية العلوم جامعه القادسية

وهي جزء من متطلبات نيل درجه الماجستير في علوم الحياة- علم الأحياء

المجهرية

من قبل

حيدر علي حسين الجمالي

جامعه القادسية - كلية العلوم - بكالوريوس علوم الحياة 2010

بإشراف

أ.م.د. ضحى مهدي العابدي