Genome Annotation and Comparative Genome Analysis of *Klebsiella pneumoniae* Strain KP3 isolated from Patient

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Reg number: 00000327665

A thesis submitted in partial fulfillment of the requirements for the degree of

MS Industrial Biotechnology

Supervised by:

Dr. Amjad Ali

Industrial Biotechnology

Atta ur Rehman School of Applied Biosciences (ASAB)

National University of Sciences and Technology (NUST), Islamabad, Pakistan.

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Dedicated to my Beloved Parents

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All praises to Allah Almighty, the most Merciful and the most Gracious, Who is the source of all knowledge. This proud success is not only mine. I share it with a lot of people. I am truly grateful to them for providing all the small steppingstones of counsel and assistance, to bring this research up to this level.

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Mahnoor Masood

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List of Abbreviations

UTI Urinary Tract Infections

NCBI National Center for Biotechnology Information

LPS Lipopolysaccharide

MDR Multi Drug Resistance

VFDB Virulence Factor Database

CGE Center For Genomic Epidemiology

OMP Outer Membrane Protein

KP *Klebsiella pneumoniae*

GAR Global Antimicrobial Resistance

RND Resistance-Nodulation-Cell Division

ABC ATP-binding cassette

SMR Small Multidrug Resistance

MFS Major Facilitator Superfamily

SNPs Single Nucleotide Polymorphisms

MLST Multi Locus Sequence Typing

ROS Reactive Oxygen Species

CARD Comprehensive Antibiotic Resistance Database

AMR Antimicrobial Resistance

ESBL Extended Spectrum Beta Lactamase

BLAST Basic Logic Alignment Search Tool

PN Pyelonephritis

cKP Classical *K. pneumoniae*

hvKP Hypervirulent *K. pneumoniae*

Abstract

Klebsiella pneumoniae is a gram-negative, nosocomial bacteria reported to be the second most common causative agent of Urinary tract infections. UTIs are one of the most common extra-intestinal infections occurring across the world. About 300 million infections are reported worldwide annually. Virulence factors of *K. pneumoniae* contribute in causing UTIs. Over the period abuse of antibiotics has developed multi-drug resistant leading to the rise in number of different infections including UTIs caused by *K. pneumoniae* in Pakistan. In depth genomic studies need to be conducted to learn more about such strains. In this study, a local isolate of *K. pneumoniae* KP3 was characterized, gone through pangenome and comparative genome analysis with global strains to get the insights of the genetic diversity, resistance, and virulence profile among *K. pneumoniae* strains across the world. The local isolate KP3 was identified to be multi-drug resistant virulent strain with a genome size of 4.9Mb having 5067 coding sequences. The strain carried many resistance genes as *TEM-1, tolC, CRP* etc. responsible for causing resistance against tetracycline, rifamycin, phenicol, fluoroquinolone etc., more than hundred virulence factors including *rmpA, rcsA hlyE,, pgaC* etc and five plasmids two of which harbor resistance genes for Carbapenems and β-Lactams. MLST analysis showed KP3 belongs to ST5. Pangenome analysis of selected 47 strains identified 14,520 genes with 343 core genes, 2061 accessory genes and 11,364 unique genes indicating an open pangenome system and a close relation to a strain KpC5 from USA. Comparative analysis exhibited KP3 had a unique resistance and virulence profile which is somewhat related to other strains in causing UTIs. Furthermore, phylogenetic analysis revealed that KP3 is evolutionarily related to the reference genome HS11286 of *K. pneumoniae* and a UTI causing strain ATCCBAA_2146 from USA. This genetic information will be helpful in future for effective control of this MDR pathogen and develop targeted therapeutics**.**

Chapter 1

Introduction

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen, one of very common nosocomial agents responsible for causing different diseases across the world (Clegg and Murphy, 2016). It has highly diverse phylogenetic and genomic distribution, also classified as one of the most commonly human disease-causing agent. Urinary tract infections are considered to be one of the most common infections in humans and *Klebsiella pneumoniae* is known to be the second most common etiological agent for both nosocomial and community acquired urinary tract infections (Florea et al., 2023).

Different number of factors are involved in establishing UTIs including invasion and colonization of host defense mechanism, anatomical factors and the virulence factors of causative agents (Nicolle, 2002). Much like UPEC, *Klebsiella pneumoniae* also has the capability to deviate from the commensal role in the intestine flora. It has the ability to establish persistence in the urinary tract and act as a storage for virulence factors to initiate the disease (Aslam et al., 2022). Host inflammatory response is triggered by breaching of *K. pneumoniae* in the sterile environment. As a result, host secretes pro-inflammatory cytokines, an influx of neutrophils and the epithelial cells exfoliation.

Antibiotics have been used as a treatment for years. Antibiotics are becoming ineffective because of their constant abuse which has led to multi-drug resistance (MDR). Subsequently it is leading to a very prominent increase in MDR organisms including *Klebsiella pneumoniae* and many more. *Klebsiella pneumoniae* has been known to be one of the major MDR organisms (Kwiatkowski et al., 2022). It is a major health threat because of its recurrence in underdeveloped countries including Pakistan. It has been reported to increase morbidity, mortality, and health care expenses.

Klebsiella pneumoniae is being studied globally for the strains involved in causing urinary tract infections. These studies will prove to be helpful in coming years to leave traditional medication behind and design pathogen specific drugs (Kline et al., 2011). Not enough studies are available on local *K. pneumoniae* strains. Therefore, it is crucial to design a surveillance program which will monitor the prevalence and antibiotic resistance as well as optimize the management of urinary tract infections in Pakistan.

1.1 Our Contribution

The current study was designed with the primary aim of evaluating the antibiotic resistance profile of local UTI causing *K. pneumoniae* strain. Moreover, it also covers the elucidation of factors participating in disease severity particularly the virulence factors.

In the current study a draft genome of local UTI causing *K. pneumoniae* was characterized and gone through comparative genome analysis. By performing comparative genome analysis, we will be able to identify the phylogenetic relation between the local and globally available UTI causing *K. pneumoniae* strains also observe the genomic diversity among these strains. In our study we mainly focused on the pathogenicity of *Klebsiella pneumoniae*, ongoing global resistance and susceptibility trends, current treatment strategies, prophylactic approaches and alternative strategies in preventing UTIs. The findings of this study will definitely help in rapid diagnosing and treating *Klebsiella pneumoniae* and combating against antimicrobial resistance in Pakistan.

1.2 Research Objectives

1 Isolation and Characterization of the local UTI causing *Klebsiella pneumoniae* KP3 isolate.

2 Pangenome analysis among globally available UTI causing *Klebsiella pneumoniae* strains.

3 Comparative genome analysis of *Klebsiella pneumoniae* strains for investigating genetic diversity, virulence and antibiotic resistance.

CHAPTER 2

Literature Review

2.1 Urinary Tract Infections

Urinary tract infections (UTI) is an infection that arises anywhere within the urinary system including kidneys, ureters, urethra and urinary bladder (Tan and Chlebicki, 2016) . The presence of bacteria in urine up to $>10^5$/ml indicates urinary tract infection (Smelov et al., 2016) . Urinary tract infections are one of the most common extra-intestinal infections that occurs when the disease causing strains spread from the host intestine to the urinary tract where the strains gain new virulence properties that are responsible for colonization at mucosal surfaces of host organism and invade aseptic urinary tract (Abo Zaid et al., 2019; Lara-Isla et al., 2017).

Urinary tract infections are of different types and are characterized as nosocomial and community acquired infections(Behzadi et al., 2019). There is a wide range of pathogens that can cause UTI by colonizing in the urinary tract including Gram-positive and Gram-Negative bacteria and few certain fungi (Smelov et al., 2016). Primary etiological agent for causing UTIs is Uropathogenic *Escherichia coli* (UPEC) but there are many other pathogens as well that can be associated with UTIs including *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Salmonella* spp., *Staphylococcus saprophyticus, Streptococcus* spp., *Enterococcus faecium* and many more including some fungal agents as well (Conover et al., 2015; Kline and Lewis, 2016). If urinary tract infections are left untreated and undiagnosed they can be responsible for septic shock, bacteremia infections and triggering sepsis in the host (Kline and Lewis, 2016).

2.2 Epidemiology of UTIs

Approximately 300 million urinary tract infections occur worldwide annually (Gaston et al., 2021). Urinary tract infections are the most common infections in United

States. These infections can be of different severity level from mild to severe sepsis, with 20 to 40% mortality rate (Jia et al., 2021). UTIs are a huge economic burden along with being a huge global burden. UTIs are responsible for causing significant morbidity in infant boys, older men and specially females of all ages. Frequency is affected by sex and age. Furthermore UTIs are more frequent in females as compared to males (Ejrnæs et al., 2011).

Epidemiological studies have indicated that annual incidence of UTIs in women is about 12% and 50% of all women will definitely experience at least one UTI by the age of 32 years (Behzadi et al., 2019). Generally it is estimated 50 to 70% of women will experience UTIs somewhere during their lifetime, 30 to 48 % women will have a recurrent UTI despite getting a proper antibiotic treatment (Kot, 2019). *Escherichia coli* are responsible for causing 75% of uncomplicated and 65% of complicated UTIs worldwide. According to a recent study done in Timergara District Hospital, Timergara, Pakistan *E. coli* is the major cause of UTIs, other reported pathogens were *Klebsiella* spp., *Enterobacter* spp., *Proteus mirabilis* (Bullens et al., 2022).

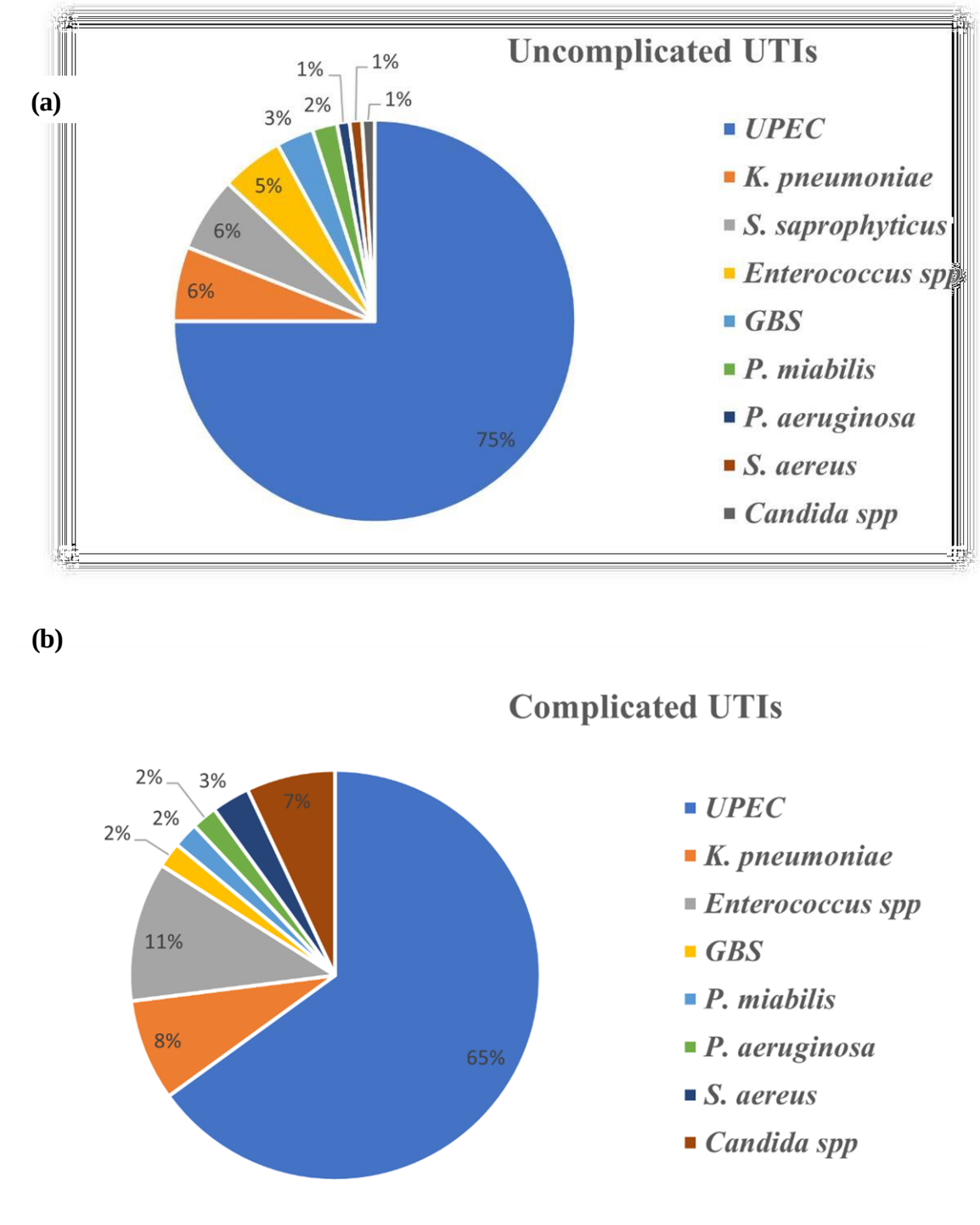

Figure 1 A global overview of the prevalence of urinary tract infections (UTIs) in relation to the microorganisms responsible for these infections, with a specific focus on two categories: (a) Uncomplicated UTIs and (b) Complicated UTIs. The data sources for this figure are derived from a comprehensive study by (Medina and Castillo-Pino, 2019). This figure provides a comprehensive depiction of the relative prevalence of UTI-causing agents in both uncomplicated and complicated cases, offering valuable insights into the global landscape of UTIs.

2.3 Microbial Spectrum Involved in UTIs

Urinary tract infections are ranked second amongst the most dominant infectious diseases across the world. There is a vast range of etiological microbial agents but *Escherichia coli* is the leading cause in both uncomplicated and complicated UTIs. 70 to 90% of community-acquired and 40 to 50% of nosocomial UTIs are mainly caused by Uropathogenic *Escherichia coli* in all age groups (Togawa et al., 2015) . Other uropathogens responsible for developing uncomplicated UTIs are *Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcus* spp., Group B *Streptococcus* (GBS), *Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida* spp., in order of prevalence **Figure 1**. (Conover et al., 2015; Forsyth et al., 2018; Kline et al., 2011).

Enterococcus spp., are the most prevalent uropathogens responsible for causing complicated Urinary tract infections followed by *K. pneumoniae, Candida* spp., *P. mirabilis, S. aeruginosa,* GBS and *P. aeruginosa* (Chen et al., 2013; White et al., 2017). Worldwide phylogenetic and pangenomic analysis revealed *E. coli* strains belong to polygroups represented as A, B1,B2,C, D,E, F and *Escherichia* cryptic clade I (Luo et al., 2012; Müştak et al., 2015). E and F are considered as new groups where F is designated as a sister group of B2 (Iranpour et al., 2015). As far as for commensal strains are concerned they are associated with group A or B1 (Bharadwaj et al., 2021; Mukherjee, 2013).

2.4 Classification of UTIs

Classification and characterization of urinary tract infections is based on different clinical manifestations, severity level, microbiological classification of pathogens,

complicating factors, infection's location in host's urinary tract and the presence or absence of symptoms (Medina and Castillo-Pino, 2019).

2.4.1 Clinical Manifestations

Clinical expressions of UTIs are classified as cystitis, urosepsis, pyelonephritis, urethritis and male adnexitis (male accessory gland infections; e.g. prostatitis) (Hälleberg Nyman et al., 2011; Smelov et al., 2016). Cystitis is defined as infection of the urinary tract infection. Bacterial infection responsible for causing swelling of kidneys is pyelonephritis. It is an acclivous spreading of infection from bladder to kidneys (Sabih and Leslie, 2023). Urosepsis is indicative bacteremia of UTI origin (Dreger et al., 2015). Inflammation of urethra is urethritis while acute and chronic infections of male urogenital tract are called as male adnexitis (Baijwan and Dhyani, 2023).

2.4.2 Severity Levels of UTIs

Severity levels of clinical manifestations of urinary tract infections are mild, moderate, severe and sepsis **Table 1**. Urosepsis is severe as compared to cystitis pyelonephritis where pyelonephritis is more severe in comparison to cystitis. Severity grading for urosepsis includes sepsis, severe sepsis and septic shock. Whereas pyelonephritis presents mild and moderate infections that are treatable with antimicrobials where severe pyelonephritis needs proper therapy and hospitalization (Fang et al., 2007; Hälleberg Nyman et al., 2011; Smelov et al., 2016).

2.4.3 Classification based on Risk Factors

Risk factors for Urinary tract infections are characterized as complicated and uncomplicated infections. In case of uncomplicated UTIs mostly healthy people are

affected that have no urinary tract abnormalities (Kolman, 2019; Pietrucha-Dilanchian and Hooton, 2016) . Complicated UTIs are mainly caused because of factors effecting host's defense or urinary tract including immunosuppression, urinary obstruction, renal transplantation, renal failure, neurological disease leading to urine retention, pregnancy and indwelling catheters (Khan et al., 2020; Kolman, 2019). There are chances of having recurring UTIs in patients and require proper therapy (Mody et al., 2017).

Pyelonephritis of the upper UTI and cystitis of the lower UTI may be present as complicated or uncomplicated infections depending upon the risk factors (Kostakioti et al., 2012). Different risk factors are associated with different types of UTIs. Risk factors for cystitis are any history of urinary tract infection, female gender, genetic susceptibility, sexual activity, obesity, diabetes and vaginal infection (Knottnerus et al., 2013; O'Brien et al., 2015) ORENUC system is used for phenotyping the risk factors of UTIs that comprises of six groups **Table 2** (Smelov et al., 2016).

Table 1 Clinical manifestations of different types of UTIs and severity grading.

Acronym	Clinical diagnosis	Clinical symptoms	Grade of severity
CY-1	Cystitis	Dysuria, frequency, urgency, suprapubic pain; sometimes unspecific symptoms	1
PN-2	Mild and moderate pyelonephritis	Fever, flank pain, CVA tenderness; sometimes unspecific symptoms of CY	2
PN-3	Severe pyelonephritis	As PN-2, nausea and vomiting with or without symptoms of CY	3
US-4	Urosepsis (simple)*	Temperature > 38°C or 36°C; heart rate > 90 beats/min; respiratory rate > 20 breaths/min or $PaCO_2$ < 32mm Hg; WBC > 12,000 cells/mm^3 or < 4000 cells/mm^3 or ≥ 10% immature(band) forms; with or without symptoms of CY or PN.	4
US-5	Severe urosepsis*	As US-4, in addition with organ dysfunction, hypoperfusion or hypotension. Hypoperfusion and perfusion abnormalities but not limited to lactic acidosis, oliguria or acute alteration of mental stress.	5
US-6	Urosepsis shock*	As US-4 or US-5, in addition with hypotension despite acute fluid resuscitation along with the presence of perfusion abnormalities but not limited to lactic acidosis oliguria or acute alteration in mental stress.	6
CY = cystitis; PN = pyelonephritis; US = urosepsis; WBC = white blood cells			

Table 2 Host risk factors in UTIs categorized according to the ORENUC system (Johansen et al., 2011)

Phenotype	Category of risk factor	Examples of risk factor
O	No known risk factor	– Otherwise healthy premenopausal women.
R	Risk factors for recurrent urinary tract infection but no risk of more severe outcome.	– Sexual behavior (frequency, spermicide) – Hormonal deficiency in post menopause – Secretor type of certain blood groups – Well-controlled diabetes mellitus
E	Extra urogenital risk factors with risk of more severe outcome.	– Prematurity, new born – Pregnancy – Male gender – Badly controlled diabetes mellitus – Relevant immunosuppression (not well defined)
N	Nephropathic diseases with risk of more severe outcome.	– Relevant renal insufficiency (not well defined) – Polycystic nephrology – Interstitial nephritis (e.g. due to analgesics)
U	Urologic risk factors with risk of more severe outcome, which can be resolved during therapy.	– Ureteral obstruction due to a ureteral stone – Well-controlled neurogenic bladder disturbances – Transient short-term external urinary catheter – Asymptomatic bacteremia
C	Permanent urinary catheter and unresolvable urologic risk factors with risk of more severe outcome.	– Long-term external urinary catheter – Unresolvable urinary obstruction – Badly controlled neurogenic bladder disturbances

2.4.4 Microbiological Characteristics of Pathogens

Essential UTI causing pathogens for 70-95% of complicated UTIs and 5-10% of uncomplicated UTIs are *E. coli* and *S. saprophyticus*. Other agents causing UTIs include *Klebsiella* spp., and *Proteus mirabilis* isolated occasionally. Microbiological spectrum for complicated UTIs includes E.coli and different gram-positive and gram-negative *including P. aeruginosa*, *Enterobacter* spp., *Citrobacter* spp., *Staphylococcus* spp., and *Enterococcus* spp., (Wagenlehner et al., 2013).

2.4.5 Classification based on Infection's Source

UTIs are one of the most common pathological conditions throughout the world in both health and community sector (Lee et al., 2018). Nosocomial UTIs goes up to 40% amongst all hospital acquired infections. Risk of getting an infection is much increased for patients with catheters or those having urological treatments and elderly male patients staying for long in hospitals (Tan and Chlebicki, 2016). Originating point of causative agents are moist sites in hospital environment (Gupta et al., 2017).

2.4.6 Classification based on Presence and Absence of Symptoms

Lower UTIs, cystitis, upper UTIs and pyelonephritis are symptomatic with clear symptoms involving kidneys and bladder. Whereas in comparison asymptomatic infections show growth of 10^5 bacteria or more in a single sample of urine in men or two consecutive female urine samples is classified asymptomatic bacteremia (Cai et al., 2016a; Nicolle, 2005).

2.5 Pathogenesis

The pathogenesis of mucosal infections involve a few steps including attachment of bacteria to the epithelium, colonization of urinary tract leading to tissue damage and in some cases there is clear dissemination and invasion (Vasudeva and Madersbacher, 2014). Host factors and virulence properties play an important role in depicting different stages of bacteria (Mak and Kuo, 2006). Attachment and internalization of bacteria are very important steps in pathogenesis. Adhering capacity for bacteria is dependent on site of infection in urinary tract also on the presence and absence of fundamental structural and functional peculiarities (Spaulding and Hultgren, 2016).

Fimbriae adhesions of bacteria helps in the attachment to urothelium and colonization in the urinary tract (Jhang and Kuo, 2017). Contamination of periurethral regions with pathogens residing in gut leads to uncomplicated UTIs, followed by colonization in urethra and spread to the bladder (McLellan and Hunstad, 2016; Qiao et al., 2013; Robino et al., 2014).

Formation of IBC (Intracellular bacterial communities), biofilm like masses are formed by bacterial access to epithelial cell cytoplasm of bladder (Darouiche et al., 2011; Nielsen et al., 2017). Extracellular bacteria can be cleared by inflammatory responses whereas bacteria that evade the immune system go through multiplication and form biofilms (Schwartz et al., 2011). These bacteria cause tissue damage by producing toxins and proteases that helps in survival of bacteria by releasing essential nutrients.

After colonization of bladder, bacteria may cause upper UTI or pyelonephritis by ascending towards the ureters and colonizing the kidneys (Spaulding and Hultgren, 2016). For complicated UTIs a pathogen follows the same steps except the host bladder is compromised for instance in catheterization. Catheterization induces an immune response that causes accumulation of fibrinogen on catheter. This leads to UTIs when bacteria adhere to catheter following the formation of biofilm (Conover et al., 2015)

2.6 Routes of Infection

There are 3 major routes of infection which lead to UTIs including hematogenous, lymphatic and ascending routes. The most common route for establishing UTI is ascending route (Copp et al., 2013). Microbial pathogens originating from rectal flora and urethra enter into the urinary tract infection in ascending route. Pyelonephritis mostly occurs because of uropathogen ascending from bladder to renal pelvis via the

ureter. Kidney infections caused through hematogenous route is not very common in healthy individuals (Busch and Kadri, 2020). Sometimes the pathogen uses the lymphatic route for entering the host urinary tract from adjacent organs (Mody et al., 2017).

2.7 *Klebsiella pneumoniae* as a Uropathogen

Klebsiella pneumoniae is a very common multi-drug resistant (MDR), opportunistic pathogen responsible for causing mainly nosocomial infections such as UTIs, pneumonia and some blood stream infections (Florea et al., 2023; Togawa et al., 2015). It also has the ability to cause community associated infections including meningitis, liver abscess and endophthalmitis in healthy individuals (Russo and Marr, 2019). Now *Klebsiella pneumoniae* is also classified as a unique circulating pathotype along with the classical *Klebsiella pneumoniae* (cKP), as a hypervirulent *K. pneumoniae* because of a rare case reported in Taiwan in 1980's ((Fang et al., 2007; Wyres et al., 2020). Some of the main characteristics of different classes of K. pneumoniae are mentioned in **Table 3**.

Table 3 Main characteristic of cKP, hvKP, and MDR-hvKP (Song et al., 2021).

Characteristics	Classical K. pneumoniae (cKP)	Hypervirulent K. pneumoniae (hvKP)	Multidrug resistant hvKP (MDR-hvKP)	References
Infections	**Acquisition:** nosocomial	**Acquisition:** community	**Acquisition:** nosocomial and community	(Hoashi et al., 2019; Hu et al., 2020; Russo and Marr, 2019)
	Host: immunocompromised patients	**Host:** healthy adults	**Host:** usually immunocompromised patients	
	Geographic region: the whole world	**Geographic region:** Southeast Asia	**Geographic region:** Asia (especially China)	
	Infectious sites: urinary tract infections, pneumoniae, blood stream infection usually polymicrobial at sites of infection	**Infectious sites:** pyogenic liver abscess, meningitis, endophthakmitis, necrotizing fasciitis; usually monomicrobial at sites of infection	**Infectious sites:** pyogenic liver abscess, bloodstream infections, urinary tract infections	
	Metastasis: uncommon	**Metastasis:** Common		
Phenotypes	Non-hypermucoviscosity and string < 5mm	Hypermucoviscosity and string ≥ 5mm	Hypermucoviscosity or non-hypermucoviscosity	(Bassetti et al., 2018)
Common serotypes	K1 – K79	K1, K2, K5, K16, K20, K54, K57, KN1	K1, K2, K16, K20, K54, K62, K64, K47	(Yang et al., 2021; Zhan et al., 2017)
Siderophores	*Enterobactin, yerseniabactin*	*Enterobactin, yersiniabactin, salmochelin* and *aerobactin*	*Enterobactin, yersiniabactin, salmochelin and aerobactin*	(Lam et al., 2018; Russo et al., 2015; Wyres et al., 2020);

2.8 Virulence Factors of *Klebsiella pneumoniae*

Klebsiella pneumoniae expresses different virulence genes that play a significant role in initiating a urinary tract infection. The pattern of virulence factors for UTIs will be different from the virulence factors of pneumonia. Virulence factors are classified as cell surface virulence factors and secreted virulence factors. These factors include adhesins, serum resistance, lipopolysaccharides (LPS), polysaccharide capsule, outer-

membrane proteins (OMPs), iron uptake receptors as siderophores and secretory toxins **Table 4** (Kot, 2019; Zubair et al., 2019).

Pathogenesis depends a lot on these virulence factors. These factors play an important role in colonization, bacterial adherence and persistence despite the host having a very effective immune mechanism (Schlager et al., 2002; Zubair et al., 2019). In vitro and in vivo models were established and used to investigate the interaction between the bacterial cells and host **Figure 2**.

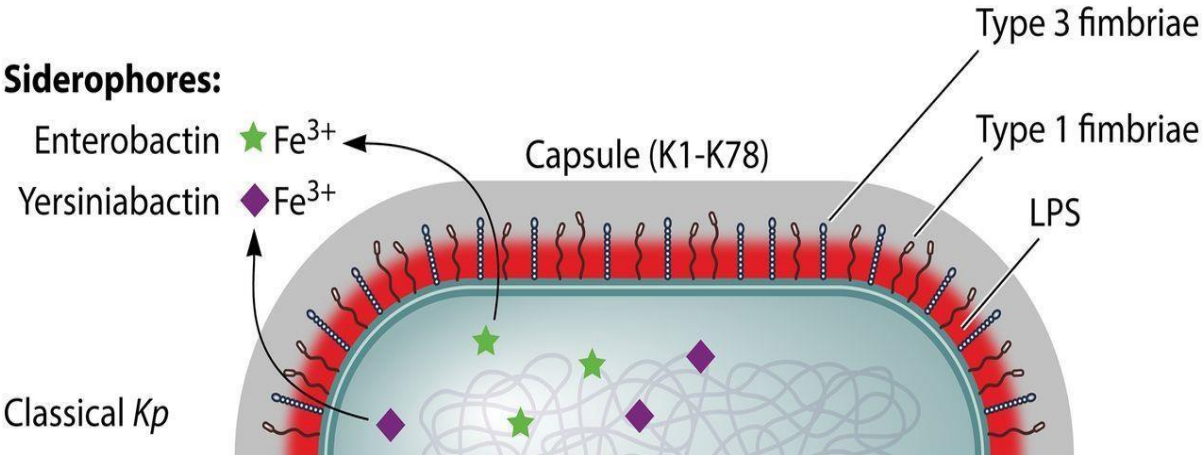

Figure 2 Virulence factors exhibited by *Klebsiella pneumoniae* are illustrated, highlighting the features of siderophore (Enterobactin and Yersiniabactin), fimbrae (Type 1 and Type 3), capsule (K1-K78), and lipopolysaccharide (LPS) composition. These virulence attributes play pivotal roles in the pathogenicity of *K. pneumoniae*, contributing to its ability to establish and sustain infections in host organisms (Paczosa and Mecsas, 2016)**.**

Table 4 Virulence factors of *Klebsiella pneumoniae* and their roles in pathogenesis (Clegg and Murphy, 2016; Paczosa and Mecsas, 2016).

Virulence factors	Role in pathogenesis
Capsule	Inhibit and evade phagocytosis by host cells, induces dendritic cell maturation, neutralizes antibacterial activity of host defense.
LPS	O antigen: Provides serum resistance.
	Lipid A: Activates inflammatory response.
Siderophore	Scavenge essential iron for survival, hypermucoviscous phenotypes have been linked to increased iron-binding activity.
Adhesins	Type 1 fimbriae: Involved in the formation of intracellular bacterial communities.
	Type 3 fimbriae: Important for biofilm formation on biotic and abiotic surfaces, role in biofilm formation on urinary catheters in vivo remains to be elucidated.
OMPs	Outer membrane protein A (OmpA): Binds to bronchial epithelial cells, DCs and macrophages, leading to enhanced cytokine production. May inhibit cytokine production and increase bacterial resistance to antimicrobial peptides such as α-defensin.
	Peptidoglycan-associated lipoprotein (Pal): Protection against neutrophil phagocytosis, killing by neutrophils and serum components.
	Murein lipoprotein (LppA): Enhances integrity and selective impermeability cell membrane, strengthens *K. pneumoniae* against anionic detergents and certain antibiotics.

2.8.1 Adhesins

Adhesins are designated as fimbrial or afimbrial depending upon their presence on surface glycoprotein known as fimbriae. Pilus/fimbria act as ligands for glycolipid and glycoprotein receptors present on uroepithelial cells (Florea et al., 2023). *Klebsiella*

pneumoniae encodes some specific adhesins that target a wide range of host receptors (Behzadi et al., 2019).

Different types of adhesins for *K. pneumoniae* are classified as type 1 fimbriae and type 3 fimbriae **Figure 3**. Type 1 fimbriae show adherence to different mannose-containing structures of host cells. FimH are present at the terminal end and plays its part in fimbrial adhesion to mast cell and promotes mannose sensitive binding. FimA acts by binding to bladder and promotes biofilm formation and cellular invasion of the bladder. It also binds to other abiotic surfaces. Some other minor submits are functionally similar to UPEC Fim subunits like *FimC*, *D*, *F* and *G* (Paczosa and Mecsas, 2016). *FimK* is an additional gene identified in *K. pneumonia* were absent in UPEC. Fim switches play its role in facilitating the expression in Type 1 fimbriae in UTIs but not in lungs or GIT (gastrointestinal tract) (Stahlhut et al., 2012).

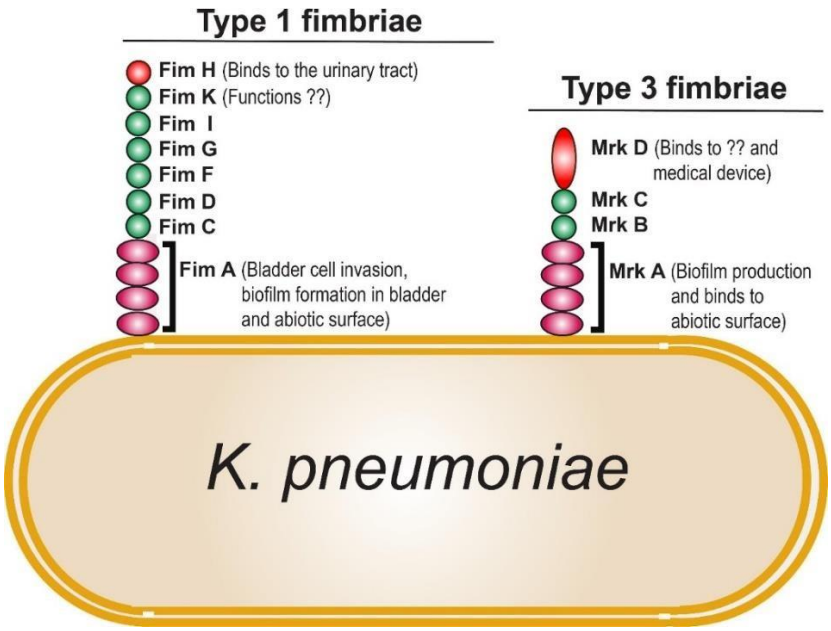

Figure 3 The adherence mechanisms of Type 1 and Type 3 fimbriae in *Klebsiella pneumoniae* are summarized, highlighting their roles in host cell attachment and the unique gene FimK's involvement in urinary tract infections (UTIs) (Paczosa and Mecsas, 2016).

Synthesis of Type 3 fimbriae is mediated by the expressions of *mrk,, B, C* and *D* genes. Just like Type 1 fimbriae, Type 3 fimbriae are present and synthesized by most *Klebsiella pneumoniae,* but Type 3 is not sensitive to mannose receptors. For Type 3 fimbriae specific host receptors are yet to be investigated **Table 5**.

Table 5 Adhesins of *Klebsiella pneumoniae* in host cell receptor (Govindarajan and Kandaswamy, 2022)

	FimG	Controls the fimbriae length and associated with FimH.
Type 3 fimbriae	**MrkA**	Adheres abd biofilm formation on abiotic surfaces like glass and plastic
	MrkD	Binding receptors in host cells are yet to be investigated. Studies have shown that MrkD posibility to bind with medical devices.
Types of adhesins	**Pili subunits/ Surface proteins**	**Receptor surface/ host cell/ abiotic suface**
Type 1 fimbriae	**FimA**	Bladder cell invasion, formation of biofilm on bladder cells and biotic surfaces like glass and plastic.
	FimK	Binding receptors in the host cell and pili subunit function are yet to be investigated.
	FimH	Binds to the mannose-binding receptors in the urinary tract.
	FimC	Encodes for periplasmic chaperon protein.
	FimD	Encodes usher protein.
	FimF	Controls the fimbriae length and associated with FimH.

2.8.2 Capsule

Capsule is a polysaccharide matrix coating the cell is an important factor in *Klebsiella pneumoniae* virulence and is condidered to be the most studied factor of *K. pneumoniae*. *Klebsiella pneumoniae* without the capsule are less virulent as compared to the capsular *K. pneumoniae*. Capsules in *K. pneumoniae* are comprised of strain-

specific polysaccharides known as K antigens i.e., K1 and K2, up K78. K1 and K2 strains have been reported to be more virulent than other serotypes. Whereas K2 strains are the most prevelent strains of K. pneumoniae followed by K1 (Yeh et al., 2010; Yu et al., 2008). Capsule play different roles as a virulence factor in K. pneumoniae **Figure 4** (Papakonstantinou et al., 2015). It prevents phagocytosis and opsonophagocytosis of the bacteria by host immune system, obstructs bactericidal action of antimicrobial peptides for instance human beta defensins 1 to 3 (Casadevall and Pirofski, 2009). It also blocks complementing components like C3 from interacting with membrane which leads to prevention of complement-mediated opsonization and lysis. Most importantly it avoids the activation of immune response (Dogan et al., 2021).

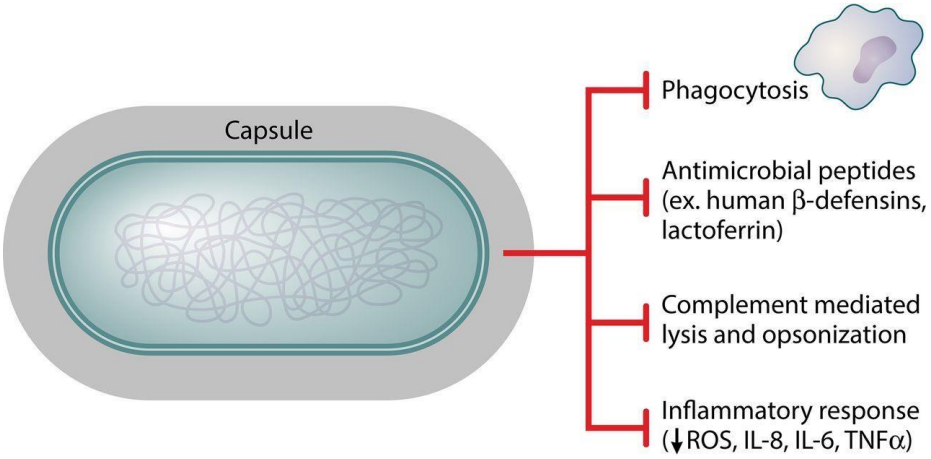

Figure 4 The critical role of the capsule in *Klebsiella pneumoniae*'s virulence is illustrated. This capsular polysaccharide coating shields the bacterium from immune defenses by preventing phagocytosis, neutralizing antimicrobial peptides, and blocking complement activation (Paczosa and Mecsas, 2016).

2.8.3 Siderophores

Small iron-chelating compounds with high affinity secreted by bacteria and fungi are called siderophores. Their purpose is to help the organism in accumulating iron. Iron is a very limited compound and not easily available to *K. pneumoniae* in the host during the infection mainly because of the immune respnse by the host where iron

is an important compound for *K. pneumoniae* pathogenesis (Gal-Mor and Finlay, 2006). *K. pneumoniae* acquies iron by section of siderophores. Siderophores have a high affinity of stealing the iron from iron-chelating proteins of the host (Bachman et al., 2012; Miethke and Marahiel, 2007).

There are more than one siderophores produced by *K. pneumoniae* that are used in optimizing colonization in different tissues (Bachman et al., 2012; Zhu et al., 2021). Different siderophores expressed in *Klebsiella pneumoniae* are yersiniabactin, enterobactin, salmochelin and aerobactin. The affinity of siderophore in *K. pneumoniae* ranges from enterobactin with the highest to aerobactin with the lowest **Figure 5** (Hsieh et al., 2008). Enterobactin is the main siderophore used by *K. pneumoniae* (Bachman et al., 2012). Yersiniabactin was discovered in Gram-negative *Yersinia* but has been since then identified in *Klebsiella pneumoniae* as well (Hsieh et al., 2008; Rijavec et al., 2008). Salmochelin is a c-glycosylated form of enterobactin (Russo et al., 2015). Aerobactin is a citrate-hydroxamate siderophore. This is not commonly expressed in classical nosocomial *K. pneumoniae*. Approximately it is expressed in only 6% of the classical strains (Bachman et al., 2012; Busch and Kadri, 2020).

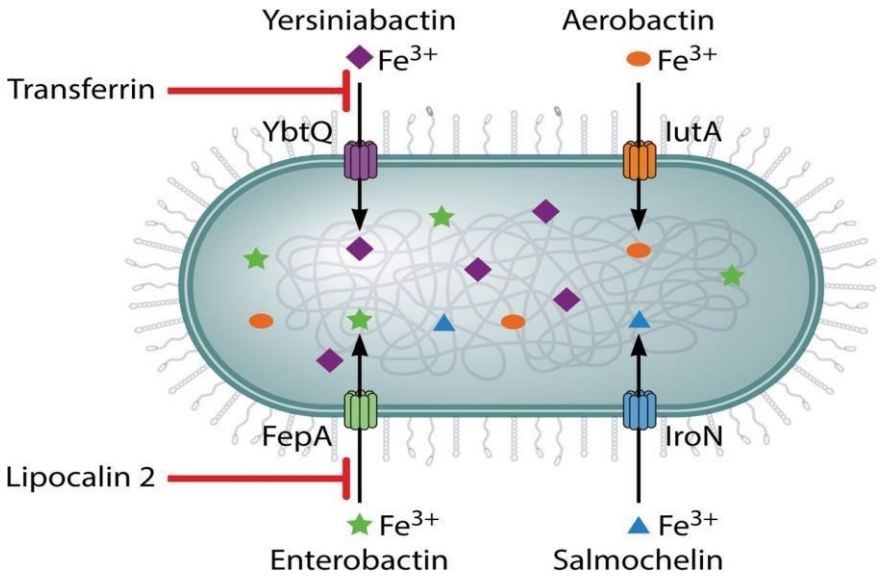

Figure 5 Illustration of multiple siderophores in *Klebsiella pneumoniae*, with enterobactin being the primary one. Other siderophores include yersiniabactin, salmochelin, and aerobactin, each with varying affinities for iron. Aerobactin is less common in nosocomial *K. pneumoniae* strains, present in only about 6% of them (Paczosa and Mecsas, 2016).

2.8.4 Lipopolysaccharides

Lipolysaccharides which are a major component of the outer cell membrane of Gram-negative bacteria are also known as endotoxin (Kong et al., 2012). LPS plays both beneficial and non-beneficial roles for *K. pneumoniae* in case of an infection i.e., it is a very important factor protecting from humoral defense systems but it can also act as a strong immune activator **Figure 6**. LPS are mainly comprised of an O antigen, core oligosaccharide and lipid A. Genes encoding these components are wb, waa and lpx gene clusters (Majumdar et al., 2016). Lipid A gets inserted into bacterial membrane and activates inflammation. *K. pneumoniae* can modify lipid A to make it less inlfammatory and also acts as a barrier against bactericidal actions of cationic antimicrobial peptides (Cai et al., 2016a). O antigen being the outermost subunit of LPS protects against complement binding i.e., Clq binding to bacteria, that inhibits subsequent activation of component pathway. Instead facilitates C3b binding away

from outer membrane that reverts bacterial lysis (Regué et al., 2004; Shankar et al., 2004).

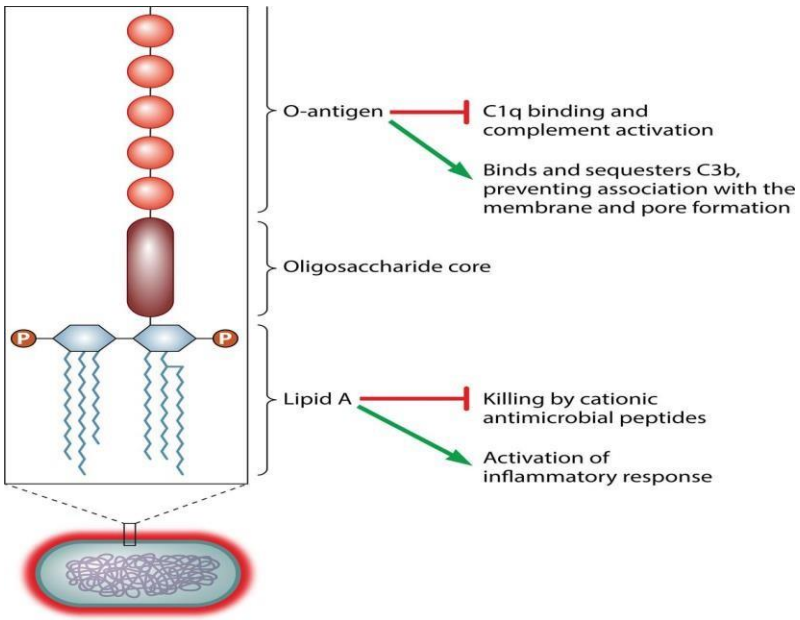

Figure 6 Role of LPS in *Klebsiella pneumoniae* in defense against the immune system and triggering immune responses. It consists of three parts: O antigen, core oligosaccharide, and lipid A (Paczosa and Mecsas, 2016).

2.8.5 Outer membrane proteins (OMPs)

Several outer membrane ptoteins have been identified in *Klebsiella pneumoniae* that play an important role in virulence of the organism (Pichavant et al., 2003). These outer membrane proteins include outer membrane protein A (OmpA), petidoglycan-associatwd lipoprotein (Pal) and murein lipoprotein (LppA). These OPMs are encoded by genes *OmpA*, *Pal* and *LppA* (Hsieh et al., 2008). OmpA playa a vital role in virulence of *K. pneumoniae*, by providing protection against innate immune response of the host (Jeannin et al., 2005). Studies have characterized peptidoglycan-associated lipoprotein (Pal) and murein lipoprotein (LppA) minimally. The role of these proteins are mainly to provide protection against neutrophil phagocytosis by serum components and neutophils (Hsieh et al., 2013). These proteins also contribute in selective

impermeability of cell membrane in a capsule and LPS independent manner along with providing strength against certain antibiotics and anionic detergents (March et al., 2013).

2.9 Antibiotic treatment of UTIs

Klebsiella pneumoniae is responsible for affecting a large population every year therefore is a major target for antimicrobial therapy. A lot of factors are involved in considering the antibiotic therapeutic strategy such as gender, patient's age, history, underlying infections, clinical presentations and site of infection (Ejrnæs et al., 2011). Another important factor while selecting the antimicrobials is local resistance profile. Commonly used antimicrobialaused for treating UTIs are fluoroquinolones, fosfomycin tromethamine, nitofurantoin, β-lactams and trimethoprim. Use of these antimicrobials is based on their pharmonkinetic profiles, their spectrum of activity and their tolerance (Jancel, 2002). Fluoroquinolones like prulifoxacin, ciprofloxacin and levofloaxin are used for the treatment of uncomplicated cystitis (Cai et al., 2016b).

Some specific antiobiotics used and susceptible against classical *Klebsiella pneumoniae* strain causing UTIs are meropenem, entapenem (100%), ceftazidime, cefoxitin, cefotaxime (94%), gentamicin (98%), amoxicillin, levofloxacin (88%), imipenem (86%), ciprofloxacin (84%). Whereas for hypervirulent *Klebsiella pneumoniae* the most effective antimicrobials are fostomycin (93.5%), imipenem (74.2%), meropenem entapenem (64%). So far imipenem has been reported to be the most effective against ESBL (extended spectrum beta-lactamase) producing *Klebsiella pneumoniae* strains (İDiL et al., 2020). For uncomplicated UTI most effective drugs are the combination drug trimethoprim and sulfamethoxazole, trimethoprim, β-lactams, nitrofurantoin, fosfomycin tromethamine and fluoroquinolones (Gupta, 2002). In case

of complicated UTIs the recommended antibiotic is amoxicilin-clavulanic acid (Kot, 2019). As the use of antibiotics have increased exessively for treating UTIs, it has resulted in the increase of antibiotic resistance as well. This has lead to a lot of organisms being MDR (multi-drug resistant) organisms like *Klebsiella pneumoniae* (Ren et al., 2022).

2.10 Global Antimicrobial Resistance

Antibiotic resistance soectrum has changed a lot within few years and is continuously changing and is going to pose a major threat to the public health sector (Alvarez-Uria et al., 2018). Antimicrobual resistance in few organisms are a major threat specifically *Escherichia coli* and *Klebsiella pneumoniae* belonging to Enterobacteriaceae (Shrivastava et al., 2018). In developing countries the resistance to antimicrobials is significantly higher as compared to developed countries such as resistance to fluoroquinolones is 55.5-85.5% in developing countries whereas in developed countries the resistance rate is approximately 5.1-32.0% ((Kim et al., 2018; Kothari et al., 2023).

2.11 Whole Genome Sequencing

Whole Genome Sequencing (WGS) of Klebsiella pneumoniae is closely tied to the broader development of genomics and sequencing technologies. Here is a brief overview of the key milestones in the history of WGS for *Klebsiella pneumoniae*, Before the advent of high-throughput sequencing technologies, scientists relied on traditional molecular biology techniques to study *Klebsiella pneumoniae* genetics. These methods were labor-intensive and focused on specific genes or genetic elements. The development of NGS technologies, such as Illumina sequencing, marked a significant advancement in genomics. These technologies allowed for the rapid and

cost-effective sequencing of entire genomes, including those of bacterial pathogens like *Klebsiella pneumoniae*. In the mid-2000s, the first whole genome sequences of *Klebsiella pneumoniae* strains were published. These early sequences provided valuable insights into the genetic makeup of this pathogen. Research into Klebsiella pneumoniae continues, with ongoing efforts to better understand its genomics, pathogenesis, and mechanisms of antibiotic resistance. WGS remains a valuable tool in these endeavors.

CHAPTER 3

Materials And Methods

3.1 Study Approval and Sample Collection

KP3 isolate was isolated from Fauji Foundation hospital, Rawalpindi, Pakistan after initial MDR confirmation and was brought to ASAB labs for further identification. The strain KP3 was isolated from the urine sample of a 65year old patient who was suffering from urinary tract interaction. The isolate KP3 was brought to the Integrated Biology Lab, ASAB for initial identification (cell/colony morphology, molecular and biochemical characterization) and to perform the antibiotic sensitivity assay.

3.2 Preliminary Identification

3.2.1 Colony Morphology

The isolate KP3 was streaked on MacConkey Agar plates; a selective media for *gram-negative s*pecies and were incubated at 37°C. After 24 hours of incubation, the shape, colony size, texture and margin were recorded and crosschecked with Bergey's Manual of Systematic Bacteriology (Bergey's Manual of systematic Bacteriology 2005)

3.2.2 Colony Morphology

To know about the cell morphology gram staining was performed. For Gram staining a smear of a pure culture was prepared on a glass slide. The glass side was then stained with Crystal violet dye (primary dye) for two minutes. Then the slide was stained with iodine solution for two minutes. Excessive stain is removed by flooding the slide with water. Purple stained were decolorized with 70% ethanol for about 1 minute. The stain was then stained with Secondary dye i.e., safranin for 45 seconds. Finally, the slide was washed, air dried and examined under the microscope.

3.3 Biochemical Characterization

After the preliminary identification, biochemical tests were performed to phenotypically characterize the isolated KP3.

3.3.1 Catalase Test

Catalase enzyme, an antioxidant enzyme is used by bacteria for the protection of cells from oxidative damage by reactive oxygen species (ROS). Catalase enzyme uses hydrogen peroxide (H_2O_2), a non-radical ROS, as its substrate and catalyze it into O_2 and H_2O. Fresh colony of isolate KP3 was placed on a slide by a sterilized loop and 3% of H_2O_2 was applied to observe the bubble formation.

3.3.2 Lactose Fermentation Test

Gram negative bacteria that have the ability to ferment lactose are identified by Lactose fermentation test. *Klebsiella pneumoniae* has the ability to ferment lactose producing acid end products which results in the decrease in pH of the medium. A positive test consists of a color change from pink to red, indicating a pH change from basic to acidic. The isolate of KP3 was streaked on MacConkey Agar plates; a differentiating agar that differentiates the gram-negative bacteria based on their lactose metabolism and were incubated at 37° C for 24 hours.

3.4 Antibiotic Susceptibility Assay

The isolate KP3 was tested against 15 antibiotics; Sulphamethoxazole/Trimethoprim (SXT), Cefepime (FEP), Ciprofloxacin (CIP), Vancomycin (VA), Enoxacin (EN), Gentamicin (CN), Piperacillin (PRL), Kanamycin (K), Amoxycilin/Clavulanic Acid 2:1 (AMC), Meropenem (MEM), Ampicillin (AMP), Amikacin (AK), Ceftriaxone (CRO), Imipenem (IPM), Chloramphenicol (C) for

antibiotic susceptibility assay using Kirby-Bauer Disc Diffusion method. Briefly, the bacterial culture at turbidity of 0.5 McFarland was prepared from an overnight culture by mixing the pure colony of KP3 isolate in 1ml of normal salinity. 50µl of bacterial suspension was then poured. The glass spreader is used for spreading the suspension over Mueller-Hinton Agar (MHA) media plates to create a media lawn. The plates then incubated at 37°C for about 24 hours. After incubation, zones of inhibitions were recorded and examined according to the guidelines of Clinical and Laboratory Standards Institute ('CLSI', 2019).

3.5 Glycerol Stock Preparation

Aqueous solution of Glycerol acting as a cryoprotectant is used for preserving microbial culture at low temperatures. For broth culture, the liquid broth medium was inoculated with fresh culture/fresh single isolate colony of KP3 in a culture tube and incubated in a shaking incubator at 37°C to ensure proper aeration and nutrient availability overnight. Shaking incubation avoids bacterial clumping at the bottom of the culture tube. After incubation culture tubes are checked for bacterial growth. The broth became cloudy from the bacterial growth. Then about 0.5ml bacterial culture from LB broth was mixed in 0.5ml of 80% sterile glycerol. The cryovials were then gently vortexed and frozen using liquid nitrogen before it was transferred to -80°C freezer.

Table 6 Concentration of antibiotics used for antibiotic susceptibility assay.

No.	**Class of Antibiotics**	**Antibiotics**	**Concentration (µg)**
1	Sulfonamides	Sulphamethoxazole/ Trimethoprim (SXT)	25
2	Cephalosporin	Ceftriaxone (CRO)	30
3		Cefepime (FEP)	30
4	Fluoroquinolones	Ciprofloxacin (CIP)	5
5		Enoxacin (EN)	10

6	Glycopeptides	Vancomycin (VA)	5
7	Aminoglycosides	Gentamicin (CN)	10
8		Amikacin (AK)	30
9		Kanantcin (K)	30
10	Penicillin	Piperacillin (PRL)	100
11		Ampicillin (AMP)	25
12		Amoxycillin / Clavulanic Acid 2:1 (AMC)	30
13	Carbapenem	Meropenem (MEM)	10
14		Imipenem (IPM)	10
15	Amphenicol	Chloramphenicol (C)	30

3.6 Molecular Identification

3.6.1 DNA Extraction

The genomic DNA of KP3 isolate was extracted by the GeneJet Genomic DNA Purification Kit (Thermo Fischer Scientific) using the self-optimized manufacturer's protocol. The pellet from the fresh broth culture was suspended in 180µL of digestion solution followed by addition of 20µL of Proteinase K Solution. The sample was incubated at 56°C using a shaking water bath for almost 30 minutes. Then 20µL of RNase A solution was added and incubated at room temperature followed by the addition of 200µL of lysis solution to the sample and about 400µL of 50% ethanol. The prepared lysate was transferred to a GeneJet Genomic DNA Purification Column inserted in a collection tube. The column was centrifuged for 1 min at 6,000 × g. The purification column was placed into a new 2mL collection tube followed by the addition of 500µL of Wash Buffer I. Then it was centrifuged for 1 minute at 8,000 × g and 500µL of Wash Buffer II was added to the purification column and centrifuged for 3 minutes at maximum speed. The purification column was transferred to a sterile 1.5mL

micro centrifuge tube and about 100µL of elute buffer was added to the center of the purification column membrane to elute genomic DNA. It was remove incubated for 2 minutes at room temperature and was centrifuge for 1 minute at 8,000 × g. The purification column was discarded and the purified DNA was stored at -20°C.

3.6.2 DNA Quantification and Integrity

Extracted DNA of the KP3 isolate was quantified using Qubit 2.0 fluorometer. For the quality and integrity analysis of extracted DNA, Agarose Gel Electrophoresis was performed using 1% agarose gel, with 1kb ladder.

3.7 Whole-Genome Sequencing, Assembly and Annotation

Whole genome sequencing of strain KP3 was performed Pusing Illumina Hi seq 2500 platform. Genomic libraries were prepared through Nextera XT Library Prep Kit at MicrobesNG, Institute of Microbiology and Infection, University of Birmingham (Edgbaston, Birmingham). The quality for the sequenced genomes were assessed by FASTQC (Andrews, 2010). The raw reads trimmed by Trimmomatic v0.30 followed by genome assembly done by QIAGEN CLC Genomics Workbench V.20.0.4. Annotation of the assembled genome was done by RAST annotation tool (Aziz *et al*., 2008) and then cross checked by Prokka (Seemann, 2014).

3.8 *In silico* Pathogen Identification

Isolate KP3 was confirmed to be *Klebsiella pneumoniae* by using the generated contigs using the KmerFinder 3.2 at The Center of Genomic Epidemiology (Larsen *et al*., 2014).

3.9 Reference Based Assembly

K. pneumoniae reference strain HS11286 complete genome (GenBank assembly accession GCA_000240185.2) was retrieved from NCBI database. The sequence files were aligned by using QIAGEN CLC Genomics Workbench V .20.0.4 alignment tool. Newly generated multi-fasta .fna file of UTI causing KP3 contigs were in the specified order as that of the *K. pneumoniae* reference genome HS11286.

3.10 Draft Genome Submission to NCBI

After the assembly and alignment, the draft genome sequence of *Klebsiella pneumoniae* KP3 was submitted to NCBI database. The raw reads of *K. pneumoniae* KP3 were submitted to the Sequence Read Archive (SRA).

3.11 Sequence Retrieval

Total 46 sequences of UTI causing *Klebsiella pneumoniae* including whole and draft genome along with proteomes were retrieved from the website of NCBI (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/klebsiella%20pneumoniae) based on the host diseases, for comparative and pangenome analysis on 25th October 2022.

3.12 Pangenome Analysis

All 47 UTI causing *K. pneumoniae* genomes were subjected to in-house pipeline PanRV to identify the highly conserved regions (Naz et al., 2019). PanRV consists of four modules out of which we have used one module; the pangenome estimation module (PGM). PGM calculates pan, core, shell and cloud genes amongst multiple genomes in less time and less space complexity by making use of integrated rapid large-scale prokaryotic pangenome analysis pipeline, Roary. Before the

pangenome estimation, all the genome fasta files were annotated by using Prokka as the input for PGM is gff file. While using PanRV different plots are obtained which gives information about the pan, core and unique genes. Moreover, a plot can be seen in the output which gives information about the addition of new genes. For the analysis, default parameters (95% minimum percentage identity for BLASTp and 99% sequence identity for core genes classification) were used.

3.13 Multi-Locus Sequence Typing (MLST) of *Klebsiella pneumoniae* strains

Multi-locus sequence typing (MLST) is a DNA sequence-based typing method used for characterizing isolates of bacterial strains using the sequences of internal fragments of six or seven well conserved house-keeping genes. Allelic variation at every locus is characterized and by comparing the set of alleles with the other profiles of isolates in the database a sequence type (ST) is assigned to all of the seven loci. MLST profile of all of the complete genomes and UTI causing KP3 was checked using the MLST tool version 2.0 at CGE (https://www.genomicepidemiology.org/services/) at default parameters (Larsen *et al*., 2012).

3.14 Identification and Comparative Analysis of Antibiotic Resistance Genes

To analyze the genetic basis of resistance and epidemiology of resistant genes in our local *Klebsiella* isolate KP3 Resistance gene identifier (RGI) tool at Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/analyze/rgi) (McArthur *et al*., 2013, Zankari *et al*., 2012) was used to check the resistance genes in the genome. The selection criteria for CARD database was set at perfect and strict with

95% identity (a widely accepted default setting in CARD RGI and similar tools because it strikes a good balance between sensitivity and specificity for identifying antibiotic resistance genes in bacterial genomes) to identify the acquired antimicrobial resistant genes. Resistance genes for all other selected strains were also identified on the same pattern. Predicted acquired resistance genes of KP3 isolate were compared with all of the complete genomes including reference genome (n=46) retrieved from NCBI for the comparison between resistance profiles and for identifying any novel resistance genes in the local strain KP3.

3.15 Identification and Comparative Analysis of Virulence Genes

Virulence factors are responsible for a pathogen's ability to damage or infect its host tissues. Identifying the virulence factors help in understanding a pathogen's invasion of the host cell, evasion of the immune system and initiation of an infection. The virulence factors for local KP3 isolate were identified through Virulence factor database (VFDB) (Chen *et al*., 2005). Virulence factors for all other selected strains were also identified by VFDB. Predicted virulence factors of local KP3 isolate were compared with all of the *Klebsiella pneumoniae* strains (n=46) retrieved from NCBI to check for any unique virulence factors in local isolate KP3.

3.16 Identification and Comparative Analysis of Plasmids

Plasmids are specific extrachromosomal DNA segments that have the ability to self-replicate. The plasmids may contain genes for virulence factors antimicrobial resistance. Plasmidfinder v.2 at CGE (https://www.genomicepidemiology.org/services/) was used to identify the plasmids for local strain *Klebsiella pneumoniae* KP3 (Carattoli *et al*., 2014). Identified plasmids were also checked for the presence of any antibiotic resistance and virulence genes.

Then identified plasmids in KP3 were compared with the global *Klebsiella pneumoniae* strains which were also identified by Plasmidfinder v.2.

3.17 SNP based Phylogeny

CSI Phylogeny 1.4 at CGE (https://www.genomicepidemiology.org/services/) at default parameters was used to deduce the SNP based phylogenetic relationship of local isolate KP3 with *the Klebsiella pneumoniae* strains from various different geographical regions for investigating evolutionary history, relationships, and functional aspects of organisms (Kaas *et al*., 2014). CSI Phylogeny points out SNPs against the simple reference genome *Klebsiella pneumoniae* HS11286, filters out the SNPs, executes site validation and concludes a phylogeny based on conjugated alignment of high-quality SNPs. Visualization of circularized SNP tree of 47 (reference strain n=1, global strains n=46 *Klebsiella pneumoniae* genomes is done via Interactive Tree of Life (iTOL) v.2 (Letunic and Bork, 2021).

CHAPTER 4

Results

4.1 Characterization of Local Isolate KP3

The local isolate KP3 showed certain growth on MacConkey Agar plates, visible colonies of KP3 were seen after incubating it at 37°C for 24 hours. Further analysis was performed for checking morphological and biochemical characteristics of the cultured colonies. Pink, mucoid, flat and rough KP3 colonies were observed in preliminary identification. The isolate was observed to be Gram-negative. The results of biochemical test revealed the colonies were catalase positive. On MacConkey agar lactose positive colonies were observed after 24 hours at 37°C. Therefore, based on biochemical and morphological characterization the KP3 was identified as *Klebsiella pneumoniae*.

4.2 Phenotypic Resistance Profile

AST analysis provided the phenotypic resistance profile of local strain KP3. The isolate KP3 was resistant against 10 antibiotics and susceptible to 5 of the antibiotics. Resistance was observed against Cefepime (30µg), Ciprofloxacin (5µg), Vancomycin (5µg), Enoxacin (10µg), Piperacillin (100µg), Kanamycin (30µg), Amoxycillin/Clavulanic Acid 2:1 (30µg), Ampicillin (25µg), Ceftriaxone (30µg) and Chloramphenicol (30µg). Whereas the isolate showed susceptibility to Sulphamethoxazole/Trimethoprim (25µg), Gentamicin (10µg), Meropenem (10µg), Amikacin (30µg), Imipenem (10µg). Resistant and susceptible antibiotics with their concentrations and zones of inhibition are mentioned in **Table 7.**

Table 7 Resistant and susceptible antibiotics against KP3 isolate.

No	Class of Antibiotics	Antibiotics	Concentration (µg)	Zone Diameter (mm)
1	Phenicol	Chloramphenicol (C)	30µg	0
2	Cephalosporin	Cefepime (FEP)	30µg	0
3		Ceftriaxone (CRO)	30µg	0
4	Fluoroquinolones	Ciprofloxacin (CIP)	5µg	0
5		Enoxacin (EN)	10µg	0
6	Glycopeptides	Vancomycin (VA)	5µg	0
7	Penicillin	Piperacillin (PRL)	100µg	0
8		Ampicillin (AMP)	25µg	0
9		Amoxycillin/ Clavulanic Acid 2:1 (AMC)	30µg	0
10	Aminoglycosides	Kanamycin (K)	30µg	0
11		Gentamicin (CN)	10µg	16mm
12		Amikacin (AK)	30µg	20mm
13	Carbapenem	Imipenem (IPM)	10µg	33mm
14		Meropenem (MEM)	10µg	14mm
15	Sulfonamides	Sulphamethoxazole/ Trimethoprim (SXT)	25µg	12mm

4.3 DNA Extraction and Quantification

GeneJet Genomic DNA Purification Kit (Thermo Fischer Scientific) extracted the genomic DNA of isolate KP3. The extracted DNA of isolate KP3 was quantified to be 19.7ng/µl.

4.4 Whole Genome Sequencing, Assembly and Annotation

1190506 paired end reads were yielded by the Illumina Sequencing Platform 2500. Generated paired end reads were assembled into 440 contigs by De novo assembly. Alignment of local isolate KP3 was checked with the reference genome *K. pneumoniae* HS11286 (GenBank assembly accession (GCA_000240185.2) through QIAGEN CLC Genomics Workbench V.20.0.4. Several insertion and deletion sequences were identified in the isolate KP3 at different positions via reference-based genome assembly **Table 8.**

Table 8 Genomic characteristics of local strain KP3.

Genomics Characteristics	
Size	4872042
Number of Contigs	440
GC content	50.9
N50	27507
L50	54
CDS	4522
Longest contig size	93628
Smallest contig size	504
Mean sequence	11072.8
tRNA	64
rRNA	5
tmRNA	1

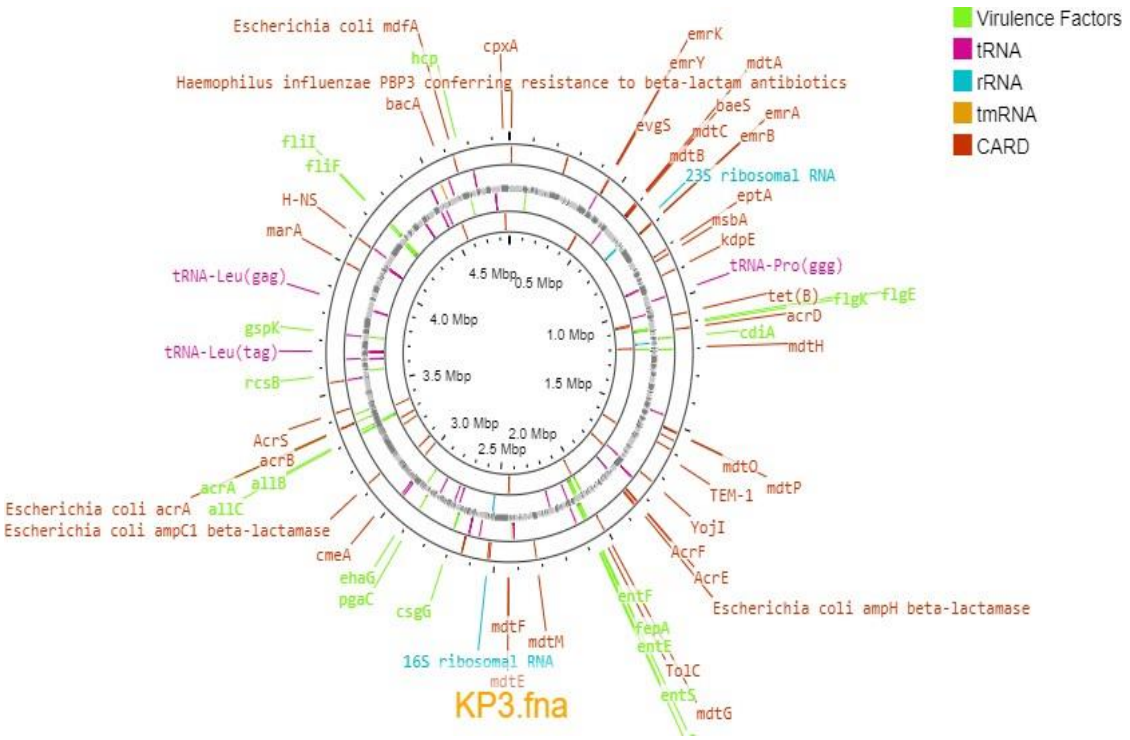

Figure 7 Circular visualization of the KP3 genome with genomic annotation, antibiotic resistance, and virulence genes showing some acquired antibiotic resistance genes from *E. coli*.

4.5 Sequence Submission to NCBI

Draft genome sequence of the local isolate KP3 was submitted to NCBI database after assembly and alignment. Raw reads of the local *Klebsiella pneumoniae* isolate KP3 were submitted to the Sequence Read Archive (SRA). Detailed are mentioned in **Table 9**.

Table 9 Sequence Submission to NCBI.

Submissions	
Accession number	JAPDVV000000000.1
Bio sample	SAMN31437005
Bio project	PRJNA893947

4.6 *Klebsiella pneumoniae* genome Retrieval

A total of 46 complete genome sequences of *Klebsiella pneumoniae* responsible for causing urinary tract infections were retrieved out of total 1332 complete genomes from across the world from NCBI database. All of the strains included in this study were reported to cause urinary tract infections in humans as we had noticed our strain to be UTI causing. 46 proteomes of completely sequences *Klebsiella pneumoniae* genomes were also retrieved from NCBI database on 25th October 2022.

4.7 Multi Locus Sequence Typing analysis of *Klebsiella pneumoniae* Strains

The MLST analysis of selected UTI causing strains (n=47) done using the scheme of seven housekeeping genes comprising *gapA, infB, mdh, pgi, phoE, rpoB, tonB*. MLST analysis revealed 25 different sequence types among 47 selected strains which showed high genetic diversity among *K. pneumoniae* genomes. Most recurrent sequence type was ST231 but isolate KP3 belonged to ST5 which identified clear genomic diversity and unique characteristics **Figure 8**. Some other STs shared by other strains were ST11 (n=7), ST101 (n=4), ST147 (n=3), ST258 (n=2), ST15 (n=2). Some different STs also found in studied strains as ST14 (n=1), ST307 (n=1), ST1440 (n=1), ST240 (n=1), ST661 (n=1) etc **Table 10**.

Table 10 Multi Locus Sequence Type of UTI causing *K. pneumoniae* strains.

Housekeeping Genes	**Sequence Types (STs)**	**No. of Isolates**
gapA, infB, mdh, pgi, phoE, rpoB, tonB	ST231	8
	ST11	7

	ST101	4
	ST147	3
	ST15, ST258	2
	ST5, ST14, ST16, ST307, ST2096, ST23, ST1440, ST512, ST225, ST323, ST29, ST2856, ST240, ST395, ST507, ST661, ST147, ST941, ST340, ST244	1 isolate from each profile

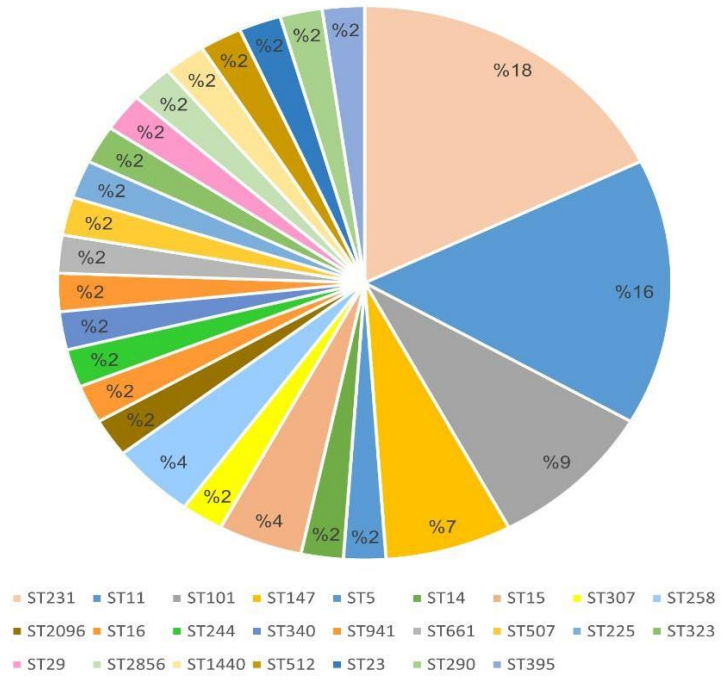

Figure 8 Pie chart illustrating sequence type diversity among UTI causing *K. pneumoniae* strains with ST231 being the most prevalent, followed by ST11, with our strain KP3 belonging to the ST5 sequence type.

4.8 Pangenome Analysis of UTI causing *Klebsiella pneumoniae* global strains

PanRV estimated total of 17520 genes in 47 UTI causing global *K. pneumoniae* strains, with 343 core genes conserved among 99 to 100% strains, 3752 soft core genes among 95 to 99% strains, 2061 accessory genes among 15 to 95% strains and 11364 unique genes among < 15% strains. **Figure 9 (a)**. Pangenome analysis also revealed *K. pneumoniae* shows an open pangenome as the number of unique genes increase **Figure 9 (c)** and total number of genes increases with adiition of new genes into the pangenome **Figure 9 (b)**. Phylogenectic tree of pangenome analysis visualized through iTOL showed close evolutionary relation between KP3 and KpC5 a strain from Dallas, USA **Figure 10**.

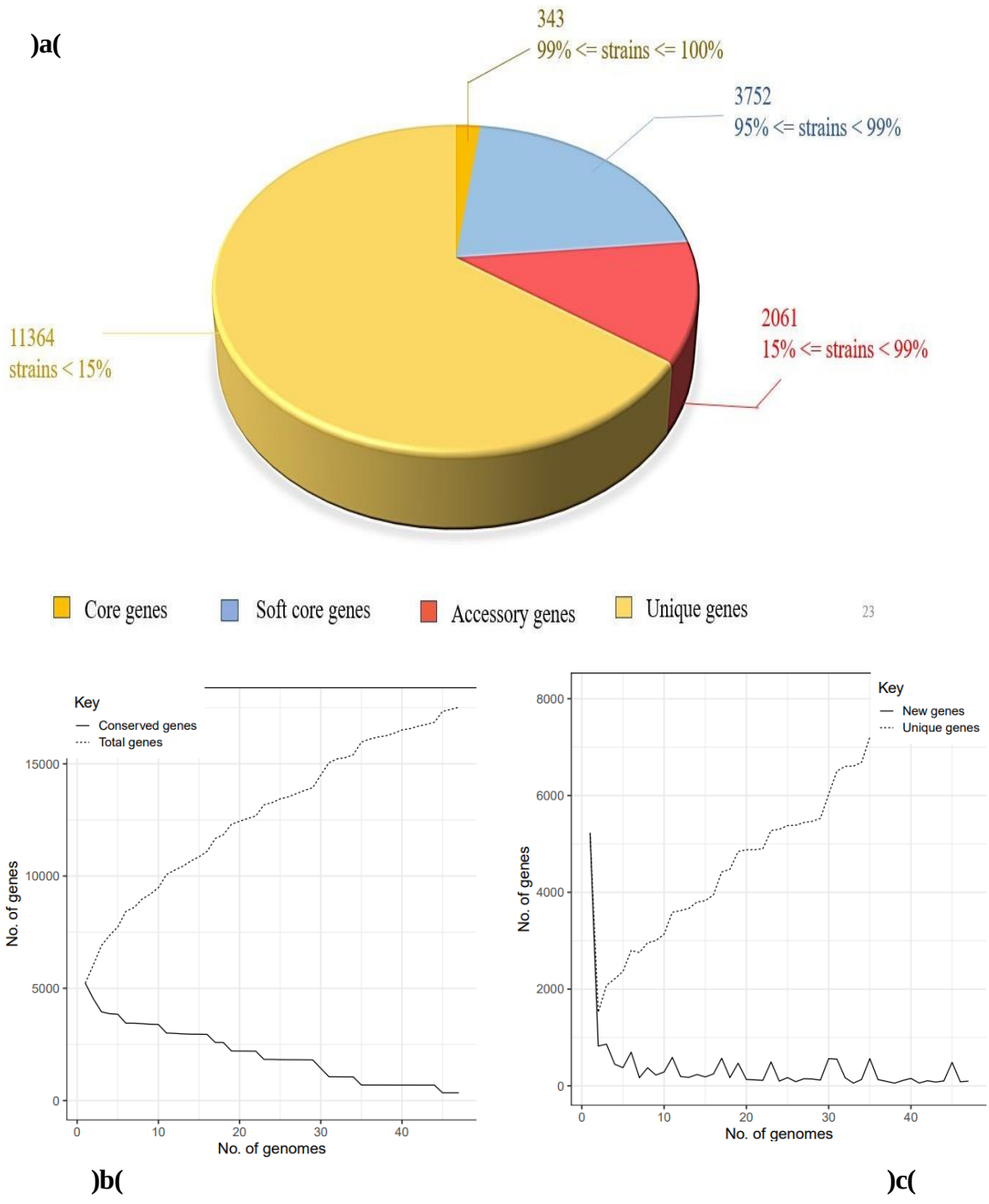

Figure 9 Comprehensive view of the *Klebsiella pneumoniae* pangenome (a) A pie chart displaying the distribution of gene clusters within the pangenome (b) A graph illustrating the growth in total genes and decrease in core genes with the inclusion of additional *K. pneumoniae* genomes (c) A graph showing the increase in unique genes as more *K. pneumoniae* genomes are included emphasizing the open pan-genome nature of the species.

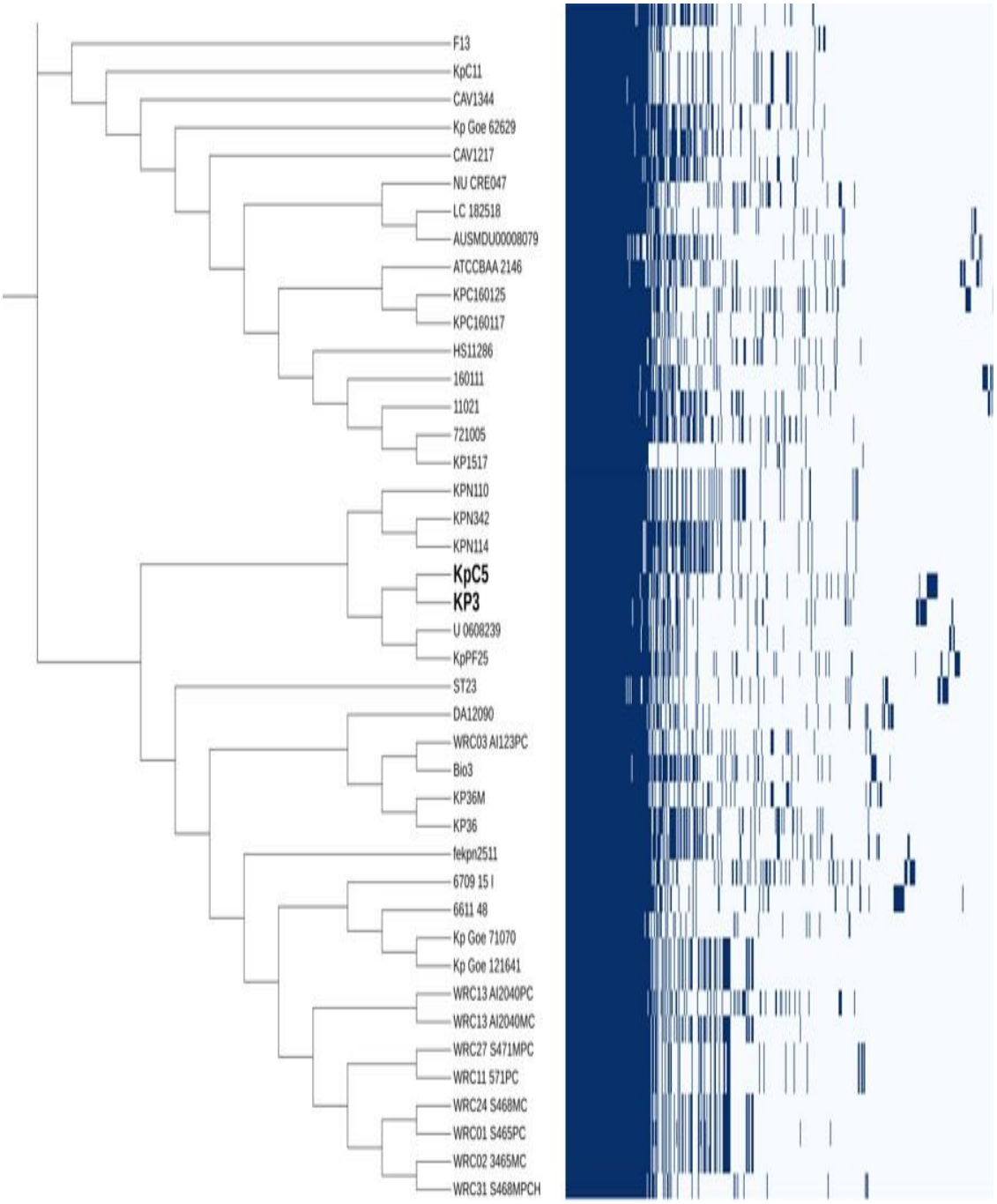

Figure 10 Pangenome tree and matrix comparing the presence/absence of core and accessory genes among *K. pneumoniae* strains, highlighting the close relationship between our strain KP3 and KpC5 from Dallas, USA.

4.9 Identification and Comparative analysis of Plasmids

5 plasmids were identified in the local KP3 isolate named as pKP3_1, pKP3_2, pKP3_3, pKP3_4 and pKP3_5 by PlasmidFinder **Table 11**. Further analysis was carried out to check the presence of antibiotic resistant and virulence genes. No virulence genes were identified in plasmids found in the isolate KP3. 2 of the plasmids pKP_3 (IncFII) and pKP_4 (IncFIA) were found to carry resistance against β-Lactams and Carbapenems **Figure 11**. IncF is the most common plasmid type found in the majority of the studied strains which are responsible for carrying resistance against major antibiotic groups such as Carbapenems and β-Lactams.

Table 11 Plasmids identified in UTI causing *Klebsiella pneumoniae* strain KP3.

Plasmids	**Most Similar Plasmid**	**Length**	**Identity Coverage**
pKP3_1	IncX3	328bp	100%
pKP3_2	Col8282	207bp	100%
pKP3_3	IncFII	262bp	96.18%
pKP3_4	IncFIA	388bp	99.74%
pKP3_5	IncFIB (AP001918)	548bp	98.91%

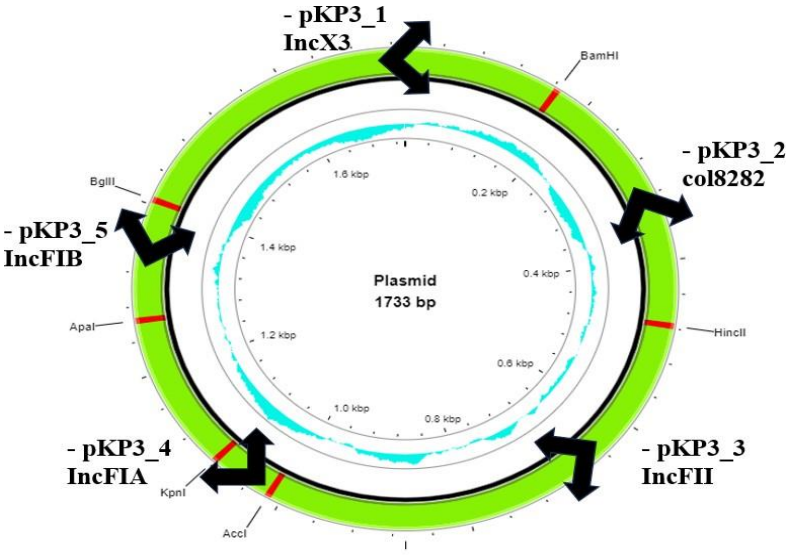

Figure 11 Visual representation of plasmids detected in the KP3 Isolate, comprising five distinct plasmids classified as IncX3, Col8282, IncFII, IncFIA, and IncFIB (AP001918).

4.10 Identification and Comparative Analysis of Resistance Genes

Different antibiotic resistance genes were identified by CARD in UTI causing KP3 isolate working against several classes of antibiotics including tetracycline (*emrY, TolC, E. coli acrA, acrB, H-NS, marB*), macrolide antibiotics (*evgA, TolC, H-NS*), fluoroquinolones (*emrA, emrR, evgA, acrB, mdtH, AcrE, marA*), phenicol antibiotic (*catI, marA, E. coli acrA, acrB*), nucleoside antibiotic (SAT-2), rifamycin antibiotic (*TolC, marA, acrA, acrB*), cephalosporin (*TEM-1, AcrE, TolC, CTX-M-15, H-NS, marA*), phosphonic acid antibiotics (*mdtG*), aminoglycoside antibiotics (*acrD, TolC, cpxA*), penam (*evgA, TEM-1, acrE, TolC, CTX-M-15, H-NS, marA*), nitroimidazole (*msbA*) which showed similarity to some extent with phenotypic antibiotic resistance profile oof KP3. RND, ABC, MFS antibiotic efflux pump encoding antimicribial genes were also identified which are responsible for providing resistance against aminoglycoside, nitroimidazole, fluoroquinolone, cephamycin, penam, cephalosporin, macrolide antibiotic, glycylcycline, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, disinfecting agents and antiseptics. *Klebsiella pneumoniae* adopts

different resistance mechanisms against antibiotics such as antibiotic inactivation, antibiotic efflux, reduced permeability to antibiotic, antibiotic target replacement, antibiotic target alteration. Some of the common resistance genes among globally studied *K. pneumoniae* strains are given in **Table 12**.

Table 12 Common Resistance Genes among UTI causing *K. pneumoniae* strains**.**

Antibiotic Resistance Genes	**AMR Gene Family**	**Drug Class**	**Resistance Mechanism**
rsmA	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, phenicol antibiotic	Antibiotic Efflux
TEM-1	TEM beta-lactamase	Monobactam, Cephalosporin. Penam	Antibiotic Activation
H-NS	Major facilitator superfamily (MFS) antibiotic efflux pump	Macrolide antibiotic, Fluoroquinolone antibiotic, Cephalosporin	Antibiotic Efflux
emrR	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic Efflux
marA	General Bacterial Porin with reduced permeability to beta-lactams	Cephamycin, Tetracycline antibiotic, Rifamycin antibiotic, Penem, disinfecting agents and antiseptics	Antibiotic Efflux, Reduced permeability to antibiotic
CRP	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, Fluoroquinolones, Penam	Antibiotic Efflux
msbA	ATP-binding cassette (ABC) antibiotic efflux pump	Nitroimidazole antibiotic	Antibiotic Efflux
CTX-M-15	CTX-M-beta-lactamase	Cephalosporin, Penam	Antibiotic Inactivation

vanG	Glycopeptide resistance gene cluster, Van ligase	Glycopeptide antibiotic	Antibiotic target alteration

4.11 SNP Based Phylogenetic Analysis of UTI causing *K. pneumoniae* Strains

Single nucleotide polymorphism based phylogenetic analysis gave us an evolutionary relation between globally studied different UTI causing *K. pneumoniae* strains. Visualization of SNP based phylogenetic tree by iTOL with the reference genome of *K. pneumoniae* HS11286 divided the studied strains into 3 clades. UTI causing local *K. pneumoniae* strain KP3 showed the closest relation to the reference strain HS11286 with 83.28% similarity. KP3 was also observed to be closely related to ATCCBAA_2146, a strain reported from the USA. This strain also shows 83.28% similarity with KP3. KP3 and ATCCBAA_2146 share similar resistance and virulence profile but different MLST profiles which identified the genetic connections and evolutionary history between these strains. Circularized SNP based phylogenetic tree showing 47 *K. pneumoniae* strains is given in **Figure 12**.

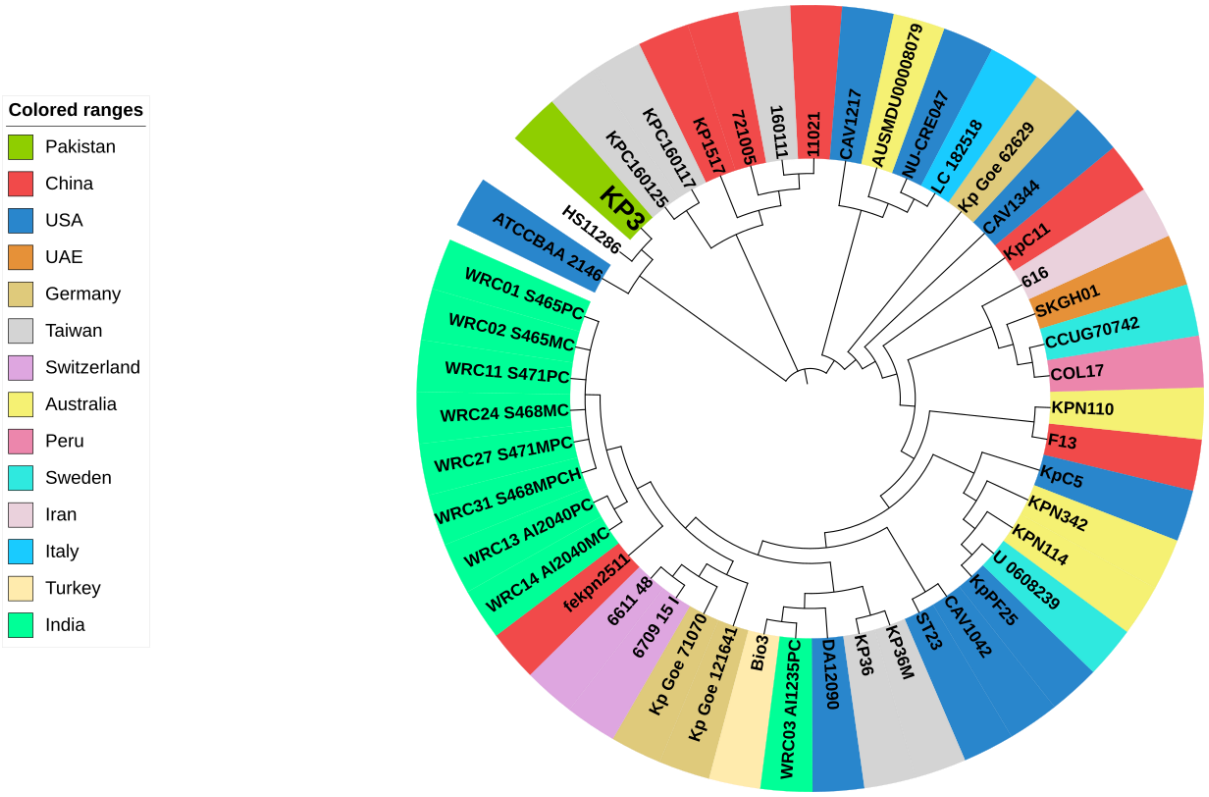

Figure 12 Circularized SNP Phylogenetic Tree encompassing 47 *K. pneumoniae* Genomic Strains. This tree is accompanied by a color-coded key that provides location information for each strain, aiding in geographical reference and understanding of their genetic relationships.

4.12 Identification and Comparative Analysis of Virulence Genes

VFDB predicted over 100 different types of virulence factors in local UTI causing *K. pneumoniae* strain KP3 which included both secreted and structural genes. These virulence factors belong to different gene clusters encoding for iron acquisition system, immune invasion, autotransporter proteins and toxins, biofilm production, serum resistance, iron acquisition systems, fimbriae, flagella, outer membrane proteins, nutritional factors, secretion system etc Figure. Virulence factors of other studied strains were also checked by VFDB and further compared with the virulence factors of KP3. It was observed that majority of the predicted virulence factors were common

between KP3 and UTI causing globally selected *K. pneumoniae* strains. Some of the common virulence factors among studied strains are given in **Table 13**.

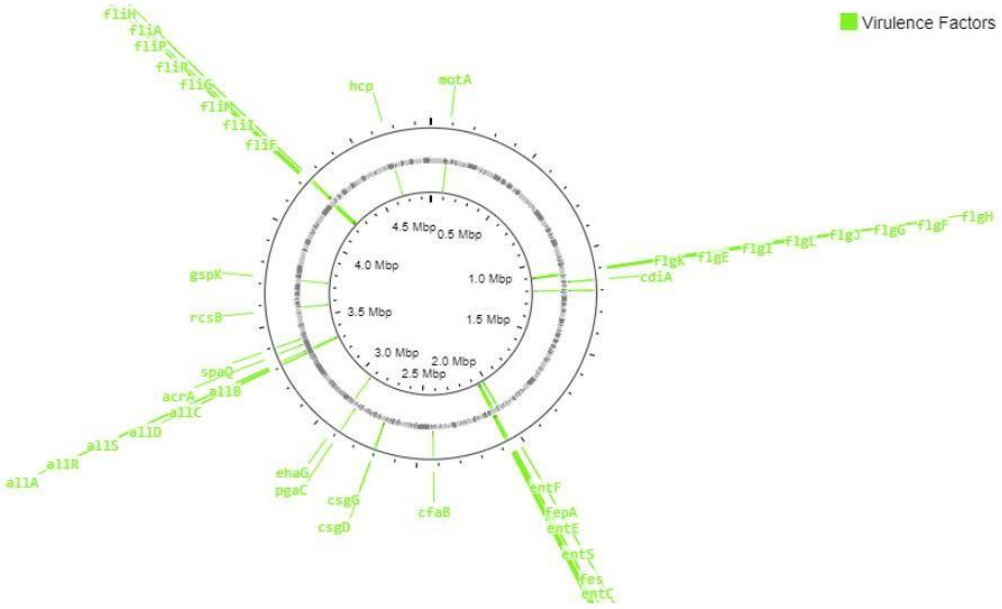

Figure 13 Virulence factors in local UTI causing *K. pneumoniae* strain KP3. VFDB analysis identified over 100 virulence factors in KP3, including secreted and structural genes related to various functions like iron acquisition, immune evasion, toxins, and more. These factors were compared with those in other strains, showing significant commonality with globally selected *K. pneumoniae* strains.

Table 13 A comparison of UTI causing KP3 virulence factors with the virulence factors of other UTI causing *K. pneumoniae* global strains.

KP3	**F13**	**KpC5**	**ST23**	**CAV1042**
Adhesins	Adhesins	Adhesins	Adhesins	Adhesins
Flagella	Flagella	Flagella	Flagella	Flagella
OMPs	OMPs	OMPs	OMPs	OMPs
-	Toxin	Toxin	Toxin	Toxin

ATP	ATP	ATP	ATP	ATP
Invasion	Invasion	Invasion	Invasion	Invasion
Biofilm formation	Biofilm formation	Biofilm formation	Biofilm formation	Biofilm formation
-	Antiphagocytosis	-	Antiphagocytosis	Antiphagocytosis

*OMPs; Outer membrane protein

*ATP; Autotransporter protein

CHAPTER 5

Discussion

Ranked as the second most prevalent bacterial infections in humans, urinary tract infections (UTIs) are predominantly attributed to the activity of uropathogenic *Escherichia coli* (UPEC) (Rosen and Klumpp, 2014). Other than UPEC other pathogens are also involved in causing UTIs such as *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcu*s spp, Group B *Streptococcus* (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida* spp in order of prevalence (Medina and Castillo-Pino, 2019). *Klebsiella pneumoniae* is reported to be the second most common pathogen in causing urinary tract infections. These strains are recognized for their capacity to elicit urinary tract diseases, they also inhabit the human intestinal tract as constituents of the normal microbial community.

The spectrum of UTI severity spans from mild manifestations to severe sepsis, with mortality rates ranging between 20% and 40% (Zhu et al., 2021). The classification of UTIs encompasses both community acquired and nosocomial infections. These infections exhibit diverse clinical presentations, varying from symptomatic to asymptomatic occurrences, while also encompassing the distinction between complicated and uncomplicated cases. Additionally, recurrent UTIs afflict a significant proportion of females. These recurrent occurrences persist despite antibiotic treatment, frequently involving the same pathogenic agent that initiated the initial infection.

Parallel to the uropathogenic *Escherichia coli*, *Klebsiella pneumoniae* takes a comparable stance in the domain of urinary tract infections. *Klebsiella pneumoniae* is often an agent of nosocomial infections, can evoke a range of disease severities, mirroring the array from mild manifestations to severe, life-threatening outcomes. In the realm of *Klebsiella pneumoniae*-induced UTIs, the distinction between uncomplicated and complicated presentations holds significance, mirroring the

spectrum observed in *Escherichia coli*-driven infections. Recurrent UTIs caused by the persistence of the same pathogenic strain are a huge challenge because antibiotic therapies despite their application frequently fail to eradicate the underlying infection properly allowing the causative strain to persist and trigger subsequent episodes.

The recurrence rates of urinary tract infections have increased the antimicrobial resistance among *Klebsiella pneumoniae* strains. This leads to an increase in the economic burden of these infections throughout the world. *K. pneumoniae* strains harbors extragenetic material (on PAIs) that codes for genes that contribute to UTI pathogenesis which makes them different from the commensal strains of the *K. pneumoniae* (Alteri et al., 2009). The transferable plasmid is responsible for the wide spread of the virulent MDR genes of *Klebsiella pneumoniae*. The emergence of these strains carrying ESBL genes has been well documented worldwide (Kot, 2019; Peirano et al., 2011). Detailed work has been carried out on different *K. pneumoniae* lineages around the world but not much known about local *K. pneumoniae* lineages in Pakistan including their genetic attributes, their resistance and virulence profiles, the genetic diversity of *K. pneumoniae* strains in Pakistan and the recent antimicrobial resistance trends.

Therefore, in this study we have reported the WGS of a local multidrug resistant UTI causing isolate KP3 and conducted a detailed comparative genome analysis with complete genome sequences of the global UTI causing *K. pneumoniae* strains (n=47) with the purpose to get insightful information regarding the genomic divergence and correlate virulence factors and antibiotic resistance profiles. The WGS and comparative genome analysis of local UTI causing isolate KP3 revealed that it is a virulent multidrug resistant strain with resistance determinants and virulence factors similar to those that were reported in the *K. pneumoniae* strains around the world. The strain KP3 possess

mobile genetic elements harboring the resistance gene as well as virulence gene responsible for disease severity and resistance against certain antibiotics specifically β-Lactams and carbapenems.

The local *K. pneumoniae* isolate KP3 belongs to sequence type 5 which is currently one of the most prevalent extraintestinal *K. pneumoniae* lineage. Phylogenetic analysis of selected *K. pneumoniae* strains using distinct phylogenetic markers including 16s rRNA, pangenome, single nucleotide polymorphism (SNP) provided consistent branching patterns suggesting similar phylogeny for UTI causing *K. pneumoniae* strains. The local KP3 isolate is evolutionary related to *K. pneumoniae* reference strain HS11286 and a strain ATCCBAA_2146 (Chapter 5) reported from USA. Both strains have similar virulence and resistance profile, suggesting a common ancestral UTI causing *K. pneumoniae* strain. Moreover the pangenome of the UTI causing *K. pneumoniae* is an open pangenome (Wang et al., 2022) and further addition of WGS of the strains may further increase its size. Our estimation suggested a total of 17520 genes and 11,364 unique genes in the *K. pneumoniae* pangenome depicting high genomic diversity among the studied *K. pneumoniae* strains and that its evolving through acquisition of the genes and diversification.

The escalation of antibiotic resistance among *Klebsiella pneumoniae* strains has grown to a critical concern on a global scale, posing substantial challenges for the healthcare department. The local *Klebsiella pneumoniae* isolate KP3 has emerged as a formidable multidrug resistant strain, displaying resistance to a range of antibiotics routinely employed for treating urinary tract infections in Pakistan. The outcomes of phenotypic antibiotic susceptibility testing revealed resistance across 10 distinct antibiotics, concordant with the results of genotypic analysis. Primarily, the isolate KP3 harbors resistant genes within both its plasmid and genomic structure, conferring

resistance against this spectrum of antibiotics. Urinary tract infections resulting from multidrug resistant *Klebsiella pneumoniae* strains play a very critical role in shaping the therapeutic strategies. The implications extend beyond individual cases, affecting the viability of empiric therapy aimed for diverse microbial agents responsible for causing UTIs. Furthermore, the phenomenon of co-selection is accentuated, wherein pathogens with inherent resistance attributes are preferentially favored. This not only exacerbates the challenge of treatment but also perpetuates the cycle of antibiotic resistance.

In contrast to previous studies conducted on *Klebsiella pneumoniae* our local strain has demonstrated a different and unique Sequence Type (ST5) which has not been reported before this (Zhou et al., 2020). Although the resistance mechanism identified in KP3 is similar to the resistance mechanism of globally reported UTI causing strains i.e., *K. pneumoniae* carries very prominent resistance against β-Lactams and carbapenems (Dunn et al., 2019). Same is the case with virulence factors, KP3 carries similar virulence factors as other globally reported *K. pneumoniae* strains for UTI (Karampatakis et al., 2023).

Plasmid analysis of UTI causing *K. pneumoniae* isolate revealed that the local strain carries 5 plasmids designated as pKP3-1 (IncX3), pKP3-2 (Col8282), pKP3-3 (IncFII), pKP3-4 (IncFIA) and pKP3-5 (IncFIB (AP001918) with high sequence similarity to already reported plasmids of *K. pneumoniae* (Al-Marzooq et al., 2015). Two of the plasmids pKP-3 (IncFII) and pKP-4 (IncFIA) were found to carry resistance against β-Lactams and Carbapenems. Several factors are involved in resistance genes dissemination among causative pathogens whereby the plasmid mediated HGT of MDR is the most significant mechanism.

Urinary tract infections are commonly encountered infections in Pakistan predominantly among females of all age groups and antibiotic resistance is rapidly growing. This has become one of the most urgent threats to public health worldwide. The emergence of new resistant bacteria is on the rise, endangering the efficacy of existing antibiotics and the resistance patterns are spreading continuously. *Klebsiella pneumoniae* is one of the most common multi-drug resistant infectious bacteria and are mostly resistant to β-Lactam antibiotics. Over a period of time, a steady rise in number of *K. pneumoniae* isolates in Pakistan's population has been recorded. Therefore, there is an urgent need to control this fast-growing antimicrobial resistant pathogen through approaches alternative to antibiotics for the future.

Whole Genome Sequencing (WGS) has significantly enhanced our understanding of bacterial pathogens like Klebsiella pneumoniae by offering in-depth genetic insights. Nevertheless, it comes with several limitations for studying Klebsiella pneumoniae including, analyzing WGS data demands specialized bioinformatics expertise and can be computationally intensive. This complexity poses a challenge for smaller laboratories or institutions lacking the necessary resources. WGS generates vast datasets that may be difficult to store and manage, particularly for labs without dedicated infrastructure. The expenses associated with sequencing equipment, reagents, and computational resources for analysis can be substantial, making WGS less accessible to certain researchers and healthcare facilities. While WGS offers extensive genetic information, deciphering the functional implications of genetic variations can be intricate. Not all identified genetic changes through WGS will have a known or understood impact on the organism's biology. The accuracy and representativeness of WGS results depend on the quality of the initial bacterial isolate and the sampling process. An unrepresentative isolate can lead to biased conclusions. Although the time

required for WGS has reduced significantly, it may still be slower than other diagnostic methods like PCR, which can provide rapid results in specific clinical situations. The accuracy of genetic variant interpretation relies on the availability and completeness of reference databases. Incomplete or outdated databases can constrain the interpretation of WGS data.

CHAPTER 6

Conclusion and Future Prospects

The comparative analysis of the KP3 isolate whole genome has unveiled a concerning picture, it is a multi-drug resistant and virulent strain responsible for urinary tract infections (UTIs). This strain has been identified as part of Sequence Type 5 (ST5) and is closely related to the reference strain HS11286 and the USA-based strain ATCCBAA_2146. Additionally, our pangenome analysis has revealed a dynamic genetic landscape in *Klebsiella pneumoniae*, with an open pangenome reflecting high genetic diversity, continually evolving through the addition of new genome.

The rising tide of antibiotic resistance, exacerbated by the misuse of antibiotics, underscores the pressing need for alternative strategies to combat UTIs in Pakistan. Specifically, the development of vaccines and novel drugs is imperative for the effective control and management of UTIs. Ongoing monitoring of UTIs is essential to track the prevalence of *Klebsiella pneumoniae* in the region and to keep a close eye on antibiotic resistance trends.

In the future, there is a critical need to expand our efforts in whole genome sequencing of *K. pneumoniae*. Estimating the pangenome size of this species will enable us to comprehensively study its population structure and diversity. This information will be invaluable in the healthcare sector for implementing measures to control and prevent the further spread of UTIs.

Moreover, it is imperative to recognize UTIs as a social issue, as they not only impact an individual's health but also disrupt their daily life, affecting quality of life, occupation, and relationships. By addressing UTIs holistically, we can work towards safeguarding public health and well-being in our community.

CHAPTER 7

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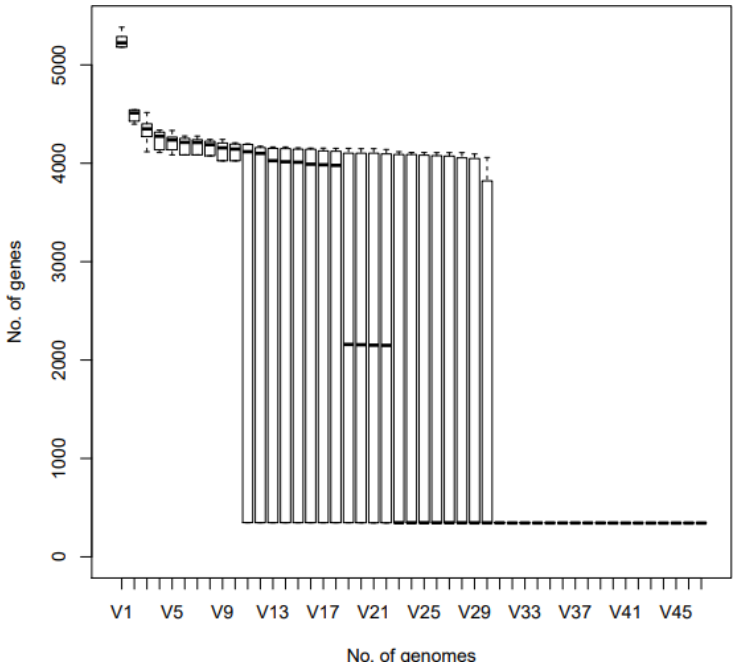
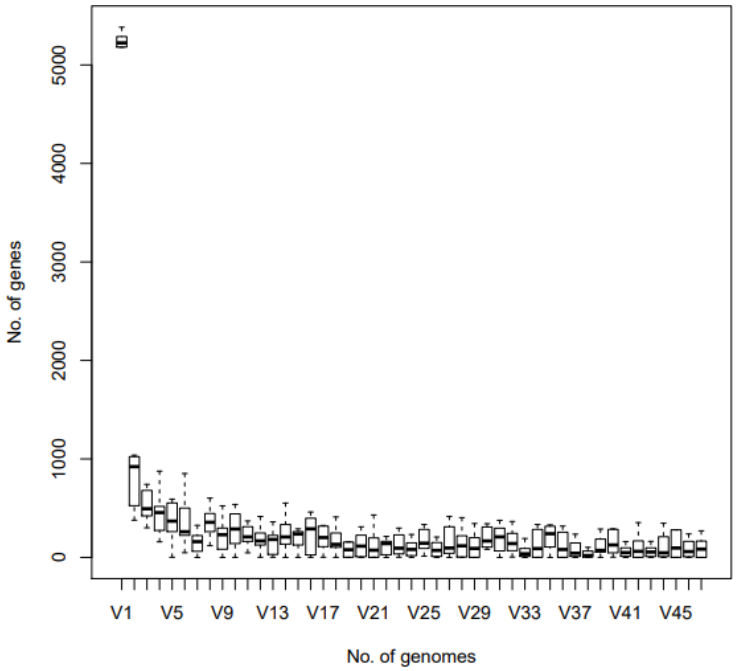
CHAPTER 8

Supplementary Information



Supplementary Figure 1 Graph representing number of Conserved Genes oberved in *Klebsiella pneumoniae* pangenome analysis.



Supplementary Figure 2 Graph representing number of new genes identified in isolate KP3.

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Mahnoor Masood

Reg number: 00000327665

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MS Industrial Biotechnology

Supervised by:

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