MAKERERE  **UNIVERSITY**

GENOMIC DETERMINANTS OF VIRULENCE AND ANTIMICROBIAL RESISTANCE IN COLISTIN RESISTANT *KLEBSIELLA PNEUMONIAE* ISOLATES FROM MULAGO NATIONAL REFERRAL HOSPITAL

MAXIMILIAN ALEX KILANGI MAGULYE, BSc, MD (Dar)
(REG.NO. 2020/HD07/21544X)

SUPERVISORS

DR. ERIC KATAGIRYA (MBChB, M.Med)

DR. SAVANNAH MWESIGWA (BSc, MSc, PhD)

DR. BEATRICE ACHAN (B.D.S, M.Med, MRes, PhD)

A DISSERTATION SUBMITTED TO THE
DIRECTORATE OF RESEARCH AND GRADUATE TRAINING
IN PARTIAL FULFILLMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN IMMUNOLOGY AND CLINICAL MICROBIOLOGY OF MAKERERE UNIVERSITY

AUGUST 2023

DECLARATION

I, **Maximilian Alex Kilangi Magulye**, declare that this dissertation is my own original work andthat it has not been submitted or presented to any institution or university for any award.

Signature …… Date 29/08/2023

This thesis has been submitted for examination with the approval of the following supervisors

Signature…… Date…August 29, 2023……

Dr. Eric Katagirya (MBChB, MMED)

Signature…… Date29th August 2023……

Dr. Savannah Mwesigwa (BSc, MSc, PhD)

Signature... Date…August 29, 2023……

Dr.Beatrice Achan (B.D.S, M.Med, MRes, PhD)

DEDICATION

My friend once told me, “Only four groups of people will ever read your dissertation; You, Your supervisors, Your Examiners, Your unlucky girlfriend who has to act as an unpaid proofreader for you. Nobody else will ever read it or care what it says”.

Therefore, I dedicate this dissertation to myself; it stands as a monument to my determination, tenacity, and strength.

ACKNOWLEDGEMENT

While dissertations are researched and written alone, they are not entirely solitary endeavours and pretending so would be disingenuous. Indeed, completion of this dissertation would not have been possible without the professional and personal support of a "village" of magnificent individuals. A complete list of everyone who had a hand in the completion of this dissertation would likely be longer than the dissertation itself. I only say thank you all! However, it would be unconceivable if I do not salute the following extraordinary persons in particular;

Dr. Eric Katagirya: As my supervisor, he ensured that my work stayed on course, he went through my research proposal and elevated it with his formatting expertise and eagle eye for detail, guided me on experiments and dissertation write-up. Indeed, I am so grateful for his genomics expertise, valuable advices and remarkable suggestions during the whole period of studies and research. His contribution to my study is unmatched. Thank you very much Eric, You are the fountain of knowledge and expert scientist and I have learnt much from you. May you always walk the glorious road to success and achieve greatness.

I am beholden to my co-supervisors for their expertise and guidance throughout this process. Dr. Savannah Mwesigwa, a pillar in genomics, his unrelenting expertise in my study remains valuable today and ever. Dr. Beatrice Achan, a forthright giant and illustrious mycology scholar, her carrier has impacted and inspired me.

My deep gratitude and appreciation to Dr. Simon Sekyanzi for his kindness of granting me permission to carry out my research using his stored samples. May your kindness and passion of health research persist. I also thank Reuben Maghembe, for his friendship as we had numerous life discussions. But most importantly being an unofficial molecular biology mentor. Our paths in academia may cross many more times in the future.

While in the Clinical Microbiology lab and Molecular Biology/Genomics lab, I encountered a group of jolly and fabulous lab technologists and technicians. They are clearly the unsung heroes who, for reasons I have never understood, have almost always preferred to stay in the background. I thank them all for their immeasurable support and advice on many of my failed experiments.

I would be remiss in not mentioning my colleagues with whom we studied together, in particular Gideon Nsubuga, for exceptional class leadership and his treasured support. It is his kind help and support that have made my study and life in Uganda a wonderful time. My colleague, Moses Levi Ntayi, we had insightful immunology discussions, I appreciate! Thanks to Robert Maximilian, a charming person, for the cherished time we spent together in social settings.

I am also deeply thankful to my girlfriend. Her name cannot be disclosed. I admire her positive outlook and her ability to smile despite the situation. For some unknown reason, she always thought I knew everything and very unfortunately, she let me know this. But I am now convinced that it is mainly this sense of pride that instilled in me confidence and courage to ride out the storms of Immunology and Clinical Microbiology inferiority. Her words of encouragement and push for tenacity ring in my ears. I fully thank her for sticking by my side even when I was irritable and depressed.

I owe my warm thanks to my family, especially my parents, Alex Kilangi and Sweetbertha Donant, for their unconditional love and for motivating me to higher achievement.

I would grossly fail in my duty if I do not acknowledge authors I haven't mentioned or cited directly but whose works have nonetheless influenced me.

Last but definitely not least, I would like to give my self credits for being patient and resilient during the period of my study which was marred by COVID-19 pandemic.

Laying it on the line, the last days of writing this not-so-much masterpiece felt like resurfacing from the ashes.

Like a phoenix.

Now I can breathe.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS/ACRONYMS

AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
BLAST	the Basic Local Alignment Search Tool
BSI	Bloodstream infections
CARD	The Comprehensive Antibiotic Resistance Database
CLSI	Clinical and Laboratory Standards Institute
CPS	Capsular Polysaccharide
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic Acid
ESBL	Extended Spectrum β-lactamase
GBDP	Genome BLAST Distance Phylogeny
GPP	Global Priority Pathogens
HGT	Horizontal Gene Transfer
ICE	Integrative and Conjugative Elements
ICU	Intensive Care Unit
IS	Insertion sequences
LPS	Lipopolysaccharide
MDR	Multidrug resistance
MeDuSa	Metagenomic Data Utilization and Analysis
MGE	Mobile Genetic Elements
MHA	Muller-Hinton Agar
MLST	Multilocus Sequence Typing
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
PATRIC	Pathosystems Resource Integration Centre
PCR	Polymerase chain reaction
PDB	protein Data Bank

PDR	Pandrug-resistant
SNP	Single nucleotide polymorphism
SPAdes	St. Petersburg genome assembler
TYGS	Type Strain Genome Server
UniProt	the Universal Protein Resource
UTI	Urinary tract infections
VFDB	The Virulence Factor Database
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively Drug Resistant

DEFINITION OF KEY TERMS AND CONCEPTS

K.pneumoniae: Belongs to the enterobacteriaceae family and is described as gram negative encapsulated, non-motile, facultattively anaerobic bacteria.

Draft genome: sequence of genomic DNA having lower accuracy than the finished sequence; some segments are missing or in the wrong order or orientation. Though the DNA sequence has enough accuracy and continuity to allow initial genomic analysis and annotation

Full/Whole genome: the complete genomic DNA sequence of a cell

Whole genome sequencing is the process of determining the entirety or nearly the entirety of the genomic DNA sequence of a cell at a single time, providing the most comprehensive characterization of the genome.

Antimicrobial Susceptibility testing: Testing of a strain of *Klebsiella pneumoniae* for its resistance to one or more antibiotic drugs

Multi-drug resistance (MDR): *Klebsiella pneumoniae* strain that is resistant to at least one agent from three or more antibiotic classes

Extensively drug resistance: *Klebsiella pneumoniae* strain that is resistant to at least one agent in all but two or fewer antimicrobial categories

Pan-drug resistance (PDR): *Klebsiella pneumoniae* strain that is resistant to all antimicrobial compounds

ABSTRACT

Introduction*:* Multidrug resistance and high virulence in *K. pneumoniae* are of particular concern and have emerged as critically alarming threats to the global community. Worringly, *K.pneumoniae* has become resistant to colistin, the last resort antibiotic for carbapenem resistant *K. pneumoniae* making the treatment of the infections more challenging. The objectives of this study were to determine hypermucoid *K.pneumoniae* isolates, delineate the virulence and antimicrobial resistance determinants of colistin resistant *K.pneumoniae* and determine the phylogenetic relatedness of each *K.pneumoniae* strain.

Methodology: All 31 archived multidrug resistant K. pneumoniae isolates were retrieved and instantly inoculated on MacConkey agar for recovery followed by re-identification using conventional biochemical methods. The isolates were then subjected to phenotypic characterization of the hypermucoid *K.pneumoniae* using string test. Additionally, the isolates were subjected to antibiotic susceptibility against 16 antibiotics.. Genomic DNA isolation from the overnight culture of the isolates was performed using CTAB method. Then library preparation and sequencing were done using illumina novaseq platform.

Results: The isolates were found to be multidrug resistant and colistin resistance was detected in 7/31 (22.58%) isolates. The sequence analysis of the commonly known genes involved in colistin resistance revealed several non-synonmous mutations in nucleotide sequences of pmrA/pmrB, phoP/phoQ and mgrB genes rendering resistance to colistin. Interestingly, the isolates also harbored mcr-1 gene in the chromosome. More so, the isolates harbored several virulent genes as several virulence factors genes such as fim genes for adherence, enterobactin, salmocherin aerobactin, clpvi for effector delivery system or waaA genes for LPS were found in colistin resistant *K. pneumoniae* strains.

Conclusion: The study clearly show the occurrence of colistin resistance and high virulence genes in clinical isolates which is alarming and warrants attention as it can lead to untreatable and invasive K. pneumoniae infections. It is therefore imperative to conduct active surveillance in order to prevent the transmission and spread of highly viurulent or multidrug resistant strains, focusing not only on antimicrobial resistance but also on identifying virulence determinants.

CHAPTER ONE: INTRODUCTION

1.0 Background

Klebsiella pneumoniae (K. pneumoniae) is a species from the family Enterobacteriaceae, which represents a diverse group of Gram negative bacteria abundant in nature and commonly found in soil, water and other surfaces. In human it forms part of the gastrointestinal flora. In the general community, 5% to 38% of individuals carry the organism in their stool and 1% to 6% in the nasopharynx (Ashurst & Dawson, 2022). In hospitalized patients, colonization rates in nasopharynx rise to 19% while it can be as high as 77% in the gastrointestinal tract, (Chang et al., 2021).

Classical *K. pneumoniae* is an opportunistic pathogen associated with myriad of serious hospital-associated infections primarily in immunocompromised individuals and with other comorbidities like diabetes, cancer and organ transplantation, elderly and neonates, accounting for about one third of all Gram-negative infections overall (Navon-Venezia *et al.*, 2017). These infections include urinary tract infections (UTI), blood stream infections (BSI), wound and soft tissue infections and respiratory tract infections like pneumoniae.

Moreover , K. pneumoniae is one of the species recognized as ESKAPE pathogens. The acronym ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeuginosa and Enterobacter species) denotes environmental or commensal bacteria that cause opportunistic infections in hospitalized or immunocompromised patients and have the ability to escape or evade killing by antibiotics and to rapidly develop multiple drug resistance. This resistance against antimicrobials leads to limited therapeutic options, resulting in increasing difficulties of treatments and death.

Recent research has uncovered sporadic evolution of *K. pneumoniae* accompanied with acquisition of additional genetic traits underlying the emergence of novel strains, causing unique clinical syndrome of community-acquired, severe and tissue-invasive infection in otherwise healthy individuals that often present in multiple sites or subsequently spread (metastatic spread)(Nakamura-Silva et al., 2021). The infections include pyogenic liver abscess, endophthalmitis, meningitis and necrotizing fasciitis (Mansour et al., 2017)).

Although several studies in different parts of the world reveal the overwhelming virulence and resistance of *K. pneumoniae* to commonly used antibiotics, there is paucity of molecular details with regard to virulence and resistance especially in East Africa and Uganda is no exception. This scenario necessitate more comprehensive research particularly application of genomics that could shed light into the molecular details on *K. pneumoniae*. Therefore, this study sought to establish genetic features determining the virulence and antimicrobial resistance pattern of *K. pneumoniae* clinical isolates from Mulago National Referral Hospital in Uganda.

1.1 Statement of the problem

Klebsiella pneumoniae is now recognized as a major pathogen of global concern due to the emergence of hospital acquired infections associated with multidrug-resistance and community acquired infections due to high virulence. Several reports have revealed that carbapenem, a preferred agent for treatment of serious K.pneumoniae infections is no longer effective to some strains. Alarmingly, resistance to colistin, an antibiotic used as a last-resort treatment of carbapenem resistant *K. pneumoniae* infections have been detected in many countries, making infections caused by this bacterium untreatable by our current arsenal of antibiotics. Despite the high proportion of AMR, emergence of colistin resistant and virulent *K.pneumoniae*, only a restricted number of studies have been conducted in Uganda. More so, detection have been reliant on culture-based antimicrobial susceptibility testing (AST) and genetic typing methods, such as multi-locus sequence typing (MLST) which generally provides little data regarding the resistance mechanisms therefore hampering the efforts to clearly substantiate the possible causes of *K.pneumoniae* resistance. Whole-genome sequencing (WGS), in contrast, provides genome-wide information at the single nucleotide level that can provide insights into the genetic basis of resistance mechanisms, (Argimón et al., 2020), (Amr, 2020) as well as pathogen identity, virulence, and ancestry, with the potential to unveil some previously untapped information at least from the genetic level.

1.2 Research objectives.

1.2.1 General objective.

To delineate the genomic features underlying the virulence and antibiotic resistance determinants of colistin resistant *K. pneumoniae* isolates from Mulago National Referral Hospital

1.2.2 Specific objectives

Specific objectives of the study were;

(i) To determine hypermucoid *K. pneumoniae* isolates.
(ii) To delineate the virulence genes of *K. pneumoniae* isolates
(iii) To determine antimicrobial resistance genes of clinical *K. pneumoniae* isolates.
(iv) To determine the phylogenetic relatedness of the *K.pneumoniae* isolates.

1.3 Significance of the study

Given the public health and clinical importance of *K.pneumoniae*, the study data from WGS are important to public health surveillance about the relatedness of the pathogen to other strains to investigate transmission routes, monitor trends over time and allow the control of the threat. This study has the potential to uncover novel strains with potentially unique genotypes from SNP typing or pangenome comparative analysis. The study will also contribute to the national and international knowledge bank through provision of reference data and deeper understanding about the underlying biological mechanisms of virulence and antimicrobial resistance.

1.4 Conceptual framework

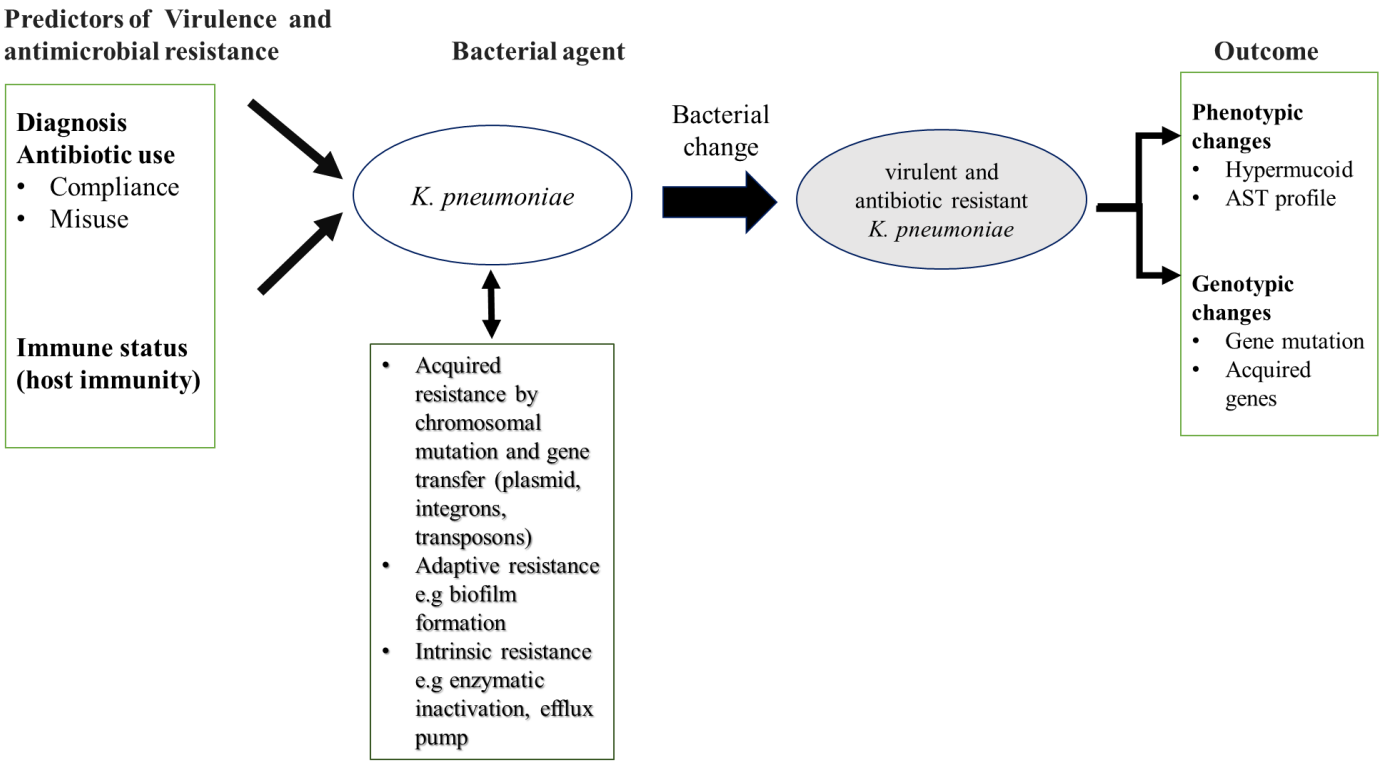


Figure 1. Conceptual framework of the study: The *K.pneumoniae* undergo changes which may result into virulent and antimicrobial resistant strains.

CHAPTER TWO: LITERATURE REVIEW

2.1 Genus Klebsiella

Bacteria of the genus *Klebsiella* was first described by Carl Friedlander in 1882 and designated in honour of the German microbiologist Edwin Klebs three years later in 1885. (Brisse et al., 2006). The genus Klebsiella belongs to the family *Enterobacteriaceae* and is described as the Gram-negative, encapsulate, non-motile, rod shape and facultative anaerobic bacteria. It is found ubiquitously in nature, including in plants, animals and human. In human the genus *Klebsiella* normally forms part of the healthy human microbiome and is particularly concentrated in the gastrointestinal tract and a few in the nasopharynx (Joseph et al., 2021). Among the *Klebsiella* species, *K. pneumoniae* is clinically important.

2.2 Taxonomy

The taxonomic hierarchy of *K.pneumoniae* is as follow

Domain: Bacteria

Phylum: Pseudomonadota

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: Klebsiella

Species: *Klebsiella pneumoniae*

2.3 Genome of *K. pneumoniae*

Typical *K. pneumoniae* genomes are large and diverse genome with an average size of ~5-6 Mbp encoding ~5000-6000 genes (Wyres et al., 2020). The core genome consists of ~1700 genes that are present in all strains regulating basic functions for survival in different environments. In

addition, *K. pneumoniae* host ~3300-4300 accessory genes that varies between strains illustrating a large adaptive capacity (Kl & Ke, 2018). These accessory genes encode specific virulence factors and antibiotic resistant enzymes and mechanisms and can be acquired by horizontal gene transfer (Martin & Bachman, 2018)

2.4 Hospital acquired and community acquired infect ions

K.pneumoniae is an important opportunistic pathogen. This pathogen is responsible for a significant proportion of hospital-acquired infections including respiratory tract infections like pneumonia, urinary tract infections, blood stream infections as well as skin and soft tissue infections accounting for about one-third of all Gram-negative infections. (Joseph et al., 2021). These infections occur primarily in immunocompromised individuals, elderly and neonates and those with other comorbidities like diabetes, cancer and organ transplantation. *K.pneumoniae* is also responsible for community acquired infections worldwide. The infections occur in otherwise healthy and immunocompetent individuals and show unique clinical syndromes and serious disseminated infections such as liver abscesses, meningitis and osteomyelitis , endophthalmis in younger and health population with ability to metastatically spread and cause high morbidity and mortality.(Zhu et al., 2021).

2.5 Virulence of Klebsiella pneumoniae

Virulence is the ability of a microorganism to cause disease in the host. The virulence is contributed by the sum of structural, biochemical, and genetic characteristics. *K.pneumoniae* expresses a variety of virulence factors such as capsule, siderophore the iron acquisition system adhesins and endotoxins which are very important in its pathogenesis. Capsule expression is a very important factor, it does two major function, preventing phagocytic uptake by the bacterium; reduce complement activation by the host immune system. Recent epidemiological studies have shown that *K. pneumoniae* causing community acquired infections have unique genetic traits; hypermucoviscosity phenotype due to heavy encapsulation and numerous virulence genes like with mucoid phenotype A (rmpA) gene, mucoviscosity-associated gene A (magA) and capsule up-regulation genes (rmpA and rmpA2). (W.-L. Yu et al., 2006). Other hypervirulence genes in *K.pneumoniae* involve the siderophore systems for Iron acquisition; Yersiniabactin (*ybt*) aerobactin (*iucABCD* and *iutA*), colibactin and salmochelin

(*iron* and *iroBCD*) (Y. Liu et al., 2021). the bacteriocin and microcin. Bacteria require iron for their replication and siderophore are the iron carriers. They can efficiently acquire iron than the host transport protein (transferrin) Adherence factor (Adhesins) like lipopolysaccharide presence and pilli or fimbriae also enhance *K. pneumoniae* virulence associated with invasive infection (Lee et al., 2017).

2.6 Antimicrobial agents

Antibiotics are the agents that are used in the treatment of bacterial infections in or on the body by either killing or inhibiting the growth and replication of bacteria. Since the discovery of Antibiotics by Alexander Fleming in 1928 and their wide clinical use in 1940, antibiotics have been the most prominent tools for the treatment of infectious diseases. Today's specialised modern medicine, like intensive care, cancer therapy and advanced surgery, rely on these potent antibacterial agents. These antibacterial agents are classified into five major groups on the basis of their mode of action (i) Cell wall synthesis inhibitors which include β-lactam antibiotics (penicillins, cephalosporins, carbapenems, and monobactams), glycopeptides (vancomycin, daptomycin, and teichoplanin) and other antibiotics such as fosfomycin and bacitracin. (ii) Protein Synthesis Inhibitors which include aminoglycosides (kanamycin and gentamicin), tetracyclines (tetracycline and tigecycline), Macrolides (erythromycin, azithromycin), and lincosamides (lincomycin and clindamycin) (iii) Antibiotics that target nucleic acid synthesis; Antibiotics inhibiting DNA synthesis such as metronidazole, quinolones and fluoroquinolones (nalidixic acid, ciprofloxacin, levofloxacin, ofloxacin and norfloxacin) and rifampin (targets RNA synthesis) (iv) Inhibitors of Tetrahydrofolate Biosynthesis/antimetabolite (Trimethoprim, sulphonamides and dapsone) (v) cell membrane disintegration such as polymyxin (Polymyxin B and Colistin) and daptomycin.

In the treatment of *Klebsiella pneumoniae* infections the relevant antimicrobial agents which are used include β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems), aminoglycosides (gentamicin, amikacin), fluoroquinolones (ciprofloxacin, levofloxacin, ofloxacin), trimethoprim/sulfamethoxazole and colistin.

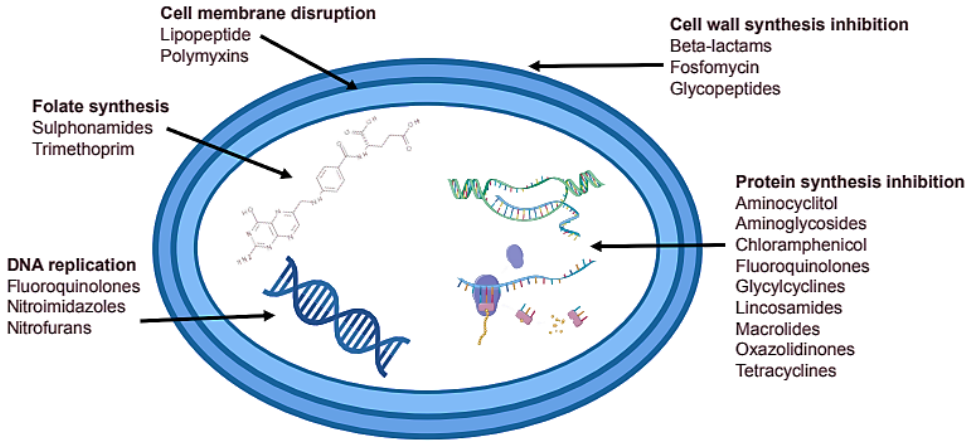


Figure 2. Mechanism of antibiotics action (Adopted from Shaikh et al. 2015 with modification) (Shaikh et al., 2015)

2.6.1 Colistin

Colistin, is an "old sort" antibiotic regarded as "last-line' drug for the treatment of multi-drug-resistant (MDR) and extensively drug-resistant Gram-negative bacteria , when no other options are available. It is a multi-component cyclic cationic polypeptide belonging to a group of antimicrobial agents called polymyxins which was originally isolated in Japan in 1947 by Y. Koyama from a broth of *Paenibacillus polymyxa* subsp. *colistinus*.(El-Sayed Ahmed et al., 2020) There are four groups of polymyxins namely Polymyxin A, B, C, D and E, of which only Polymyxin E (Colistin) and Polymyxin B are used clinically in humans. Colistin is a bactericidal antibiotic directed against selected gram-negative bacteria, including *Klebsiella species*, *Pseudomonas aeruginosa*, *Acinetobacter* species but it is ineffective against Gram-positive bacteria and mycoplasmas. The exact mechanism to explain the antibacterial action of Colistin is still elusive. However the most plausible mechanism is that, colistin targets lipopolysaccharide (LPS) of the gram-negative bacteria membrane (Velkov et al., 2013). Colistin has a strong positive charge and a hydrophobic acyl chain that give them a high binding affirnity to LPS

molecules. The cationic polypeptide of Colistin interact electrostatically with anionic LPS molecules and competitively displace divalent cations (magnesium and calcium ions) from them causing disruption of the membrane. Magnesium and calcium ions acts as stabilizers of the LPS molecules. Therefore, their disruption result in increased permeability of the cell envelope, leakage of the cell contents and subsequently, cell death. (Hamel et al., 2021). Other potential mechanisms of Colistin action have been described such as inhibition of vital respiratory enzymes and endotoxin activity of lipid A. Colistin inhibits endotoxin activity of lipid A by binding to LPS molecules and neutralizing them, therefore suppressing the induction of shock through the release of cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 8 (IL-8) (Dijkmans et al., 2015). Colistin acts via the production of reactive oxygen species (ROS) this is known as, Fenton reaction, causing damage of DNA, lipid and protein and end up with cell death (Dai et al., 2016). Another proposed mechanism is the vesicle-vesicle contact pathway which explains that colistin molecule with a hydrophobic acyl tail can enter into and cross the outer membrane and induce exchange of phospholipids between leaflets of the inner membrane and outer membrane; this leads to loss of membrane integrity, osmotic pressure compromise and cell lysis (Elias et al., 2021)

Though Colistin was available for use in gram-negative infections since 1950s it was abandoned in most parts of the world in the early 1980s because of the reported high incidence of nephrotoxicity and neurotoxicity (Tamma & Lee, 2009). However the emergence of bacteria resistant to most classes of available antibiotics and the paucity of new antimicrobial agents with activity gram-negative microorganisms have led to the reintroduction of Colistin into clinical practice for the treatment of carbapenem-resistant Gram-negative bacteria.

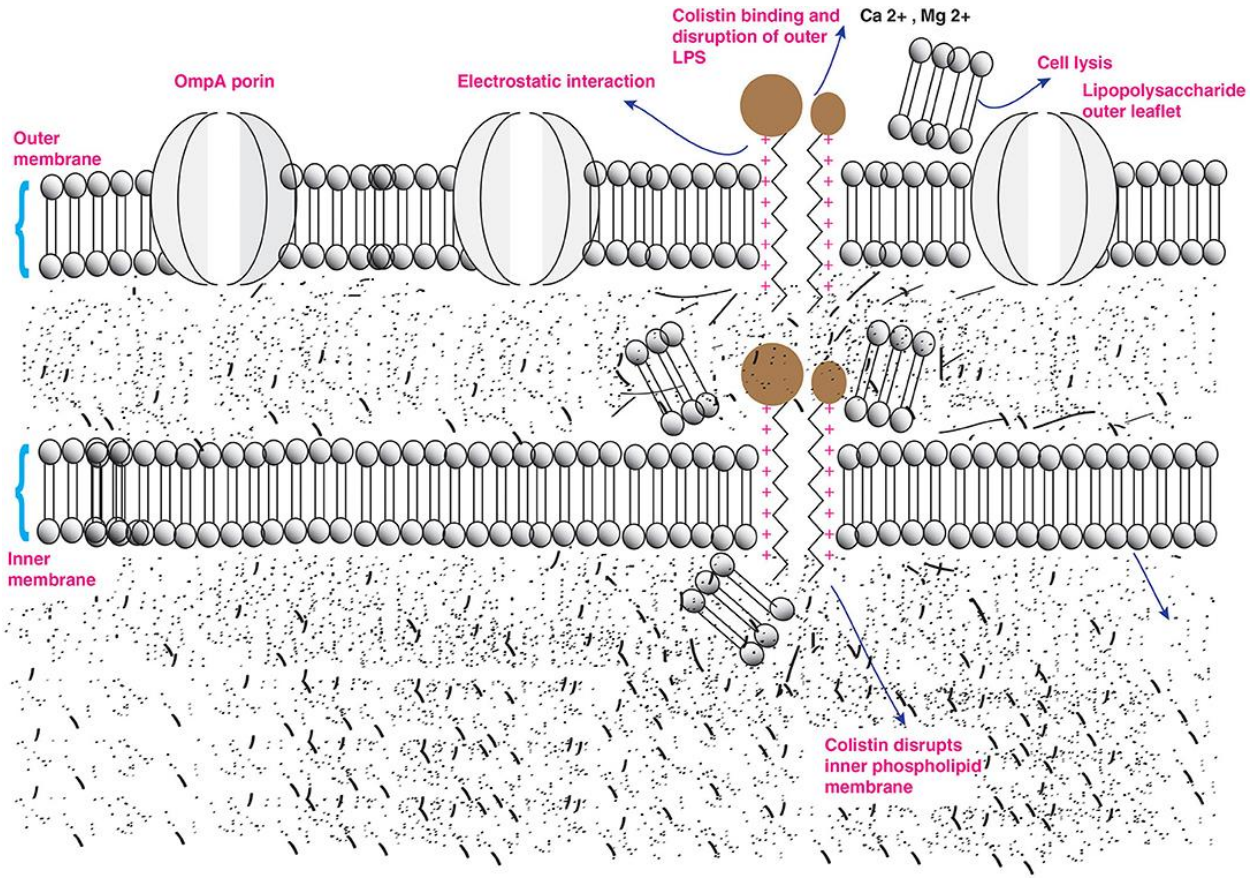


Figure 3. Mechanism of action of Colistin on a bacterial surface membrane. The cationic polypeptide of Colistin interact electrostatically with anionic LPS molecules on the bacterial cell membrane and competitively displace divalent cations (magnesium and calcium ions) from them causing disruption of the membrane and eventually leakage of the cell content and death Adopted from (Gogry et al., 2021)

2.7 Antimicrobial Resistance (AMR)

Antimicrobial resistance (AMR) is the ability of microorganisms (bacteria, fungi and viruses) to resist the action of drugs designed kill them through targeting different parts of the cell of the microorganism, which are crucial for growth and survival.(Kon & Rai, 2016). Resistance in bacteria may arise by de novo mutations or through acquisition of resistance genes from other organisms.(Wellington et al., 2013) The process of bacteria becoming resistant is natural event however it is accelerated by the misuse and overuse of antimicrobials. (Nikolich et al., 1994)

2.7.1 Genetics of antimicrobial resistance

2.7.1.1 Natural resistance

Natural resistance are either intrinsic (the genes are always expressed in the bacterial species) or induced (the genes are naturally occurring in bacteria, however they are only expressed to resistance levels after exposure to an antibiotic). The intrinsic resistance is related to the general physiology of bacteria and is controlled by chromosomes. Klebsiella spp are said to be intrinsically resistant to penicillins. Enterobacter spp are also intrinsically resistant to ampicillin, amoxicillin, amoxicillin-clavulanate, first generation cephalosporins and cefoxitin owing to the production of constitutive AmpC beta-lactamase (Emilio Bouza et al 2002)

2.7.1.2 Acquired resistance

AMR can be acquired vertically by chromosomal mutation or horizontally through acquisition of resistance genes carried by mobile genetic elements (MGE) such as plasmids, transposons, integrons and insertion sequences.

Chromosomal DNA mutation

Spontaneous mutation within chromosomal genes may confer resistance to antibiotics. Mutations may occur in genes encoding antibiotic modifying enzymes, genes encoding drug targets, genes encoding drug transporters or genes encoding regulators that control drug transporters. (Reygaert, 2018). These mutations may lead to structural and functional modifications in the cellular target ultimately antibiotic resistance. For instance chromosomal mutations are clinically important for streptomycin, rifampin, and trimethoprim and is largely involved in aminoglycoside-resistant clinical isolates. (Jacoby & Archer, 1991,Harbottle et al., 2006),. Mutation in the DNA gyrase genes are the most common mechanism of fluoroquinolone resistance (Roberts et al., 2021).

Horizontal gene Transfer (HGT)

This is the mobility of genetic material among bacteria. HGT has largely contributed to the spread of antibiotic resistant genes (ARGs) through the exchange of these resistance genes among diverse species (Le Roux & Blokesch, 2018). ARGs are not always transferred alone but can be found in mobile genetic elements (MGEs). Such MGEs include plasmids and integrative

and conjugative elements (ICEs) which are able to transfer between bacterial cells, insertion sequences, transposons and gene cassettes/integrons which are able to move within or between DNA molecules. The global dissemination of multidrug resistant bacteria carrying multidrug resistant plasmids demonstrate the rapid spread of ARG genes in these bacteria through horizontal gene transfer (Vinayamohan et al., 2022). In *K.pneumoniae*, Plasmid carry several antibiotic resistance genes. Several studies point out class I,II, III integrons as an important players in the dissemination of ESBL resistance gene and carbapenemase encoding genes causing Multidrug resistant (MDR) *K. pneumoniae*, with class I integron being most prevalent (Fahimeh et al., 2016). Also Insertion sequences are associated with the mobilization of antibiotic resistance determinants and the modulation of pathogenic characteristics. A study by Stephen Fordham et al indicate that the disruption of the mgrB gene by insertion sequences is a mechanism mediating colistin resistance in *K. pneumoniae* (Zaman et al., 2018). ICEs routinely encode a range of accessory functions, including virulence and antibiotic resistance. Three mechanisms of horizontal gene transfer are known to mediate the spread; transformation in which bacteria take up DNA from their environment, conjugation whereby bacteria directly transfer genes to another cell and transduction where bacteriophage move genes from one cell to another. ARGs are mostly associated with conjugative elements such as plasmids or transposons. The conjugation of MGEs conferring AMR has been observed in various ecosystems, from transfer between bacteria in the soil and water environment to food and healthcare associated pathogens (Davison, 1999)

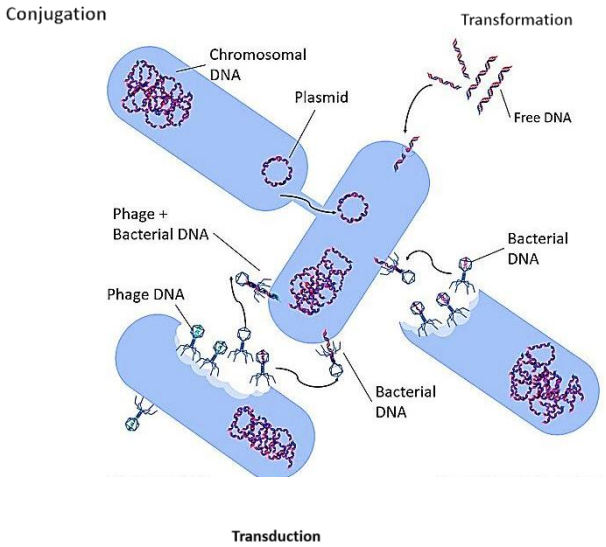


Figure 4. Mechanisms of Horizontal gene transfer (adopted from (von Wintersdorff et al., 2016)

2.7.2 Mechanism of Antimicrobial Resistance by *K. pneumoniae*

Multiple mechanisms play a role in antibiotic resistance by *K.pneumoniae;* restricted access of the antibiotic to its target either by efflux pump example several studies have shown that *K.pneumoniae* multidrug resistance is mediated by several efflux pumps; AcrAB, OqxAB and MFS efflux pumps.(Bialek-Davenet et al., 2015), reduction in number or modification of outer membrane porins (OmpK35 and OmpK36) to decrease the permeability of the membrane to the antibiotics, enzymatic inactivation of the antibiotic, for instance *K. pneumoniae* produce enzymes including extended-spectrum β-lactamases, metallo-β-lactamases, oxacillinases and carbapenemases, that hydrolize the β-lactam antibiotics (Kim et al., 2016).

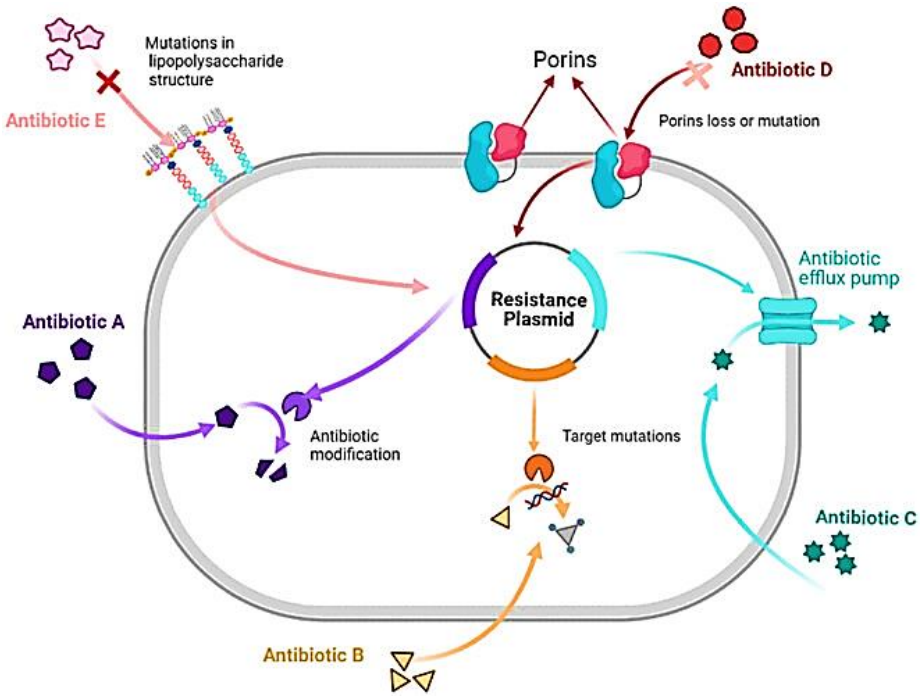


Figure 5. Mechanisms of resistance in Klebsiella pneumoniae (Adopted from (Y. Li et al., 2023)

2.7.3 Colistin Resistance in *K. pneumoniae*

Four main known mechanisms that give rise to colistin resistance include : (i) modification of the lipid A component in lipopolysaccharide (LPS) moiety (which has overall negative charge and is an initial target for Colistin) via the addition of cationic groups to the LPS. Such modification is achieved by modulation of lipid A moiety of LPS through addition addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) on lipid A that results in decrease in the negative charge on the bacterial surface. This reduces the electrostatic interaction between polycationic colistin and lipopolysaccharide resulting into subsequent reduction of lipopolysaccharide binding to colistin leading to resistance. (ii) enzymatic inactivation of Colistin (iii) activation and overexpression of a broad-spectrum efflux pump (iv) overproduction of capsular polysaccharide (CPS) in some Gram negative bacteria that hide the polymyxin binding sites and the release of CPS trapping polymyxins/Colistin. Several studies have elucidated that resistance pattern in *K. pneumoniae* is dependent on the number of capsule layers exhibited by the bacterium. Those *K. pneumoniae* strains with multiple capsule layer were observed to be more resistant to Colistin than those with few capsule layers. The expression of

multiple layers is thought to cause decreased electrostatic interaction of Colistin with its target site consequently resulting into colistin resistance. More so, a study conducted by Llobet et al., 2008 revealed that *K. pneumoniae* can shed capsular polysaccharides (CPSs) from its surface. The released CPSs can then trap or bind to polymyxins/Colistin, thereby reducing the quantity of drug that reaches the bacterial cell surface, resulting to polymyxin/colistin resistance (Llobet et al., 2008)

Lipid A modification with PEtN is mediated by either chromosomally mediated genes or plasmid-encoded genes. However L-Ara4N is mediated by genes located exclusively on the chromosome. Several studies on acquired colistin resistance in K. pneumoniae have revealed that chromosomal mutation in the regulatory genes PhoP/PhoQ, pmr A/pmrB and mgr B are important mechanisms leading to resistance (chromosomal mediated resistance). The mutation results in modification in the lipid A of the LPS on the bacterial surface, reducing the binding of Colistin. (Cheng et al., 2015)

Upon exposure to Colistin PhoP/PhoQ two-component system (PhoP/PhoQ TCS) encoded by PhoP and PhoQ genes is activated (Jaidane et al., 2018). The activation of PhoP/PhoQ TCS causes upregulation of the pmrHFIJKLM operon, that mediate the synthesis of lipid-modifying moieties L-Ara4N that binds to lipid A membrane . PhoP/PhoQ genes may also be involved in lipid modification by activation of pmrA/pmrB by pmrD (Pragasam et al., 2017)

Colistin resistance can be mediated by pmrCAB operon. Mutations in pmrA or pmrB genes results into activation of PmrA which in turn up-regulates PmrC a phosphotransferase and *arnBCADTEF-pmrE* operon (also called *pmrHFIJKLM-ugd*) that play essential role in the biosynthesis and addition of phosphoethanolamine (PEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A respectively (Olaitan et al., 2016)

MgrB is another genetic determinant that mediate Colistin resistance. MgrB is a small transmembrane protein produced upon the activation of the PhoP/PhoQ signaling system and acts as a negative regulator of this system (Haeili et al., 2017). It regulates the expression of etpB, which is related to the modification of LPS(Zafer et al., 2019). Inactivation or deletion of the mgrB gene causes the upregulation of PhoP/PhoQ system which in turn, activates the arnBCADTEF operon leading to L-Ara4N biosynthesis and consequently addition of L-Ara4N moiety to lipid A and thus enhancing colistin resistance.(Azam et al., 2021)

Plasmid mediated resistance which is basically the horizontal transfer of plasmid –borne mobile colistin resistance (mcr) genes play a critical role in the dissemination of Colistin resistance in various bacteria including *K. pneumoniae*. 10 mcr genes (mcr-1 to mcr-10) have been noted to disseminate worldwide, however only mcr-1, mcr-3, mcr-7 and mcr-10 have been reported in *K. pneumoniae*. The mcr gene encodes for the enzyme phosphoethanolamine transferase (PEtn) which adds PEtn residue to the lipid A moiety leading to a reduction of the negative charge of LPS thus decreasing the binding affirnity of colistin, consequently colistin resistance (Sun et al., 2018)

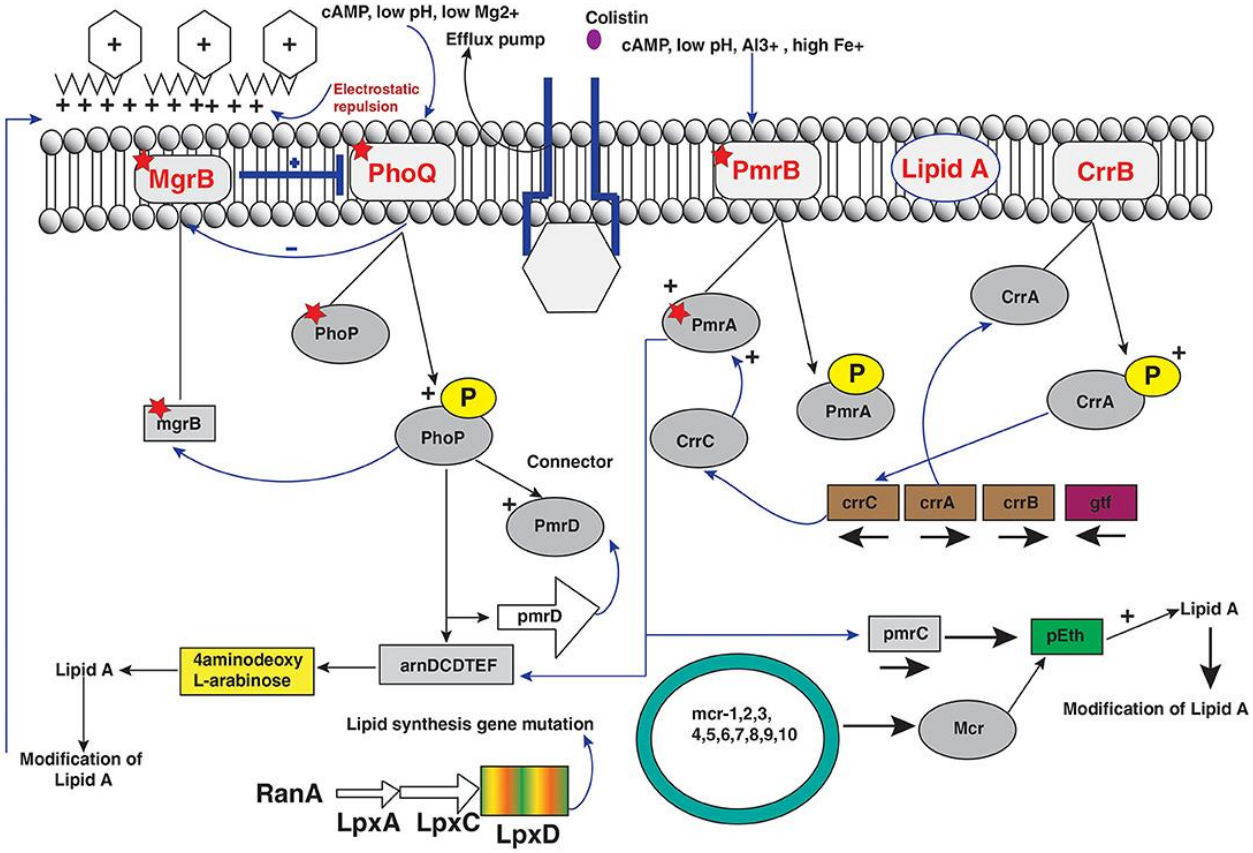


Figure 6. Colistin resistance mechanisms regulators involving chromosomal and plasmid-mediated pathways of lipopolysaccharide modifications in Enterobacteriaceae (Adopted from (Gogry et al., 2021)

2.7. 4 Antimicrobial resistance is global public health threat

After the introduction of the antibiotics in clinical use in the $1940_{S,}$ many doctors and public health professions thought that these antibiotics dubbed as "miracle drugs" are the super weapon that would give humans the final decisive victory over bacteria, and therefore it was the time to turn attention to non-infectious diseases like cancer, heart diseases, diabetes (Wall, 2020). However, we are now in a grim consequence of untreatable human pathogens because of the emergence of antimicrobial resistance by bacteria that can survive treatment with all sorts of antibiotics partly due to uncontrollable antibiotic consumption (Prestinaci et al., 2015). Suffice it to say, AMR is a silent pandemic and WHO has declared it as one of the top 10 global public health threats facing humanity with an estimation of 700000 deaths annually worldwide and every country is potentially affected (Jansen et al., 2018). In 2019 an estimated 1.27 million people died from antibiotic-resistant bacterial infections, more death than HIV/AIDS or Malaria (X. Li et al., 2022). If not properly addressed, the number is projected to grow to 10 million per year by 2050. Along with this, Infections with AMR leads to serious illnesses and prolonged hospital admissions, increases in healthcare costs, higher costs in second line drugs and treatment failures. According to different studies, it is projected that AMR could cost from $300 billion to more than $ 1 trillion annually by 2050 worldwide (Dadgostar,2019).

On 27 February 2017, the WHO published the list of global priority pathogens (GPP) – a catalog of 12 species of bacteria grouped under three priority tiers according to their antimicrobial resistance critical, high and medium (Mancuso et al., 2021) with *K. pneumoniae* amongst the first priority/critical pathogens.

In Uganda, some studies have been found to report on the problem of *K. pneumoniae* antimicrobial resistance. A study done in Uganda tertially hospitals noted the existence of carbapenem resistant *K. pneumoniae* (Ssekatawa et al., 2021). Another cross sectional study conducted by Stanley et al, in the gut of out-patients from pastoralist communities of Kasese district observed high MDR-*K.pneumonia*e isolates (Stanley et al., 2018). This staggering resistance of *K. pneumoniae* to commonly used antibiotics heralds a clarion call towards strengthening and regular AMR local and regional surveillance

2.8 Antimicrobial susceptibility testing

Several ways are used in Antimicrobial susceptibility testing (AST). The most commonly used methods are Disk diffusion method, Broth microdilution method, E-test

2.8.1 Agar diffusion method

The agar diffusion method also known as the Kirby–bauer method is a widely used technique in microbiology and remains the valuable tool in the determination of antimicrobial susceptibility. The technique involves the use of agar plates containing a solid growth medium which is inopculated with the bacterial culture of interest. Paper disks, soaked in a specific concentration of the antibiotic, are then placed on the agar surface. The antibiotic diffuses from the disk into the agar, creating a concentration gradient. The growth inhibition zone around the disk is measured and used to determine the susceptibility of the microorganism to the antibiotic. It is a cost-effective and widely standardized technique that provides useful information for guiding treatment decisions. However, this method is unreliable for colistin susceptibility testing due to poor diffusion of the large colistin molecule in the diffusion based assays.

2.8.2 Broth Microdilution method

In broth microdilution method, microorganisms are tested for the ability to produce visible growth in broth containing dilutions of the antimicrobial agent. The tubes containing antibiotics are inoculated with standardized bacterial suspension. After overnight incubation at 35^0 C, the tubes are examined for visible bacterial growth as evidenced by turbidity. Broth microdilution method is currently the recommended technique to determine susceptibility to Colistin.

2.9 Whole genome sequencing of *K.pneumoniae*

Whole-genome sequencing (WGS) is a comprehensive method of analyzing entire genomes. It uncovers the bacterial genome and allowing bioinformatics analysis to take place. WGS promises to be transformative for the practice of clinical microbiology for rapid detection and characterization of clinical relevant bacteria like *K.pneumoniae* due to its capacity in offering a vastly improved level of genomic AMR genes. In addition to bacterial identification and

molecular characterization, WGS may offer a significant source that can be used to predict the phenotype of the microbe. All these serve many purposes like choosing a beneficial treatment, identifying epidermic strains, outbreaks and tracking spread, thus likely to replace traditional typing methods, resistance gene detection and other sequence-based investigations (e.g 16 rDNA, PCR) in the near future.

CHAPTER THREE: METHODOLOGY

3.1 Study design

This was a cross-sectional study.

3.2 Study site and setting

The study used the stored *K. pneumoniae* clinical samples at the clinical microbiology laboratory, Makerere university College of Health Sciences. The samples were isolated from clinical specimens of ICU patients at Mulago National Referral Hospital (MNRH). Mulago National Referral Hospital also known as Mulago National Specialized Hospital is a tertially hospital and the largest public hospital in uganda located on Mulago Hill in the Northern part of the city of Kampala, immediately west of the Makerere university College of Health Sciences. The geographical coordinates of the hospital are 0°20'16.0"N, 32°34'32.0"E (Latitude:0.337786; Longitude:32.575550). All microbiological analysis such as phenotypic identification, bacterial culture and Antimicrobial Susceptibility testing and were carried out at clinical microbiology laboratory at the department of Medical Microbiology of Makerere university whereas DNA extraction was done the Genomics and molecular biology facilities at the department of Immunology and Molecular Biology of Makerere university college of Health Sciences. unit of molecular biology of makerere university. Library preparation and Genomic DNA sequencing were performed at Macrogen in Seoul, Republic of Korea. Bioinformatics analysis was conducted at Makerere university and in other places using a personal computer.

3.3 Study samples and population

The study used 31 stored *K. pneumoniae* samples kept at the clinical microbiology laboratory, Makerere university College of Health Sciences.

3.4 Sample size calculation.

All the stored sample isolates (31 samples) were used for hypermucoid phenotypic characterization of *K.pneumoniae* as well as for antimicrobial susceptibility testing. In the genomic section, five (6) hypermucoid and Colistin resistant K. pneumoniae were selected for

sequencing. The illumina Novaseq 6000 platform of choice yielded 4gb (4Gb is equivalent to 4 billion base pairs) of 150 bp length-paired end (PE) reads per each sample. This sequence depth is more appropriate / recommended for better recovery of strains and variants. (Gweon et al., 2019).

3.5 Sampling technique

All stored *K.pneumoniae* isolates were used for phenotypic characterization of hypermucoid strains. However purposeful sampling was employed in selecting samples for DNA extraction. Only 6 Colistin resistant *K.pneumoniae* with hypermucoid phenotype were selected for DNA extraction and sequencing.

3.6 Sample collection/ retrieval

The study sample isolates of Colistin resistant *K.pneumoniae* used were reserved at the clinical microbiology laboratory at Makerere university College of Health Sciences. The sample isolates were kept in a -80^0C freezer in a brain- heart infusion broth containing 20% glycerol.

3.7 Inclusion and Exclusion Criteria

All stored *K.pneumoniae* isolates properly labeled with the name of the organism, type of the specimen, date of identification were included for the study. However contaminated isolates and those with incomplete information and inadequate volume were excluded.

3.8 Methodological details

3.8.1. Bacterial strains and phenotypic characterization

All 31 isolates that had been phenotypically identified as *K. pneumoniae* under the "Colistin resistance among the gram negative rods study" were included in the study. They were retrieved from the -80^0C freezer, thawed and sub-cultured on MacConkey. Isolates were then re-identified as *K.pneumoniae* based on Gram negative rod morphology, negative oxidase test, negative indole test, negative motility test, positive urease test, positive simmon's citrate Agar test and

triple sugar iron test. All 31 isolates grew upon subculturing and they were all re-confirmed as K. pneumoniae on all the seven criteria set for this study.

3.8.2 String test to determine hypermucoviscosity

In order to determine hypermucoviscosity, a string test of colonies from culture media was performed for all *K. pneumoniae* isolates. A viscous string of greater than 5 mm in length when a loop is used to stretch the colony on an agar plate indicate positive test (Kumabe & Kenzaka, 2014). To perform the string test, a loopful of bacteria was streaked onto 5% sheep blood agar plate and incubated overnight overnight at 37^0C. After incubation, a single colony was picked up with a sterile loop and stretched carefully by pulling it up and away from the agar surface. If the colony produced a long, visible, slimy string that stretches for several millimeters ≥5 mm before breaking off, it was considered to be hypermucoid. On the other hand, if the string was short or non-existent, the strain was non-hypermucoid.

3.8.3 Antibiotic susceptibility Testing

Antibiotic susceptibility testing was performed by standard Kirby-Bauer disc diffusion method or broth microdilution method (in the case of Colistin) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. 16 different antibiotics tested were augmentin, piperacillin/tazobactam, cefuroxime, ceftazidime, ceftriaxone, cefepime , imipenem, fosfomycin, gentamicin, amikacin, tigecycline, chloramphenicol, ciprofloxacin, cotrimoxazole, and Colistin and antimicrobial profile among strains was reported as susceptible, resistant or indeterminate and susceptibility and resistance were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines for *Klebsiella spp*. The resistance profiles were described as multidrug resistant (MDR), extensively drug-resistant (XDR) or pan-drug-resistant (PDR)

3.8.4 DNA Extraction

A total of 6 colistin resistant *K. pneumoniae* isolates with hypermucoviscous phenotype were inoculated on Mueller Hinton Agar plates and incubated for 24 h at 37^oC. The bacterial DNA was extracted using a CTAB protocol as previously described by Willner et al, 2012 (Willner et al., 2012) Briefly, bacterial cultures were centrifuged in 4000 rpm for 6 min to pellet cellular

material. The supernatant was removed and the cell pellets were then suspended in 567 µl TE buffer, pH 8, 30 µl 10% sodium dodecyl sulfate (SDS) and 3-5 µl Proteinase K then incubated at 37°C for 1 h. Samples were then incubated for 15 min in 65°C with 100 µl of 5M NaCl prepared with sterile water and 80 µl CTAB (CTAB/NaCl solution; 4.1 g NaCl, 10 g CTAB in 100 mL sterile water).

Following incubation, DNA was purified by extraction with an equal volume of phenol/ chloroform/isoamyl alcohol (25:24:1). Supernatant which contains total genomic DNA was collected after centrifugation at 13,000 rpm and 500 µl isopropanol was added, mixed gently and kept for 12 hours in -20°C. DNA pellet was obtained by centrifuging at 2000 rpm for 20 min, washed with 500 µL 70% ethanol air-dried and re-suspended in 50 µL of nuclease free water.

For analysis of extracted DNA, gel electrophoresis was carried out. DNA purity was determined on the Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) in which the purity ratio (A260:A280) was determined by determining the absorbance value at 280 nm. DNA was quantified on the Qubit dsDNA fluorometer (ThermoFisher Scientific, Waltham, MA, USA).

3.8.5 Genomic Library preparation and sequencing.

A quality control experiment was conducted to evaluate the quantity and condition of DNA before library preparation and sequencing. Quantity of DNA was done by QuantiFluor®dsDNA system (promega, cat.#E2671) and verification of the genomic DNA integrity was done using an agilent technologies 2100 Bioanalyzer (Agilent, Part # G2939BA). For preparation of ready-to-sequence libraries of bacterial genomes, Nextera DNA XT library preparation kit (Illumina, San Diego, CA, USA) was used following manufacturers recommended protocol followed by sequencing using Illumina Novaseq 6000 platform at Humanizing Genomics, Macrogen Inc, in Seoul in South Korea. Illumina sequencing generated paired-end short reads with approx. 50-fold coverage and an average length of 150bp.

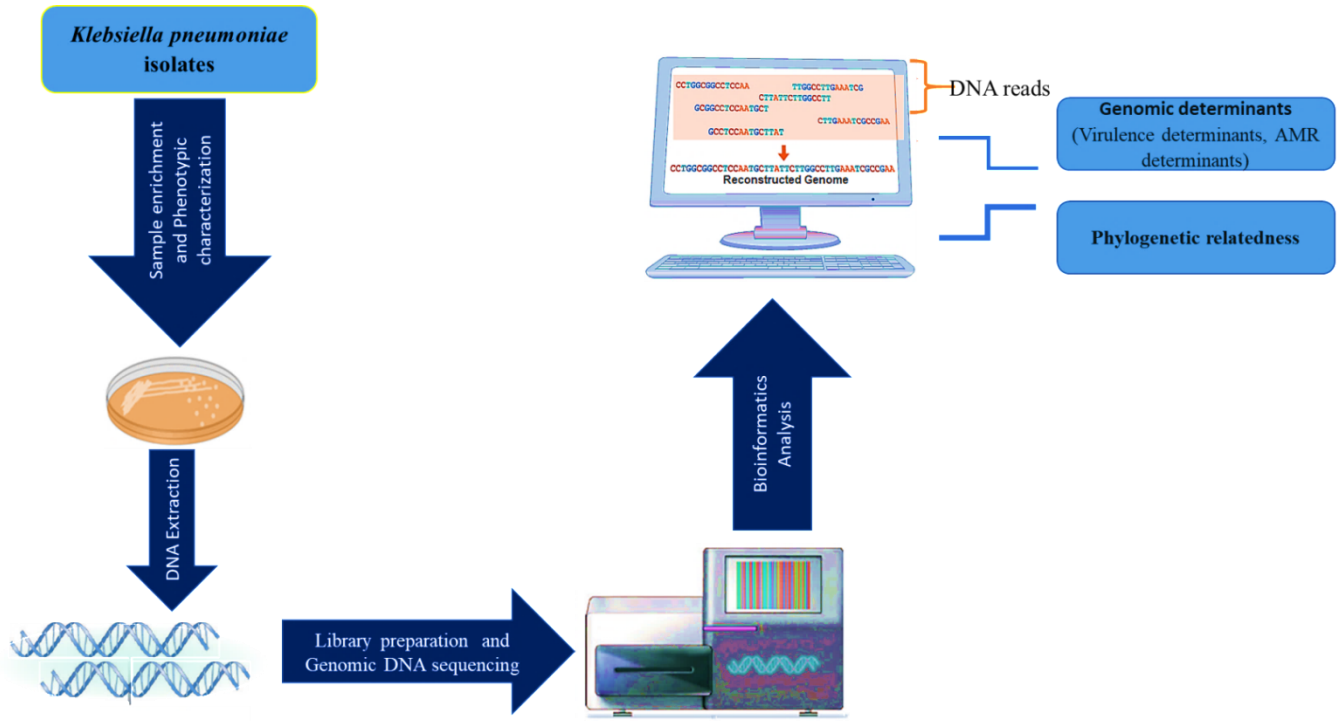


Figure 7. Schematic workflow of the whole study

3.9. Sequence data analysis

Data management involved bioinformatics analysis of sequence data in which raw data from the NovaSeq run were stored on the NovaSeq instrument under the folder output. These data usually consist of short-read sequences, sequence identifiers and quality scores stored in FASTQ format.

3.9.1 Read quality control

Sequenced raw data were quality controlled using FastQC. FastQC provides a modular set of analyses to check if the data has any problems before doing further analysis. Using Trimmomatic, raw reads were trimmed, removing adaptors and low-quality sequences (with <15 per base quality).

3.9.2 Genome Assembly

Genome assembly was approached by mapping high quality reads onto a reference genome using BWA-MEM (H. Li, 2013). Chromosomes were generated by mapping the denovo assembly-derived contigs to relevant reference genomes using the MeDuSa pipeline. Plasmids were separately denovo-assembled using plasmid SPAdes and then scaffolded with MeDuSa against closest references recovered from NCBI BLASTn and/or plasmid database (PLSDB)

3.9.3 Genome Annotation and Gene detection

The assembled genomes were annotated using the RAST server that uses subsystems technology to assign gene function. RAST was also used for the identification of protein encoding genes, rRNAs, and tRNAs.

The bacterial resistome was obtained by assessing the occurrence of antimicrobial resistance genes and plasmid replicons using the Comprehensive Antibiotic Resistance Database (CARD), ResFinder and PlasmidFinder from the Center for Genomic Epidemiology. Moreover, manual curation was done to establish individual gene polymorphism for Colistin resistance. The phenotypic effect of amino acid substitutions were predicted using polymorphism phenotyping v2 web tool (PolyPhen-2). VFDB was used for detection of virulence genes. The VirulenceFinder service from the same website was also used to identify additional virulence-specific genes.

3.9.4 Phylogenetic analysis

To determine the phylogenetic relatedness a concatenated marker gene maximum-likelihood tree was constructed using a number of *K. pneumoniae* reference genomes chosen based on BLAST similarity results, clonal complexes and sequence types. Pairwise alignment and visualization of selected genomes with the respective reference strains was achieved through the MAFFT(Katoh et al., 2019) using default parameters. The sequences were first processed with PhyloSift, the phylogenetic tree was then constructed using RAXML (Stamatakis et al., 2008), visualized and edited with Dendroscope (Huson et al., 2007). Genome-based phylogeny were first deduced from the Pathosystems Resource Integration Center (PATRIC) database (Antonopoulos et al.,

2019). Strain identification and possible closest relatives were further done using the Type Strain Genome Server (TYGS), which integrates several algorithms including genome to genome distance (Meier-Kolthoff & Göker, 2019)

3.10 Nucleotide sequence accession numbers

Whole Genome sequence data have been deposited in GenBank-NCBI, a division of the National Library of Medicine. The data are available under project accession number PRJNA985059

3.11 Ethical Approval and consent to participate

A waiver of consent was obtained from the Makerere university School of Biomedical Sciences-Research and Ethics Committee (SBS-REC). All methods were carried out in accordance with relevant guidelines and regulations

CHAPTER FOUR: RESULTS

4.1 Samples description

A total of 31 *Klebsiella pneumoniae* clinical isolates obtained from patients hospitalized at ICU wards of Mulago National Referral Hospital were used in this study. The isolates were recovered at Makerere University Clinical Microbiology Laboratory from rectal swabs of patients admitted to the Mulago Hospital ICU for advanced respiratory support with diagnoses including Meningitis, pneumonia, sepsis, hypertension, diabetic ketoacidosis, Acute kidney injury and nephrotic syndrome. The other indications for ICU admission included brain diseases such as brain tumour, post-tumour resection, post-craniotomy and haematoma evacuation and severe subarachnoid haemorrhage.

4.2 Phenotypic Resistance Profiles

Antibiotic susceptibility tests (AST) revealed high levels of resistance to commonly used antibiotics against *K. pneumoniae* infections (Table 1)

Table 1. Antimicrobial susceptibility profiles

ANTIBIOTIC	INITIAL	R	S	I	NO. OF ISOLATES	RESISTANCE PERCENTAGE
Amikacin	AMK	2	23	6	**31**	6.45%
Augmentin	AMC	14	4	13	**31**	45.16%
Cefepime	CFPM	7	4	2	**13**	53.85%
Ceftazidime	CTZ	17	13	1	**31**	54.84%
Ceftriaxone	CRO	21	9	1	**31**	67.74%
Cefuroxime	CXM	26	5	0	**31**	83.87%
Chloramphenicol	CHL	10	20	1	**31**	32.26%
Ciprofloxacin	CIPRO	17	0	14	**31**	54.84%
Colistin	CL	7	24	0	**31**	22.58%
Cotrimoxazole	CMX	26	5	0	**31**	83.87%
Fosfomycin	FOS	9	18	4	**31**	29.03%
Gentamicin	GM	15	15	1	**31**	48.39%
Imipenem	IMP	1	27	3	**31**	3.23%
Levofloxacin	LVX	9	6	12	**27**	33.33%
Piperacillintazobactam	PTZ	11	8	12	**31**	35.48%
Tigecycline	TGC	21	9	1	**31**	67.74%

The most resisted antibiotic being Cotrimoxazole (83.87%), cefuroxime (83.87%), Tigecycline (70.00%), ceftriaxone (67.74%). The least resisted antibiotics were colistin (22. 58%), Amikacin (6.45%) and Imipenem (3.23%). Among the dataset, 90.32% (28/31) were classified as MDR, i.e., resistant to at least one agent in three or more drug classes. No XDR or PDR isolates were detected.

4.3 Genome features of *K.pneumoniae* strains

In order to determine the genomic determinants of virulence and antimicrobial resistance in colistin resistant *Klebsiella pneumoniae*, whole genome sequencing using the Illumina Novaseq 6000 was performed on six isolates (MAKM-RS045. MAKM-3381, MAKM-RS081, MAKM-5490, MAKM-RS083 and MAKM-RS028). The raw reads were assembled using SPAdes v.3.15 into complete chromosome and various plasmids. General features of each genome were investigated and are summarized in Table 3.

Briefly to confirm the taxonomic placement and predict the possible closest strains, each assembly was uploaded and analyzed with the Microbial Genomes Atlas (MiGA) using TypeMat algorithm. The closest strains identified from MiGA were downloaded and used as reference genomes for chromosome level assembly with MeDUSA. While completeness was checked by BUSCO, contest16S was used to check for possible contamination. Of all six genomes of *K.pneumoniae,* we found 99.9-100% completeness and with no or negligible contamination in five genomes. Based on these observations, we concluded that the five (5) isolates are indeed *K.pneumoniae*. Moreover, upon assembly, sample MAKM-RS028 was found with 600 contigs with genome size of 15,795,720 bp, which is about 3 times the average genome of *Klebsiella pneumoniae*.

Contamination check with Contest16S revealed only one 16S rRNA, which upon BLASTn against the NCBI nucleotide database revealed 100% identity with *Citrobacter freundii*. The average genome size of *Citrobacter freundii* is known to about 5.1Mb (Wan et al., 2020), i.e. a third of the MAKM-RS028 genome. For better understanding of the cause of this large genome size, it was considered to be a metagenome, in which the reads were reassembled with metaSPAdes (v3.15.5) and binned with the BV-BRC binning algorithm, followed by assessing the quality of the bin parameters with CheckM (v1.0.18) (Table 2). Metagenomic assembly with

metaSPAdes resulted in 8130 contigs, a total of 18,204,134 bp with GC content of 53.54%. From these metagenome binning results, bin 2 which matched with *Klebsiella pneumoniae* was selected for downstream genomic analysis.

Table 2. Metagenome features

Bin	Score	Hit genome	BV-BRC ID	Completeness	Contamination	Contigs	Genome size	Mean coverage
1	2654	Citrobacter clone	200446.3	96.8	1.5	110	4724722	238.95
2	2423	*K. pneumoniae* clone	573.2411	95.7	3.7	407	5838852	87.11
3	2423	*E. coli* clone	573.241	94.7	4.9	371	5222916	62.76

The chromosome size (genome size) and the number of genes in each strain varied. The mean number of genes across all strains was 5962.4 genes and a genome length of 5335766 bp with GC content averaging 57.48%, the smallest chromosome size being 5231098 bp and largest being 5403338 (Table 3). All *K. pneumoniae* genomes obtained shows that the strains have a complete genome when compared with the reference genomes found in the data base in NCBI. https://www.ncbi.nlm.nih.gov/nuccore/?term=klebsiella%20pneumoniae%20makm.Other details such as number of plasmids, protein coding sequences, are shown in (Table 3).

Table 3. Genome features of sequenced *K. pneumoniae* strains

Strain	GenBank Accession No.	Size (bp)	No. of plasmid(s)	GC%	Total No. of Genes	No. of Protein coding sequences (CDS)
MAKM-3381	CP129541.1	5321788	4	57.58	6304	5800
MAKM-RS081	CP129536.1	5319428	3	57.35	6031	5680
MAKM-RS083	CP129612.1	5231098	3	57.61	5694	5390
MAKM-RS045	CP130497.1	5403338	1	57.46	5908	5623
MAKM-5490	CP130492.1	5403180	4	57.40	5875	5551

4.4 Phylogenetic analysis of *K.pneumoniae* strains

The evolutionary relationship of the five sequenced *K.pneumoniae* strains was inferred using the maximum likelihood proteome phylogenomic analysis and put in context with eleven (11) completely sequenced strains of *K.pneumoniae*. As shown in Fig. 8, the *K.pneumoniae* strains MAKM-RS045. MAKM-3381 and MAKM RS081 were most closely related to *K.pneumoniae* subsp. ozaenae strain ATCC 11296 whereas strain MAKM 5490, MAKM RS083 are closerly related to *K.pneumoniae* DSM 30104 and *K.pneumoniae* ATCC 13883. And what does this mean?

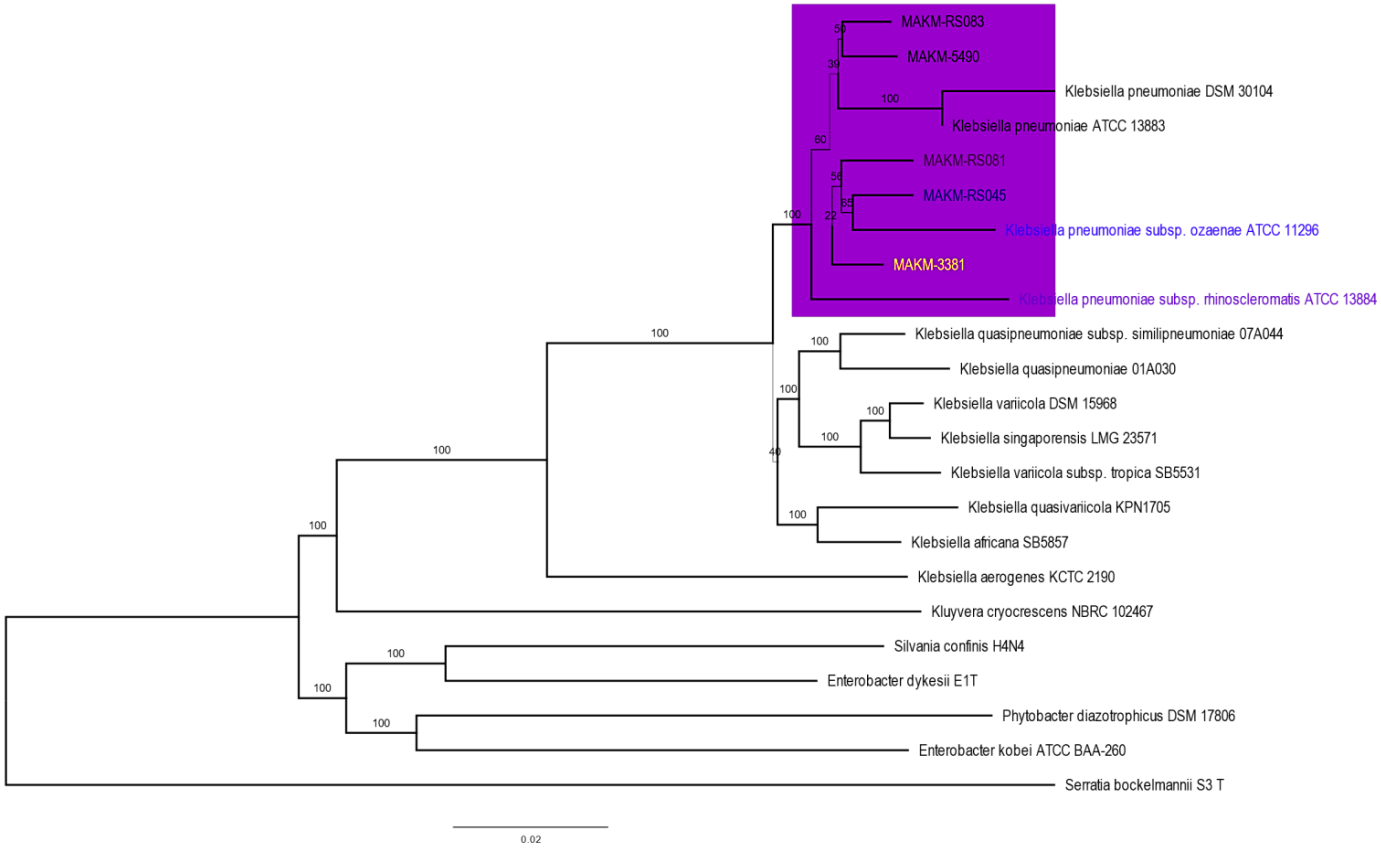


Figure 8. **Phylogenomic tree inferred with FastME 2.1.6.1 from whole-proteome-based GBDP distances.** The branch lengths are scaled via GBDP distance formula d_5. Branch values are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 81.6 %. The tree was midpoint-rooted.

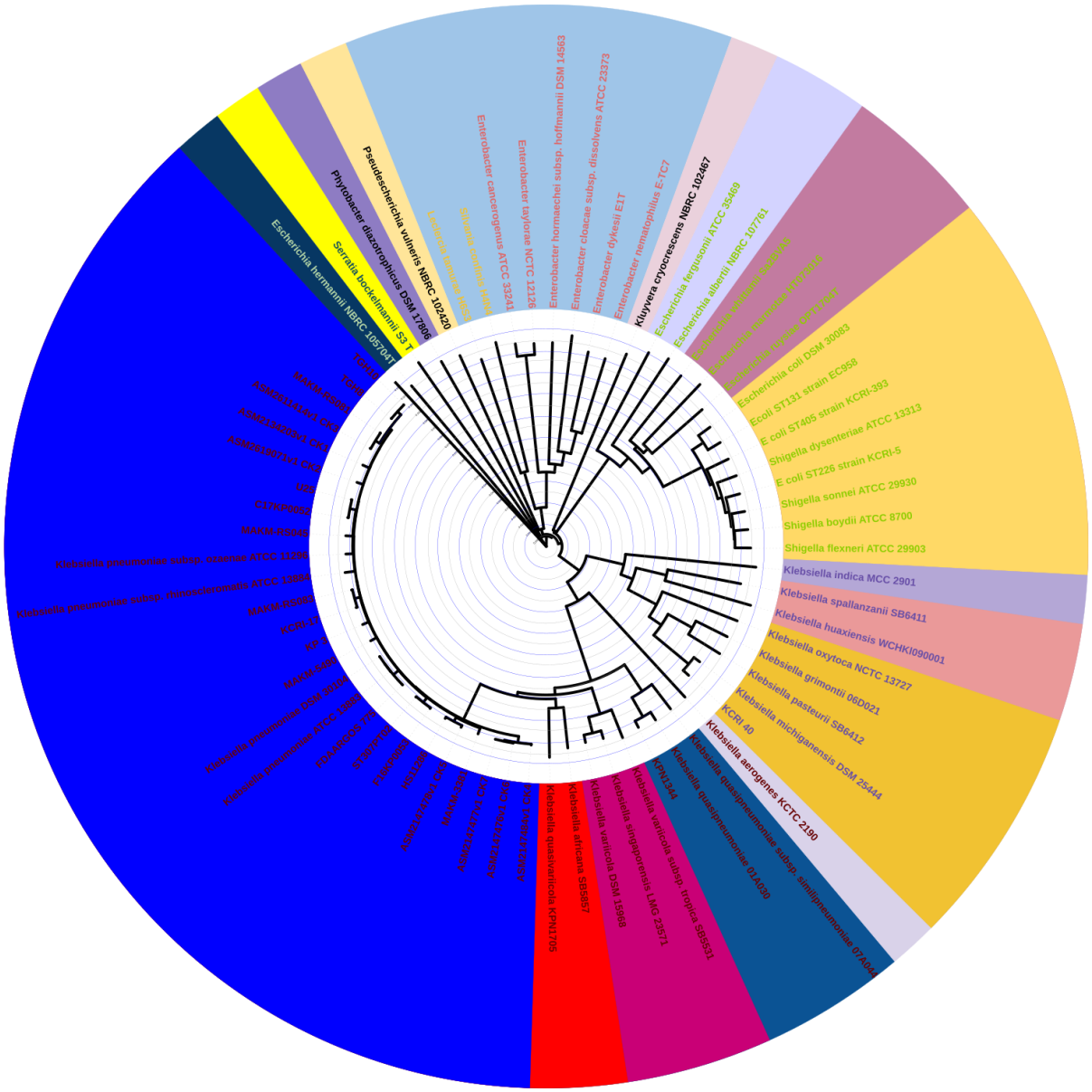


Figure 9. **Tree inferred with FastME 2.1.6.1 from GBDP distances calculated from chromosome sequences.** The branch lengths are scaled in terms of GBDP distance formula d5. The branches were deduced from GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 65.0 %. The tree comprises selected strains from other studies as well as those selected automatically by the TYGS algorithm. The tree was rooted at the midpoint.

4.5 Genetic Determinants of Resistance

4.5.1 Overall antibiotic resistance determinants

Whole genome sequence (WGS) analysis of antimicrobial resistance genes via the Comprehensive Antibiotic Resistance Database (CARD) revealed the presence of multiple resistome encoding for resistance within and between antibiotic classes. (Table 4) The isolates carried β-lactamase genes, including extended-spectrum beta lactamase (ESBL) and carbapenamese genes (*bla*SHV-1, *bla*SHV-11, *bla*TEM-1, *bla*CTX-M-15, *bla*OXA-1, CRP, Ecol_MarR_MULT, H-NS, Hinf_PBP3_BLA, Kpne_KpnE, Kpne_KpnG, Kpne_KpnH, Kpne_KpnF, Kpne_ramR, Kpne_OmpK37, marA,), Glycopeptide resistance genes (VanG), aminoglycoside resistance genes (baeR, aadA5, aadA2), Peptide resistance genes that include colistin resistance genes (ArnT, OmpA, eptB, LptD), Macrolide (Mrx, mphA), Fluoroquinolone resistance genes (adeF, emrR, rsmA, QnrS1). The isolates also had resistance genes for other antibiotic classes including msbA, tet (D), oqxA, sul2 for nitroimidazole, tetracycline, nitrofurantoin and sulfamexothazole respectively. Resistance to other antibiotic classes was conferred by: trimethoprim -*dfr*A17, *dfr*A12, fosfomycins-fosA6, Ecol_UhpT_FOF and even disinfectants and antiseptics – leuO, qacEdelta1.

Table 4. Resistance genes identified using whole-genome sequencing in 5 colistin-resistant *K. pneumoniae* using CARD

Types of Resistance Genes		Mechanism of Resistance	Number of Isolate
B-lactam resistance including ESBL, carbapenemes, cephalosporins, monobactams.	*bla*SHV-1	Antibiotic inactivation	1
	*bla*SHV-11	Antibiotic inactivation	3
	*bla*TEM 1	Antibiotic inactivation	4
	*bla*CTX-M-15	Antibiotic inactivation	4
	*bla*OXA-1	Antibiotic inactivation	1
	CRP	Antibiotic efflux	3
	Ecol_MarR_MULT	Antibiotic efflux, Antibiotic target alteration	1
	H-NS	Antibiotic efflux	3
	Hinf_PBP3_BLA	Antibiotic target alteration	1
	Kpne_KpnE	Antibiotic efflux	3
	Kpne_KpnG	Antibiotic efflux	5
	Kpne_KpnH	Antibiotic efflux	5
	Kpne_KpnF	Antibiotic efflux	5

	Kpne_ramR	Antibiotic target alteration, Antibiotic efflux	1
	Kpne_OmpK37	Reduced permeability to antibiotics	2
	marA	Antibiotic efflux, Reduced permeability to antibiotics	3
	LptD	Antibiotic efflux	5
Peptide resistance	ArnT	Antibiotic target alteration	3
	OmpA	Reduced permeability to antibiotics	3
	EptB	Antibiotic target alteration	3
	Kpne_KpnE	Antibiotic efflux	3
	Kpne_KpnG	Antibiotic efflux	5
	Kpne_KpnH	Antibiotic efflux	5
	Kpne_KpnF	Antibiotic efflux	5
	LptD	Antibiotic efflux	5
Glycopeptide resistance	vanG	Antibiotic target alteration	2
Aminoglycoside resistance	BaeR	Antibiotic efflux	5
	Kpne_KpnE	Antibiotic efflux	3
	Kpne_KpnG	Antibiotic efflux	5
	Kpne_KpnH	Antibiotic efflux	5
	Kpne_KpnF	Antibiotic efflux	5
	aadA5	Antibiotic inactivation	1
	aadA2	Antibiotic inactivation	1
Macrolide resistance	CRP	Antibiotic efflux	3
	H-NS	Antibiotic efflux	3
	Kpne_KpnE	Antibiotic efflux	3
	Kpne_KpnG	Antibiotic efflux	5
	Kpne_KpnH	Antibiotic efflux	5
	Kpne_KpnF	Antibiotic efflux	5
	Mrx	Antibiotic inactivation	2
	mphA	Antibiotic inactivation	2
Fluoroquinolone resistance	AdeF	Antibiotic efflux	5
	CRP	Antibiotic efflux	3
	emrR	Antibiotic efflux	5
	Ecol_MarR_MULT	Antibiotic efflux, Antibiotic target alteration	1
	H-NS	Antibiotic efflux	3
	Kpne_KpnG	Antibiotic efflux	5
	Kpne_KpnH	Antibiotic efflux	5
	Kpne_ramR	Antibiotic target alteration, Antibiotic efflux	1
	marA	Antibiotic efflux, Reduced	3

		permeability to antibiotics	
	rsmA	Antibiotic efflux	5
	OqxA	Antibiotic efflux	4
	QnrS1	Antibiotic target protection	2
Sulfamethoxazole resistance	*sul*2	Antibiotic target alteration	2
	sul1	Antibiotic target replacement	4
Trimethoprim resistance	*dfrA17*	Antibiotic target replacement	1
	dfrA12	Antibiotic target replacement	1
Tetracycline resistance	AdeF	Antibiotic efflux	5
	Ecol_MarR_MULT	Antibiotic efflux, Antibiotic target alteration	1
	H-NS	Antibiotic efflux	3
	Kpne_KpnE	Antibiotic efflux	3
	Kpne_ramR	Antibiotic target alteration, Antibiotic efflux	1
	marA	Antibiotic efflux, Reduced permeability to antibiotics	3
	Kpne_KpnF	Antibiotic efflux	5
	OqxA	Antibiotic efflux	4
	tet(D)	Antibiotic efflux	1
Chloramphenicol	Kpne_ramR	Antibiotic target alteration, Antibiotic efflux	1
	marA	Antibiotic efflux, Reduced permeability to antibiotics	3
	rsmA	Antibiotic efflux	5
	catI	Antibiotic inactivation	1
Rifamycin resistance	Ecol_MarR_MULT	Antibiotic efflux, Antibiotic target alteration	1
	Kpne_KpnE	Antibiotic efflux	3
	Kpne_ramR	Antibiotic target alteration, Antibiotic efflux	1
	marA	Antibiotic efflux, Reduced permeability to antibiotics	3
	Kpne_KpnF	Antibiotic efflux	5
	LptD	Antibiotic efflux	4
Fosfomycin	Ecol_UhpT_FOF	Antibiotic target alteration	1
	FosA6	Antibiotic inactivation	3
Nitrofuran antibiotic	OqxA	Antibiotic efflux	4
Nitroimidazole antibiotic	msbA	Antibiotic efflux	4
Biocides resistance (Disinfecting agents and antiseptics resistance)	Kpne_KpnE	Antibiotic efflux	3
	Ecol_MarR_MULT	Antibiotic efflux, Antibiotic target alteration	1

	Kpne_ramR	Antibiotic target alteration, Antibiotic efflux	1
	leuO	Antibiotic efflux	3
	MarA	Antibiotic efflux, Reduced permeability to antibiotics	2
	Kpne_KpnF	Antibiotic efflux	5
	qacG	Antibiotic efflux	1
	qacEdelta1	Antibiotic efflux	4

Additionally, the resistance genes of the isolates was confirmed by Resfinder. The AMR genotype of the strains are compaired with some published strains in East Africa in figure 10

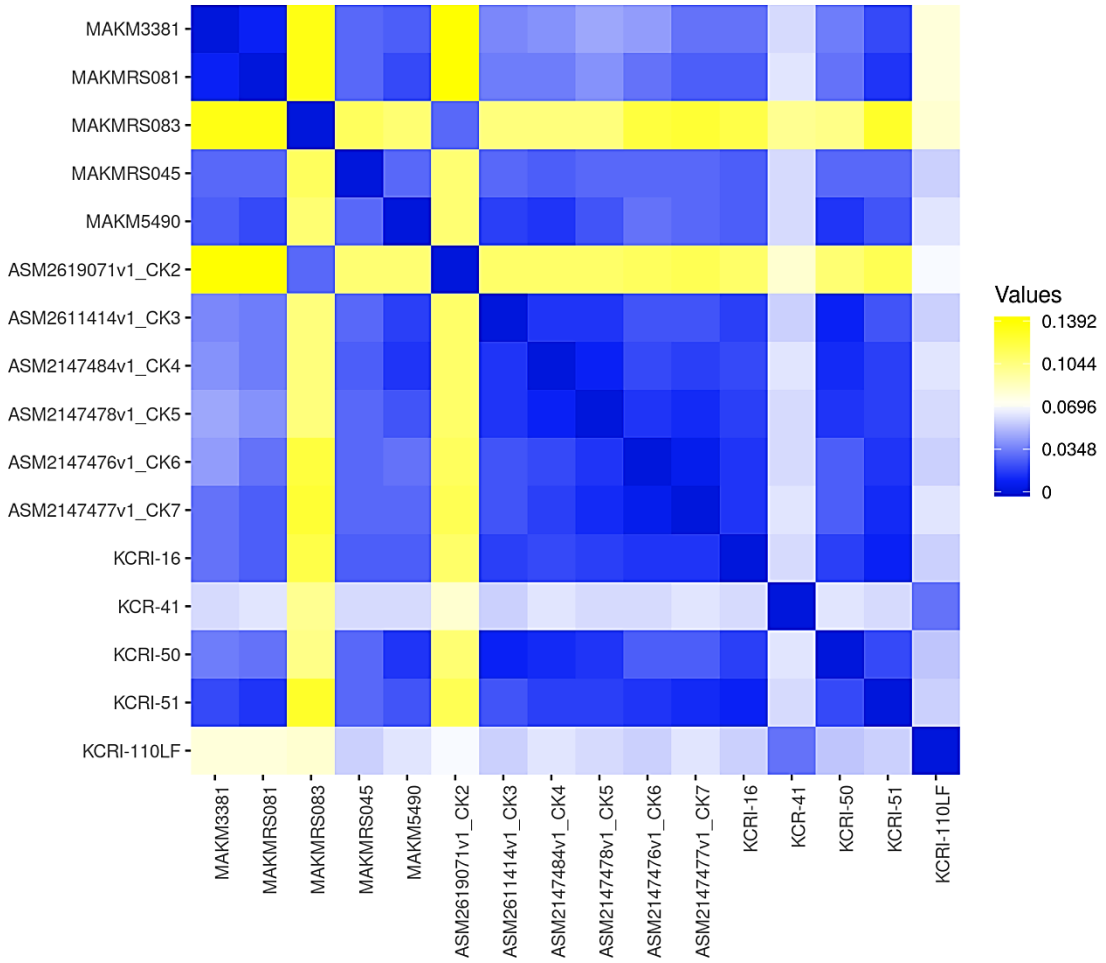
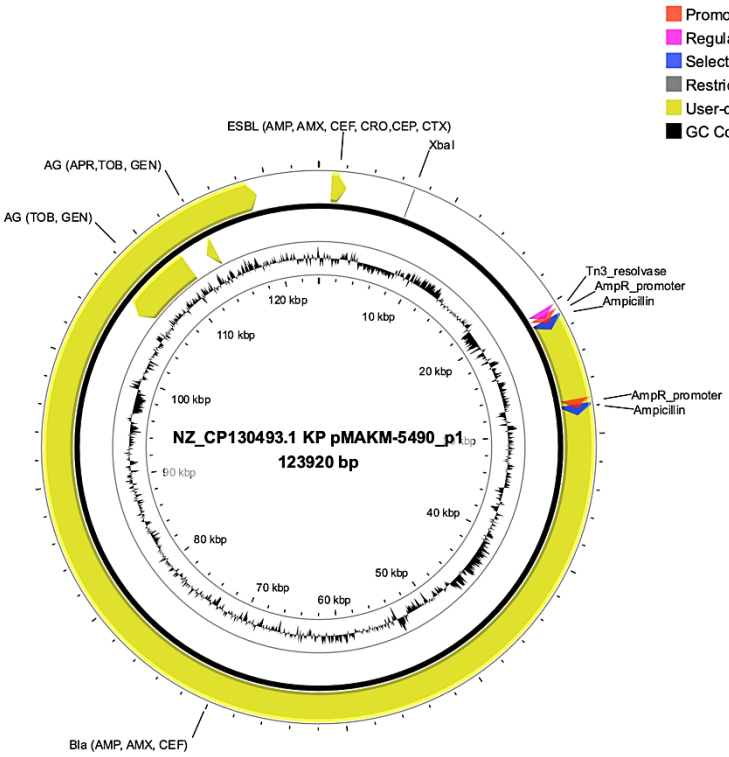
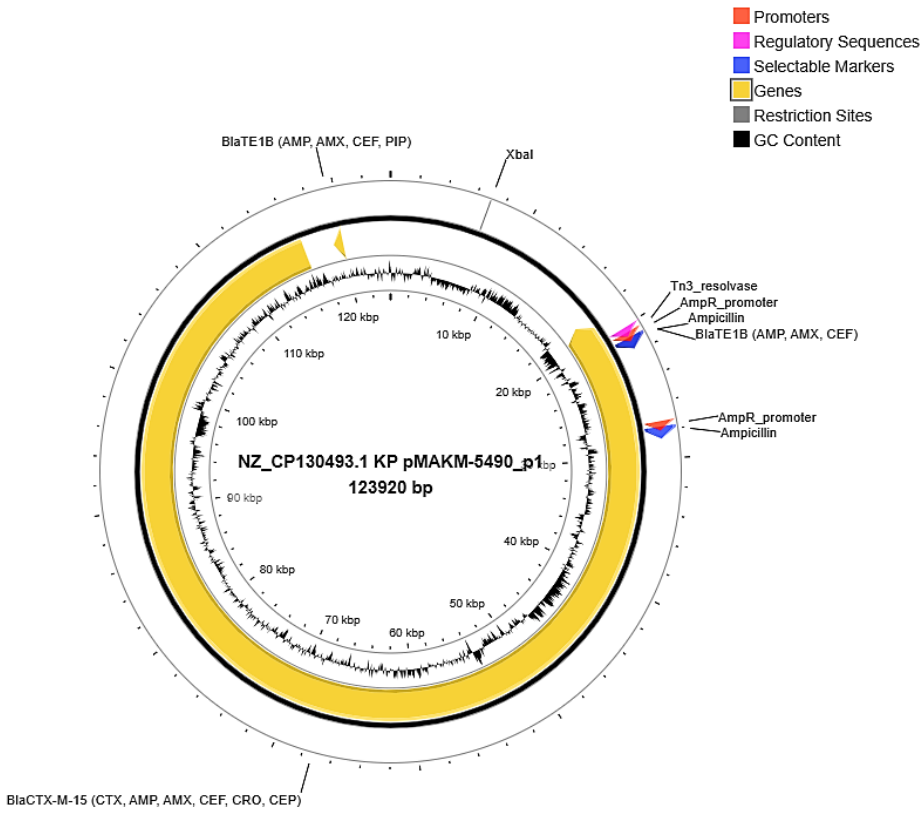


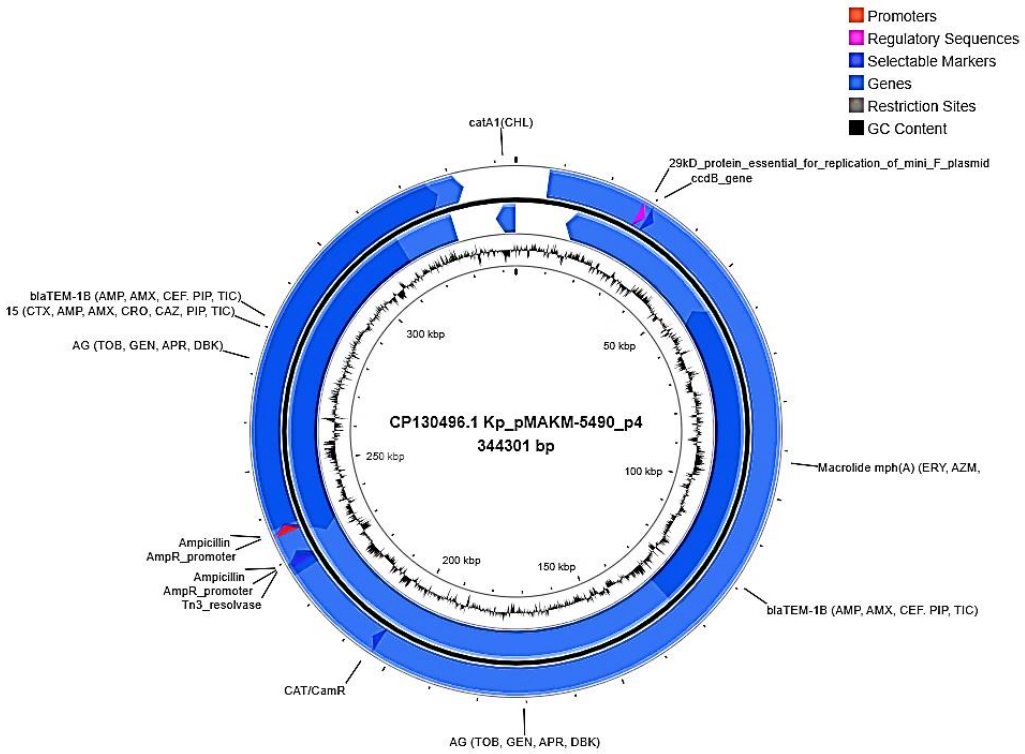
Figure 10. **A heatmap representing a pairwise correlation of the strains in comparison with those published from East Africa**. The degree of correlation increases from blue to yellow based on the number of AMR genotypes recovered from ResFinder.

4.5.2 Plasmid characterization

K. pneumoniae strains contained a total of 15 plasmids. Plasmids were found to harbor multidrug resistance genes (Figures 11,12 and 13)







Figures 11, 12,13. Plasmid circos with AMR genotypes

4.5.3 Colistin resistance determinants

Concerning genetic determinants related to colistin resistance, the BLAST results and ResFinder analysis revealed several genetic modifications in chromosomal loci. Briefly, the genomes of Colistin resistant *K. pneumoniae* strains were also investigated to decipher the genetic mechanism underlying colistin resistance through sequence analysis of TCRS genes. The sequence analysis of the commonly known genes involved in colistin resistance revealed several polymorphisms in nucleotide sequences of pmrA/pmrB and phoP/phoQ operon likely affecting the function of these proteins. There were non-synonymous deleterious mutations in pmrA (H219N, G53S), pmrB (N8T, G250C, A252G, D150V, L332M, L237R, H267P, R315P, Q331H, R256G, T157P), phoP (T151A) and phoQ (F163L, L30Q**,** H234Y, A351D).

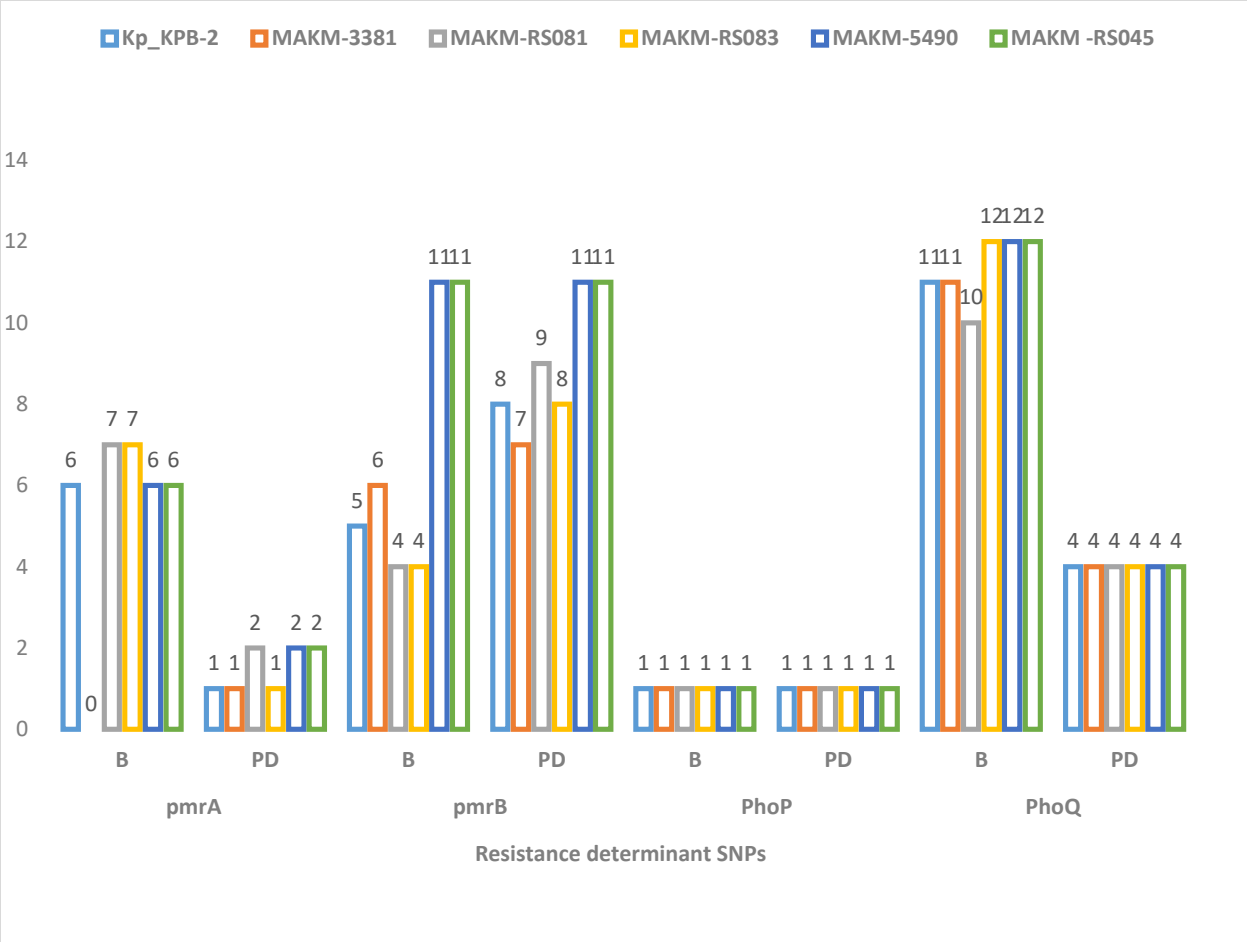


Figure14. Resistant determinant SNPS

Additionally there were non-synonymous deletereous mutations in mgrB (C28G). The insertion sequences IS1 and IS5 are highly implicated in colistin resistance, which occurs via modification or the inactivation of *mgrB* gene. The later plays role in the regulation the phoP/Q gene (Cannatelli Antonio et al., 2013). Therefore, insertion-mediated inactivation of the *mgrB* gene upregulates the *arnBCADTEF* operon, a glycosyl transferase, which adds 4-amino-4-deoxy-L-arabinose to lipids A, and PhoP/Q two component signaling pathway, resulting in resistance to colistin (Azam et al., 2021). In this work, insertion transposase genes for both of the insertion sequences were identified in all the five isolates and are located in the chromosomal DNA at different genomic positions (Table 5).

Table 5. Location of insertion transposase genes in the chromosomal DNA

Genes		Isolates				
		MAKM-3381	MAKM-RS081	MAKM-RS083	MAKM-5490	MAKM-RS045
IS1 transposase	Genomic position	335760..337757	424767..425522	497812..498509	104317..105014	104317..105014
	Accession	WP_223290842.1	WP_124057264.1	WP_223290842.1	WKU57685.1	WKU57685.1
IS5 transposase	Genomic position	2716937..2717611	441139..442107	2323866..2324834	2424129..2425097	242424129..2425097
	Accession	WP_040089720.1	WP_094316038.1	WP_077266803.1	WKU59790.1	WKU59790.1"

The gene mcr-1 gene mediates colistin target modification by adding phosphoethanolamine moiety to lipid A. This decreases the overall net charge of the bacterial outer membrane (Samantha & Vrielink, 2020) and hence the inherent affinity to colistin and related polymyxins, resulting in reduced activity of the colistin. Here all the five isolates were found to harbor three genes annotated as phosphoethanolamine transferase. Table 6, represents a summary of each protein and details of the annotations

Different genes annotated as phosphoethanolamine transferases with putative role in lipid lipopolysaccharide modification

Strain	Locus tag	Protein ID	Genomic position	Length (aa)	Annotation
MAKM3381	QY478_RS13550	WP_040088712.1	601211-1602791	525	phosphoethanolamine transferase
	QY478_RS13740	WP_301736672.1	1638183-1639823	546	phosphoethanolamine transferase EptA
	QY478_RS25220	WP_004152427.1	4053442-4055115	557	kdo(2)-lipid A phosphoethanolamine 7"-transferase
MAKMRS081	tag=QY481_RS10145	WP_004152427.1	1312964-1314637	557	kdo(2)-lipid A phosphoethanolamine 7"-transferase
	QY481_RS26690	WP_032419159.1	4621774-4623354	526	phosphoethanolamine transferase
	QY481_RS26885	WP_064114526.1	4658626-4660266	546	phosphoethanolamine transferase EptA
MAKM-RS083	QY482_08080	WKG17326.	1074882-1076555	557	kdo(2)-lipid A phosphoethanolamine 7"-transferase
	QY482_19340	WKG14661.1	3302015-3303655	546	phosphoethanolamine transferase EptA
	QY482_19530	WKG14699.1	3339283-3340863	526	phosphoethanolamine transferase
MAKM-RS045	Q3W73_08230	WKU59076.1	1658131-1659771	546	phosphoethanolamine transferase EptA
	Q3W73_08420	KU59114.1	1694926-1696506	526	phosphoethanolamine transferase
	Q3W73_25205	WKU57285.1	5081193-5082866	557	kdo(2)-lipid A phosphoethanolamine 7"-transferase
MAKM-5490	Q3W69_1099	WKU54017.1	2267434-2269014	526	hosphoethanolamine transferase
	Q3W69_1118	WKU54055.1	2304287-2305927	546	phosphoethanolamine transferase EptA
	Q3W69_21310	WKU51134.1	4407754-4409427	557	kdo(2)-lipid A phosphoethanolamine 7"-transferase

Protein sequences identified as kdo(2)-lipid A phosphoethanolamine 7"-transferase were aligned against selected known mcr-1 genes to find the possible conserved regions. Shown in figure 15, all the five sequences possess some conserved regions with plasmid-borne sequences of the family mcr-1. Although most of the regions are diverse, the conserved part portray related enzymatic role that potentially contribute to polymyxin resistance.

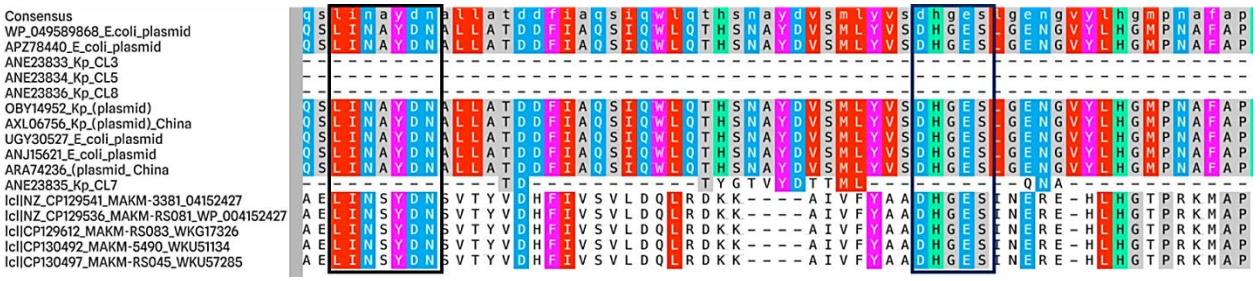


Figure 15. Part of the sequence alignment showing conserved regions between the standard known mcr-1 genes and the MAKM- strain-derived chromosomal-borne phosphoethanolamine lipid A transferases. Conserved regions are indicated in black boxes**.**

To predict the possible hit for mcr-related genes, each of the extracted proteins was analyzed for homology against multiple databases including swissprot90_2023_02, pdb70_230417, ECOD-F70_20220613, scope70_2.08, COG-KOG, NCBI-CD_3.19 and pfamA_35.0, using default parameters of Comer (Dapkūnas & Margelevičius, 2023a). Figure 16 shows the number of hits with which the chromosome-borne MAKM transferases from this study possess homology. It follows that all the genes annotated as phosphoethanolamine transferases have the potential to confer polymyxin resistance. Here it is shown that that each is significantly homologous to polymyxin resistance mcr-1 genes with up to three hits (positive score range 61-68%) of the mcr-1 family carried by plasmids within *Klebsiella* pneumoniae and other enterobacteria. All the five strains had similar hits for lipid A phosphoethanolamine transferase. Thus only one (MAKM-RS083) was chosen for representation in Figure 16.

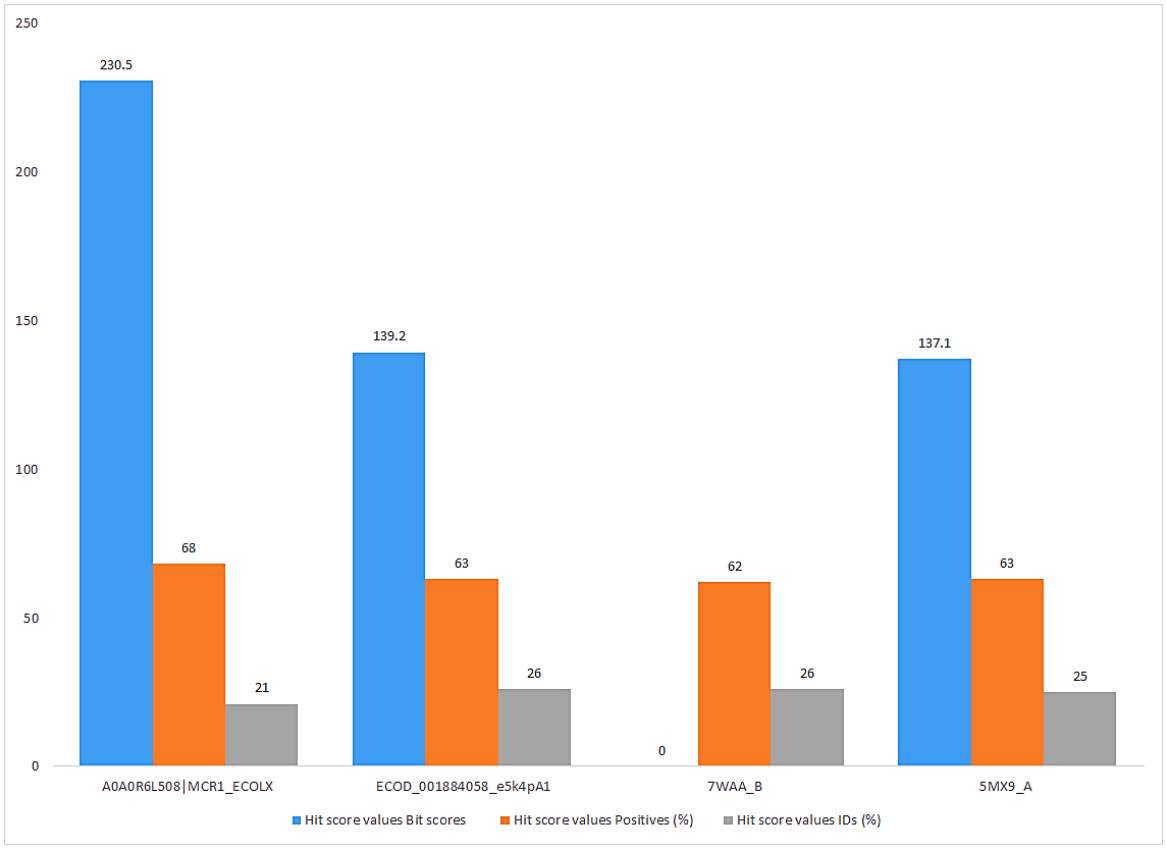


Figure 16. mcr-1 hit genes and their score values for the strain MAKM-RS083. The blue bar represents the number of bit scores for each homologous protein match from the database while the res to fhe bars represent the precentage of positively correlated as well as identities in terms of percentages.

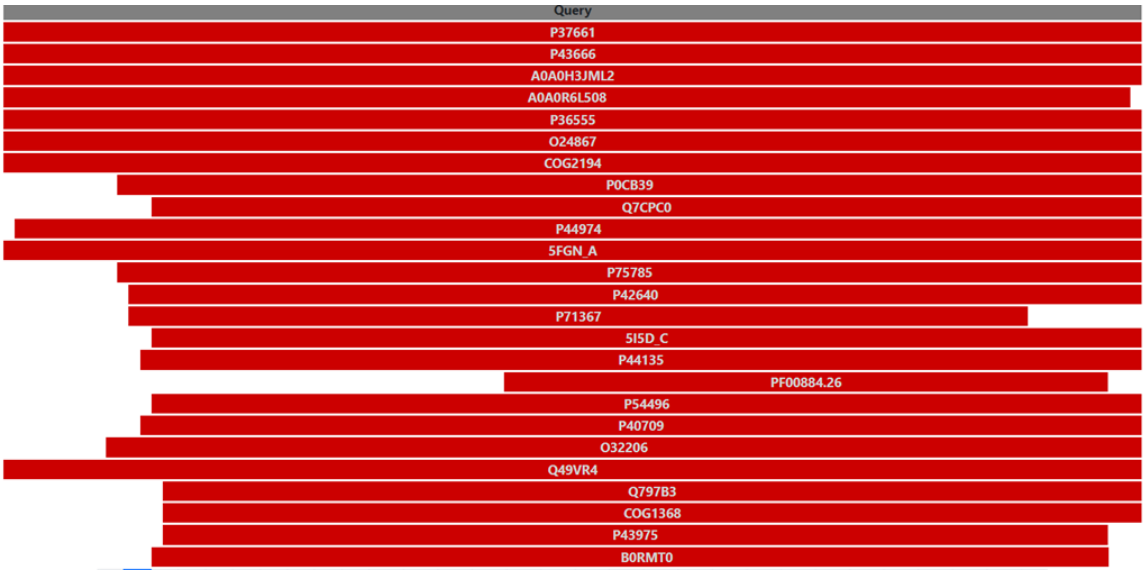


Figure 17. Domain match for mcr and other LPS biosynthesis related genes recovered from domain search.

4.6 Virulence Factors Associated with the *K. pneumoniae* Isolates

4.6.1 String test

For a string test, if the inoculation loop was able to produce a viscous string of greater than 5 mm in length while stretching a single colony away from an overnight culture agar plate, it was regarded indicative of hypermucoviscous *K. pneumoniae* isolates. Out of all *K. pneumoniae* isolates 14 had a positive string test result, indicating hypermucoviscosity phenotype.

Figure 18. String tests results: Stretching of the colonies of *K. pneumoniae* isolated from the patients'rectal swab samples resulted in the formation of a string of approximately 7 mm. (A string 5 mm or more is regarded as positive).

4.6.2 Genetic determinants of virulence

The genome sequencing results of 5 isolates were compared with VFDB. Results showed a total of 54 diverse virulence genes involved in siderophores expression (enterobactin, yersiniabactin, aerobactin, and salmochelin), adherence, biofilm formation, immune evasion, secretion system, (Table 7). The most predominant genes were the chromosomal genes were fim and ecp for adherence and biofilm formation, which were present in all isolates.

Table 7. Some important virulence detected in the study

Virulence factors	Genes
Adherence virulence factors	fimA, fimB, fimC, fimD, fimE, fiml, yagX/ecpC, yagW/ecpD, yagZ/ecpA, yagY/ecpB, yagV/ecpE, ykgK/ecpR, sfaF, focD, pilB, fleQ
Siderophore (iron-absorption factor)	Enterobactin; fepA, fepC, fepG, fepB, fepD, fepG,fes, sfaF, entA, entB, entC, entF, entE, entS
	Salmochelin; iroN
	Aerobactin; iutA
Effector delivery system	ClipV1
Lipopolysaccharide (LPS)	waaA

Table 8. Some capsular polysaccharide genes using virulence factors from Kleborate analysis

Genome Name	species	wzi	K_locus	K_type	O_locus	O_type
MAKM-3381	Klebsiella pneumoniae	-	KL110	unknown (KL110)	O3b	O3b
MAKM-5490	Klebsiella pneumoniae	wzi62	KL62	K62	O1/O2v2	O1
MAKM-RS081	Klebsiella pneumoniae	wzi104	KL51	K51	O1/O2v2	O1
MAKM-RS045	Klebsiella pneumoniae	wzi13	KL13	K13	O1/O2v1	O1
MAKM-RS083	Klebsiella pneumoniae	wzi141	KL25	K25	O5	O5

CHAPTER FIVE: DISCUSSION

Multidrug resistance and high virulence have become apparent evolutionary directions of *K. pneumoniae*, and have emerged as critically alarming threat to the global community (S. Liu et al., 2021). Recent reports revealed the coexistence of both phenotypes in *K. pneumoniae* strains in clinical settings which imperils the improvement in medical health care in consideration of the increased morbidity, mortality, length of hospitalization and poor clinical outcome, (S. Liu et al., 2022, Lan et al., 2021, Tang et al., 2020). With the co-ocurrence of colistin resistance and high virulence in some *K.pneumoniae* strains, the untreatable invasive infection are obvious. It is therefore crucial to curtail this problem through rapid detection and genetic characterization of clinical relevant bacteria like *K.pneumoniae* to curb their spread. Whole genome sequencing has emerged as a transformative tool in the field of clinical microbiology and is now increasingly employed in clinical settings and research to investigate antimicrobial resistance and virulence of bacteria for active surveillance.

Our study revealed resistance of *K. pneumoniae* isolates to colistin antibiotic. 100% of the isolates investigated matched with both genotypic and phenotypic colistin resistance indicating high probability to predict the phenotype based on genotypic resistance. The genetic determinant of colistin resistance noted in this study were pmrA, pmrB, phoP, phoQ and mgrB genes. There were significant non-synonymous deleterious mutations in these genes. Chromosomal mutation in the regulatory genes PhoP/PhoQ, pmr A/pmrB and mgr B are important mechanisms leading to resistance (chromosomal mediated resistance). The mutation results in modification in the lipid A of the LPS on the bacterial surface, reducing the binding of Colistin. (Jayol et al., 2014). Other studies' findings are in agreement with these results and implicate the high prevalence of mutation in these genes with colistin resistance.(Yusof et al., 2022, Jaidane et al., 2018, Olaitan et al., 2014). Notably numerous amino acid substitution were observed in pmrB, the findings are supported by previous studies which also observed this trend. (Jayol et al., 2014).

Despite the fact that each isolate had atleast one plasmid and fifteen plasmids in all isolates, mcr-1 gene that codes for colistion resistance was not detected on any of these plasmids. Similarly other studies have demonstrated the same previously (Masood et al., 2021). Interstingly, on the contrary, we detected mcr-1 gene on the chromosome. The results of this particular study are in agreement with other earlier reports of localization of mcr-1 gene on the chromosome (Singh et

al., 2018, H. Yu et al., 2016, Falgenhauer et al., 2016) An important explanation for this finding is that mcr-1 gene has integrated and stabilized within the chromosome. It is also important to note that we employed similar method employed by Uddin et al (Uddin et al., 2022) in detection of mcr-1 gene in *E.coli* to detect mcr-1 gene in *K.pneumoniae*. Briefly, the prediction of mcr-1 genes was done through detection of mcr-like proteins' homologous sequences through BLASTp search in several databases. The hits which the chromosome-borne transferases of *K.pneumoniae strains* MAKM from this study possessed, showed homology. It follows that all the genes annotated as phosphoethanolamine transferases were mcr-1 genes and could therefore confer colistin resistance. Again, establishing homology among proteins is vital to studies of protein function prediction, phylogenetics and evolution, protein annotation, structure and classification. In protein function prediction, accurate alignments between homologous protein sequences and their abudance are critical (Margelevičius, 2020). Despite the fact that NCBI and UniProt are leading and high quality databases which give identity or similarity sequences, they fall short in sensitive, accurate and fast homology searches. Therefore for protein analysis by homology, we used the COMER web server which is the latest high-quality tool integrating multiple databases (Dapkūnas & Margelevičius, 2023a). It employs homology search algorithm for protein analysis at the sequence, structure and functional levels and the significant hits that COMER identifies generally represent true evolutionary relationship(Dapkūnas & Margelevičius, 2023b)

In this study, we identified mutations in arnT and eptB genes. Mutations in arnT and eptB mediate colistin resistance (Mathur et al., 2018). It is therefore likely that these mutations could also explain colistin resistance in the isolates they were identified.

One particularly crucial observation is that Colistin resistant *K. pneumoniae* harbored high number of multidrug resistance genes. The high MDR genotypes in this study is possibly attributed to chromosomal mutations and plasmid carrying genes conferring resistance to β-lactams, fluoroquinolones, macrolides, aminoglycosides, among others. One of the most prevalent resistant genes were the β-lactamase genes in particular ESBL genes including ^{bla}TEM and ^{bla}CTX-M, ^{bla}SHV gene families. These finding depict a closely similar pattern from a study conducted by Liu et al, (Y. Liu et al., 2021). The high prevalence of ^{bla}CTX-M-15 and ^{bla}TEM-1 coincide with the observation from clinical *K. pneumoniae* samples isolates in a county

clinical Emergence hospital Romania (Ghenea et al., 2022) which revealed worrying increase of these ESBL genes as well as recent worldwide reports on the distribution of these ESBL genes (Zhou et al., 2015, Khosravi et al., 2013, Emeraud et al., 2021, Kakuta et al., 2020)

Fluoroquinolone resistance is multifactorial, it can result from overexpression of multi drug efflux pumps which pumps out antibiotics from bacteria thus reducing their therapeutic effect, antibiotic target alteration and reduced permeability to antibiotics (Redgrave et al., 2014, Hooper & Jacoby, 2015, Hooper & Jacoby, 2016) A specific fluoroquinolone resistant gene QnrS1 which protect antibiotic target was detected in one isolate. The detection of several efflux pump genes such as adeF, CRP, emrR, H-NS, Kpne_KpnG, Kpne_KpnH, Kpne_ramR, marA, rsmA and Ecol_MarR_MULT in the isolates offers the significant reason for their resistance. On the same note, the detected mutation in Kpne_ramR which is a repressor that regulate Ram A expression may cause upregulation of AcrAB consequently leading to alteration of antibiotic target.

Similar to other studies macrolide resistant genes mphA, Mrx and other multi drug-efflux genes were identified. Inspite of macrolide not being used in treatment of gram–negative infections, their presence in these *K.pneumoniae* isolates or any other gram-negative bacteria may serve as the reservoir of resistance genes that can be transferred to gram-positive bacteria thus enhancing resistance.

Consistent with the previous studies, fosfomycin resistance were mediated by a plasmid–encoded enzyme FosA6 (Guo et al., 2016). This enzyme breaks the epoxide ring of the molecule and therefore cause antibiotic inactivation. Another resistance gene Ecol_UhpT_FOF though rarely reported in many studies has been detected in one isolate.

Regarding sulfamethoxazole/trimethoprim; sul2 and sul1 genes were detected and confer resistance to sulfamethoxazole through antibiotic target protection/alteration (J. Li et al., 2020) whereas in trimethophoprim dfrA17 and dfrA12 genes were detected, each in one isolate. In this study, Co-trimoxazole which is a broad spectrum antibiotic containing two antimicrobial drugs (sulfamethoxazole and trimethoprim) was the highest resisted drug by *K.pneumoniae* which is consistent with other previous studies in other countries (Kashefieh et al., 2021, Hu et al., 2014, Sheykhsaran et al., 2018).Two plasmid mediated genes encoding sulfamethoxazole resistance have been detected. sul 2 gene had high frequency as it was found in four out of five isolates. This is not surprising as earlier studies identified the high prevalence of this gene

(Kashefieh et al., 2021). Also our study identified dfrA17, dfrA12 genes which mediated trimethoprim resistance through antibiotic target replacement. The detection of these genes point out the phenotypic resistance phenotype exhibited by the isolates.

Resistance to tetracycline is mediated by efflux pumps, antibiotic target alteration or reduced permeability to antibiotics. Like in other previous studies, tetD was identified as the tetracycline resistance gene (Sheykhsaran et al., 2018) . However, contrary to these earlier studies, the frequency of Tetracycline was low as it was found in only one isolate. Other multi-efflux pump genes like AdeF, Ecol_MarR_MULT, H-NS, Kpne_ramR, marA, and OqxA were also detected. Among chloramphenicol resistance, an antibiotic efflux gene rsmA was prevalent. Most importantly the commonly detected gene catI that mediate resistance through antibiotic inactivation was identified in only one isolate..

Aminoglycoside are vital in treatment of *K.pneumoniae* infections. Several mechanisms are involved in causing resistance to Aminoglycoside including efflux mechanisms, enzymatic modification of the antibiotic and impaired membrane permeability (Garneau-Tsodikova & J. Labby, 2016). In our study different multidrug efflux genes including BaeR, Kpne_KpnE, Kpne_KpnG, Kpne_KpnH, Kpne_KpnF and genes were found in high frequency. Also aadA5 and aadA2 genes that mediate resistance to aminoglycoside through antibiotic inactivation were detected in one isolate each.

Strains in our study are clustered with several other some strains that are already published from other studies or found in NCBI. (figure 9), which infer close relatedness of our strains with those from other parts. For instance; the strain MAKM-RS083 in our study is closerly related with strain KCRI-17 isolated from wound swab of a patient in Kilimanjaro, Tanzania, which may indicate similar resistance pattern. Even though some of those studies did not report colistin resistance probably due to their objectives or other shortfalls. This study serves as an indicator of the possibility of presence of colistin resistant strains in East African region.

On the other hand, recent research have directed their focus on the emerging hypervirulent *K.pneumoniae* strains due to the fact that they are associated with severe infections such as liver abscess, pneumonia and sepsis. Previous studies suggested that multidrug resistant *K. pneumoniae* strains were less virulent.(Lomonaco et al., 2018) More recently several report have suggested increasing rise of the virulent and multidrug resistant *K.pneumoniae.* Examples

include reports by Liu et al, who evaluated hypervilurent *K.pneumoniae* with colistin resistance in china where Fatima et al reported clinical *K.pneumoniae* which are virulent multidrug resistant (X. Liu et al., 2022, Fatima et al., 2021). Jia et al, also addressed this threat on his study which unveiled *K. pneumoniae* with superplasmid coharboring hypervirulence and multidrug Resistance genes (Jia et al., 2022). This study reports that the *K. pneumoniae* isolates are multidrug resistant and virulent.

Five (5) *K. pneumoniae* isolates investigated had numerous virulence genes with exception of one isolate which had only adherence virulence genes, fim genes. The adherence genes were numerous and mostly fim genes such as yagX/ecpC, yagW/ecpD, yagY/ecpB, yagZ/ecpA just to mention few (Table 7). Siderophores gene clusters encoding salmochelin, enterobactin and aerobactin were also detected. Effector delivery system T6SS was important virulence factor found in *K.pneumoniae* in this study. Siderophores are molecules used by *K. pneumoniae* and other bacteria for Iron acquisition. Several siderophore such salmochelin; iroN, enterobactin like entA, entB, entE, fepB, fepD among others and aerobactin; iutA were found in four isolates. Many studies have confirmed the involvement of aerobactin in mediating hypervirulence. The study by Bailey et al suggest that aerobactin presence enhance hypervirulence (Bailey et al., 2018), Russo et al clearly state that Aerobactin mediate virulence in hypervirulent *K.pneumoniae*.(Russo et al., 2014, Russo et al., 2015). The presence of siderophore aerobactin in *K.pneumoniae* isolates confirms their hypervirulence. Just the same as aerobactin, salmochelin is also associated with increased virulence in *K.pneumoniae* (Nicolò et al., 2022)

Previous findings associated hypermucoviscous phenotype with hypervirulent *K. pneumoniae* strain. A simple technique called string test is recommended to identify the hypermucoviscous phenotype. In this study. a number of *K. pneumoniae* were determined to be hypermucoviscous as out of all 31 tested samples only 14 had hypermucoviscous phenotype accounting to 45.1 Percent (45.1%). The results suggest that these positive *K.pneumoniae* strains may be hypervirulent. However, some studies refute that the string test cannot be used solely as a measure to determine if *K.pneumoniae* exhibits hypervirulence. Not all hypervirulent *K.pneumoniae* strains (hvKp) are hypermucoid. Findings from Russo et al. suggest genetic cause of hypervirulence in which *rmpA* and *iucA* genes are the major determinants of hypervirulence (Russo et al., 2014). rmpA is a plasmid encoded virulence gene regulating the synthesis of

capsular polysaccharides (CPS) production of large amount of CPS offers a protective layer that helps the bacteria to evade the host immune response and survive in different environments (Zhang et al., 2020). The finding of this study clearly indicate that these isolates are highly virulent though the aforementioned genes were not found. The isolate still expressed many important virulence genes. Moreso, it has now emerged that even presence of aerobactin can cause hypermucoviscosity in *K.pneumoniae* strains.

Due to resource limitations, we sequenced only six purposefully selected isolates, which although few, still give significant snapshort of genomic deteminants of virulence and colistin resistance in *K.pneumoniae*.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

The study underscored the application of Whole genome sequencing technology to uncover the genetic determinants of virulence and Colistin resistance in clinical *K. pneumoniae* isolates. The findings revealed a relatively high proportion of virulent and antimicrobial resistant genes.

The results from the sequenced *K.pneumoniae* strains showed relatively a high proportion of virulence genes. Several virulence factors genes such as fim genes for adherence, enterobactin, salmocherin aerobactin for iron acquisition, clpvi for effector delivery system or waaA genes for LPS were found in colistin resistant K. pneumoniae strains. The presence of aerobactin and salmochelin is strongly indicative of hypervirulent strains of *K.pneumoniae*.

All five sequenced Colistin resistant *K.pneumoniae* strains have genotypic determinants for colistin resistance due to mutations in pmrA, pmrB, rhoP, rhoQ and mgrB genes. Another genetic determinant of note was mcr-1 gene which was found on the chromosome. These colistin resistant isolates also had determinants encoding antimicrobial resistance genes against the investigated classes of antibiotics.

If the results of this study are interpreted in the context of the general objective which was to delineate the genomic determinants of virulence and antimicrobial resistance in colistin resistant *K.pneumoniae* isolates, then the study clearly show the co-occurrence of colistin resistance and high virulence genes in clinical isolates which is alarming and warrants attention as it can lead to untreatable and invasive *K. pneumoniae* infections. It is therefore imperative to conduct active surveillance in order to prevent the transmission and spread of highly virulent or multidrug resistant strains, focusing not only on antimicrobial resistance but also on identifying virulence determinants.

While this work encouragingly shows high level of matching between genotypic and phenotypic results, it is too early to conclude that genotype predict phenotype as it was investigated in only five isolates. More research must be done to ascertain the concordance of the results. Additionally, colistin resistance databases are still less exhaustive, in this study we relied on manual curation to establish individual gene polymorphism for colistin resistance

REFERENCES

Amr, N. G. H. R. U. on G. S. of. (2020). Whole-genome sequencing as part of national and international surveillance programmes for antimicrobial resistance: A roadmap. *BMJ Global Health*, *5*(11), e002244. https://doi.org/10.1136/bmjgh-2019-002244

Antonopoulos, D. A., Assaf, R., Aziz, R. K., Brettin, T., Bun, C., Conrad, N., Davis, J. J., Dietrich, E. M., Disz, T., Gerdes, S., Kenyon, R. W., Machi, D., Mao, C., Murphy-Olson, D. E., Nordberg, E. K., Olsen, G. J., Olson, R., Overbeek, R., Parrello, B., … Yoo, H. (2019). PATRIC as a unique resource for studying antimicrobial resistance. *Briefings in Bioinformatics*, *20*(4), 1094–1102. https://doi.org/10.1093/bib/bbx083

Argimón, S., Masim, M. A. L., Gayeta, J. M., Lagrada, M. L., Macaranas, P. K. V., Cohen, V., Limas, M. T., Espiritu, H. O., Palarca, J. C., Chilam, J., Jamoralin, M. C., Villamin, A. S., Borlasa, J. B., Olorosa, A. M., Hernandez, L. F. T., Boehme, K. D., Jeffrey, B., Abudahab, K., Hufano, C. M., … Carlos, C. C. (2020). Integrating whole-genome sequencing within the National Antimicrobial Resistance Surveillance Program in the Philippines. *Nature Communications*, *11*(1), Article 1. https://doi.org/10.1038/s41467-020-16322-5

Ashurst, J. V., & Dawson, A. (2022). Klebsiella Pneumonia. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK519004/

Azam, M., Gaind, R., Yadav, G., Sharma, A., Upmanyu, K., Jain, M., & Singh, R. (2021). Colistin Resistance Among Multiple Sequence Types of Klebsiella pneumoniae Is Associated With Diverse Resistance Mechanisms: A Report From India. *Frontiers in Microbiology*, *12*, 609840. https://doi.org/10.3389/fmicb.2021.609840

Bailey, D. C., Alexander, E., Rice, M. R., Drake, E. J., Mydy, L. S., Aldrich, C. C., & Gulick, A. M. (2018). Structural and functional delineation of aerobactin biosynthesis in hypervirulent Klebsiella

pneumoniae. *Journal of Biological Chemistry*, *293*(20), 7841–7852. https://doi.org/10.1074/jbc.RA118.002798

Bialek-Davenet, S., Lavigne, J.-P., Guyot, K., Mayer, N., Tournebize, R., Brisse, S., Leflon-Guibout, V., & Nicolas-Chanoine, M.-H. (2015). Differential contribution of AcrAB and OqxAB efflux pumps to multidrug resistance and virulence in Klebsiella pneumoniae. *Journal of Antimicrobial Chemotherapy*, *70*(1), 81–88. https://doi.org/10.1093/jac/dku340

Brisse, S., Grimont, F., & Grimont, P. A. D. (2006). The Genus <Emphasis Type="Italic">Klebsiella</Emphasis>. *The Prokaryotes*, 159–196. https://doi.org/10.1007/0-387-30746-X_8

Cannatelli Antonio, D'Andrea Marco Maria, Giani Tommaso, Di Pilato Vincenzo, Arena Fabio, Ambretti Simone, Gaibani Paolo, & Rossolini Gian Maria. (2013). In Vivo Emergence of Colistin Resistance in Klebsiella pneumoniae Producing KPC-Type Carbapenemases Mediated by Insertional Inactivation of the PhoQ/PhoP mgrB Regulator. *Antimicrobial Agents and Chemotherapy*, *57*(11), 5521–5526. https://doi.org/10.1128/aac.01480-13

Chang, D., Sharma, L., Dela Cruz, C. S., & Zhang, D. (2021). Clinical Epidemiology, Risk Factors, and Control Strategies of Klebsiella pneumoniae Infection. *Frontiers in Microbiology*, *12*, 750662. https://doi.org/10.3389/fmicb.2021.750662

Cheng, Y.-H., Lin, T.-L., Pan, Y.-J., Wang, Y.-P., Lin, Y.-T., & Wang, J.-T. (2015). Colistin Resistance Mechanisms in Klebsiella pneumoniae Strains from Taiwan. *Antimicrobial Agents and Chemotherapy*, *59*(5), 2909–2913. https://doi.org/10.1128/aac.04763-14

Dadgostar, P. (2019). Antimicrobial Resistance: Implications and Costs. *Infection and Drug Resistance*, *12*, 3903. https://doi.org/10.2147/IDR.S234610

Dai, C., Tang, S., Velkov, T., & Xiao, X. (2016). Colistin-Induced Apoptosis of Neuroblastoma-2a Cells Involves the Generation of Reactive Oxygen Species, Mitochondrial Dysfunction, and Autophagy. *Molecular Neurobiology*, *53*(7), 4685–4700. https://doi.org/10.1007/s12035-015-9396-7

Dapkūnas, J., & Margelevičius, M. (2023a). The COMER web server for protein analysis by homology. *Bioinformatics*, *39*(1), btac807. https://doi.org/10.1093/bioinformatics/btac807

Dapkūnas, J., & Margelevičius, M. (2023b). The COMER web server for protein analysis by homology. *Bioinformatics*, *39*(1), btac807. https://doi.org/10.1093/bioinformatics/btac807

Davison, J. (1999). Genetic Exchange between Bacteria in the Environment. *Plasmid*, *42*(2), 73–91. https://doi.org/10.1006/plas.1999.1421

Dijkmans, A. C., Wilms, E. B., Kamerling, I. M. C., Birkhoff, W., Ortiz-Zacarías, N. V., van Nieuwkoop, C., Verbrugh, H. A., & Touw, D. J. (2015). Colistin: Revival of an Old Polymyxin Antibiotic. *Therapeutic Drug Monitoring*, *37*(4), 419. https://doi.org/10.1097/FTD.0000000000000172

Elias, R., Duarte, A., & Perdigão, J. (2021). A Molecular Perspective on Colistin and Klebsiella pneumoniae: Mode of Action, Resistance Genetics, and Phenotypic Susceptibility. *Diagnostics*, *11*(7), Article 7. https://doi.org/10.3390/diagnostics11071165

El-Sayed Ahmed, M. A. E.-G., Zhong, L.-L., Shen, C., Yang, Y., Doi, Y., & Tian, G.-B. (2020). Colistin and its role in the Era of antibiotic resistance: An extended review (2000–2019). *Emerging Microbes & Infections*, *9*(1), 868–885. https://doi.org/10.1080/22221751.2020.1754133

Emeraud, C., Figueiredo, S., Bonnin, R. A., Khecharem, M., Ouzani, S., Leblanc, P.-E., Jousset, A. B., Fortineau, N., Duranteau, J., & Dortet, L. (2021). Outbreak of CTX-M-15 Extended-Spectrum β-Lactamase-Producing Klebsiella pneumoniae ST394 in a French Intensive Care Unit Dedicated to COVID-19. *Pathogens*, *10*(11), Article 11. https://doi.org/10.3390/pathogens10111426

Fahimeh, H., Nasim, S., Pegah, A., Mojgan, R., Neda, M., & Reza, A. M. (2016). *Assessment Of The Prevalence Of Class I And Ii Integrons Of Escherichia Coli And Klebsiella Neumoniae Isolates From Hospitals Of Hamadan*. *23*(381), 193–201.

Falgenhauer, L., Waezsada, S.-E., Gwozdzinski, K., Ghosh, H., Doijad, S., Bunk, B., Spröer, C., Imirzalioglu, C., Seifert, H., Irrgang, A., Fischer, J., Guerra, B., Käsbohrer, A., Overmann, J., Goesmann, A., & Chakraborty, T. (2016). Chromosomal Locations of mcr-1 and blaCTX-M-15 in Fluoroquinolone-Resistant Escherichia coli ST410. *Emerging Infectious Diseases*, *22*(9), 1689–1691. https://doi.org/10.3201/eid2209.160692

Fatima, S., Liaqat, F., Akbar, A., Sahfee, M., Samad, A., Anwar, M., Iqbal, S., Khan, S. A., Sadia, H., Makai, G., Bahadur, A., Naeem, W., & Khan, A. (2021). Virulent and multidrug-resistant Klebsiella pneumoniae from clinical samples in Balochistan. *International Wound Journal*, *18*(4), 510–518. https://doi.org/10.1111/iwj.13550

Garneau-Tsodikova, S., & J. Labby, K. (2016). Mechanisms of resistance to aminoglycoside antibiotics: Overview and perspectives. *MedChemComm*, *7*(1), 11–27. https://doi.org/10.1039/C5MD00344J

Ghenea, A. E., Zlatian, O. M., Cristea, O. M., Ungureanu, A., Mititelu, R. R., Balasoiu, A. T., Vasile, C. M., Salan, A.-I., Iliuta, D., Popescu, M., Udriștoiu, A.-L., & Balasoiu, M. (2022). TEM,CTX-M,SHV Genes in ESBL-Producing Escherichia coli and Klebsiella pneumoniae Isolated from Clinical Samples in a County Clinical Emergency Hospital Romania-Predominance of CTX-M-15. *Antibiotics*, *11*(4), Article 4. https://doi.org/10.3390/antibiotics11040503

Gogry, F. A., Siddiqui, M. T., Sultan, I., & Haq, Q. Mohd. R. (2021). Current Update on Intrinsic and Acquired Colistin Resistance Mechanisms in Bacteria. *Frontiers in Medicine*, *8*. https://www.frontiersin.org/articles/10.3389/fmed.2021.677720

Granata, G., & Petrosillo, N. (2017). Resistance to Colistin in Klebsiella pneumoniae: A 4.0 Strain? *Infectious Disease Reports*, *9*(2), Article 2. https://doi.org/10.4081/idr.2017.7104

Guo, Q., Tomich, A. D., McElheny, C. L., Cooper, V. S., Stoesser, N., Wang, M., Sluis-Cremer, N., & Doi, Y. (2016). Glutathione-S-transferase FosA6 of Klebsiella pneumoniae origin conferring fosfomycin resistance in ESBL-producing Escherichia coli. *Journal of Antimicrobial Chemotherapy*, *71*(9), 2460–2465. https://doi.org/10.1093/jac/dkw177

Gweon, H. S., Shaw, L. P., Swann, J., Maio, N. D., AbuOun, M., Niehus, R., Hubbard, A. T. M., Bowes, M. J., Bailey, M. J., Peto, T. E. A., Hoosdally, S. J., Walker, A. S., Sebra, R. P., Crook, D. W., Anjum, M. F., Read, D. S., Stoesser, N., & Consortium, on behalf of the R. (2019). The impact of sequencing depth on the inferred taxonomic composition and AMR gene content of metagenomic samples. *Environmental Microbiome*, *14*. https://doi.org/10.1186/s40793-019-0347-1

H, E., I, U.-H., S, M., A, Z., & M, M. J. (2013). Detection of extended-spectrum β-lactamases in Klebsiella pneumoniae: Comparison of phenotypic characterization methods. *Pakistan Journal of Medical Sciences*, *29*(3). https://doi.org/10.12669/pjms.293.3576

Haeili, M., Javani, A., Moradi, J., Jafari, Z., Feizabadi, M. M., & Babaei, E. (2017). MgrB Alterations Mediate Colistin Resistance in Klebsiella pneumoniae Isolates from Iran. *Frontiers in Microbiology*, *8*. https://www.frontiersin.org/articles/10.3389/fmicb.2017.02470

Hamel, M., Rolain, J.-M., & Baron, S. A. (2021). The History of Colistin Resistance Mechanisms in Bacteria: Progress and Challenges. *Microorganisms*, *9*(2), Article 2. https://doi.org/10.3390/microorganisms9020442

Harbottle, H., Thakur, S., Zhao, S., & White, D. G. (2006). Genetics of Antimicrobial Resistance. *Animal Biotechnology*, *17*(2), 111–124. https://doi.org/10.1080/10495390600957092

Hooper, D. C., & Jacoby, G. A. (2015). Mechanisms of drug resistance: Quinolone resistance. *Annals of the New York Academy of Sciences*, *1354*(1), 12–31. https://doi.org/10.1111/nyas.12830

Hooper, D. C., & Jacoby, G. A. (2016). Topoisomerase Inhibitors: Fluoroquinolone Mechanisms of Action and Resistance. *Cold Spring Harbor Perspectives in Medicine*, *6*(9), a025320. https://doi.org/10.1101/cshperspect.a025320

Hu, L., Zhong, Q., Shang, Y., Wang, H., Ning, C., Li, Y., Hang, Y., Xiong, J., Wang, X., Xu, Y., Qin, Z., Parsons, C., Wang, L., & Yu, F. (2014). The prevalence of carbapenemase genes and plasmid-mediated quinolone resistance determinants in carbapenem-resistant Enterobacteriaceae from five teaching hospitals in central China. *Epidemiology & Infection*, *142*(9), 1972–1977. https://doi.org/10.1017/S0950268813002975

Huson, D. H., Richter, D. C., Rausch, C., Dezulian, T., Franz, M., & Rupp, R. (2007). Dendroscope: An interactive viewer for large phylogenetic trees. *BMC Bioinformatics*, *8*(1), 460. https://doi.org/10.1186/1471-2105-8-460

Jacoby, G. A., & Archer, G. L. (1991). New Mechanisms of Bacterial Resistance to Antimicrobial Agents. *New England Journal of Medicine*, *324*(9), 601–612. https://doi.org/10.1056/NEJM199102283240906

Jaidane, N., Bonnin, R. A., Mansour, W., Girlich, D., Creton, E., Cotellon, G., Chaouch, C., Boujaafar, N., Bouallegue, O., & Naas, T. (2018). Genomic Insights into Colistin-Resistant Klebsiella pneumoniae from a Tunisian Teaching Hospital. *Antimicrobial Agents and Chemotherapy*, *62*(2), 10.1128/aac.01601-17. https://doi.org/10.1128/aac.01601-17

Jansen, K. U., Knirsch, C., & Anderson, A. S. (2018). The role of vaccines in preventing bacterial antimicrobial resistance. *Nature Medicine*, *24*(1), Article 1. https://doi.org/10.1038/nm.4465

Jayol, A., Poirel, L., Brink, A., Villegas, M.-V., Yilmaz, M., & Nordmann, P. (2014). Resistance to Colistin Associated with a Single Amino Acid Change in Protein PmrB among Klebsiella pneumoniae Isolates of Worldwide Origin. *Antimicrobial Agents and Chemotherapy*, *58*(8), 4762–4766. https://doi.org/10.1128/aac.00084-14

Jia, X., Zhu, Y., Jia, P., Liu, X., Yu, W., Li, X., Xu, Y., & Yang, Q. (2022). Emergence of a Superplasmid Coharboring Hypervirulence and Multidrug Resistance Genes in Klebsiella pneumoniae Poses New Challenges to Public Health. *Microbiology Spectrum*, *10*(6), e02634-22. https://doi.org/10.1128/spectrum.02634-22

Joseph, L., Merciecca, T., Forestier, C., Balestrino, D., & Miquel, S. (2021). From Klebsiella pneumoniae Colonization to Dissemination: An Overview of Studies Implementing Murine Models. *Microorganisms*, *9*(6), Article 6. https://doi.org/10.3390/microorganisms9061282

Kakuta, N., Nakano, R., Nakano, A., Suzuki, Y., Masui, T., Horiuchi, S., Kakuta, R., Tsubaki, K., Ogawa, M., & Yano, H. (2020). Molecular characteristics of extended-spectrum β-lactamase-producing Klebsiella pneumoniae in Japan: Predominance of CTX-M-15 and emergence of hypervirulent clones. *International Journal of Infectious Diseases*, *98*, 281–286. https://doi.org/10.1016/j.ijid.2020.06.083

Kashefieh, M., Hosainzadegan, H., Baghbanijavid, S., & Ghotaslou, R. (2021). The Molecular Epidemiology of Resistance to Antibiotics among *Klebsiella pneumoniae* Isolates in Azerbaijan, Iran. *Journal of Tropical Medicine*, *2021*, e9195184. https://doi.org/10.1155/2021/9195184

Katoh, K., Rozewicki, J., & Yamada, K. D. (2019). MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics*, *20*(4), 1160–1166. https://doi.org/10.1093/bib/bbx108

Khosravi, A. D., Hoveizavi, H., & Mehdinejad, M. (2013). Prevalence of Klebsiella pneumoniae Encoding Genes for Ctx-M-1, Tem-1 and Shv-1 Extended-Spectrum Beta Lactamases (ESBL) Enzymes in Clinical Specimens. *Jundishapur Journal of Microbiology*, *6*(10), Article 10. https://doi.org/10.5812/jjm.8256

Kim, J., Jo, A., Chukeatirote, E., & Ahn, J. (2016). Assessment of antibiotic resistance in Klebsiella pneumoniae exposed to sequential in vitro antibiotic treatments. *Annals of Clinical Microbiology and Antimicrobials*, *15*(1), Article 1. https://doi.org/10.1186/s12941-016-0173-x

Kl, W., & Ke, H. (2018). Klebsiella pneumoniae as a key trafficker of drug resistance genes from environmental to clinically important bacteria. *Current Opinion in Microbiology*, *45*. https://doi.org/10.1016/j.mib.2018.04.004

Kon, K. V., & Rai, M. (Eds.). (2016). *Antibiotic resistance: Mechanisms and new antimicrobial approaches*. Elsevier, Academic Press.

Kumabe, A., & Kenzaka, T. (2014). String test of hypervirulent Klebsiella pneumonia. *QJM: An International Journal of Medicine*, *107*(12), 1053–1053. https://doi.org/10.1093/qjmed/hcu124

Lan, P., Jiang, Y., Zhou, J., & Yu, Y. (2021). A global perspective on the convergence of hypervirulence and carbapenem resistance in Klebsiella pneumoniae. *Journal of Global Antimicrobial Resistance*, *25*, 26–34. https://doi.org/10.1016/j.jgar.2021.02.020

Le Roux, F., & Blokesch, M. (2018). Eco-evolutionary Dynamics Linked to Horizontal Gene Transfer in Vibrios. *Annual Review of Microbiology*, *72*(1), 89–110. https://doi.org/10.1146/annurev-micro-090817-062148

Lee, C.-R., Lee, J. H., Park, K. S., Jeon, J. H., Kim, Y. B., Cha, C.-J., Jeong, B. C., & Lee, S. H. (2017). Antimicrobial Resistance of Hypervirulent Klebsiella pneumoniae: Epidemiology, Hypervirulence-Associated Determinants, and Resistance Mechanisms. *Frontiers in Cellular and Infection Microbiology*, *0*. https://doi.org/10.3389/fcimb.2017.00483

Li, H. (2013). *Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM*. https://doi.org/10.48550/arXiv.1303.3997

Li, J., Bi, W., Dong, G., Zhang, Y., Wu, Q., Dong, T., Cao, J., & Zhou, T. (2020). The new perspective of old antibiotic: In vitro antibacterial activity of TMP-SMZ against Klebsiella pneumoniae. *Journal of*

Microbiology, Immunology and Infection, *53*(5), 757–765. https://doi.org/10.1016/j.jmii.2018.12.013

Li, X., Fan, H., Zi, H., Hu, H., Li, B., Huang, J., Luo, P., & Zeng, X. (2022). Global and Regional Burden of Bacterial Antimicrobial Resistance in Urinary Tract Infections in 2019. *Journal of Clinical Medicine*, *11*(10), Article 10. https://doi.org/10.3390/jcm11102817

Li, Y., Kumar, S., Zhang, L., Wu, H., & Wu, H. (2023). Characteristics of antibiotic resistance mechanisms and genes of Klebsiella pneumoniae. *Open Medicine*, *18*(1). https://doi.org/10.1515/med-2023-0707

Liu, S., Ding, Y., Xu, Y., Li, Z., Zeng, Z., & Liu, J. (2022). An outbreak of extensively drug-resistant and hypervirulent Klebsiella pneumoniae in an intensive care unit of a teaching hospital in Southwest China. *Frontiers in Cellular and Infection Microbiology*, *12*, 979219. https://doi.org/10.3389/fcimb.2022.979219

Liu, S., Galat, V., Galat4, Y., Lee, Y. K. A., Wainwright, D., & Wu, J. (2021). NK cell-based cancer immunotherapy: From basic biology to clinical development. *Journal of Hematology & Oncology*, *14*(1), Article 1. https://doi.org/10.1186/s13045-020-01014-w

Liu, X., Wu, Y., Zhu, Y., Jia, P., Li, X., Jia, X., Yu, W., Cui, Y., Yang, R., Xia, W., Xu, Y., & Yang, Q. (2022). Emergence of colistin-resistant hypervirulent Klebsiella pneumoniae (CoR-HvKp) in China. *Emerging Microbes & Infections*, *11*(1), 648–661. https://doi.org/10.1080/22221751.2022.2036078

Liu, Y., Lin, Y., Wang, Z., Hu, N., Liu, Q., Zhou, W., Li, X., Hu, L., Guo, J., Huang, X., & Zeng, L. (2021). Molecular Mechanisms of Colistin Resistance in Klebsiella pneumoniae in a Tertiary Care Teaching Hospital. *Frontiers in Cellular and Infection Microbiology*, *11*, 673503. https://doi.org/10.3389/fcimb.2021.673503

Llobet, E., Tomás, J. M., & Bengoechea, J. A. (2008). Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. *Microbiology*, *154*(12), 3877–3886. https://doi.org/10.1099/mic.0.2008/022301-0

Lomonaco, S., Crawford, M. A., Lascols, C., Timme, R. E., Anderson, K., Hodge, D. R., Fisher, D. J., Pillai, S. P., Morse, S. A., Khan, E., Hughes, M. A., Allard, M. W., & Sharma, S. K. (2018). Resistome of carbapenem- and colistin-resistant Klebsiella pneumoniae clinical isolates. *PLOS ONE*, *13*(6), e0198526. https://doi.org/10.1371/journal.pone.0198526

Mancuso, G., Midiri, A., Gerace, E., & Biondo, C. (2021). Bacterial Antibiotic Resistance: The Most Critical Pathogens. *Pathogens*, *10*(10), Article 10. https://doi.org/10.3390/pathogens10101310

Mansour, W., Haenni, M., Saras, E., Grami, R., Mani, Y., Ben Haj Khalifa, A., el Atrouss, S., Kheder, M., Fekih Hassen, M., Boujâafar, N., Bouallegue, O., & Madec, J.-Y. (2017). Outbreak of colistin-resistant carbapenemase-producing Klebsiella pneumoniae in Tunisia. *Journal of Global Antimicrobial Resistance*, *10*, 88–94. https://doi.org/10.1016/j.jgar.2017.03.017

Margelevičius, M. (2020). COMER2: GPU-accelerated sensitive and specific homology searches. *Bioinformatics*, *36*(11), 3570–3572. https://doi.org/10.1093/bioinformatics/btaa185

Martin, R. M., & Bachman, M. A. (2018). Colonization, Infection, and the Accessory Genome of Klebsiella pneumoniae. *Frontiers in Cellular and Infection Microbiology*, *0*. https://doi.org/10.3389/fcimb.2018.00004

Masood, K. I., Umar, S., Hasan, Z., Farooqi, J., Razzak, S. A., Jabeen, N., Rao, J., Shakoor, S., & Hasan, R. (2021). Lipid A-Ara4N as an alternate pathway for (colistin) resistance in Klebsiella pneumonia isolates in Pakistan. *BMC Research Notes*, *14*(1), 449. https://doi.org/10.1186/s13104-021-05867-3

Mathur, P., Veeraraghavan, B., Devanga Ragupathi, N. K., Inbanathan, F. Y., Khurana, S., Bhardwaj, N., Kumar, S., Sagar, S., & Gupta, A. (2018). Multiple mutations in lipid-A modification pathway &

novel fosA variants in colistin-resistant Klebsiella pneumoniae. *Future Science OA*, *4*(7), FSO319. https://doi.org/10.4155/fsoa-2018-0011

Meier-Kolthoff, J. P., & Göker, M. (2019). TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nature Communications*, *10*(1), 2182. https://doi.org/10.1038/s41467-019-10210-3

Nakamura-Silva, R., Oliveira-Silva, M., Furlan, J. P. R., Stehling, E. G., Miranda, C. E. S., & Pitondo-Silva, A. (2021). Characterization of multidrug-resistant and virulent Klebsiella pneumoniae strains belonging to the high-risk clonal group 258 (CG258) isolated from inpatients in northeastern Brazil. *Archives of Microbiology*, *203*(7), 4351–4359. https://doi.org/10.1007/s00203-021-02425-0

Navon-Venezia, S., Kondratyeva, K., & Carattoli, A. (2017). Klebsiella pneumoniae: A major worldwide source and shuttle for antibiotic resistance. *FEMS Microbiology Reviews*, *41*(3), 252–275. https://doi.org/10.1093/femsre/fux013

Nicolò, S., Mattiuz, G., Antonelli, A., Arena, F., Di Pilato, V., Giani, T., Baccani, I., Clemente, A. M., Castronovo, G., Tanturli, M., Cozzolino, F., Rossolini, G. M., & Torcia, M. G. (2022). Hypervirulent Klebsiella pneumoniae Strains Modulate Human Dendritic Cell Functions and Affect TH1/TH17 Response. *Microorganisms*, *10*(2), Article 2. https://doi.org/10.3390/microorganisms10020384

Nikolich, M. P., Hong, G., Shoemaker, N. B., & Salyers, A. A. (1994). Evidence for natural horizontal transfer of tetQ between bacteria that normally colonize humans and bacteria that normally colonize livestock. *Applied and Environmental Microbiology*, *60*(9), 3255–3260. https://doi.org/10.1128/aem.60.9.3255-3260.1994

Olaitan, A. O., Diene, S. M., Kempf, M., Berrazeg, M., Bakour, S., Gupta, S. K., Thongmalayvong, B., Akkhavong, K., Somphavong, S., Paboriboune, P., Chaisiri, K., Komalamisra, C., Adelowo, O. O., Fagade, O. E., Banjo, O. A., Oke, A. J., Adler, A., Assous, M. V., Morand, S., … Rolain, J.-M. (2014).

Worldwide emergence of colistin resistance in Klebsiella pneumoniae from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator mgrB: An epidemiological and molecular study. *International Journal of Antimicrobial Agents*, *44*(6), 500–507. https://doi.org/10.1016/j.ijantimicag.2014.07.020

Olaitan, A. O., Morand, S., & Rolain, J.-M. (2016). Emergence of colistin-resistant bacteria in humans without colistin usage: A new worry and cause for vigilance. *International Journal of Antimicrobial Agents*, *47*(1), 1–3. https://doi.org/10.1016/j.ijantimicag.2015.11.009

Patel, G., Huprikar, S., Factor, S. H., Jenkins, S. G., & Calfee, D. P. (2008). Outcomes of Carbapenem-Resistant Klebsiella pneumoniae Infection and the Impact of Antimicrobial and Adjunctive Therapies. *Infection Control & Hospital Epidemiology*, *29*(12), 1099–1106. https://doi.org/10.1086/592412

Pragasam, A. K., Shankar, C., Veeraraghavan, B., Biswas, I., Nabarro, L. E. B., Inbanathan, F. Y., George, B., & Verghese, S. (2017). Molecular Mechanisms of Colistin Resistance in Klebsiella pneumoniae Causing Bacteremia from India—A First Report. *Frontiers in Microbiology*, *7*. https://www.frontiersin.org/articles/10.3389/fmicb.2016.02135

Prestinaci, F., Pezzotti, P., & Pantosti, A. (2015). Antimicrobial resistance: A global multifaceted phenomenon. *Pathogens and Global Health*. https://doi.org/10.1179/2047773215Y.0000000030

Redgrave, L. S., Sutton, S. B., Webber, M. A., & Piddock, L. J. V. (2014). Fluoroquinolone resistance: Mechanisms, impact on bacteria, and role in evolutionary success. *Trends in Microbiology*, *22*(8), 438–445. https://doi.org/10.1016/j.tim.2014.04.007

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology*, *4*(3), 482–501. https://doi.org/10.3934/microbiol.2018.3.482

Roberts, M. G., Burgess, S., Toombs-Ruane, L. J., Benschop, J., Marshall, J. C., & French, N. P. (2021). Combining mutation and horizontal gene transfer in a within-host model of antibiotic resistance. *Mathematical Biosciences*, *339*, 108656. https://doi.org/10.1016/j.mbs.2021.108656

Russo, T. A., Olson, R., MacDonald, U., Beanan, J., & Davidson, B. A. (2015). Aerobactin, but Not Yersiniabactin, Salmochelin, or Enterobactin, Enables the Growth/Survival of Hypervirulent (Hypermucoviscous) Klebsiella pneumoniae Ex Vivo and In Vivo. *Infection and Immunity*, *83*(8), 3325–3333. https://doi.org/10.1128/iai.00430-15

Russo, T. A., Olson, R., MacDonald, U., Metzger, D., Maltese, L. M., Drake, E. J., & Gulick, A. M. (2014). Aerobactin Mediates Virulence and Accounts for Increased Siderophore Production under Iron-Limiting Conditions by Hypervirulent (Hypermucoviscous) Klebsiella pneumoniae. *Infection and Immunity*, *82*(6), 2356–2367. https://doi.org/10.1128/iai.01667-13

Samantha, A., & Vrielink, A. (2020). Lipid A Phosphoethanolamine Transferase: Regulation, Structure and Immune Response. *Molecular Mechanisms in Integral Membrane Enzymology*, *432*(18), 5184–5196. https://doi.org/10.1016/j.jmb.2020.04.022

Shaikh, S., Fatima, J., Shakil, S., Rizvi, S. Mohd. D., & Kamal, M. A. (2015). Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi Journal of Biological Sciences*, *22*(1), 90–101. https://doi.org/10.1016/j.sjbs.2014.08.002

Sheykhsaran, E., Bannazadeh Baghi, H., Soroush Barhaghi, M. H., Alizadeh, N., Memar, M. Y., Etemadi, S., & Ghotaslou, R. (2018). The rate of resistance to tetracyclines and distribution of tetA, tetB, tetC, tetD, tetE, tetG, tetJ and tetY genes in Enterobacteriaceae isolated from Azerbaijan, Iran during 2017. *Physiology and Pharmacology*, *22*(3), 205–212.

Singh, S., Pathak, A., Kumar, A., Rahman, M., Singh, A., Gonzalez-Zorn, B., & Prasad, K. N. (2018). Emergence of Chromosome-Borne Colistin Resistance Gene mcr-1 in Clinical Isolates of

Klebsiella pneumoniae from India. *Antimicrobial Agents and Chemotherapy*, *62*(2), 10.1128/aac.01885-17. https://doi.org/10.1128/aac.01885-17

Ssekatawa, K., Byarugaba, D. K., Nakavuma, J. L., Kato, C. D., Ejobi, F., Tweyongyere, R., & Eddie, W. M. (2021). Prevalence of pathogenic Klebsiella pneumoniae based on PCR capsular typing harbouring carbapenemases encoding genes in Uganda tertiary hospitals. *Antimicrobial Resistance & Infection Control*, *10*(1), Article 1. https://doi.org/10.1186/s13756-021-00923-w

Stamatakis, A., Hoover, P., & Rougemont, J. (2008). A Rapid Bootstrap Algorithm for the RAxML Web Servers. *Systematic Biology*, *57*(5), 758–771. https://doi.org/10.1080/10635150802429642

Stanley, I. J., Kajumbula, H., Bazira, J., Kansiime, C., Rwego, I. B., & Asiimwe, B. B. (2018). Multidrug resistance among Escherichia coli and Klebsiella pneumoniae carried in the gut of out-patients from pastoralist communities of Kasese district, Uganda. *PLOS ONE*, *13*(7), e0200093. https://doi.org/10.1371/journal.pone.0200093

Sun, J., Zhang, H., Liu, Y.-H., & Feng, Y. (2018). Towards Understanding MCR-like Colistin Resistance. *Trends in Microbiology*, *26*(9), 794–808. https://doi.org/10.1016/j.tim.2018.02.006

Tamma, P. D., & Lee, C. K. (2009). Use of Colistin in Children. *The Pediatric Infectious Disease Journal*, *28*(6), 534. https://doi.org/10.1097/INF.0b013e3181ac4980

Tang, M., Kong, X., Hao, J., & Liu, J. (2020). Epidemiological Characteristics and Formation Mechanisms of Multidrug-Resistant Hypervirulent Klebsiella pneumoniae. *Frontiers in Microbiology*, *11*. https://www.frontiersin.org/articles/10.3389/fmicb.2020.581543

Uddin, M. B., Alam, M. N., Hasan, M., Hossain, S. M. B., Debnath, M., Begum, R., Samad, M. A., Hoque, S. F., Chowdhury, M. S. R., Rahman, M. M., Hossain, M. M., Hassan, M. M., Lundkvist, Å., Järhult, J. D., El Zowalaty, M. E., & Ahmed, S. S. U. (2022). Molecular Detection of Colistin Resistance mcr-1 Gene in Multidrug-Resistant Escherichia coli Isolated from Chicken. *Antibiotics*, *11*(1), Article 1. https://doi.org/10.3390/antibiotics11010097

Velkov, T., Roberts, K. D., Nation, R. L., Thompson, P. E., & Li, J. (2013). Pharmacology of polymyxins: New insights into an 'old' class of antibiotics. *Future Microbiology*, *8*(6), 711–724. https://doi.org/10.2217/fmb.13.39

Vinayamohan, P. G., Pellissery, A. J., & Venkitanarayanan, K. (2022). Role of horizontal gene transfer in the dissemination of antimicrobial resistance in food animal production. *Current Opinion in Food Science*, *47*, 100882. https://doi.org/10.1016/j.cofs.2022.100882

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M., & Wolffs, P. F. G. (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology*, *7*. https://www.frontiersin.org/articles/10.3389/fmicb.2016.00173

Wall, S. (2020). Prevention of antibiotic resistance – an epidemiological scoping review to identify research categories and knowledge gaps. *Global Health Action*. https://www.tandfonline.com/doi/full/10.1080/16549716.2020.1756191

Wan, K. H., Park, S., Hess, B. M., Neff, M. J., Booth, B. W., & Celniker, S. E. (2020). Complete Genome Sequence of the Citrobacter freundii Type Strain. *Microbiology Resource Announcements*, *9*(19). https://doi.org/10.1128/MRA.00240-20

Wellington, E. M., Boxall, A. B., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., Johnson-Rollings, A. S., Jones, D. L., Lee, N. M., Otten, W., Thomas, C. M., & Williams, A. P. (2013). The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases*, *13*(2), 155–165. https://doi.org/10.1016/S1473-3099(12)70317-1

Willner, D., Daly, J., Whiley, D., Grimwood, K., Wainwright, C. E., & Hugenholtz, P. (2012). Comparison of DNA Extraction Methods for Microbial Community Profiling with an Application to Pediatric Bronchoalveolar Lavage Samples. *PLOS ONE*, *7*(4), e34605. https://doi.org/10.1371/journal.pone.0034605

Wyres, K. L., Lam, M. M. C., & Holt, K. E. (2020). Population genomics of Klebsiella pneumoniae. *Nature Reviews Microbiology*, *18*(6), Article 6. https://doi.org/10.1038/s41579-019-0315-1

Yu, H., Qu, F., Shan, B., Huang, B., Jia, W., Chen, C., Li, A., Miao, M., Zhang, X., Bao, C., Xu, Y., Chavda, K. D., Tang, Y.-W., Kreiswirth, B. N., Du, H., & Chen, L. (2016). Detection of the mcr-1 Colistin Resistance Gene in Carbapenem-Resistant Enterobacteriaceae from Different Hospitals in China. *Antimicrobial Agents and Chemotherapy*, *60*(8), 5033–5035. https://doi.org/10.1128/aac.00440-16

Yu, W.-L., Ko, W.-C., Cheng, K.-C., Lee, H.-C., Ke, D.-S., Lee, C.-C., Fung, C.-P., & Chuang, Y.-C. (2006). Association between rmpA and magA Genes and Clinical Syndromes Caused by Klebsiella pneumoniae in Taiwan. *Clinical Infectious Diseases*, *42*(10), 1351–1358. https://doi.org/10.1086/503420

Yusof, N. Y., Norazzman, N. I. I., Hakim, S. N. W. A., Azlan, M. M., Anthony, A. A., Mustafa, F. H., Ahmed, N., Rabaan, A. A., Almuthree, S. A., Alawfi, A., Alshengeti, A., Alwarthan, S., Garout, M., Alawad, E., & Yean, C. Y. (2022). Prevalence of Mutated Colistin-Resistant Klebsiella pneumoniae: A Systematic Review and Meta-Analysis. *Tropical Medicine and Infectious Disease*, *7*(12), Article 12. https://doi.org/10.3390/tropicalmed7120414

Zafer, M. M., El-Mahallawy, H. A., Abdulhak, A., Amin, M. A., Al-Agamy, M. H., & Radwan, H. H. (2019). Emergence of colistin resistance in multidrug-resistant Klebsiella pneumoniae and Escherichia coli strains isolated from cancer patients. *Annals of Clinical Microbiology and Antimicrobials*, *18*(1), 40. https://doi.org/10.1186/s12941-019-0339-4

Zaman, T. U., Albladi, M., Siddique, M. I., Aljohani, S. M., & Balkhy, H. H. (2018). Insertion element mediated mgrB disruption and presence of ISKpn28 in colistin-resistant Klebsiella pneumoniae isolates from Saudi Arabia. *Infection and Drug Resistance*, *11*, 1183. https://doi.org/10.2147/IDR.S161146

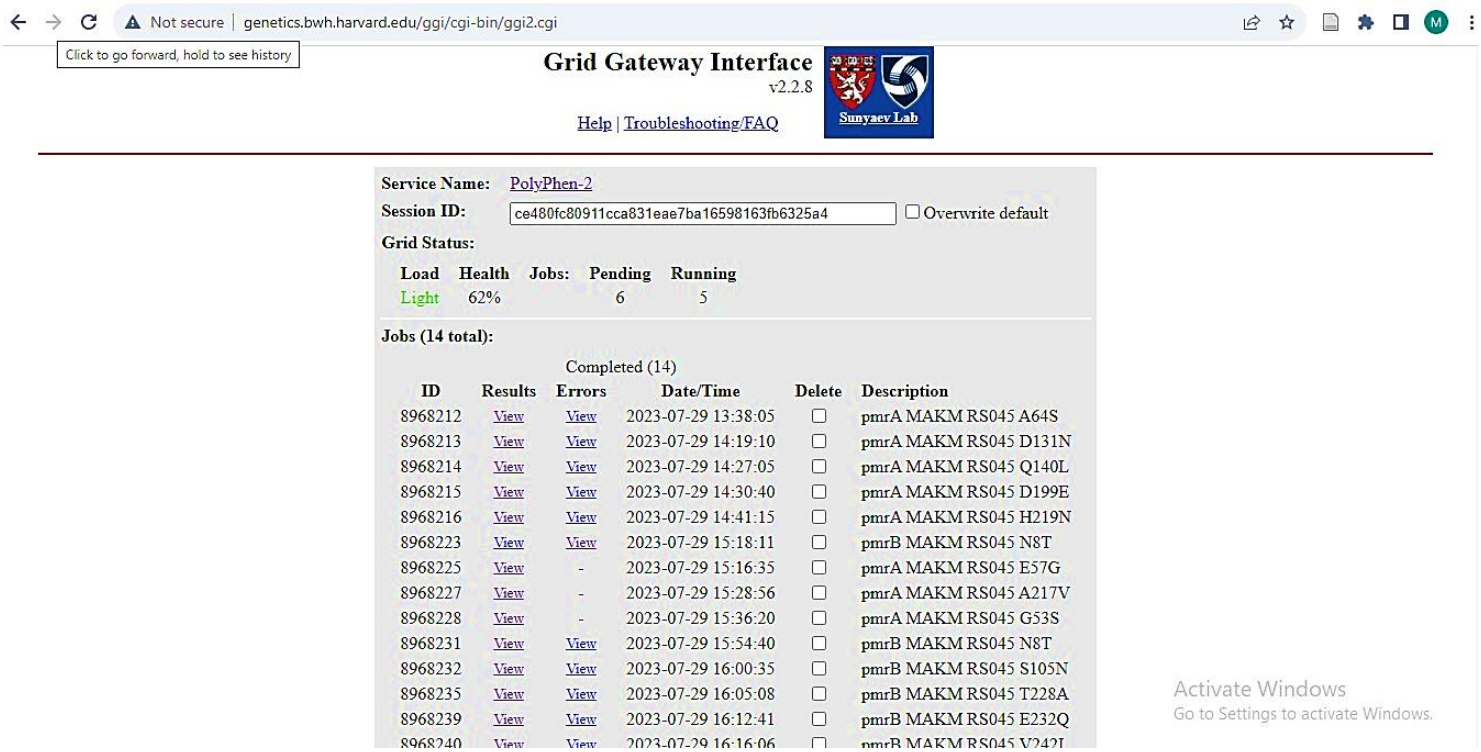
Zhang, Y., Jin, L., Ouyang, P., Wang, Q., Wang, R., Wang, J., Gao, H., Wang, X., Wang, H., & China Carbapenem-Resistant Enterobacteriaceae (CRE) Network. (2020). Evolution of hypervirulence in carbapenem-resistant Klebsiella pneumoniae in China: A multicentre, molecular epidemiological analysis. *Journal of Antimicrobial Chemotherapy*, *75*(2), 327–336. https://doi.org/10.1093/jac/dkz446

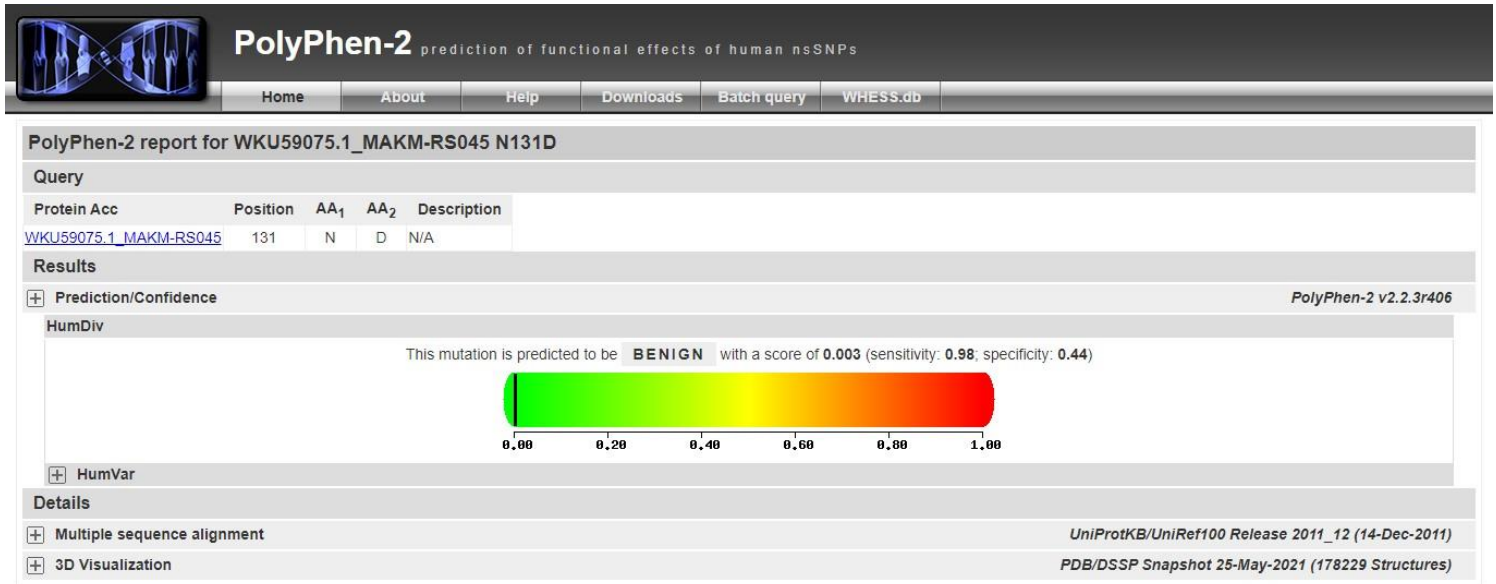
Zhou, K., Lokate, M., Deurenberg, R. H., Arends, J., Lo-Ten Foe, J., Grundmann, H., Rossen, J. W. A., & Friedrich, A. W. (2015). Characterization of a CTX-M-15 Producing Klebsiella Pneumoniae Outbreak Strain Assigned to a Novel Sequence Type (1427). *Frontiers in Microbiology*, *6*. https://www.frontiersin.org/articles/10.3389/fmicb.2015.01250

Zhu, J., Wang, T., Chen, L., & Du, H. (2021). Virulence Factors in Hypervirulent Klebsiella pneumoniae. *Frontiers in Microbiology*, *0*. https://doi.org/10.3389/fmicb.2021.642484

APPENDICES

APPENDIX A: SNP detection in PhoP/Q, PmrA/B, mgrB by PolyPhen-2





PolyPhen-2 prediction of functional effects of human nsSNPs

Home | About | Help | Downloads | Batch query | WHESS.db

PolyPhen-2 report for WKU54057.1_MAKM-5490 N131D

Query

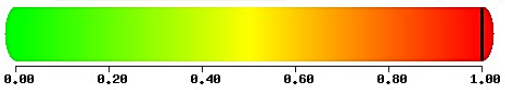
Protein Acc	Position	AA_1	AA_2	Description
WKU54057.1_MAKM-5490	131	N	D	N/A

Results

Prediction/Confidence — *PolyPhen-2 v2.2.3r406*

HumDiv

This mutation is predicted to be **PROBABLY DAMAGING** with a score of **1.000** (sensitivity: **0.00**; specificity: **1.00**)



HumVar

Details

Multiple sequence alignment — *UniProtKB/UniRef100 Release 2011_12 (14-Dec-2011)*

3D Visualization — *PDB/DSSP Snapshot 25-May-2021 (178229 Structures)*

Software & web support: ivan adzhubey — Web design & development: biobyte solutions

PolyPhen-2 prediction of functional effects of human nsSNPs

Home | About | Help | Downloads | Batch query | WHESS.db

PolyPhen-2 report for WKU54057.1_MAKM-5490 T8N

Query

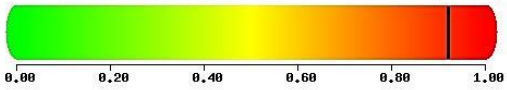
Protein Acc	Position	AA_1	AA_2	Description
WKU54057.1_MAKM-5490	8	T	N	N/A

Results

Prediction/Confidence — *PolyPhen-2 v2.2.3r406*

HumDiv

This mutation is predicted to be **POSSIBLY DAMAGING** with a score of **0.922** (sensitivity: **0.81**; specificity: **0.94**)



HumVar

Details

Multiple sequence alignment — *UniProtKB/UniRef100 Release 2011_12 (14-Dec-2011)*

3D Visualization — *PDB/DSSP Snapshot 25-May-2021 (178229 Structures)*

Software & web support: ivan adzhubey — Web design & development: biobyte solutions

APPENDIX 2: Example of virulence factors genes detected in one strain using VFDB

VFDB: Virulence Factors of Bacterial Pathogens

[Your input] NZ_CP129541.1 Klebsiella pneumoniae strain MAKM-3381chromo…						
VFG002415 (yagX/ecpC) E. coli common pilus usher EcpC [ECP] [Escherichia coli …	2526	0.0	87.6% (2197/…	2	2191208-219…	2526-20
VFG002416 (yagW/ecpD) polymerized tip adhesin of ECP fibers [ECP] [Escherichia…	1644	0.0	89.6% (1422/…	0	2189575-219…	1644-58
VFG002414 (yagZ/ecpA) E. coli common pilus structural subunit EcpA [ECP] [Esch…	588	0.0	90.1% (516/5…	0	2194489-219…	584-12
VFG002412 (yagY/ecpB) E. coli common pilus chaperone EcpB [ECP] [Escherichia …	669	0.0	88.9% (552/6…	0	2193759-219…	669-49
VFG002417 (yagV/ecpE) E. coli common pilus chaperone EcpE [ECP] [Escherichia …	756	e-175	85.8% (626/7…	0	2188920-218…	732-3
VFG001443 (ompA) outer membrane protein A [OmpA] [Escherichia coli O18:K1:H…	1041	e-157	85.6% (868/1…	15	1394941-139…	9-371, 406-1…
VFG002413 (ykgK/ecpR) regulator protein EcpR [ECP] [Escherichia coli O157:H7 st…	591	e-146	86.4% (502/5…	0	2195147-219…	591-11
VFG000923 (fepA) ferrienterobactin outer membrane transporter [Enterobactin] […	2241	e-138	83.1% (2005/…	4	1120992-112…	271-317, 365…
VFG000933 (entB) isochorismatase [Enterobactin] [Escherichia coli CFT073]	858	e-131	82.8% (656/7…	8	1828249-182…	858-68
VFG000934 (entA) 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase [Enterobact…	747	e-110	86.1% (397/4…	0	1827498-182…	743-283
VFG000925 (fepC) ferrienterobactin ABC transporter ATPase [Enterobactin] [Esche…	816	e-110	82.7% (550/6…	0	1836752-183…	91-755
VFG000932 (entE) 2,3-dihydroxybenzoate-AMP ligase component of enterobactin …	1611	5e-98	82.8% (759/9…	0	1829133-182…	1592-916, 86…
VFG000928 (fepG) iron-enterobactin ABC transporter permease [Enterobactin] [Es…	993	2e-85	80.1% (673/8…	2	1835827-183…	155-993
VFG044165 (entS) enterobactin exporter, iron-regulated [enterobactin] [Escheric…	1251	4e-83	82.8% (667/8…	2	1833406-183…	1151-1036, 5…
VFG000930 (entF) enterobactin synthase multienzyme complex component, ATP-…	3882	4e-74	82.9% (1436/…	0	1837792-183…	3608-3471, 2…
VFG000876 (fimD) Outer membrane usher protein fimD precursor [Type 1 fimbria…	2637	2e-66	80.4% (709/8…	0	4646129-464…	2297-1714, 9…
VFG000182 (xcpR) general secretion pathway protein E [xcp secretion system] [Ps…	1509	7e-51	89.5% (205/2…	0	2362293-236…	349-383, 892…
VFG000924 (fepB) ferrienterobactin ABC transporter periplasmic binding protein […	957	4e-46	83.2% (367/4…	0	1832227-183…	133-458, 568…
VFG000575 (mgtC) Mg2+ transport protein [MgtBC] [Salmonella enterica subsp. e…	696	9e-35	80.4% (295/3…	0	584013-584379	38-404
VFG000926 (fepD) ferrienterobactin ABC transporter permease [Enterobactin] [Es…	1017	3e-34	80.9% (516/6…	0	1834807-183…	151-586, 781…
VFG000935 (iroN) salmochelin receptor IroN [IroN] [Escherichia coli CFT073]	2178	5e-33	83.5% (319/3…	0	1120992-112…	274-565, 805…
VFG000619 (iutA) aerobactin receptor IutA [Aerobactin] [Shigella flexneri 2a str. …	2196	1e-30	82.1% (284/3…	0	1280001-128…	172-455, 838…
VFG000936 (iutA) ferric aerobactin receptor precusor IutA [Aerobactin] [Escheric…	2280	1e-30	82.1% (284/3…	0	1280001-128…	256-539, 922…
VFG000931 (entC) isochorismate synthase 1 [Enterobactin] [Escherichia coli CFT0…	1188	1e-24	82.6% (433/5…	0	1830765-183…	1154-893, 76…
VFG000873 (fimA) Type-1 fimbrial protein, A chain precursor [Type 1 fimbriae] [Es…	606	1e-21	82.1% (165/2…	0	4650205-465…	245-45
VFG000871 (fimB) Type 1 fimbriae Regulatory protein fimB [Type 1 fimbriae] [Esc…	603	2e-20	83.5% (212/2…	0	4651986-465…	566-404, 119…
VFG002480 (tssH-5/clpV) Clp-type ATPase chaperone protein [T6SS-1] [Burkholder…	3039	7e-20	84.9% (433/5…	0	1014288-101…	680-727, 122…
VFG000142 (waaC) 3-deoxy-D-manno-octulosonic-acid (KDO) transferase [LPS] [Ps…	1068	2e-17	82.9% (131/1…	0	3987745-398…	191-34
VFG013346 (galU) glucosephosphate uridylyltransferase [LOS] [Haemophilus influ…	888	2e-17	80.8% (173/2…	0	138017-138230	44-257
VFG002048 (gspE) general secretion pathway protein E [T2SS] [Shigella dysenteri…	1494	7e-17	80.5% (289/3…	0	2362470-236…	511-672, 919…
VFG000875 (fimC) Chaperone protein fimC precursor [Type 1 fimbriae] [Escherich…	726	7e-17	82.4% (211/2…	0	4648749-464…	460-268, 234…
VFG002050 (gspG) general secretion pathway protein G [T2SS] [Shigella dysenteri…	456	3e-13	87.4% (76/87)	0	2364726-236…	49-135
VFG044159 (fes) enterobactin/ferric enterobactin esterase [enterobactin] [Esche…	1203	1e-12	91.0% (142/1…	0	1841699-184…	1145-1096, 1…
VFG014984 (algW) AlgW protein [Alginate regulation] [Pseudomonas aeruginosa P…	1170	2e-11	89.6% (103/1…	0	2336881-233…	696-633, 693…
VFG000872 (fimE) Type 1 fimbriae Regulatory protein fimE [Type 1 fimbriae] [Esc…	597	2e-11	86.1% (255/2…	0	4650918-465…	563-452, 527…
VFG000168 (pchD) pyochelin biosynthesis protein PchD [Pyochelin] [Pseudomonas…	1644	6e-11	88.7% (63/71)	0	1829372-182…	1377-1307
VFG000879 (fimH) FimH protein precursor [Type 1 fimbriae] [Escherichia coli CFT…	912	3e-10	77.1% (344/4…	0	4644128-464…	599-154
VFG001248 (fleQ) transcriptional regulator FleQ [Flagella] [Pseudomonas aerugin…	1473	1e-09	90.4% (227/2…	0	3374430-337…	724-796, 717…
VFG045346 (llpA) immunogenic lipoprotein A [llpA] [Vibrio vulnificus YJ016]	810	4e-09	91.1% (51/56)	0	2299259-229…	637-692

VFG000141 (waaA) lipopolysaccharide core biosynthesis protein WaaP [LPS] [Pseu…	1278	4e-09	87.5% (63/72)	0	3977302-397…	990-919
VFG018241 (luxS) S-ribosylhomocysteinase [AI-2] [Vibrio cholerae O1 biovar El Tor…	519	2e-08	81.5% (110/1…	0	4953637-495…	151-285
VFG012337 (sfaF) S fimbriae outer membrane usher SfaF [S fimbriae] [Escherichia…	2679	2e-08	82.0% (178/2…	0	4646078-464…	2393-2263, 1…
VFG000912 (focD) F1C fimbrial usher [F1C fimbriae] [Escherichia coli CFT073]	2679	2e-08	82.0% (178/2…	0	4646078-464…	2393-2263, 1…
VFG000874 (fimI) Fimbrin-like protein fimI precursor [Type 1 fimbriae] [Escherich…	540	2e-08	86.3% (120/1…	0	4649237-464…	540-446, 197…
VFG002049 (gspF) general secretion pathway protein F [T2SS] [Shigella dysenteria…	1200	6e-08	86.4% (114/1…	0	2364444-236…	990-1047, 11…
VFG002076 (clpV1) type VI secretion system AAA+ family ATPase [HSI-I] [Pseudom…	2709	6e-08	85.0% (147/1…	2	1188243-118…	2153-2053, 7…
VFG000143 (waaF) heptosyltransferase I [LPS] [Pseudomonas aeruginosa PAO1]	1038	6e-08	92.2% (83/90)	0	3988145-398…	835-784, 350…
VFG000133 (algA) phosphomannose isomerase / guanosine 5'-diphospho-D-mannos…	1446	6e-08	96.0% (144/1…	0	3815222-381…	568-604, 116…
VFG000112 (pilB) type 4 fimbrial biogenesis protein PilB [Type IV pili] [Pseudomo…	1701	6e-08	81.8% (202/2…	0	2362896-236…	1141-1274, 1…