Prevalence of Colistin Resistance among MDR Gram Negative Bacteria from Tertiary Care Hospitals

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2023

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A thesis submitted in partial fulfillment of the requirements for the

Degree of

Doctor of Philosophy

In

Microbiology

By

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Islamabad

2023

Dedication

I want to dedicate this research work to the most important and amazing human being in my Life

*"**My Ammi and Abbu**" for always supporting me through every thick and thin. To my Best Friend "**Mehreen**" for always been so understanding and bearing my mood swings throughout these years. To my Sister "**Ashja Raneen**" for always helping and teasing me at the same time. To my Brother's, (**Qaizar, Zeeshan, Hassam and Abeer**) for their compassion and support.*

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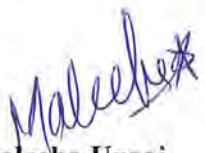

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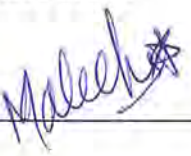
Student / Author Signature: 

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List of abbreviations

Abbreviation	Full form
AgNP	Silver based nanoparticles
AMR	Antimicrobial resistance
ARA	L-arabinose
ARG	Antimicrobial Resistance Genes
bp	Base pair
BV-BRC	Bacterial and Viral Bioinformatics Resource Centre
Cam	Chloramphenicol
CARD	Comprehensive Antimicrobial Resistance Database
CC	Clonal Complex
CDA	Capital Development Authority Capital Hospital from Islamabad
CDT	Combined disk test
CFU	Colony Forming Unit
CGE	Center of Genomic Epidemiology
CIT	Trisodium citrate
CLSI	Clinical Laboratory and Standard Institute
CMS	Colistin Methatesodium Salt
CPR	Carbapenem resistant
CPS	Capsular polysaccharide
CR	Colistin resistant
CTAB	Cetyltrimethyl ammonium bromide
DAB	L-α-γ-di-aminobutyric acid
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EntB	Enterobactin
ESBL	Extended Spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FimH	Type-1 Fimbrial-adhesin-encoding gene
GLU	D-glucose
ICEkp	Integrative and Conjugative Element *K. pneumoniae*
IL-8	Interleukin-8
IM	Inner Membrane
IMP	Imipenem-type carbapenemases
IND	Indole test
IroN	Salmochelin
IS	Insertional sequence
ISO	International Organisation for Standardization
Iuc	Aerobactin
KPC	*K. pneumoniae* carbapenemases
K-serotype	Capsular Polysaccharides
LAMP	Loop mediated isothermal amplification
LAra4N	4Amino4-deoxy-L-arabinose
LB	Luria Bertani

LDC	L-lysine
LPS	Lipopolysaccharide
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MBL	Metallo β-lactamases
mcr	Mobile colistin resistance gene
MDR	Multidrug resistance
mg	Milli gram
MH	Military Hospital Rawalpindi
MHA	Muller Hinton agar
MHB	Muller Hinton broth
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
MMS	MICRONAUT MIC-Strip
MS	Mass Spectrometry
MTS	MIC Test Strip
NDM	New Delhi Metallo β Lactamases
ODC	L-ornithine
OM	Outer membrane
OMP	outer membrane porins
ONPG	2-nitrophenyl-ß-Dgalactopyranoside
PBP	Penicillin-binding proteins
PCR	Polymerase Chain Reaction
PDR	Pan drug resistant
PEA	Phosphoethanolamine
PGMI	Federal Government Polyclinic Hospital
PIMS	Pakistan Institute of Medical Science Hospital
qRT-PCR	Real Time quantitative PCR
RmpA/RmpA2	Mucoid phenotype regulatory genes
ROS	Reactive oxygen species
rpm	Rotations per minute
ST	Sequence Type
TCS	Two Component System
TDA	Tryptophane Deaminase
TNFα	Tumour Necrosis Factor
TSI	Triple Sugar Iron
UAE	United Arab Emirates
URE	Urea
USA	United States of America
UTI	Urinary Tract Infections
VIM	Verona integron encoded Metallo β-lactamases
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensive Drug Resistant
ybt	Yersiniabactin,

Acknowledgments

All the praises and thanks are for the **Almighty Allah**, the Compassionate, the Merciful, the Ultimate source of all the knowledge and wisdom, who gave us thoughts, health and talent to complete this project. All the praises to His **Holy Prophet Muhammad (S.W.H), w**ho enabled us to recognize our Creator.

I feel my first and foremost obligation to express my grateful appreciation and thanks to my supervisor/mentor **Prof Dr. Rani Faryal.** Her illustrious advice, keen interest and encouragement were real source of inspiration during the completion of this thesis.

I am very much thankful to **Mehreen Arshad** for providing me with the opportunity to work in such a prestigious Laboratory at Stanley Manne Research group, Northwestern University Chicago.

I would like to Thank **Higher Education Commission of Pakistan** for their financial support through Indigenous scholarship and IRSIP scholarship.

I take this opportunity to say thanks to my best friends **Mehreen** and **Azeema Umair**. The time that we have been together are the best time I've had in my life. Thanks for always being supportive to me…thanks for bearing with my whining and my mood swings. I cannot imagine that I could have completed this work without your support. You girls are my best asset, the friends I am proudest to have…the friends I love most. I would also like to acknowledge Hira, Rabail, Marbaila, and Maryam Siddique for always listening and understanding. The bond we share is precious and near to my heart. I would like to thank Zinan for always there for me whenever needed (indeed a life saver). You are a beautiful kind-hearted human being. Thank you Haziq, Rumaisa, Iqra, Nurgus, Rafia, Sabir, Saba, Saman, Amna I am blessed to have you all in my life.

I would like to Thank Aspen kremer, and Alima Sanjwani for giving me beautiful memories of Chicago I hope we will meet again soon. It was a wonderful and memorable time with you guys. Thanks, Alima, for listening to my long conversations and bearing me with my emotional breakdowns. Thank you Joon waun, you were always a helping and encouraging person. I will try my best to keep you updated about my journey.

Meanwhile I cannot forget to appreciate the great help, advices and support rendered by **Dr. Khadeeja, Dr. Mehmood** and all the members of Prof. Dr Faryal's Research group

for helping me get through the difficult times, and for all the emotional support, camaraderie, entertainment, and caring they provided.

We cannot remain without expressing our heartiest gratitude to our **Parents and Family** who made devotion to my studies and prayed for my success. I would like to thank my one and only Aunt (Ainee), for always been there whenever needed. Thank you for helping me especially with the new dresses, gifts and instant favors.

Maleeha Urooj

Abstract

Antibiotic resistance among Gram-negative bacterial pathogen is a global menace and is considered, a silent pandemic associated with higher morbidity and mortality worldwide. With an increase in antimicrobial resistance (AMR), and few new available drug, older toxic drug "colistin" is consider as last-resort for the treatment of these resistant pathogens. Gram-negatives, overcome the lethal effects of this drug via modification of outer membrane (OM). The mechanism responsible for colistin resistance has not been deciphered completely yet. However, resistance is thought to be associated with the plasmid-mediated carriage of mobile colistin-resistant genes (*mcr*), or chromosomal alterations in lipopolysaccharide (LPS) modification genes, or efflux pump. These modifications affect colistin binding to its target site or extrusion of drug. The emergence of colistin resistance and its gradual increase in clinical Gram-negatives, compelled research community to comprehend and explore other mechanisms responsible for resistance.

Increase in colistin resistant (CR) Gram-negatives have been reported from multiple countries and recent studies from Pakistan highlighted the emerging resistance to colistin in Gram-negatives with detection of *mcr* gene. Based on previous reports from Pakistan, where low *mcr* gene prevalence with much higher phenotypic resistance indicated that there are other possible mechanisms for resistance in MDR Gram-negatives which need to be elucidate for better understanding of this emerging threat. Hence, there is a need for a genome-based study to gain insight of underlying colistin resistance mechanism among Gram-negatives in Pakistan. This study aims to investigate the prevalence of colistin resistance among Gram-negative bacteria and to decipher molecular mechanism of resistance in *mcr* negative CR MDR Gram negative bacteria.

A total 566 clinical specimens were collected from patients visiting 4 major tertiary care hospitals which were, Military Hospital (MH), Rawalpindi, Pakistan Institute of Medical Science Hospital Islamabad (PIMS), Federal Government Polyclinic Hospital Islamabad (PGMI), and Capital Development Authority (CDA) Capital Hospital from Islamabad. Isolation and identification of Gram-negative bacteria was carried out using standard cultural, biochemical techniques and API10S kit. Phenotypic colistin resistance was evaluated using modified broth microdilution method and Minimum inhibitory concentration (MIC) were interpreted as per "European Committee on Antimicrobial

Susceptibility Testing" (EUCAST) guidelines. Antimicrobial susceptibility profiling of CR Gram-negatives against a panel of 10 commonly used antibiotics was done by using Kirby-Bauer disc diffusion assay. The inhibition zone breakpoints were interpreted as per "Clinical Laboratory and Standard Institute" (CLSI) guidelines. For detection of plasmid mediated *mcr* genes, polymerase chain reaction (PCR) approach was used. For the understanding of other potential molecular mechanisms of resistance, whole genome sequencing (WGS) of selected *mcr* negative Gram-negative isolates was carried out. Whole genome data analysis was done using various *in silico* tools and online databases which includes Bacterial and Viral Bioinformatics Resource Center (BV-BRC), Comprehensive Antimicrobial Resistance Database (CARD), Pathogenwatch, Pasteur and ResFinder Database. Phage red recombinase system was used to knock out transporter gene (*YjiJ*) using *E. Coli* UTI89 to assess this novel approach to mitigate resistance gene.

A total 528 Gram-negatives were identified from 566 collected clinical specimens, out of which 70% were *Enterobacteriaceae.* The most predominant member of *Enterobacteriaceae* was *E. coli* (46.6%), followed by *K. pneumoniae* (43%). In case of non-*Enterobacteriaceae, A. baumannii* (50.9%), and *P aeruginosa* (37.8%) were dominant. Higher number of both *Enterobacteriaceae* and non-*Enterobacteriaceae* isolates were recovered from male patients, and from age group III. Majority of the *Enterobacteriaceae* members were isolated from urine specimens while in case of non-*Enterobacteriaceae*, pus and blood were the dominant specimen type.

Prevalence of phenotypic colistin resistance among Gram negative was 31% (161/525). Highest colistin resistance was observed in *K. pneumoniae* (88/161), followed by *P. aeruginosa* (26/161) and *A. baumannii* (15/161). Among all CR, 84% displayed MDR phenotype against panel of tested antibiotics. While 74.53% resistance was observed to last line empirical drug "carbapenem". Most of the CR Gram-negatives were from male patients (71.4%), highest percentage were from age group II (30.4%), while most common specimen type were urine (26.7%), blood (22.4%) and pus (19.9%).

Carriage of plasmid-mediated colistin resistance genes was assessed among CR-Gram negative isolates (n=161). The carriage of *mcr* genes was low among CR-Gram negatives (12%), as percentage positivity of *mcr*-1, *mcr*-2 and *mcr*-3 gene were 0.6%, 9% and 5.5% respectively. Highest *mcr*-2 (6%) and *mcr*-3 (2%) gene carriage was detected among CR-*K. Pneumoniae* followed by *P. aeruginosa,* and *A. baumannii.*

To assess the underlying mechanism of colistin resistance, selected *mcr* negative CR-Gram-negatives (*n*=23) were characterized using WGS. In all the 23 sequenced genomes, 100 non-synonymous mutations were detected in various Lipid A modification genes, of which 65 mutations were novel. Majority of the novel variations were in CR-*E. coli* and CR-*E. hormaechei* genomes. A total of 18 variations were predicted to have deleterious impact and were annotated to be preset in various crucial protein domains. Apart from carrying multiple mutations, the acquisition of putative phosphoethanolamine (PEA) and glycosyl transferase genes via phage mediated integration was also predicted in CR-Gram negative pathogens.

Microbial Genome characterization further revealed the carriage of *bla*NDM1, *bla*NDM-5 and *bla*NDM-7 gene along with other crucial carbapenemase, Extended Spectrum β-lactamase (ESBL), and AMR determinants. The potential carriage of diverse siderophores, adhesion, invasion and biofilm associated determinants were also identified in MDR CR strains depicting a potentially convergent hypervirulent and MDR genotype emergence. The acquisition of these AMR and virulent determinants might be associated with mobile genomic elements as various (Insertional sequence) IS elements, plasmid replicons, conjugative elements and CRISPR-Cas regions were predicted in the sequenced genomes.

Multi Locus Sequence Typing (MLST), predicted that most of the CR-Gram-negative isolates belonged to globally distributed high risk MDR lineages. The identified STs were, ST-11 and ST-2096 in *K. pneumoniae*, ST-448 and ST-617 in *E. coli* while ST-93 in *E. hormaechei*. Some potentially emerging high risk MDR clonal lineages were also observed these were ST-1503, ST-208 in CR *A. baumannii. S. maltophilia* was identified to carry a novel ST in this study as remained unmatched with available ST in Pasteur, Center of Genomic Epidemiology (CGE) and Pathogenwatch databases.

In current study, a novel therapeutic approach was used to render drug resistant strain drug sensitive strain for enhance eradication of resistant bacteria with conventional drugs. A single colony on chloramphenicol containing agar plates was detected after knock out of *yjiJ* transport gene, where targeted gene was replaced with chloramphenicol (Cam^R) selective marker gene. However, despite phenotypic inactivation and gene inactivation, a PCR based detection *yjiJ* gene was seen in the transformed colony which suggested possible multiple copy nature of this *yjiJ* transporter gene in *E. coli* model genome. This

approach had limited success, however such technique for development of anti-infective therapy.

The current study reported much higher phenotypic colistin resistance compared to a low plasmid mediated *mcr* gene carriage among Gram negative bacterial isolates pointing toward the possible role of chromosomal mechanism in resistance. This is the first study to provide a snapshot of multiple underlying chromosomal mechanisms in CR MDR Gram negative isolates, thought to be associated with LipidA modification as were having novel non-synonymous variations, with deleterious impact on crucial protein domains of Two component system (TCS) associated proteins, and transferases, putative PEA and glycosyl transferases using WGS approach. Moreover, the detection of convergent high-risk MDR+hypervirulent clonal lineages in these CR isolates reflects the emergence of potential pan drug resistant with highly pathogenic potential in clinical setting, require immediate attention. To cope this emerging threat therapeutic potential of membrane associated transporter genes needs to be explored as a possible option against MDR bacteria.

Chapter 1 Introduction

1.1. Gram-Negative Bacterial Infections

Gram-negative bacteria are a major public health problem nowadays as the infection caused by these pathogenic microorganisms are sometimes life-threatening, hence are responsible for high morbidity and mortality across the globe (Oliveira & Reygaert, 2022). Among Gram-negative bacteria, majority of the clinical infections are associated with two major groups which include members of *Enterobacteriaceae* (*Escherichia, Klebsiella, Citrobacter, Enterobacter*, *Shigella, Salmonella)* and non-fermenter Gram-negatives (*Acinetobacter baumannii*, *Alcaligenes, Burkholderia cepacian, Moraxella, Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia)* (Surgers *et al.,* 2017; Weiner-Lastinger *et al.,* 2020). Member of *Enterobacteriaceae* being widespread in the community are responsible for about 80% of clinically associated infections, which include diarrhea, endotoxic shock, meningitis, pneumonia, sepsis, and urinary tract infections (UTI) among others (Ye *et al.,* 2018). Non-fermenter, on the other hand, usually have low prevalence compared to *Enterobacteriaceae* but are associated with life-threatening nosocomial infections like ventilator associated pneumoniae, endocarditis, meningitis, catheter associated UTI, soft tissue infections, burn and wound infections, especially among immunocompromised patients (Weiner-Lastinger *et al.,* 2020). In past, Gram-positive bacteria were considered to be the major culprits for hospital acquired infections (Waterer & Wunderink, 2001). With advancement of medical techniques, invasive procedures, indwelling devices, intra-abdominal tubing, and ventilators, a steady increase in Gram-negative bacterial infections occurred (Kaye *et al*., 2015). The situation was further exacerbated with the advent of antimicrobial agents and their widespread use in clinical settings, which led to the selection of drug resistant Gram-negative bacteria that has now become a public health concern (Fernández-Martínez *et al.,* 2022).

1.2. Multi-Drug Resistance in Gram-Negative Bacteria

The discovery of penicillin a ‘wonder drug’ in 1928, initiated a golden era that revolutionized modern medicines. Penicillin belongs to the family of bactericidal drugs, the β lactam antibiotics which also include cephalosporins, monobactams, and carbapenem. All these

antibiotics possess β lactam ring and have a similar mode of action where these drugs target penicillin binding proteins (PBP), a main constituent of Gram-negative bacterial cell wall and cause cell death (Lima *et al.,* 2020). But soon Gram-negative bacteria evaded the lethal effects of these bactericidal agents via various mechanisms like target site modification, overproduction of efflux pumps, reduction in membrane permeability, acquisition of mutations in regulatory genes, and most importantly by production of various β lactamases which can hydrolyse β lactam ring (Knowles, 1985; Vasilenko, 2021; Silago, 2021). β lactamases break the azetidinone ring (hydrolyzing the amide bond and generating amino and carboxyl groups) to make the β lactam drug ineffective (Majiduddin *et al*., 2002; Bush & Bradford, 2020). The spread of β lactamase producing Gram-negative bacterial pathogens led to discovery of broad spectrum antimicrobials, their introduction and use in the clinical settings to treat β lactamase producers (Bassetti & Righi, 2015a). More and more antimicrobial agents were discovered and synthesized to fulfill the demand of the medical community. Excessive and over the counter use of these 'magic bullets' not only in hospital settings but also in veterinary, poultry, and even in the agriculture sector introduced as well as elicited the emergence of multi drug resistant (MDR) bacterial strains (Hawkey, 2015; Mulani *et al.,* 2019). MDR bacterial pathogens showed non susceptibility to one or more drugs from at least three different antimicrobial classes, which are now classified by using CLSI guidelines (Bassetti & Righi, 2015b).

According to the 2014 report of World Health Organization (WHO), Gram-negative bacteria were documented for majority of MDR infections worldwide. The European Centre for Disease Prevention and Control in 2019, reported 670000 MDR cases only in Europe due to Gram-negative pathogens which ultimately led to 33000 deaths annually (Binsker *et al.,* 2022b). In recent years, Gram-negative bacteria have evolved so rapidly under the pressure of antimicrobial agents, they are now almost resistant to the majority of routinely used antimicrobials, thus limiting the therapeutic options for the treatment of infections caused by these MDR bugs (Hansen, 2021).

Among the important Gram-negative MDR pathogens, the resistance rate is much higher among '*Klebsiella pneumoniae, Enterobacter, Acinetobacter baumannii,* and*' Pseudomonas aeruginosa,* contributing the "ESKAPE" list of pathogens, which are considered as "critical or priority pathogens". These pathogens together with other members of *Enterobacteriaceae* like '*E. coli, Citrobacter, Salmonella, Shigella, and Stenotrophomonas'* are responsible for the majority of hospital associated recalcitrant infection with limited treatment options especially when β lactamases are involved, therefore, are consider as a threat to public health (Xu *et al.,* 2014; Mulani *et al.,* 2019; Paul *et al.,* 2022). β lactam hydrolysing enzymes are widely dispersed especially among Gram-negatives and are classified into various groups based on their structural and functional characteristics by Ambler, (1980), and Bush & Jacoby, (2010) classification schemes respectively.

The highly reported AmpC β lactamases confer resistance to penicillins', 1st, 2nd, and 3rd generation cephalosporins, and monobactam which belong to Class C as per Ambler classification and are carried on the chromosome or mobile genetic elements. On the other hand, ESBL are carried on mobile genetic elements (Plasmids, transposons, and integrons) that are widely disseminated among Gram-negative bacterial species. These β lactamases belong to Ambler Class A and confer non-susceptibility against penicillin, narrow and broad-spectrum cephalosporins, and even to β lactamase inhibitors. TEM, SHV, and CTX-M are also important ESBL, which are more common compared to others GES, SFO, VEB, PER, and BEL, with wide distribution globally among MDR pathogens (Bush & Bradford, 2020). With the increase in prevalence of ESBL producing Gram-negative bacterial infection, medical community was diverted to the use of broad-spectrum β lactam drug carbapenem, as a first-line empirical treatment. Carbapenem belongs to the β lactam group of antibiotics which includes imipenem, meropenem, and ertapenem. These are broad-spectrum antimicrobial agents and are stable against the hydrolysing effect of ESBL or AmpC enzyme. (Lee *et al.,* 2017; Rodriguez-Bano *et al.,* 2018; Ting *et al.,* 2018). Being effective against β lactamase producing Gram-negative pathogens, frequent use of carbapenem as a first line empirical regimen potentiated the emergence of resistance to this potent drug (Sheu *et al.,* 2019).

1.3. Carbapenem Resistance Among MDR Gram-Negative Bacteria

After the development of carbapenem resistant (CPR) pathogens, such infection are even more difficult to treat which become associated with long hospital stays, high economic cost especially with other comorbid conditions with even higher mortality risk (Tabak *et al.,* 2019). These CPR bacteria are, therefore, considered to pose more therapeutic challenge leading to treatment failures (McCann *et al.,* 2018; Lodise *et al.,* 2022). These bacteria counter lethal effects of carbapenem via various mechanisms which include loss of outer membrane porins *e.g*., OMPs in *Enterobacteriaceae* and OprD in *P. aeruginosa*, overexpression of various efflux pumps e.g., Mex in *P. aeruginosa*, and AcrAB-TolC in *Enterobacteriaceae* while most importantly production of carbapenem hydrolysing enzymes termed as "Carbapenemases" (Bush & Bradford, 2020; Heo, 2021).

Apart from a few exceptions in the family *Enterobacteriaceae* like *Proteus, Morganella,* and *Providencia,* which are intrinsically resistant to imipenem, all the other members have acquired carbapenem resistance via acquisition of carbapenemase genes. CPR pathogens particularly '*K. pneumoniae, E. coli, Enterobacter, A. baumannii,* and *P. aeruginosa'* are considered as critical pathogens due to the acquisition of various carbapenemases. Various such pathogens have different carbapenemases like *K. pneumoniae* carbapenemases (KPC's), Metallo β lactamases (MBL's), Imipenem type carbapenemases (IMP), Verona integron encoded Metallo β lactamases (VIM) and OXA-type carbapenemases, which confer resistance to all commonly used antimicrobial agents like penicillins', cephalosporins, fluoroquinolones, aminoglycoside and even to β lactamase inhibitors (Bassetti & Righi, 2015a; Tumbarello *et al.,* 2018). Most of the carbapenemase genes are thought to be carried by mobile genetic elements (plasmids and transposons), hence are widely and easily disseminated among bacterial species escalating morbidity and mortality rate worldwide (Sekyere *et al.,* 2016a).

The *Enterobacteriaceae* and other Gram-negative pathogens, beside carbapenemases have other mobile genetic elements together with other resistant determinants, thus responsible for the emergence of extensive drug-resistant (XDR) and even pan drug-resistant (PDR)

"superbugs" (Vannice *et al.,* 2019). The pattern of dissemination of β lactamases especially ESBL's and carbapenemases may vary between Gram-negative bacteria depending upon various factors like human host and pathogen associated factors, but one of the prominent factors is antibiotic pressure (Bush & Bradford, 2020). The frequent use of β lactam antibiotics especially carbapenem for treating MDR Gram-negative infection, has led to the resistance as its low concentration is a factor in development of resistance. The enormous amount of antibiotic substances released into the environment by humans has likely sped up the rate at which bacteria evolve (Baquero, 2001). Horizontal gene transfer between phylogenetically distinct bacteria as well as between pathogens and non-pathogenic bacteria can be accelerated by prolonged exposure of bacterial cells to low concentrations of antibiotics. Numerous studies have shown a direct connection between environmental antibiotic subinhibitory concentrations and bacterial resistance developed under selective pressure(Tello *et al*., 2012; Larsson & Flach, 2022; Mancuso *et al*., 2023). The escalating number of carbapenemases that have now disseminated globally threaten the therapeutic regimens for the treatment of serious infections. The lack of new drugs in the antibiotic armamentarium and continuous upsurge of resistance against the first-line empirical drug "carbapenem" has led to the reintroduction of older but toxic antimicrobial agents like aminoglycoside, tigecycline, and polymyxins (colistin), for treatment of infections by CPR Gram-negative bacteria (Hadjadj *et al.,* 2021; Heo, 2021; Apanga *et al.,* 2022).

1.4. Colistin: A Last-Resort Antibiotic for the Management of CPR Pathogens

Colistin, a polymyxin E antibiotic was considered as the last-resort antibiotic because it is highly potent against CPR Gram-negative bacterial pathogens (Doi, 2019). Colistin is a non-ribosomally synthesized, macrocyclic poly-peptide drug. It was first discovered in 1949 from a Gram-positive soil bacterium *Bacillus polymyxa* sub-specie *colistinus* (Falagas & Kasiakou, 2005). Followed by its initial discovery, colistin was rapidly adopted therapeutically for the treatment of various infections like UTI, diarrhea, eye, and ear infections, and even for decontamination of the bowel.

Apart from its therapeutic use in human, colistin was also used as a prophylactic agent in animal farming and as a growth promoter in the poultry industry in Japan, the United States, and Europe (Bialvaei & Kafil, 2015a). Being discovered in 1949, the pharmacokinetic and pharmacodynamic properties of colistin were not fully explored until prompted by its rampant use. Later in 1970, various adverse nephrotoxic and neurotoxic effects were reported which led to the abandonment of this lipopeptidyl drug for other safer alternative therapeutic options available at that time (Karaiskos *et al.,* 2017). But later in 1990's, after the emergence of life-threatening MDR Gram-negative infections in the community and hospitals led the re-introduction of toxic but potent older drug, which is now considered as a last-resort drug for the treatment of MDR and even XDR pathogens (Ahmed, 2021).

Commercially, two forms of colistin are available, one is colistin sulfate while the other is colistin methatesodium salt (CMS). Colistin sulfate is cationic, administered orally or topically, and is more toxic compared to colistin methatesodium salt which is an anionic inactive prodrug, usually administered parenterally and aerosolized form (Bialvaei & Kafil, 2015b). The intravenous formulation of CMS has been used in cystic fibrosis patients and is very effective, especially in treating infections caused by MDR Gram-negative bacteria (Sekyere *et al.,* 2016a).

1.4.1. The Spectrum of Activity

Being narrow-spectrum antibiotic, Colistin is effective against most of the MDR Gram-negative bacterial pathogens (*K. pneumoniae, E. coli, Enterobacter, Citrobacter, P. aeruginosa, A. baumannii,* and *Stenotrophomonas).* There are a few exceptions among Gram-negative bacteria where it is ineffective as these pathogens are intrinsically resistant to colistin like *Burkholderia, Proteus, Providencia, Morganella, Serratia,* and *Vibrio cholera.* All the Gram-positive and anaerobic bacteria are also resistant to colistin (Ahmed *et al*., 2020; Ahmed, 2021).

1.4.2. Colistin Structure and Mechanism of Action

Colistin is a deca peptide antibiotic, composed of a heptapeptide cyclic ring attached with a tripeptide amino acid sidechain linked with an acylated fatty acid chain at its N terminus (Figure.1). The L-α-γ-di-aminobutyric acid (DAB), an important constituent of the tripeptide chain is responsible for the cationic nature of the drug while the hydrophobic fatty acid tail is responsible for the toxicity and antimicrobial activity of the colistin (Bialvaei & Kafil, 2015b).

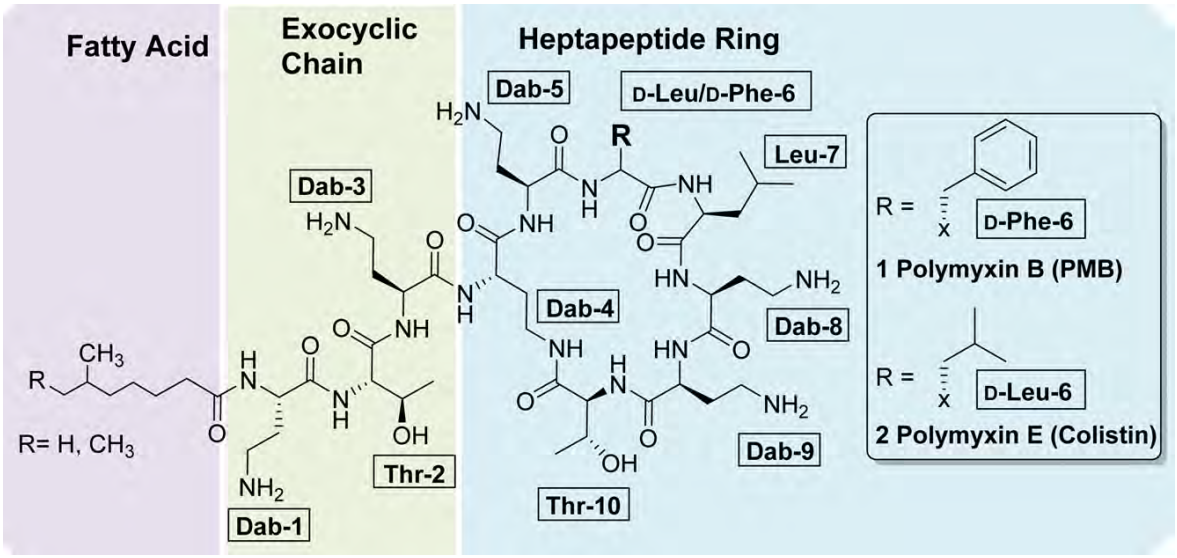

Figure 1.1: Structural composition of Colistin (Gallardo-Godoy *et al.,* 2016)

Being potent against MDR Gram-negatives, the actual bactericidal mechanism of action of colistin is still unclear, it is thought to act by many different mechanisms. It is speculated that colistin mainly interacts with the bacterial membrane in a surfactant or detergent like manner (Gurjar, 2015). Being cationic, colistin primarily targets the negatively charged LPS membrane of Gram-negative bacteria. The cationic tripeptide amino acid chain of colistin interacts with the anionic cell membrane and leads to the membrane destabilization by displacing calcium (Ca^{2+}) and zinc (Zn^{2+}) ions. The disruption of the Gram-negative OM further facilitates the piercing of fatty acid tail into cracks, which lead to self promoted uptake of colistin inside the cell. The hydrophilic amino acid in colistin further facilitates its interaction with lipopeptides of the inner membrane, which causes the disintegration of the membrane (Velkov *et al.,* 2010). The membrane destabilization mechanism of colistin results in the leakage of periplasmic and cytoplasmic content, which ultimately causes cell death (Velkov *et al.,* 2013).

Lipid A is the main antigenic component of the LPS in Gram-negative bacteria, it acts also as an endotoxin which elicits immune response. The immune response due to this endotoxin induce release of inflammatory cytokines like tumour necrosis factor (TNFα) and interleukin-8 (IL-8), which can lead to septic shock during infection. Colistin on the other hand not only destabilizes the outer membrane but also inhibits the release of lipid A molecules by binding and neutralizing the endotoxic effect ultimately suppressing the induction of shock (Andrade *et al.,* 2020a). Another mechanism of colistin mediated killing is via production of reactive oxygen species (ROS) like hydroxyl (OH^-), superoxide (O_2^-), and hydrogen peroxide (H_2O_2). The initial breaching of the outer membrane by colistin generates oxidative stress with release of free radicals like O_2^-. In the presence of superoxide dismutase, the O_2^- is converted into H_2O_2 and oxygen. Oxidation of ferrous ion to ferric iron by H_2O_2 ,further intensifies the oxidative damage to various important cell organelles due to modification of proteins, lipids, and DNA which causes cell death (Ahmed *et al.,* 2020).

1.4.3. Emerging Resistance Against Colistin

Being abandoned in the 1980s due to its associated neuro and nephrotoxicity, the use of colistin in the veterinary and poultry sector remained common as a prophylactic agent and growth promoter. After three long decades of its discontinuation in clinical settings, colistin was revived as a last line drug by WHO (2007), for the treatment of recurrent and persistent infection by MDR CPR Gram-negative bacteria (Hamel *et al.,* 2021a). The genomic evolution especially concerning survival strategies against the harmful effect of antimicrobial agents especially against antibiotics is very much pronounced in Gram-negative bacteria (Martínez, 2012). As they have many versatile strategies, resistant Gram-negatives have managed to escape from the microbicidal effects of this last-resort drug too. The use of low sub inhibitory concentration of colistin in farm animals has attributed to the selection and emergence of CR strains in these settings (Poirel *et al.,* 2017). In recent years, various studies have been reported on the emerging colistin resistance among farm animals (Hille *et al.,* 2018; Wang *et al.,* 2018; Clemente *et al.,* 2019). The animal to human transmission of resistant strains via horizontal gene transfer or through the food chain might have a role in the extensive spread of resistance among Gram-negative pathogens in clinical settings (Marshall & Levy, 2011; Matli *et al.,* 2022). The upsurge in MDR Gram-negative infections in clinical settings also started as the scarcity of available drugs for the treatment favoured the use of colistin which placed selective pressure on Gram-negative bacteria and hence colistin resistance developed (Binsker *et al.,* 2022b).

1.4.4. Mechanism of Colistin Resistance

The mechanism of colistin resistance is considered to be very complex and multifactorial. Development of resistance to this drug is majorly associated with the modification of Gram-negative bacterial OM via replacement of phosphate group with cationic moieties [Phosphoethanolamine (PEA) or 4Amino4-deoxy-L-arabinose (LAra4N)] ultimately reducing the net negative charge of the LPS, which reduces or inhibit the binding of the drug (Sekyere *et al.,* 2016a). For the resistance, it was seen that the addition of LAra4N moieties in the outer membrane is more effective as compared to PEA's addition as the former provides more cationic charge to the outer membrane than the latter, which hinders the

colistin binding efficiently (Quiroga *et al.,* 2019). Other colistin resistance mechanisms include loss of LPS, porin loss, overexpression of efflux pumps, polysaccharide capsule production and enzymatic breakdown of colistin (Ahmed *et al.,* 2020).

Intrinsic resistance to colistin has been reported among some of the Gram-negative bacteria (*Burkholderia cepacia*, *Brucella*, *Campylobacter, Chromobacterium, Edwardsiella*, *Legionella, Morganella morganii, Neisseria*, *Proteus*, *Providencia*, *Serratia*, *Vibrio cholera)* and Gram-negative anaerobic cocci due to the presence of LAra4N moieties in LPS which reduces the overall negative charge by inhibiting the binding of colistin (Kaye *et al.,* 2016). On the other hand, various MDR Gram-negative pathogens (*E. coli, K. pneumoniae, Enterobacter, A. baumannii*, and *P. aeruginosa*), are now considered "Critical priority pathogens" due to their ability to resist a number of antibiotics and transfer antibiotic resistance determinants to other bacteria. It is believed that CR is either due to acquisition of chromosomal mutations in Two-component system (TCS) genes (*PhoP/PhoQ*, *PmrA/PmrB*, *ColR/ColS, ParR/ParS*, *Arn* operon, *Lpx* operon), their regulators (*MgrB, PmrD, crr, PagP)* and other accessory genes (*EptA, EptC, PmrK, OpgE*) which modify the LPS through the addition of LAra4N or PEA moieties (Jeannot *et al.,* 2017). Historically, the acquisition of chromosomal mutations under colistin selective pressure was thought to be the primary cause of resistance to this drug but later in late 2015, the initial discovery of a plasmid borne mobile colistin resistance gene *"mcr-1",* from food animals in China changed the perspective and now plasmid-mediated dissemination of resistant determinants is considered as more devastating, especially in humans (Liu *et al.,* 2016a; Nang *et al.,* 2018). These genes encode for PEA transferase, which inserts PEA at 4'-phosphate group of lipid A, reducing the negative charge of the outer membrane (Liu *et al.,* 2016b).

The presence of *mcr* genes on the plasmid make their dissemination easy and widely in various Gram-negative bacteria in various environments and settings like farm animals (Oh *et al.,* 2020), poultry (Wang *et al.,* 2019), migratory birds (Mohsin *et al.,* 2016), vegetables (Liu & Song, 2019), wastewater (Hayashi *et al.,* 2019), hospital sewage (Zhao *et al.,* 2017) and even from soil (Oliveira *et al.,* 2019) plus aquatic environments (Cherak *et al.,* 2021)

across 30 countries in five continents, rendering it as threat to the global public health (Binsker *et al.,* 2022b).

1.4.5. Exploration of Mechanism of Colistin Resistance

Broth microdilution based test is still regarded as the gold standard for assessing phenotypic colistin resistance as both EUCAST and CLSI recommend to use ISO-20776 standard broth microdilution method (Chew *et al.,* 2017a; Jayol *et al.,* 2018). Despite being frequently used, this test for AMR surveillance has severe limitations. The high levels of chemical contamination in samples can lead to false negative results, and the cultivation phase is laborious, requiring lengthy incubation time, several steps, and validation assays. Due to all these issues, an alternate to this traditional broth microdilution method are explored and now genotypic methods to identify colistin resistance in Gram-negative bacteria are utilized along with this assay (Haeili *et al.,* 2019).

The complex and sophisticated mechanisms by which Gram-negative bacteria protect themselves against this last-resort medication are now researched to determine the basis of such high drug resistance (Hamel *et al.,* 2021b). Historically, mechanisms of colistin resistance were explored using intrinsically resistant Gram-negatives like *Proteus* and *Serratia* focusing on lipid A composition using traditional assays like chromatography, electron microscopy and mass spectroscopy (Sud & Feingold, 1970; Weber *et al.,* 1979). Xu *et al.,* (2018) reported that lipid A modification via the addition of PEA moiety exist in *mcr*-gene positive bacterial strains by using matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS) assay.

More rapid methods like conventional PCR and real time quantitative (qRT)-PCR are now adopted to understand the mechanism of resistance to colistin in phenotypic resistant Gram-negative isolates by targeting both chromosomal and plasmid mediated lipid A modification genes along with assessment of transcript levels of these genes in both mutant and wild type isolates (Sekyere *et al.,* 2016b). In contrast to culture-based techniques, PCR based molecular methods are quick and easily identify a variety of resistance genes, even in bacteria which are difficult to grow in a laboratory conditions (Luby *et al.,* 2016). According

to CLSI and International Organisation for Standardization (ISO), PCR based detection of the gene does not necessarily indicate tolerance but further need expressional analysis.
In order to identify the factors causing phenotypic antibiotic resistance and effectively manage infections by antibiotic resistant bacteria, it is crucial to understand the dynamics of the bacterial genome (Kwong *et al.,* 2015). For bacterial strain characterization and epidemiological investigations, Whole genome sequencing (WGS) is currently the best method available (Aruhomukama *et al.,* 2019a).

WGS is the most precise and accurate molecular method for analysing genome of a specific organism and its antimicrobial resistance genes (ARG's) (Irfan *et al.,* 2022). An organism's entire genome can be screened along with ARG's, their copy numbers, mutations, and new resistance genes. The most widely used WGS platforms globally are Illumina (Illumina, Inc.), Nanopore MinIOn (Oxford Nanopore Technology), and PacBio® HiFi technology (Pacific Bioscience). The WGS is robust and comprehensive technique where resistance mechanism present in the bacterial genome is easy to analyse it completely. By comparing the type of plasmid harbouring resistance genes and chromosomal resistance genes, isolate's evolutionary ancestry and its relationships to other isolates can be determined. The data produced by WGS also aid in identifying the source in epidemic investigations (Roodsari *et al.,* 2014; Jain *et al.,* 2019).With time, WGS costs has reduced but still need skilled personnel for data extraction and analysis (Anandan *et al.,* 2015; Bose *et al.,* 2022).
The WGS technique has playing a significant role in identifying novel and rare mutations in *EptA* and *ArnT* genes, which are associated with colistin resistance in addition to other novel variants of Fosfomycin resistance genes in CPR *K. pneumoniae* (Aris *et al*., 2020). A recent study by Elias *et al.,* (2022), on genomic data was used to explore phylogenomic diversity among chromosomal colistin resistance gene in *K. pneumoniae* isolates.

Also WGS data, is helpful to detect novel mutations and genes as reported by Ahsan *et al.,* (2022), who discovered new point mutations linked to colistin resistance in *A. baumannii* (ATCC-17978) when exposed to sub-inhibitory amounts of colistin. Further, this work on mice models also found that the colistin tolerance developed independently of *LpxA*, *LpxB*,

LpxC, *LpxD*, and *PmrAB*, however, reduction in the pathogenicity with less severity in infections with proinflammatory immune response was seen. Nowadays, *mcr* monitoring programs use the high throughput sequencing technologies (Hamel *et al.,* 2021b) along with PCR based screening for detection of novel molecular mechanisms (Liu *et al.,* 2016c; Xavier *et al.,* 2016a; Wang *et al.,* 2020).

The computational and bioinformatics tools are employed for analysing the molecular mechanism of resistance, which predict potential resistance mechanisms by detecting mutations in genome and gene acquisition from other microorganisms. There may be additional mechanisms involved in polymyxin resistance, as initial targets found to be responsible for colistin resistance are now insufficient to explain resistance in different isolates (Aruhomukama *et al.,* 2019a). Genome based approaches should be used to analyse a variety of worldwide datasets of bacterial genomes in order to detect and forecast novel mutations for resistant mechanism in these emerging pathogens. Such data can also be used to develop therapeutic interventions targeting both existing and possible acquired mutations in genes that might be causative for antibiotic drug resistance (Hamel *et al.,* 2021b; Irfan *et al.,* 2022).

Beside additional barrier provided by outer membrane, the MDR efflux pumps are also involved in AMR. Therefore, development of novel antimicrobial medicines for Gram-negative bacteria are still particularly challenging (Li *et al.,* 2015). CR Gram-negatives therapeutic options are restricted due to their high potential for acquisition of mobile AMR genes and rapid genetic mutations in chromosomal genes too.

Now various other therapeutic options for treatment of Gram-negatives are utilized. As colistin have synergistic effect with a variety of other substances including antibiotics and adjuvants, it is still used for treatment as it lowers the amount of colistin needed for therapy, reduces its toxicity, combats colistin resistance, and ensures maximum therapeutic effectiveness (Hu *et al.,* 2019). Various broad spectrum and toxic antimicrobials (carbapenems, rifampicin, tigecycline, and Fosfomycin) in combination with colistin have

been used especially for the treatment by colistin resistant pathogens' infections (Tascini *et al.,* 2013a; Park *et al.,* 2019a; Almutairi, 2022a).

Researchers have examined variety of other approaches too more recently, like CRISPR-cas9 approach (He *et al.,* 2021), monoclonal antibodies (Rosini *et al.,* 2020), nanoparticles (Muenraya *et al.,* 2022a), natural chemicals (Foda *et al.,* 2022), herbal extracts (Xu *et al.,* 2022a), phage's (Mousavi *et al.,* 2021) and antimicrobial peptides (Chosidow *et al.,* 2023). However, all these approaches showed promising results in *in vitro* and in animal models but still need to pass clinical trials to be implemented for clinical use.

Another important concern is the genomic potential of CR MDR Gram-negative bacteria to change as they are so adaptable in their resistance mechanism, they might become resistant to these new drugs too soon after their approved for clinical use. Because bacteria quickly developed resistance after the use of antibiotics, we cannot entirely rely on the development of newer antibiotics (Sharma *et al.,* 2022a).

1.4.6. Global Emergence of Colistin Resistance

Extensive colistin use in the veterinary sector and the emergence of *mcr* determinants in farm animals suggested that the selective pressure may have induced the spread of these genes among Gram-negatives in recent years (Hamel *et al.,* 2021a). The presence of these CR genes in the animals and food chains are now considered as potential hot spots for *mcr* genes as the highest colistin resistance is reported among food-producing animals from China, Taiwan, Switzerland, and Poland with very extensive farming industry (Morris & Cerceo, 2020). In recent years, the highest colistin resistance in Gram-negatives is detected among *E. coli* strains isolated from farm animals (Andrade *et al.,* 2020a). Higher consumption of colistin in livestock and agriculture sector is also found to be associated with high resistant rates, especially in Greece and Italy too, where colistin resistance among CPR *K. pneumoniae* was 20.8% and 43% respectively (Petrosillo *et al.,* 2019). The frequent use of drugs in livestock and selection of zoonotic strains have been closely associated which can be directly and indirectly transmitted from animal to human through food chain. (Marshall & Levy, 2011). Apart from animal to human transmission via the food chain,

migration and human travel from countries like Asia, Africa, and South America with high colistin resistance rates in Gram-negative bacteria (*mcr-1* prevalent regions) to rest of world is the major factor for its worldwide dissemination (WHO, 2021). In recent years, the emergence of colistin resistance in clinical setting might be attributed to the increased use of this drug for the treatment of CPR Gram-negatives (Morris & Cerceo, 2020).

Resistance rate of colistin differs among Gram-negative pathogens, mostly highest colistin resistance rate is reported in *K. pneumoniae,* and *A. baumannii* (Li *et al.,* 2019a). According to a report by European Antimicrobial Resistance Surveillance Network, about 29% of CPR *K. pneumoniae* showed non-susceptibility to colistin in different countries from Europe. A study from Dubai, United Arab Emirates (UAE), reported higher colistin resistance among CPR *K. pneumoniae* isolates from 5 major hospitals (Li *et al.,* 2019a). From Egypt, 21.3% colistin resistance among MDR *P. aeruginosa* was reported (El-Baky *et al.,* 2020).

Colistin resistance is emerging as a big therapeutic challenge due to not only acquisition of chromosomal mutations and plasmid-mediated colistin-resistant determinants but also due to co-carriage of other antimicrobial determinants like *NDM, OXA-48, CTX-M, SHV, Tet*, *CatB*, *etc.* Hence, CR pathogens are also becoming CRF MDR Gram-negative bacteria with the potential to transform into a Pan drug-resistant superbug (Hussein *et al.,* 2021).

AMR is a catastrophe for developing countries especially with over burdened health care system especially post COVID-19 pandemic era (Arshad *et al.,* 2021; Elmahi *et al.,* 2022; Sulis *et al.,* 2022). Pakistan being a developing country, its situation is further worrisome. Up to now few reports on MDR Gram-negative bacteria especially in clinical settings are available in literature, in which high prevalence of colistin resistant bacteria is recorded with detection of *mcr* genes from local setting, present it as a grave health concerns (Khan *et al.,* 2019; Uppal *et al.,* 2022). As excessive, over the counter use of broad-spectrum and high priority antimicrobial agents in various sectors like agriculture (Mehmood *et al.,* 2021), veterinary (Umair *et al.,* 2022), poultry industry (Mohsin *et al.,* 2019; Umair *et al.,* 2021), and even in healthcare setting (Mustafa *et al.,* 2022), has further led to development of resistance against the last empirical drug 'carbapenem' which was used prior to colistin.

Later, many reports on carbapenem MDR Gram-negatives not only globally but also from Pakistan were published (Ejaz *et al.,* 2022). The upsurge of carbapenem resistance especially in critical priority pathogens like *K. pneumoniae*, *E. coli*, *Enterobacter*, *Citrobacter*, *Acinetobacter*, and *Pseudomonas* followed by the introduction of colistin in clinical setting proved as 'last nail in the coffin' as resistance against last-resort drug have been emerging in these MDR priority pathogens too (Imtiaz *et al.,* 2021; Ahsan *et al.,* 2022) . Although both chromosomal and plasmid mediated determinants play crucial role in development of colistin resistance but in Pakistan, studies only focused on *mcr* gene detection using targeted and traditional molecular based approaches like PCR, Sanger sequencing *etc.* (Mohsin, Azam, *et al.,* 2019; Hameed *et al.,* 2020; Imtiaz *et al.,* 2021; Furqan *et al.,* 2022a; Shafiq *et al.,* 2022). Despite being detected in food chain and healthcare system, the prevalence of these mobile genes is quite low and not justifying as the main mechanism responsible for colistin resistance in our country. Therefore, there is need to understand the other possible mechanisms of colistin resistance which is highly prevalent in our community and hospitals by using more comprehensive-genome based approaches (Hameed *et al.,* 2019c, 2020; Li *et al.,* 2021a). Such approach will fulfil the gaps in understanding the genetic basis for CR in local settings. Also, it would assess significance of WGS for epidemiological surveillance of emerging clinical threat, their development and transmission in the community. Furthermore, novel therapeutics and diagnostic targets would be identified for better intervention, containment, and control of CR MDR Gram-negatives

Aim of The Study

In this era of escalating CR in Pakistan and globally, the understanding of molecular mechanisms underlying acquired colistin resistance via mobile and chromosomal genes in MDR Gram-negative bacterial pathogens is of dire need. Also, potential co-carriage of AMR and virulent determinants in CR priority pathogens is required to explore their role in enhancing the AMR.

The aim of the current study is to assess the prevalence of colistin resistance among MDR Gram-negatives and to elucidate the potential molecular mechanisms underlying colistin resistance among CR MDR Gram-negatives. This study is also designed to evaluate a novel therapeutic approach for treatment MDR in model organism.

Objectives

1. Determination of colistin resistance prevalence among Gram-negatives from four tertiary care hospitals of Islamabad/Rawalpindi.
2. Detection of plasmid-mediated colistin resistance genes in phenotypic colistin-resistant isolates.
3. Elucidation of the molecular mechanism associated with colistin resistance and MDR phenotype among CR MDR Gram-negative bacteria.
4. Exploration of anti-infective therapy as a potential therapeutic option targeting drug transporter gene (*YjiJ*) by using single gene knock-out model.

In humans, Gram-negatives are responsible for 80% of the infections such as diarrhea, meningitis, urinary tract infections, endotoxic shock, pneumonia, sepsis, and others (Oliveira & Reygaert, 2021). However, due to ability of acquiring resistance to antibiotics, these pathogens pose a great threat to human health globally. The development of drug resistance in Gram-negatives to the majority of antibiotics including β lactams, carbapenems, fluoroquinolones, and aminoglycosides, became alarmingly high worldwide (Shrivastava *et al.,* 2018). In this situation, the medical community decided to reconsider the use of colistin in hospitals throughout the world, where compared to colistin no other less toxic or effective antibiotic was available for the treatment of MDR Gram-negative infections (Kasiakou *et al.,* 2005; Spellberg *et al.,* 2011; Nation *et al.,* 2014; Bialvaei & Kafil, 2015b).

Among the two known polymyxins (B and E) for therapeutic purposes, colistin is used more extensively worldwide due to its better availability (Lee & Doi, 2014). Colistin a polycationic peptide produced by "*Bacillus polymyxa"* (Falagas & Kasiakou, 2005; Gallardo-Godoy *et al.,* 2016) and has extremely rapid bactericidal effect. For past few decades, it is available in its inactive form (CMS) also, which is less toxic compared colistin sulphate form (Li *et al.,* 2001; Bergen *et al.,* 2006; Poudyal *et al.,* 2008). However, still it has nephrotoxic and neurotoxic effects on the body, it was replaced by the aminoglycosides in 1970s'. But soon after the emergence of MDR Gram-negative pathogens against aminoglycosides also, it regained its importance now as a last line drug against these bacteria in late 1980s'. It was reintroduced for treatment of infections, as salvage therapeutic option for human health setting against deadly infections caused by *P. aeruginosa, K. pneumonia* and *A. baumannii* (Falagas & Kasiakou, 2005; Li *et al.,* 2005, 2006; Landman *et al.,* 2008). As colistin was discontinued and reintroduced later due its toxicity, little is known about its pharmacological information. However, few of the initial studies had some information on its dosage, without proper knowledge of pharmacology and pharmacokinetics of the drug in light of modern medicine (Markou *et al.,* 2008; Plachouras *et al.,* 2009). It was used initially for the treatment of the Cystic fibrosis patients inhalation therapy for the lung infection with MDR strains but at this time no recommendation for dosage were available (Garonzik *et al.,* 2011; Ratjen *et al.,* 2006; Tsuji *et al.,* 2019). The Food and Drug Administration (FDA) in

USA approved dosage for Colistin as in base form while it is available as colistin methanesulfonate salt (DeRyke *et al.,* 2010; Lim *et al.,* 2010).

Colistin has bactericidal effect and one of the proposed mechanism widely accepted for its activity as antimicrobial is binding of positively charged L-di-aminobutyric acid with the negatively charged phosphate groups in Gram-negative`s outer membrane lipid A which also act as antigen (Plachouras *et al.,* 2009; Deris *et al.,* 2014). After binding of colistin, it causes the destruction of three-dimensional structure of LPS layer by displacement of Ca^{2+} and Mg^{2+} ions. The hydrophobic terminal acyl chain of colistin is then inserted, leading to its expansion in OM monolayer. Because of OM's permeabilization, colistin can pass through OM. In case if colistin is used with other hydrophilic antibiotics like β lactam, gentamicin, rifampicin, meropenem, and tigecycline develop synergistic effect with it (Bolla *et al.,* 2011). By adding hydrophilic groups to the fatty acid chains, colistin causes the loss of stability of phospholipid bilayer of the inner membrane (IM) and it causes failure in cell integrity with cell lysis (Velkov *et al.,* 2010).

The bactericidal effect of colistin is largely caused by the solubilization of the bacterial cell membrane, but another possible mechanism found is vesicle-vesicle interaction. Following the entry of colistin through the outer membrane, lipid exchanges between the inner and outer membranes occur, resulting in structural changes in the membranes with loss of phospholipids and osmotic imbalance (Kaye *et al.,* 2016).

Free radical build up associated with the oxidative stress brought on by colistin damages DNA, protein, and lipid due to oxidation which results in bacterial death due to extensive changes in membrane proteins, enzymes and even genome. Colistin also acts on lipid A's endotoxin and inhibit essential respiratory enzymes. Colistin inhibits the lipid A of LPS action by attaching to and neutralizing the LPS molecules, which in turn cause formation of cytokines TNF-α and IL-8 to suppress the shock response (Falagas & Kasiakou, 2005; Ahmed *et al.,* 2020).

Despite being largely discontinued for medical use in most of the world in the early 1980s, colistin has been used constantly in veterinary medicine to treat Gram-negative bacterial

infections in animals, as prophylactic therapy and growth promoter (Sun *et al.,* 2018). Colistin was administered is huge variety of animal, including pigs, poultry, cattle, sheep, goats, laying hens, and rabbits, as well as milk producing animals throughout the European Union (Pardon *et al.,* 2012; Gelbíčová *et al.,* 2019). Such widespread use of colistin has prompted the development and spread of commensal and pathogenic AMR bacteria in the intestines of animals used for food production (Kempf *et al*., 2016). Colistin is not well absorbed by the digestive system, it is released in to environment which places selection pressure not only on the gut microbiota but also on organisms in the natural habitats (Rhouma *et al.,* 2016). One report from Italy, indicate that CR *E. coli* are found in wild rabbits and hares which had no exposure or previously treated with colistin (Dotto *et al.,* 2014). Another explanation could be that the wildlife is a possible natural environmental reservoir for colistin resistance, it might be contributing to its spread to other animals and/or humans via food chains (Andrade *et al.,* 2020b).

Resistance to colistin can be intrinsic property of the bacteria or can also be acquired through various means like chromosomal mutations or acquisition of genes. Intrinsic resistance to colistin has been well documented among some of the Gram-negative bacteria (*Burkholderia cepacia*, *Brucella*, *Campylobacter, Chromobacterium, Edwardsiella*, *Legionella, Morganella morganii, Neisseria*, *Proteus*, *Providencia*, *Serratia*, *Vibrio cholera*, and Gram-negative anaerobic cocci), and is due to the presence of LAra4N moieties in LPS which reduces the overall negative charge, hence binding of colistin do not take place (Kaye *et al.,* 2016). On the other hand, various MDR Gram-negative bacterial critical priority pathogens (like *E. coli, K. pneumoniae, Enterobacter spp, A. baumannii*, *P, aeruginosa*), have acquired different mechanisms to resist lethal effects of colistin. Transferase enzymes encoded by *Ugd, ArnB, ArnC, ArnA, ArnD, ArnT, ArnE* and *ArnF* genes are required for biosynthesis of LAra4N for its addition to LPS. ArnT links the LAra4N to the 4′phosphate group of lipid A, while PEA transferases (EptA, EptB, EptC and OpgE) add PEA to the 1′phosphate group on the lipid A. In addition to above mentioned chromosomally encoded genes, many other genes are responsible for the regulation, biosynthesis and addition of LAra4N and PEA in response to environmental signals (Raetz *et al.,* 2007). Acquisition of chromosomal

mutations by various genes of TCS namely PhoPQ and PmrAB (*PhoP/PhoQ*, *PmrA/PmrB*) effect directly/indirectly resistance development. Other drug resistance imparting operons are A*rn* operon and *Lpx* operon, two component genes TCS and their regulators (*MgrB, PmrD, crr, PagP)* can modify LPS by adding sugar moiety either LAra4N or PEA (Jeannot *et al.,* 2017). In different Gram-negatives, either all mechanisms or some of the discussed mechanisms play role in drug resistance development.

The operon PmrAB in Gram-negatives, encodes TCS which modifies the 1′-phosphate group of lipid A. Due to mutation in the *PmrA* or *PmrB* gene, there is activation of either response regulator PmrA or PmrB kinase which lead to overexpression of LPS modifying enzymes and hence colistin resistance. In Enterobacterales, missense mutations detected in *PmrA* genes in *E.coli* led to change in amino acid (G53S), which was later experimentally confirmed to mediate colistin resistance (Janssen *et al.,* 2020).

Another important TCS in Gram-negatives is PhoQ sensor kinase, which respond to low environmental Mg^{2+} concentrations, changes in pH, and the presence of antimicrobial peptides by activation of the PhoP response regulator. PhoP response regulator controls the expression of genes involved in magnesium transport and modification of LPS. PhoPQ, and also contribute to colistin resistance by indirectly activating the PmrAB TCS via PmrD regulatory gene (Rubin *et al.,* 2015). Missense mutations and deletions in *PhoP* gene have been identified which encodes the REC domain, trans regulatory cytoplasmic domains and inter domain regions of PhoP (Dagher *et al.,* 2020).

The expression of both PhoPQ proteins is tightly regulated by *MgrB* gene (negative feedback regulator gene), which encodes a small transmembrane protein MgrB that exerts negative feedback to the PhoPQ TCS (Lippa & Goulian, 2009). When mutations occur in *MgrB* gene, it upregulates phoPQ operon as well as activate another pmrHFIJKLM operon, all these changes effects lipid A modification (Cannatelli *et al.,* 2013). Also deletion in *MgrB* gene results in upregulation of the PhoP regulated gene in *E. coli* and results in colistin resistance (Lippa & Goulian, 2009). In case of *K. Pneumoniae,* and *K. oxytoca,* MgrB inactivation is thought to be the most common mechanism for colistin resistance too

(Giani *et al.,* 2015; Poirel *et al.,* 2015). Beside missense mutations and deletions, other alterations like nonsense mutations are also seen in *MgrB* gene, which results premature termination (Yap *et al.,* 2022)

Mutation of *crrB* operon in another TCS have been seen to play role in colistin resistance in *K. pneumonia*. This TCS comprise of *crrA* (regulatory protein) and *crrB* gene (sensor kinase). The CrrAB operon regulates the expression of a glycosyltransferase-like protein, involved in lipid A modification (Wright *et al.,* 2015). Amino acid substitutions in the CrrB protein has been reported to increase colistin resistance in *K. pneumonia*. The mutation of the *crrB* gene results in increased autophosphorylation of CrrB protein, it subsequently activate pmrHFIJKLM operon, *PmrC* and *PmrE* genes, results in modification of LPS (Cheng *et al.,* 2016).

Some Gram-negatives such as *Citrobacter, Enterobacter,* and *Salmonella* possess a RamA regulator that effects the expression of various lipid A biosynthesis genes. Alteration in *RamA* gene results in reduce susceptibility to colistin. In *K. pneumoniae* , RamA activates various lipid A biosynthesis genes *LpxC, LpxO,* and *LpxL2* after binding to these, ultimately changing interaction of various enzymes and kinases (Majumdar *et al.,* 2016).

Beside modifications in the lipid A, capsular polysaccharide (CPS) has been seen to play an important role in colistin resistance. The upregulation of capsular biosynthesis gene in *K. pneumoniae* results in less colistin target sites and hence reduced uptake of this drug (Campos *et al.,* 2004). Resistance was also found to be associated with the number of CPS layers in *K. pneumoniae,* strains with multiple layers CPS were more resistant compared to the ones with few CPS layers (Formosa *et al.,* 2015).

Some of the studies have also demonstrated the role of efflux-pump systems in colistin resistance. In *Enterobacteriaceae*, activation of some important efflux pumps (KpnEF, AcrAB and Sap proteins), results in increased level of resistance towards colistin. Mutations in AcrAB-TolC pump in *E. coli* showed eightfold increase in colistin MIC (Srinivasan & Rajamohan, 2013). In case of *A. baumannii*, presence and associated mutations in EmrAB efflux pump both were responsible for colistin resistance (Lin *et al.,* 2017). In case of *P.*

aeruginosa, MexXY-OprM pump was functionally linked with LPS modification for resistance development against last line drug (Puja *et al.,* 2020).

Historically, acquisition of chromosomal mutations under colistin selective pressure were thought to be the primary cause of colistin resistance but later in 2016, after the discovery of a mobile colistin resistance gene *mcr-1,* from a food animal in China, posed global public health concern (Liu *et al.,* 2016a). Being borne on plasmid, its spread and dissemination was rapid and was observed in various Gram-negative bacteria especially in members *Enterobacteriaceae.* These *mcr-1* positive Gram-negative bacteria were isolated from multiple sources like farm animals (Oh *et al.,* 2020), poultry (Wang *et al.,* 2019), migratory birds (Mohsin *et al.,* 2016), vegetables (Liu & Song, 2019), waste water (Hayashi *et al.,* 2019), hospital sewage (Zhao *et al.,* 2017), soil (Oliveira *et al.,* 2019) and aquatic environments (Cherak *et al.,* 2021) from about 30 countries across five continents (Binsker *et al.,* 2022b).

After the initial discovery of *mcr-1* gene in *E. coli* from China, various *mcr-1* gene variants, together with nine *mcr* gene homologues were detected which were not restricted to any host but were widely disseminated into various environment worldwide (Ahmed *et al.,* 2020). *mcr-2* gene was first reported in *E. coli* isolated from bovine and porcine sources in Belgium (Xavier *et al.,* 2016b), *mcr-3* from porcine *E.coli* from China (Yin *et al.,* 2017), *mcr-4* in *Salmonella* and *E. coli* from Belgium, Italy and Spain (Carattoli *et al.,* 2017), *mcr-5* in *S. enterica* from poultry and food samples from Germany (Borowiak *et al.,* 2017), *mcr-6* gene in porcine *Morexella* from Great Britain (AbuOun *et al.,* 2017), *mcr-7* gene in poultry *K. pneumoniae* isolates from China (Yang *et al.,* 2018), *mcr-8* gene in livestock and human *K. pneumoniae* from China (Wang *et al.,* 2018), *mcr-9* in patient *S. enterica* from USA (Carroll *et al.,* 2019), with a recent addition of *mcr-10* in clinical *E. roggenkampii* from China (Wang *et al.,* 2020). Such wide spread of *mcr* gene and its variants from these sources poses a serious concern for global public health.

Colistin is used as last-resort treatment for CPR MDR Gram-negative bacterial infections, a major contributor of morbidity and mortality worldwide (Parisi *et al.* 2015). The colistin use

for treatment of human infections, differs in various countries, some countries like Japan and South Africa have banned on use of colistin clinical setting. While other regions of the world such as Europe and Australia, colistin is administrated to treat infection and is only available in its parenteral formulation (CMS). In the countries including USA, Brazil, Malaysia, and Singapore, clinicians either prescribe colistin or the parenteral formulation of polymyxin B (Nation *et al.,* 2014). Each country has its own policy on usage of colistin, still majority of the previous publications show low to high resistance rates. (Falagas *et al.,* 2010).

In Europe, colistin-resistant strains were reported in early 2000 (Gales *et al.,* 2006). The SENTRY antimicrobial monitoring program from multiple countries recorded that with exception of *Klebsiella spp*. resistance to the polymyxins was low in other Gram-negative isolates from Asia Pacific to Latin American regions (Gales *et al.*, 2011). Globally various studies have been carried out to determine the prevalence of colistin resistance in clinical Gram-negatives. In Italy, colistin resistance was reported 43% in CPR *K. pneumoniae* isolates collected from 21 hospitals (Monaco *et al.,* 2014). Studies from USA, showed less than 5.5% resistance rates among *P. aeruginosa* and *A. baumannii* (Landman *et al.,* 2005; Keen *et al.,* 2010). A high colistin resistance (25.8%), was also reported from Romania (Mezghani Maalej *et al.,* 2012). The European Centre for Disease Prevention and Control (ECDC) in 2017, found an overall 8.5% colistin resistance in Europe. In their report, 88.5% CR was from Greece and Italy. Another ECDC report by its European Antimicrobial Resistance Surveillance Network (2018b), showed increased prevalence (29%) of CR *K. pneumoniae* cases in carbapenem resistants.

A Tunisian epidemiological study by National Medical Facility, reported increase in prevalence of CR *K. pneumoniae* from 3.57% in 2002 to 9.68% in 2013 (Battikh *et al.,* 2017). A French retrospective study on multiple intensive care units (ICU), identified an overall 4.4% prevalence of CR *A. baumannii* (Mahamat *et al.,* 2016). A study by Lu *et al.* (2018) on bloodstream infection patients from China detected low 0.4% CR isolates but were majorly hypervirulent *K. pneumoniae*. CR rates were identified much higher from Dubai, UAE (27%), and Italy (43%) (Moubareck *et al.,* 2018).

Clinical *E. coli* isolates often do not exhibit colistin resistance, however, show a low to moderate CR rate of 0.2–0.6%. Environmental studies, however, showed an alarmingly high frequency of CR *E. coli* in livestock and the general environment which are considered to be major contributor of CR (Li *et al.,* 2019c).

There is currently a scarcity of data on the precise processes underlying polymyxin resistance in *S. maltophilia* including heteroresistance or adaptive colistin resistance. Because of the high genetic diversity in the clinical isolates of *S. maltophilia,* detection of phenotypic AST has issue in term of accuracy and reliability. It is still difficult to determine the MIC in clinical isolates as MIC breakpoints for colistin for *S. maltophilia* are not established (Betts *et al.,* 2014; Gherardi *et al.,* 2015; Martínez-Servat *et al.,* 2018).

Colistin resistance is considered as a global public health issue as it affects both developed and developing countries. In Pakistan, the first *in vitro* colistin resistance among Gram-negative bacteria was reported in 2014 from Karachi. This study was carried out at Indus Hospital Karachi from total 885 clinical isolates, in which 3 isolates were colistin resistant (2 *E. coli*, 1 *A. baumannii*) (Bashir & Ahmed, 2016). In 2017, a study from Aga Khan University (Karachi), 40/251 CRE isolates had colistin MIC of ≥4 µg/mL and overall, 15.9% colistin resistance. In this study, 50% of the CR isolates were *K. pneumoniae* (Qamar *et al.,* 2017). In 2017, Azam *et al.,* (2017), reported first time detection of *mcr-1* gene in a clinical *E. coli* isolated from a patient admitted in a tertiary care hospital from Faisalabad. Plasmid mediated *mcr-1* gene carriage was also observed in *A. baumannii* and *P. aeruginosa* clinical isolates from Peshawar (Hameed *et al.,* 2019a). A multiple hospitals based study from Lahore, reported 4 CR *K. pneumonia* isolates from a total of 35 ESBL producing *K. pneumoniae* while *mcr-1* gene was identified in all 4 CR isolates (Bilal *et al.,* 2020). A study by Hameed *et al.* (2020) from Peshawar, identified higher colistin resistance among *E. coli* (23.3%), and *K. pneumoniae* (40%), majority of the CR isolate were also MDR with low *mcr-1* gene prevalence which was in six CR *E. coli* and 4 CR *K. pneumoniae.*

In a hospital-based study from Rawalpindi/Islamabad, 15% colistin resistance was seen among *K. pneumoniae* while, *mcr-1* and *mcr-2* gene detection was 12% and 9%,

respectively(Imtiaz *et al.,* 2021). Another hospital based cross-sectional study from Rawalpindi, observed high (10.4%) colistin resistance among CPR *K. pneumoniae* isolates while *mcr-1* gene was identified in only ten isolates(Furqan *et al.,* 2022a). An ICU based cross sectional study from Karachi, identified a higher colistin resistance rate among study isolates as 85% of the total isolated *K. pneumoniae* were found resistant (Syed *et al.,* 2022). According to a global meta analysis by Sameni *et al.,* (2022), CR *K. pneumoniae* prevalence was 17.46% in Pakistan. These studies from Pakistan, indicate that colistin resistance is emerging in this region. Combating AMR in already overburdened and low-resourced healthcare system in Pakistan is not only difficult but is of utmost importance (Iqbal *et al.,* 2022). The surveillance system is not comprehensive and there are still gaps not only in clinical diagnosis and reporting of AMR in the country which is indicated by relatively little literature on colistin resistance's prevalence (Saleem *et al.,* 2022).

The routine microbiological culturing provide a mean to isolate CR bacterial strains from multiple sources which includes environmental, foods, animal as well as clinical specimens but require broth microdilution protocol and PCR based method to confirm its colistin resistance (Chew *et al.,* 2017b). Due to large molecular size of polymyxins, it is poorly diffusible in agar therefore, agar dilution and disc diffusion methods not recommended any more by the joint CLSI-EUCAST document (Momin *et al.,* 2017). Colistin E-test strips are also used but these have been ruled out by the CLSI-EUCAST joint committee because of poor diffusibility in agar (Nordmann *et al.,* 2016).

For determination of colistin MIC, broth microdilution, is considered as a gold standard. Colistin's broth microdilution, however, is linked to problematic methodology. Colistin binds to the polystyrene in polystyrene trays leads to reduction in concentration in broth affecting the MIC values (Singhal *et al.,* 2018). Attempts have been made to stop this by adding a surfactant, like polysorbate-80, to the test system (Sader et al., 2012). But later it has been demonstrated that colistin has a synergistic effect and that surfactants do not improve assay performance. In order to address these issues, a joint CLSI-EUCAST working group have decided, the use of polystyrene microtitre plates and the sulphate salt of colistin

without the addition of surfactants in accordance with ISO standard 20776-1 as a reference broth microdilution (BMD) method (http://www.eucast.org/ast_of_bacteria/guidance_documents/). For *Enterobacteriaceae*, *Pseudomonas aeruginosa,* and *Acinetobacter spp*, EUCAST has recommend colistin breakpoints for susceptible ≤2 mg/L; resistant >2 mg/L previously, but in 2017 was revised as MIC ≥4mg/L for resistance in EUCAST Breakpoint Tables version 7.1 (2017). CLSI has no colistin breakpoint for *Enterobacteriaceae*, however, it has an epidemiological cut off value of 2 mg/L for *Raoultella ornithinolytica, E. aerogenes* and *E. coli*. According to CLSI, the colistin breakpoints for *P. aeruginosa, and Acinetobacter* spp is: susceptible ≤2 mg/L, intermediate =4 mg/L, and resistant ≥8 mg/L (Satlin *et al.,* 2020). Although, broth microdilution is a reference method it poses many challenges when used in clinical laboratories being laborious and time consuming (Poirel *et al.,* 2017).

Alternate to broth microdilution method, MICRONAUT MIC-Strip (MMS) and MIC Test-Strip (MTS) are commercially available rapid test to determine MIC against colistin. These strips comprise of 12 well plastic strip with 11 different concentrations of colistin and can be used for testing single isolate. MTS are gradient paper strips with increasing colistin concentrations. MTS is similar to E-test while MMS testing is broth based (Matuschek *et al.,* 2018). Although, these tests are not expensive and easy to use, the performance of these non-automated systems is ambiguous as various studies identified discrepancies among MIC when compared with reference method (Vourli *et al.,* 2017). Another drawback of these methods is a limited concentration range which limits the detection of CR isolates with low MIC, also have comparatively low precision in case some bacteria like *S. maltophilia, P. aeroginosa* and *A. baumannii* (Depka *et al.,* 2020).

Due to ease in usage compared to the reference broth microdilution method, automated methods are gaining attention for colistin susceptibility testing. The automated available commercial systems for determining MIC of colistin are MicroScan, BD Phoenix, Sensititre and Vitek 2. Among these, the MicroScan and Sensititre have been tested to be100% sensitivity for detecting *mcr*-producing *Enterobacteriaceae*. The detection of colistin

resistance by BD Phoenix standard is quite comparable to broth microdilution assay. However, BD Phoenix and Phoenix 100™ showed good sensitivity for phenotypic assessment of colistin however, are have less sensitivity for *mcr* gene detection (Hong *et al.,* 2019). BD-Phoenix also have low sensitivity for testing non-fermenters, which have much higher MICs(Carretto *et al.,* 2018; John Osei Sekyere, 2019a).

Rapid Polymyxin NP test is a novel test for determination of colistin resistance designed by Nordmann *et al.* (2016), with a sensitivity and specificity of 99.3% and 95.4%, respectively. This is colorimetric assay uses glucose metabolism to identify the growth of Enterobacterales at a certain polymyxin concentration. As a result of acid production linked to the metabolism of glucose, phenol red, a pH indicator, changes from orange to yellow, also was indicator of polymyxin resistance (Yainoy *et al.,* 2018). Several investigations have found this test an excellent assay for detecting colistin resistance even from blood cultures with a sensitivity of 100% (Malli *et al.,* 2019). Despite a higher sensitivity from blood samples, CR Gram-negatives detection from stool samples had lower sensitivity (Przybysz *et al.,* 2018).

Apart from the *Enterobacteriaceae*, Rapid polymyxin NP test was also developed for detection of colistin resistance among non-fermenters especially *A. baumannii* and *P. aeruginosa* (Lescat *et al.,* 2019). For *A. baumannii*, pH indicator phenol red was used to detect colour change from red to orange/yellow, but for *P. aeruginosa,* bromocresol purple was used as pH indicator which changes colour from green to violet (Sadek *et al.,* 2020). Lescat *et al.* (2019), found that the Rapid Polymyxin NP for *P. aeruginosa* had 100% sensitivity but lower specificity of 95%. However, for *A. baumannii*, inconsistencies regarding both specificity and sensitivity were observed (Malli *et al.,* 2021).

Another novel phenotypic method was designed to detect *mcr* positive Gram-negatives. In Coli-spot test, colistin solution (8mg/L) was used instead of disk. Isolate with >5 mm clear zone of inhibition, was interpreted as colistin susceptible and colonies growing within the inhibition zone are interpreted as CR (Jouy *et al.,* 2017).

Four EDTA based assays were designed by Esposito *et al.* (2017) to detect *mcr* positive isolates from various sample sources test. These assays rely on zinc ion chelation which is required for enzymatic activity of the plasmid mediated *mcr*-1 PEA transferase. The other was Combined disk test (CDT), in which 2 colistin disks (10 µg) were used in which one of the disks was treated with EDTA (10 µL of 100 mmol/L). A variation of ≥3 mm between the colistin impregnated disk and the colistin EDTA impregnated disk was interpreted as positive result for *mcr*-1. The sensitivity and specificity measured by the CDT were 96.7% and 89.6%, respectively. However, a more recent investigation found that CDT's sensitivity and specificity were 12% and 65.2% respectively. Therefore, additional evaluation studies are still required to validate the CDT (Clément *et al.,* 2018).

The second chelator-based test is Colistin MIC reduction test but unlike CDT, this test uses non-cation adjusted Muller Hinton broth (MHB) supplemented with EDTA solution (80 µg/mL). It was thought that adding Ca^{2+} and Mg^{2+} would reduce the inhibitory effects of EDTA, and may encourage the action of suspected PEA transferases in *E. coli* (Esposito *et al.,* 2017). Despite using various concentration of EDTA, this approach was unable to effectively identify *mcr* producers in Enterobacterales isolates (Büdel *et al.,* 2019).

The third approach was a modified version of Rapid Polymyxin NP test, supplemented with EDTA (80 µg/mL), for the detection of *mcr* positive Gram-negatives. No colour change was interpreted as positive test for *mcr* gene because of the presence of EDTA. This test demonstrated 96.7% sensitivity and 100% specificity for the detection of *mcr*-1 positive CR *E. coli* isolates(Esposito *et al.,* 2017).

The fourth chelator based approach was alteration of Zeta potential test. This test was based on detection differences of surface-membrane ionic charges in the presence of EDTA (80µg/ml) for detection of *mcr*-1 producing Gram-negatives. A Zeta potential ratio (Rzp = ZP+EDTA/ZP-EDTA) is used as a measure presence/absence of *mcr*-1 producers. Colistin susceptible isolates were recorded with greater anionic surface charges (−21.54 and −44.21 mV) while CR were recorded with lesser anionic surface charges (−4.20 to −19.34 mV) due

to the presence of PEA, LAra4N or galactosamine on Lipid A (Esposito *et al.,* 2017). This test had 95.1% sensitivity and 100% specificity, however, no inhibitory effect of EDTA was detected on *mcr*-1 producer *K. pneumoniae* (Leshaba *et al.,* 2022).

Apart from varied sensitivity and discrepancies associated with results, phenotypic methods additionally are time consuming, error prone and show less *mcr* specificity and sensitivity (Nordmann *et al.,* 2016; Bardet *et al.,* 2017). Hence molecular diagnostics techniques are now widely used and accepted as are rapid, efficient and have much higher specificity and sensitivity for the detection of colistin resistance. These methods are considered ideal for detection of *mcr* producing Gram-negatives with much higher specificity and sensitivity than phenotypic methods (Leshaba *et al.,* 2022).

The most popular molecular tool utilised by clinical laboratories to comprehend the resistance mechanisms in phenotypic resistant strains is the PCR based approach like conventional PCR and qRT-PCR (Sekyere *et al.,* 2016c). Conventional PCR was the first tools used for identification of *mcr*-1 gene from swine *E. coli* isolates (Liu *et al.,* 2016c). For detection of multiple genes and variants in a single reaction, real time and multiplex PCR assays are designed. These methods are used to amplify the *mgrB*, *phoPQ*, *pmrAB*, *pmrHFIJKLM*/*arnBCADT*, and *mcr* genes, major genes considered for colistin resistance. Additionally, in order to ascertain the rate at which each resistance gene is expressed in the resistant phenotype, qRT-PCR(Sekyere, 2019b).

Nijhuis *et al.* (2016) used qRT-PCR, for the detection of *mcr*-1 gene in *E. coli* and suggested that this method was 100% sensitive. A quantitative Taqman® PCR assay designed by Chabou *et al.* (2016), for detection of *mcr*-1 genes qualitatively and quantitatively in *E. coli* and *K. pneumonia*. For detection of *mcr-1*, *mcr-2* and/or *mcr-3* in *Enterobacteriaceae*, SYBR® Green-based qRT-PCR was designed by Bontron, Poirel, and Nordmann, (2016). The *mcr* genes were detected in bacteria isolated from human stool sample with 100% sensitivity and specificity. Dona *et al.* (2017) also designed a SYBR® Green real-time assay,

however, this assay from stool samples had low sensitivity for detection of *mcr-1* gene due to factors such PCR inhibitors.

Microarray is another molecular diagnostic technique for detection of both β lactamases and *mcr*-1/-2 genes in bacterial cultures. Multiple ligation reactions CT103XL microarray, simultaneously detect ESBL's, *mcr*-1 and 2 genes as well as many variants (*mcr*-1.1, *mcr*-1.2, *mcr*-1.3 up to *mcr*-1.7) in *Enterobacteriaceae* strains isolated from human, animals, and environment sources (Bernasconi *et al.,* 2017). The capability of this microarray diagnostic technique has advantage that it can be upgraded with new or developing resistance genes and variations. However, due to its cost and expertise requirement this technique not feasible for laboratories with limited resources (Sekyere, 2019b).

A nucleic acid based approach, Amplidiag CarbaR+MCR assay was designed in 2019. This molecular technique was able to screen both co-carriages of carbapenemase and *mcr*-1/*mcr*-2 genes from both rectal swabs and bacterial cultures with sensitivity and specificity 92% to 100% and 86% to 100%, respectively. This assay, however, was not able to detect GES carbapenemase producing *P.* aeruginosa having *mcr-1* and *mcr*-2 (Girlich *et al.,* 2019). Additionally, the assay was effective as within 3 hours diagnosis was possible from cultivated bacteria (Holma *et al.,* 2021).

Another nucleic based molecular method is Loop-mediated isothermal amplification (LAMP). Two commercially available LAMP instruments are Eazyplex® and SuperBug, are used for detection of *mcr*-1 from cultured bacteria within ≤30 minutes but not other target genes (Imirzalioglu *et al*., 2017; Zou *et al*., 2017). Multiplex LAMP assay is an advance LAMP assay based on restriction endonuclease use for the identification of several other *mcr* genes. The 2 version of multiplex LAMP, one LAMP systems detect *mcr*-2 & 5 while other LAMP system to amplify the *mcr*-1, 3, & 4 genes (Zhong *et al.,* 2019).

Although molecular based detection assays such as Microarray, LAMP, multiplex PCR, and real time PCR are rapid and accurate. However, all these molecular detection techniques have limitations including the fact that they can only identify specific, well known genes

like *mcr* genes, which are also carried by colistin susceptible strains. Therefore, such techniques are unable to detect the unknown causative molecular mechanism of colistin resistance (Osei & Asante, 2018). The development of next generation sequencing technologies has made it possible to determine the genomes of pathogens through high-throughput, parallel sequencing of DNA fragments rapidly and economically (Waddington *et al.,* 2022). Also WGS enables a wider detection of potential novel or altered colistin resistance genes, as well as specified resistance profile that alerts for a potential colistin resistance mechanism (Hahm *et al.,* 2022).

WGS provides details up to single nucleotide level in the genome of an organism, which can be utilised for identification of pathogen, ancestry , its virulence and also genetic basis for drug resistance (Vegyari *et al.,* 2020). Such WGS of bacterial strain enable quick detection of both chromosomal and plasmid-mediated resistance. WGS using high throughput sequencing along with bioinformatics analyses have made possible the comprehensive analysis of various genes, their mutations and possible mechanisms for pathogenicity and AMR. Nowadays, WGS based surveillance is employed to understand the transmission of pathogen.

This approach has enables us to map spread of resistance under One-Health perspective which was not previously possible (Aruhomukama *et al.,* 2019b). Additionally, by using bioinformatics tools analysis are quick as enormous datasets is already available for comparison, rapid detection and risk assessment (Aanensen *et al.,* 2021). Hence, WGS is also used for understanding the genomic basis for colistin resistance. Novel *mcr*-3 gene variants were identified in *Aeromonas* specie through such sequencing analysis (Eichhorn *et al.,* 2018). A genomic study by Peter *et al.,* (2018), identified hyper mutator genotype in a CR *Citrobacter werkmanii* co-carrying novel Metallo β lactamse gene using MiSeq Illumina sequencing platform. The genome characterization of first clinical case of CR *S. Enteritidis* from South Africa via Illumina MiSeq platform revealed absence of *mcr* gene but found genomic alterations already reported colistin resistance genes with a novel colistin resistance

genes (Rule *et al.,* 2019). A study by Lalaoui *et al.,* (2019) used WGS to identify *mcr* genes and chromosome alterations in *MgrB* and *PmrB* genes for polymyxin resistance.

In an outbreak of CR *A. baumannii* in India, WGS approach was utilized to trace the transmission pathways (Miltgen *et al.,* 2021). A study by Bir *et al.,* (2022) reported multiple genomic alterations in lipid A modification genes associated with colistin resistance in two XDR *K. pneumoniae* isolates using WGS approach. An African study recently dissected genomic potential of CR *A. baumannii* isolate using sequencing approach. Sequencing found that it is a potentially high-risk *A. baumannii* clone having carriage of crucial AMR and virulent determinants along with lipid biosynthesis gene mutations (Qamar *et al.,* 2017; Shamina *et al.,* 2020; Gharaibeh *et al.,* 2022; Nogbou *et al.,* 2022; Syed *et al.,* 2022).

The genome of two ST 131 CR *E. coli* isolated from surgical wound's abscess and peritoneal fluid after WGS were having carriage of plasmid-mediated AmpC β lactamase gene (*bla*CMY-2) and *mcr*-1 gene (Ortiz de la Tabla *et al.,* 2017). The genomic analysis of MDR *S. maltophilia* with high levels of resistance to colistin and meropenem revealed presence of *mcr*-5.3, *mcr*-8.2, and β lactamase encoding genes with absence of plasmid. This study further identified 12 genes linked to seven different types of efflux pumps, which were believed to be crucial in the development and spread of colistin resistance in this emerging pathogen (Li *et al.,* 2019).

The rise of CR bacteria and their management is major global problem. Consequently, it is a therapeutic challenge to control these colistin-resistant bacteria carrying the lactamases. To combat the CR bugs, there are many new promising strategies such as nano based, photodynamic based, CRISPR-Cas based and phage based strategy. Also, lots of research is in progress for the development of new molecules, repurposing of the existing drugs and combination treatments using colistin (Sharma *et al.,* 2022b).

It has been suggested that the nano based strategy might be better option for treatment of infections resistant to colistin. According to a study, in which silver based nanoparticles (AgNP) were used against pan drug resistant (PDR) *A. baumannii*, a higher MIC value was

found when colistin and AgNPs were given in combination as compared to when either colistin or AgNPs were given. These results indicated that colistin plus AgNP combination had a synergistic effect, hence low concentration of colistin was effective but with nanoparticles (Muenraya *et al.,* 2022).

Combinatorial therapy is a combinations of colistin and other antimicrobial drugs has been found to be effective against CR bacteria with MDR phenotype (Almutairi, 2022b). Colistin is frequently used in conjunction with other antibiotics including tigecycline, meropenem, gentamicin, or Fosfomycin (Dizbay *et al.,* 2010). A patient with ventilator associated pneumonia by CR bacteria was successfully treated with combination therapy of colistin with vancomycin and rifampicin (Tascini *et al.,* 2013b). A recent study was conducted to ascertain the efficacy of colistin and rifampicin in combination for the treatment of *CR A. baumannii,* although microbiological response was 100% but uncertainty in clinical response was observed. Hence, a randomised clinical trial was initiated to confirm the effectiveness of this combination medication (Park *et al.,* 2019). The synergistic effect is seen to differ with species and strain type, hence personalised therapy based on *in vitro* synergy testing is suggested for treatment. Another recent study revealed that monotherapy with individual antimicrobials was ineffective, however, triple combination therapy of polymyxin B, rifampicin, and amikacin enhanced *in vitro* bacterial killing with 1.7 log decline in colony counts in a mouse model infected with CR and CPR *K. pneumoniae* (Aye *et al.,* 2020).

Research into the potential of "repurposing" of currently available medications has received much interest for its application in the treatment of CR bacteria (Almutairi, 2022b). Ellipticine, a naturally occurring alkaloid, and its analogues were first suggested as potential anti-cancer (Stiborová *et al.,* 2001) but later on it was found to show activity against CR *E. coli*. Hence, it is now recognized as a potent chemical for treatment of resistant pathogens (Lu *et al.,* 2020). It is discovered that CR Gram-negative infections could be treated by combining the anthelmintic medication “niclosamide” with the antibiotic colistin (Domalaon *et al.,* 2019). CR Gram-negatives can be eliminated by combining anthelmintic nonantibiotic

molecules with colistin too (Domalaon *et al*., 2019). Also combination of colistin and anticancer medication "PFK-158" is found to work together synergistically to treat CR *Enterobacteriaceae* (Zhang *et al.,* 2019).

A study demonstrated that ursolic acid can inhibit the efflux pump and restore colistin sensitivity in MDR CR *E. coli* and *K. pneumoniae*. In an *in vitro* time kill assay, ursolic acid was found to increase the bactericidal activity of colistin by 4-4.5 logs with decrease in the bioburden in *in vivo* zebra fish by 1-1.5 logs (Sundaramoorthy *et al.,* 2019).

The CRISPR/Cas9 method is investigated to either simultaneously remove many plasmids in one step or to remove a plasmid containing *mcr*-1 gene in a stepwise way to convert colistin resistant bacteria into sensitive strain (Wang *et al.,* 2019). Recently, Khambhati *et al.* (2022) proposed using CRISPR-assisted phage genome editing to create phage variants that could help combating drug resistance.

Therapeutic potential use of phage's is recently researched for treatment of CR pathogens. A cocktail of three phage's namely Phieco32virus, Myoviridae, and Podoviridae was found to be effective against meropenem and CR strains with reduction in the bacterial load by 3 log in 2 hours (Manohar *et al.,* 2019). By horizontally transferring the *mcr*-1 gene and inactivating the *mgrB* chromosomal gene, Hao *et al.* induced colistin resistance in CR *K. pneumoniae*. These recombinant isolates now carrying *mcr*-1 with in active *mgrB* chromosomal gene were tested against lytic phage NJS1 for treatment. This phage was effective in both *in vitro* and *in vivo* model (biofilms or moth larvae) in reducing CR bacteria load compared to the wild-type strains (Hao *et al.,* 2019). Another, combination of two phage's vB_AbaM_ISTD and vB_AbaM_NOVI isolated from wastewaters, also showed bactericidal activity against CPR MDR bacterial pathogens. CR isolates were also found to be sensitive to both of the NOVI and ISTD phage's (Vukotic *et al.* 2020). A lytic phage IsfAB78 was tested against clinical isolates of MDR CR *A. baumannii* and was seen to significantly reduce (19–87%) MDR *A. baumannii* resistant bacteria in biofilm structures by after 40 min of incubation (Ebrahimi *et al.,* 2021).

Although all these therapeutic options have shown potential results in preliminary studies but still their application is constrained by a lack of clinical data, which require additional *in vivo* studies. Bacteria are known to develop defence mechanisms against antibiotics within a very short period of time, as a result of evolutionary selection pressures (Baym *et al.* 2016). There are many reports on development of drug resistant not only to antibiotics, but also to other therapeutic approaches like enzyme inhibitors and bacteriophages (Sundaramoorthy *et al.,* 2022).

As colistin resistance is emerging as a big therapeutic challenge. The acquisition of chromosomal alterations and plasmid mediated CR genes along with co-carriage of AMR genes (NDM, OXA-48, CTX-M, SHV, Tet, CatB) with virulent determinants in MDR Gram-negative bacteria further exaggerated the problem (Hussein *et al.,* 2021). Knowing the fundamental clinical plus epidemiological traits and associated factors contributing to the establishment of colistin resistance is crucial to understand these most serious and life-threatening problems seen in healthcare settings. Also, there is need for identification of new drug targets for the development of novel antibacterial agents against these emerging pathogens. High throughput sequencing approaches and genome based analysis are not only beneficial in uncovering the potential mechanisms of resistance but also provide crucial details of potential genomic determinants that can be used as potential target for future research.

Although colistin resistance is a worldwide issue, but it has a disproportionately negative impact on low and middle income countries, where it poses a threat to health care systems. Recent studies in Pakistan have highlighted the increasing colistin resistance rate among Gram-negative isolates but these findings are still inconsistent as data is analysed from smaller sample size, lack of suitable methodologies for detection and most importantly lack of comprehensive genomic analysis by high throughput sequencing.

The current study was a retrospective study carried out from September 2018 to December 2021 in the Medical Microbiology and Molecular Medicine Laboratory, Department of Microbiology, Quaid-i-Azam university Islamabad (Pakistan) and from June 2022 to December 2022 in Arshad Lab at the Stanley Manne Children's Research Institute at Lurie Children's Hospital affiliated with Northwestern University, Chicago, USA. Clinical specimens (blood, bronchial lavage, catheters, fluids, pus, sputum, urine, and wound swab) of the patients visiting MH Rawalpindi, PIMS Hospital, PGMI Hospital and CDA Capital Hospital from Islamabad were collected with patients' consent and data.

3.1. Ethical Approval

The study was carried out after approval of bioethics committees of departments involved in this research work, which were Bio ethic committee of Quaid-i-Azam University, National University of Medical Sciences (No: 06/R&D/03 NUMS; Date: 2-9-2017) and Shaheed Zulfikar Ali Bhutto Medical University (No: F.1-1/2015/ERB/SZABMU/; Date: 13-11-2017).

3.2. Inclusion Criteria

The clinical specimens included in the study were collected from patients clinically suspected of Gram-negative bacterial infections irrespective of patient's age or gender and have not taken any antibiotics in last three months.

3.3. Exclusion Criteria

Patients suffering from infections caused by other bacterial agents rather than Gram-negative bacteria, immune disorders and metabolic diseases were excluded.

3.4. Isolation and Biochemical Identification of Gram-Negatives

Both conventional and rapid biochemical identification schemes were adopted for the identification of the Gram-negatives from clinical specimens. For the recovery of the bacterial isolates, clinical specimens were inoculated on MacConkey agar, Cystein lactose electrolyte deficient (CLED) agar, blood agar, and chocolate agar. After preparation of

media, media was poured into petri plates and incubated at 37℃ for 18-24 hours. All the recipes used for the media preparation are presented in Appendix I.

Gram staining was used to differentiate bacteria into two major groups based on cell wall composition. As the cell wall composition of Gram-positive and Gram-negative differ in peptidoglycan content. The primary stain (Crystal violet) is retained by Gram-positive bacteria as it forms complex with the mordant (iodine) and even after the application of decolourizer the complex remains bound to the thick peptidoglycan leaving the bacterial cells stain blue. On the other hand, due to thin peptidoglycan layer the primary stain and mordant complex is washed away by the action of decolourizer, the cell walls when treated with secondary stain, it appears pink under microscope. The isolated and purified colonies obtained on the agar media were Gram stained and processed for identification of bacterial species.

First, **s**mear of purified colony was prepared by adding a drop of normal saline on sterilized glass slide. By using sterilize loop, single pure colony from overnight bacterial culture was picked and mixed with the saline to make smear and was heat fixed. The smear was treated with primary stain for about a minute and rinsed with tape water. Gram's Iodine was then applied to the smear for around 30 seconds. After rinsing, the smear was treated with decolourizer for roughly 10 seconds and then promptly washed with tape water. Finally, the smear was flooded with secondary stain, and left for about 40 seconds followed by rinsing with tape water. These smears were then left to dry at room temperature. These stained smears were examined under a bright field microscope at 100X oil immersion lens.

3.4.1. Growth on MacConkey Agar

For the assessment of fermentation ability of the Gram-negatives, purified bacterial isolates were inoculated on MacConkey agar. The chemical composition of MacConkey agar makes it both selective and differential media for the isolation of Gram-negatives. The media constituents have crystal violet and bile salts which makes it selective for Gram-negatives growth by inhibiting Gram-positive bacteria. whereas, the differential property is due to the presence of sugar (lactose), the fermentation of which differentiate Gram-negatives. The

members of family *Enterobacteriaceae* ferment lactose and produce pink coloured colonies while member of *non-Enterobacteriaceae* are non-fermenters which produce opaque/colourless colonies on the MacConkey agar. The colour of the colonies is due to the production of acid after fermentation of lactose, which is detected by change in neutral red indicator. MacConkey agar (Sigma) was prepared in distilled water followed by autoclaving at 121°C for 15 min (15 psi). Media was allowed to cool at room temperature and then poured into petri plates under sterile conditions. Clinical specimens were inoculated on agar plates via streak plate method followed by incubation at 37 °C for 24 hr.

3.4.2. Oxidase Test

The oxidase test was used to differentiate Gram-negatives into *Pseudomonas* (oxidase positive) from other non lactose fermenting Gram-negative bacteria like *Acinetobacter* (oxidase negative). Most of the other Gram-negative diplococci (*Neisseria, Moraxella*) and spiral curved rods (*Campylobacter, Helicobacter pylori, Vibrio cholera*) give positive oxidase test. In this test, bacteria with cytochrome oxidase enzyme use oxygen to generate energy by converting it into hydrogen peroxide and water via transfer of electron transport chain. This oxidation by indophenol oxidase enzyme is detected by Kovac's Reagent (tetra-methyl-p-phenylenediamine dihydrochloride) in these bacteria. In this reaction, the colourless reagent act as artificial electron donor which after oxidation by the enzyme changes to a coloured end product (dark blue or purple).

For oxidase test, filter paper strips were soaked in commercially available 1% Kovac's reagent (Sigma) and air dried. Single isolated colony from an overnight culture was carefully picked by the help of sterile loop. These colonies were rubbed on the treated filter paper and results were noted. A positive oxidase test was shown by a change in the colour of the filter paper from white to dark blue or purple within 15–20 seconds, whereas a negative oxidase test was indicated by no colour change.

3.4.3. Triple Sugar Iron Test

Triple sugar Iron (TSI) test is based on the principle of lactose fermentation leading to production of hydrogen sulfide(H_2S) and gas production by Gram-negative bacteria. This test was carried out to distinguish between enteric Gram-negative bacteria. TSI agar contains three sugars, which includes lactose (1%), sucrose (0.1%) and glucose (0.1%). In case of bacteria that do not ferment supplemented sugars and only utilize peptone for their growth, produce alkaline by products while those bacteria that utilize sugars produce acidic products. The production of acidic and alkaline by-products by the bacteria is detected by the presence of a pH indicator, phenol red. Apart from the sugars, the agar is also supplemented with ferrous sulfate, which basically is used to show reduction to hydrogen sulfide colourless, gas that was detected by an indicator sodium thiosulfate.

The TSI agar slants were made by dissolving media in distilled water followed by autoclaving at 121℃ for 15 minutes at 15psi. After sterilization, about 6-7mL of the agar was poured into10mL sterile glass tubes placed in a rack. The rack was then carefully tilted to make slant with good surface area and deep butt (1 inch). For TSI test, 18-24 hours old culture of isolates used from which single bacterial colony was picked by sterile inoculating needle for both the slants and butt inoculation. These slants were then incubated at 37℃ and the results were analysed after 18 hours.

3.4.4. Indole Test

The deamination of tryptophane (amino acid) by the action of the tryptophanase enzyme is an important biochemical test for the identification of enteric bacteria. This reaction leads to the production of various intermediate products like pyruvic acid, ammonia and especially indole. For this test, peptone water was used as inoculating medium as the peptone is a rich source of tryptophane, thus provides a substrate for the action of tryptophanase enzyme produced by the bacteria. The production of the indole was the detected by the Kovac's reagent. Peptone water was prepared in 1L of distilled water followed by autoclaving (121℃ for 15 minutes). About 5mL of the sterile peptone water was transferred into 10mL test tube, inoculated by a single isolated colony using sterilize loop and incubated at 37℃ of 18-24

hours. After incubation, 0.5mL of Kovac's Reagent was added to the tube and the formation of red coloured ring on the surface of media was checked, as it's indication of indole production.

3.4.5. Citrate Utilization Test

This test was used to distinguish among coliforms like *Enterobacter* species from faecal coliforms (*E. coli)*. This test checks citrate utilization by bacterial isolates as a carbon and energy source. Although citrate is generated during the Krebs cycle, it can also be taken up by the bacteria from an exogenous source with the help of bacterial permeases. The metabolism of citrate leads toward the formation of oxaloacetate, which further is metabolized to pyruvic acid and carbon dioxide (CO_2). The water and sodium ion in the media ultimately reacts with CO_2 and form sodium bicarbonate or alkaline end-products, which were detected by the change in the colour of the media.

Simon citrate media was used to screen the bacterial strains' ability to metabolize citrate as a sole carbon source supplied in the media. Simon citrate agar was weighed and dissolved in 1 mL of distil water followed by autoclaving (121℃ for 15 min). The slants were prepared as mentioned previously**.** A 24 hours old pure culture of the isolate was taken by sterilized needle and streaked on slant surface. These tubes were incubated at 37℃ for 18-24 hours and results were noted. The media contained bromophenol blue as pH indicator, with pH drop with change in media colour from green to blue. It was considered as positive citrate test if blue colour while no change in colour indicated the negative citrate test.

3.4.6. Rapid Identification via API 10S Test Strip

API10S identification test strips (BioMerieux Inc) were used for the rapid identification of *Enterobacteriaceae* and non-fastidious Gram-negative rods. The API10S kit was supplied with 10S disposable strips, incubating boxes and test regents, the results were interpreted as per result table provided by manufacturer. This kit contains dehydrate substrate in 10 microtubes, which were incubated with 18 hours old bacterial suspension for rapid identification. The substrates available for biochemical testing were 2-nitrophenyl-ß-D-

galactopyranoside (ONPG), D-glucose (GLU), L-arabinose (ARA), L-lysine (LDC), L-ornithine (ODC), trisodium citrate (CIT), sodium thiosulfate (H_2S), urea (URE), L-tryptophane (TDA), L-tryptophane (IND) and GLU tube-for NO_2 production. L-tryptophane is used as substrate in both Tryptophan deaminase test (TDA), and Indole test (IND). TDA test is used for the detection of the tryptophan deaminase enzyme (detected by adding Ferric chloride). While IND test is used for production of indole from tryptophan by the enzyme tryptophanase (Indole is detected by adding Kovac's reagent).

The bacterial suspensions were made from isolated bacterial colony in 5mL of API suspension media. The homogenized bacterial suspensions prepared were immediately used for the test and carefully added into microtubes of the strip. To avoid bubble formation, the strip was tilted, and the inoculum was added at the end of the cupule. For CIT test, both tubes and cupule were filled, whereas for rest of the tests only tubes were filled with suspension. Mineral oil was added in the cupule for LDC, ODC, H_2S and URE tests to create an anaerobic environment. After inoculation, these stripes were placed in the incubation box covered with lid and incubated at 37℃ for about 18-24 hours.

Results were interpreted by using the reading table provided with the kit. These results for the spontaneous tests based on colour change on majority of test, while for TDA, IND and NO_2 tests their respective microtubes were treated with reagents provided along with the kit. For TDA test, a drop of TDA reagent was added in to the microtube, change in colour indicated a positive test. For IND test, a drop of JAMES reagent and change in colour was observed. While for NO_2 Test, a drop of each of the two reagents NIT 1 and NIT 2 was added to the cupule and results in the microtubes were noted after 2-5 minutes.

3.5. Modified Broth Microdilution Method for Phenotypic Colistin Resistance

For phenotypic colistin resistance detection, broth-microdilution method was used to check the MIC against colistin. Colistin sulfate (Sigma Aldrich) stock solution was prepared by adding 6.51mg to 1 mL of distilled water to get 5120mg/L stock concentration. For working solution, 1 mL of stock solution was diluted in 9 mL of distilled water to get the final

concentration of 512 mg/L. Aliquots (1mL) were made in sterile glass tubes, these glass tubes were carefully sealed with paraffin and capped to store at 4℃.

Muller Hinton broth (MHB) was prepared with 1 litre of distilled water followed by sterilization 121℃ for 15 minutes. This media was allowed to cool to 2-8℃ before the addition of cations (Mg^{2+}, Ca^{2+}). The cation content of MHB media was adjusted by adding 1mL of Mg^{2+}stock solution (10mg/L) and 2mL of Ca^{2+} stock solution (20mg/L) before use in broth microdilution assay. The recipes of stock solutions are provided in Appendix II.

A fresh 2mL nutrient broth (NB) was inoculated with a single purified isolated bacterial colony. These tubes were incubated at 37℃ on shaking incubator for about 18-24 hours. The turbidity of bacterial suspension was adjusted and matched with 0.5 MacFarland standard (OD_{600}=0.08-0.1; CFU=1.5x10^8). The suspension was diluted (1:100) with sterile MHB for further analysis in polystyrene based 96-well microtiter plates per EUCAST guidelines.

All the Gram-negative bacterial isolates were tested against 10 different dilutions of colistin in two replicates to reduce the chance of random error. In each row, 100µL MHB was added into 10 wells. In 1st well, 50µL of colistin working solution (512mg/L) was added to MHB and after proper mixing 50µL was then aspired and dispensed to the 2nd well. It was repeated till 10th well and from 10th well 50µL antibiotic containing MHB was aspired and discarded. All these 10 wells were inoculated with 100µL of bacterial suspension. The 10 different dilutions obtained were 128,64, 32, 16, 8,4,2,0.5 and 0.25 mg/L. A blank was setup which was termed as growth control which was in the 11th well inoculated with 50µL test bacterial suspension. The sterility control was 50µL sterile MHB in 12th well. These plates were covered with the lid and placed on a wet paper towel. All of test microtiter plates were carefully incubated at 37℃ for 18-24 hours.

Plates were carefully examined under light to check for appearance of button-like growth in the bottom of the wells and MIC was carefully noted. These plates optical density (OD) was read on the spectrophotometer (Multiscan Go) at 600nm.

3.5.1. Resazurin Based Cell Viability Assay

This assay was used to detect the antibacterial activity of colistin against the Gram-negative bacterial isolates. For cell viability assay, 30µL of 0.015% resazurin sodium salt (Sigma) solution was added to each well containing bacterial suspension in the microtiter plate having a varied concentration of colistin sulfate salt. These plates were re-incubated for 2 hours at 37℃ and the results were noted as per colour change. The change of colour from purple to pink indicted bacterial growth.

All isolates were tested by three methods which included button like growth in microtiter plates with colistin, ODs at 600 and cell viability assay. Finally, results were carefully interpreted as per EUCAST guidelines (http://www.eucast.org) on basis of these three methods. Breakpoint tables for interpretation of MICs, version 10 (2020) of EUCAST was used and isolates having MIC value ≥4 mg/L were considered as resistant.

3.6. Antimicrobial Susceptibility Profiling via Disc Diffusion Method

In order to determine the AMR profile of CR isolates, antibiotic susceptibility testing was done against Piperacillin/Tazobactam, Cefazolin, Cefepime, Cefotaxime, Cefoxitin, Aztreonam, Imipenem, Amikacin, Ciprofloxacin, and Cefoperazone/sulbactam via Kirby Bauer disc diffusion method. Muller Hinton agar (MHA) was prepared and sterilized by autoclaving at 121℃ for 15 minutes. An isolated pure colony from overnight cultures of CR Gram-negatives was suspended in 1.0 mL of saline (0.85% NaCl) solution. These suspensions were compared with 0.5 McFarland standard equivalent to OD_{600}=0.08-0.1, 1.5 × 10^8 CFU/mL. Bacterial suspensions were spread on the MHA plates and antibiotic discs were applied. All these plates were incubated overnight at 37℃. Results were interpreted as resistant, sensitive, and intermediate after measuring zone diameter and comparison with the break points as mentioned in Appendix III as per CLSI recommendations (M100-29th Edition https://clsi.org/)

3.7. Plasmid Extraction

The phenotypically confirmed CR isolates were further analysed at molecular level for detection of mobile colistin resistance. From these CR strains, plasmids extraction was

carried out by using Gene Jet miniprep Plasmid Extraction Kit (ThermoFisher). Bacterial overnight cultures were prepared in 15mL falcon tubes. Centrifugation was carried out at room temperature for 5 minute to form pellet. The pellet was resuspended in 350µL of resuspension buffer containing the RNase. After the addition of the resuspension buffer, these tubes were inverted several times for proper mixing. The bacterial suspension was treated with 350µL of lysis buffer, the tubes were instantly inverted several times to avoid clump formation. After the lysis step, 250µL of neutralization buffer was added instantly to inhibit the action of lysis buffer. These tubes were then centrifuged at 1300 rpm for 5 minutes. After centrifugation, the supernatant transferred to membrane-filter column provided with the kit. The filter containing tubes were then centrifuged for 2 minutes, the flow through was discarded. The column was then washed with 500µL of wash buffer and the tubes were centrifuged for 2 minutes. This washing step was repeated twice. After washing, column was removed, and the filter tubes were shifted to the sterile Eppendorf. About 50-80µL of elution buffer was added to the filter and the tubes were incubated at room temperature for about 2 minutes, which were later centrifuged for 2 minutes at 13000 rpm. The membrane filters were removed, and the extracted plasmids were stored at -20℃. The purity of plasmids extracted was checked on the 0.7% agarose gel (120 minutes at 70 volt) and visualized under SynGene Transilluminator.

3.8. Polymerase Chain Reaction for the Detection of Plasmid-Mediated Mobile Colistin Resistant Genes

For the detection of plasmid-mediated mechanism for colistin resistance in CR Gram-negative isolates, three mobile colistin resistance gene (*mcr*-1, *mcr*-2, and *mcr*-3) were targeted by using PCR based method. Previously reported primers for *mcr*-1(Liu *et al.,* 2016b), *mcr*-2 (Xavier *et al.,* 2016b) and *mcr*-3 (Yin *et al.,* 2017) gene were used (Appendix IV). For amplification of these genes, 2XGreenTaq master mix (ThermoFisher) was used. Thermocycler conditions are mentioned in the Appendix V.

3.9. DNA Extraction for 16S rRNA Based Whole Genome Sequencing

The genomic DNA from CR Gram-negatives were extracted using an improved method of the standard phenol/chloroform protocol (Neumann *et al.* 1992). For DNA extraction, fresh bacterial culture was prepared. A single isolated colony was used to inoculate 1.5 mL nutrient broth and incubated at 37℃ for 24 hours in shaking incubator (250 rpm). Pellets were obtained by centrifugation for 2 minutes at 8000 rpm. These pellets were resuspended into 200 µL of Tris Ethylenediaminetetraacetic (EDTA) buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), 30 µL of 10% sodium dodecyl sulphate and 5uL of proteinase K (ThermoFisher) followed by 1hour incubation in water bath at 37℃. After incubation, 100 µL of Cetyltrimethyl ammonium bromide (CTAB) buffer plus 80 µL of 5M Sodium chloride were added and tubes were incubated at 65 ℃ for 10 minutes. Phenol chloroform isoamyl alcohol (1:1) was added in each tube followed by centrifugation at 10,000 rpm for 20 min. The upper layer was collected while the pellets were discarded, and this step was repeated twice to remove cell debris. Iso propanol (500 µL) and sodium acetate (300 µL) were added later, and tubes were incubated at room temperature for 10-15 minutes for proper precipitation of DNA followed by centrifugation at 10,000 rpm for 5 minutes. Pellets were collected and washed two times with 200 µL of ethanol. These pellets were air dried at room temperature and resuspended into the 50-100 µL of TE buffer before storage at -20℃ for later use.

The purity and yield of the DNA were assessed by calculating the A260/A280 ratios, optical density at A260 was checked for protein impurities followed by DNA concentrations determination via nanodrop spectrophotometer (Titertek Berthhod, Germany). After quantification, the selected samples were than forwarded for sequencing to Macrogen Korea and Northwestern University genomic centre (NUcore), Chicago, (USA).

3.10. Whole Genome Sequencing and Data Analysis

Genomic DNA of selected CR isolates, which were negative for *mcr* gene were sent for sequencing to decipher other molecular mechanism of CR by characterization of genome. Initially due to COVID-19 pandemic based international travel restriction and country wide

lock down, only Four DNA samples from CR isolates were sent to Korea (Macrogen), for WGS.

TruSeq Nano DNA Kit (Guide, Part # 15041110 Rev. D) was used for the library preparation of these extracted genomes. These libraries were than sequenced using MiSeq platform at 151 base pair (bp) paired end reads. The remaining 19 DNA samples were later submitted to Northwestern Sequencing Core Facility, Northwestern university, USA. A Kapa Hyper Prep kit (Roche, Pleasanton, CA, USA) was used for the DNA library preparation. These libraries were sequenced using HiSeq 4000 and 300-bp paired end reads were generated.

De novo assembly of the raw reads was carried out via SPAdes v3.12.0 and unicycler while the quality was assessed via QUAST. All these CR genomes were assembled for comprehensive genome annotation using Bacterial and Viral Bioinformatics Resource Centre (BV-BRC), and RAST tool kit (Olson *et al.,* 2023). *In silico* Multilocus sequence typing (MLST), based on house keeping gene loci was carried out using online tools which were; Pasteur Database (Jolley *et al*., 2018), Centre of Genomic Epidemiology (CGE) Database (Larsen *et al*., 2012), EnteroBase (Zhou *et al.,* 2020), BacWGSTdb (Feng *et al.,* 2021), and Pathogenwatch (Argimón *et al*., 2021). Capsular genotype was carried out using Pathogenwatch Database while in case of *E. coli,* serotypes were predicted by using, virulence Finder (Tetzschner *et al*., 2020), SerotypeFinder 2.0 (Joensen *et al*., 2015). Serotype was predicted for further clonal typing using two *in silico* tools; *Fim*-typing by FimTyper 1.0 plus (Roer *et al*., 2017), and CH-typing by CH-typer version1.0 (Roer *et al*., 2018).

3.10.1. Prediction of Antimicrobial and Virulent Determinants

"To comprehensively predict and confirm antibiotic determinants, three bioinformatics tools were used which included; ResFinder version 4.1 (Florensa *et al.,* 2022), Comprehensive Antimicrobial Resistant Database (Alcock *et al*., 2023), and Arg Anot (Gupta *et al*., 2014). While virulence genes were predicted using VirulenceFinder version 2.0 (Tetzschner *et al*.,

2020), Pasteur database (Jolley *et al*., 2018), Pathogenwatch (Argimón *et al*., 2021), and BacWGSTdb (Feng *et al.,* 2021).

3.10.2. Prediction of Mobile Genetic Elements

To confirm absence of *mcr* genes and further predict if any other mobile genetic elements, different tools were employed. Plasmid Finder.2.1 (Carattoli *et al*., 2014), was used to search for the carriage of plasmid replicon. For the prediction of CRISPR-Cas region, CRISPR Finder web tool by Grissa *et al* (2007) was used while for typing of predicted CRISPR-Cas regions CRISPRCasTyper database was used (Russel *et al*., 2020). Insertional sequences were predicted using ISFinder database (Siguier *et al*., 2006), and MobileElementFinder version 1.0.3 (Johansson *et al*., 2021). The prediction of conjugative elements and associated genes was carried out using OriTfinder database (Li *et al*., 2018). Further putative genes carriage found on the analysed whole genome, were subjected to predication if such genes existed on the mobile genetic elements and were annotated for if any role in CR using BV-BRC and NCBI database.

3.10.3. Detection of CR Chromosomal Mutation

For the detection of mutations reported for Lipid A modification genes on the chromosome for colistin resistance among these CR isolates, the sequence data for each individual gene was manually curated from the whole genome sequence data. Depending upon the bacterial specie, a set of Lipid A modification genes were selected. In case of CR *K. pneumoniae*, nucleotide and amino acid sequences of 16 chromosomal Lipid A modification genes (P*hoP, PhoQ, PmrA, PmrB, PmrD, PmrF, EptA, EptB, OpgE, PagP, RamA, ArnD, ArnE, ArnF, ArnT, MgrB*) were searched and extracted from the genomic data while in CR *E. coli* genomes the 16 targeted genes were ; *PhoP, PhoQ, PmrA, PmrB, PmrD, PmrF, EptA, EptB, EptC OpgE, PagP, ArnD, ArnE, ArnF, ArnT,* and *MgrB.* In case of CR *E. hormaechei,* sequence of 13 Lipid A modification genes (*PhoP, PhoQ, PmrA, PmrB, EptA, EptB, OpgE, PagP, ArnD, ArnE, ArnF ArnT, MgrB)* were sorted. Among *A. baumannii* 7 genes sequences were targeted which included *PmrA, PmrB, LpxA, LpxC, LpxD, LpxF,* and *LpsB*.

For assessment of sequence similarity and prediction of possible mutations, Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from National Centre for Biotechnology Information (NCBI) was used. For the mutational analysis, selected sequences were matched against reference genomes which included *K. pneumoniae* MGH-78578 (NC_009648.1), *E. coli* K12 MG1655 (U00096.2), *E. hormaechei* DSM-16691(CP017179.1), *A. baumannii* ATCC-17978 (CP000521.1) and *S. maltophilia* K279a (NC_010943.1).

3.10.4. Impact Prediction of Non-Synonymous Mutations

The impact of variations was further assessed by using PREDICTSNP database (Bendl *et al*., 2014). Protein encoding domains and features were predicted using UniProt database (https://www.uniprot.org/), InterPro database (https://www.ebi.ac.uk/interpro/) and AlphaFold database (https://alphafold.ebi.ac.uk/).

3.11. Gene Knock out Analysis Using ʎ Phage Red Recombinase System

Gene knock out analysis was carried out to explore the transporter gene as potential candidate for the anti-infective therapy for MDR CR bacteria. The gene knock-out analysis was done via using ʎ phage red recombinase as it is a quick and effective technique for disrupting chromosomal genes. The PCR primers with similarity to the desired gene were designed, which was initially developed by Datsenko and Wanner (2000). For this experiment, a UTI-associated *E. coli* (UTI89) was used as a model organism for transformation and gene knock-out experiment by targeting a transporter gene. This phage ʎ system had red recombinase was under the control of an inducible promoter which was easy to cure and had low copy number. To knock out gene using ʎ phage red recombinase system a set of primers targeting flanking regions of adjacent genes of desired site were designed to further perform recombination.

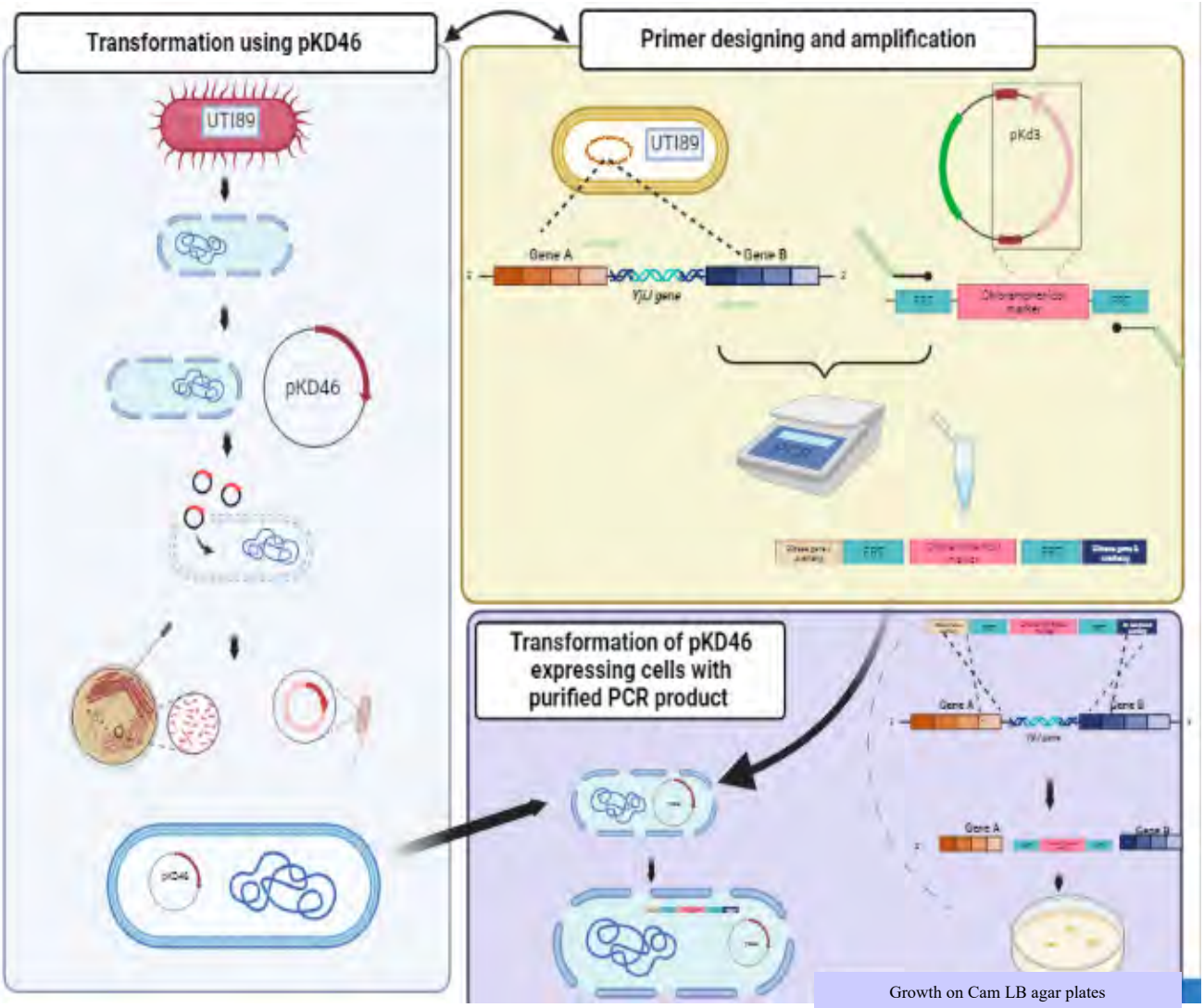

Figure 3.1: Schematic representation of gene knock out experiment using phage ʎ red recombinase system

3.11.1. Primers and PCR for Homology Extension

To knock-out a chromosomal transporter gene (*YjiJ*) in UTI89, about 50 bp overhang primers were designed for homology based-extension of genes surrounding target genes (Table 3.1).

Table 3.1: Sequence of 50 bp overhang primers for homology extension and amplification of Cam^R selective marker

Primer	Sequence (5'-3')	Red colour overhang	Product size (bp)
Forward	CTTCGCCGCCTGGCCGAGATATTTTT TTGCCTGTCCTAAGTTACCAAACAgtgt aggctggagctgcttc	50bp	1131
Reverse	CGGCCAAAACCAGCAGTAGGCTGGT CACAATTTTGATACGTTTTAACAatggg aattagccatggtcc	48 bp	

For the recombination event, target gene was replaced with resistant marker namely chloramphenicol selective marker Cam^R gene. pKD3 plasmid was used for the amplification of chloramphenicol selective marker Cam^R gene with 50bp overhang of adjacent genes as recombination site. The sequence of UTI89 *E. coli* strain, and pKD3 plasmid was obtained from NCBI data base (CP000243.1 and AY048742.1, respectively) for primer designing. For development of CMR genes with overhang a one-step PCR approach was used, in which amplification was done using 2X Apex Taq DNA polymerase Master mix (Genesee Scientific). Recipe for the reaction mixture and thermocycler conditions used to optimize the primers are provided in Appendix VI and VII respectively. The amplified product was confirmed on 1% agarose gel electrophoresis followed by purification using GeneJet PCR purification kit (ThermoFisher).

3.11.2. *DpnI* Digestion of PCR Product

DpnI was used for the removal of plasmid template from the amplified products. For the *DpnI* digestion, 50µL of the purified PCR products were treated with 1µL of *DpnI*. The mixture was mixed and incubated at 37℃ for 1-2 hours. The treated PCR products were then repurify using GeneJet PCR purification kit (ThermoFisher) and used for the transformation analysis.

3.11.3. Preparation of UTI89 Electrocompetent Cells

Electrocompetent *E. coli* UT189 cells were prepared by using Barrick's Lab protocol available online (https://barricklab.org/twiki/bin/view/Lab/ProtocolsElectrocompetentCells). The overnight culture was prepared by *E. coli* cultivating in Luria-Bertani Broth (LB) medium. In a 50mL flask, 10 mL of fresh LB medium was prepared and inoculated with 100µL of the overnight culture (OD_{600} of ~0.05). The cells were incubated for 2-3 hours in shaking incubator to attain mid-exponential phase (OD_{600} of 0.6). The bacterial cells were transferred to falcon tubes and centrifuged for 5 minutes at 6000 rpm to get the pellet. The supernatant was promptly removed, and pellets were washed with 10% glycerol (chilled). To resuspend the pellet, tubes were vortexed and then centrifuged for 3.5 minutes followed by removal of supernatant. The washing and resuspension steps of pellet with 10% chilled glycerol were repeated four times. Finally, the washed pellets were resuspended in 100µL of 10% chilled glycerol providing with 100X concentration. Cells were aliquoted (30-50µL) in cold Eppendorf and were stored at -80℃ for further use.

3.11.4. Transformation of UTI89 Competent Cells with pKD46 Plasmid via Electroporation

Phage red recombinase system expressing plasmid pKD46 was used for transforming *E. coli* competent cells by using electroporation. The electrocompetent cells were allowed to thaw on ice. About 1-3µL of extracted pKD46 plasmid (<100ng) was carefully added and mixed by gently flicking the tubes. The tubes were incubated for 10 minutes on ice. The mixture

was transferred to chilled cuvettes with gentle taping to allow it set at the bottom of the cuvette. The cuvettes were placed in electroporator, followed by electroporation at 200 ohms. The cells were immediately recovered in 1 mL fresh LB and incubated at 30℃ for 1 hour in shaking incubator. The pKD46 plasmid carried a selective marker gene for ampicillin resistance (Amp^R), therefore for the selection of transformants, 100µL of the inoculum was transferred to the ampicillin containing LB plate. These plates were incubated overnight at 30℃.

3.11.5. Development of Electrocompetent UTI89pKD46 Transformants

The UTI89pKD46 transformants were cultivated in ampicillin (Amp) containing LB overnight at shaking incubator at 30℃. In 30mL LB-Amp was inoculated with 500µl of overnight bacterial suspension in 100ml flask and incubated at 30℃ in shaking incubator for 2-3 hours until it reached OD_{600} =`0.35-0.400. For arabinose induction, 300µL of 10% arabinose was added into 30mL of the suspension to achieve 0.1% arabinose concentration. The culture was again incubated for 15 minutes in shaking incubator at 30℃. This suspension was immediately transferred to chilled 50mL falcon tubes and incubated on ice for 40 minutes. After incubation, this culture was centrifuged for 15 minutes on 1800xg at 4℃ to get the pellet. Supernatant was discarded while the pellets were resuspended in 30mL chilled 10% glycerol solution. These bacterial cells were centrifuged at 3000xg for 5 minutes. The recovered pellets were resuspended in 10% glycerol (chilled). This pellet washing step was repeated 3 times. After the final washing step, the pellets were loose and mushy, hence were carefully transferred to a chilled 15mL falcon tube to centrifuge at 3000xg for 5 minutes. After centrifugation, excessive glycerol solution was discarded, pellets were dissolved in 300µL 10% glycerol solution, aliquots (50µL) were made in cold Eppendorf tubes and stored at -80℃.

3.11.6. Transformation of Competent UTI89pKD46 with Desired Cam^R Gene Insert with Overhangs

For transforming ʎ phage red recombinase expressing UTI89 cells with PCR product, 50µl aliquots containing electrocompetent UTI89pKD46 cells were thawed on ice. About 50-

200ng (1-5µL) *DpnI* treated purified PCR product was added and mixed by flicking the tube. The mixture was transferred to chilled cuvette and electroporation was done (200 ohms). After electroporation, *E. coli* were immediately recovered in LB (1mL) and incubated at 37℃ on shaking incubator for 1.5 hours. About 200µL of the suspension was plated on LB containing Chloramphenicol (Cam) plates. These plates were incubated at 37℃ overnight.

After overnight incubation, the transformants colonies which appeared on the LB CAM plates were further confirmed using PCR. The extraction of DNA from knock-out mutants was done by boiling lysate method. A single isolated colony was carefully picked and mixed with 100µL of TE buffer. The mixture was incubated at 94℃ for 10 minutes on heat block followed by centrifugation at 12000 rpm for 5 minutes. Supernatant containing DNA was transferred into new Eppendorf and the pellet was discarded. For confirmation of *YjiJ* gene knock-out mutant, PCR was carried out using same primers which performed amplification of 50bp homology overhangs. The wild type *E. coli* (UTI89) strain was used as control. Internal primers were also designed to check knock out *YjiJ* gene in both wildtype and mutant strain along with confirmation of Cam^R gene in transformant/ knock out mutant strain. For the detection of curing of the helper plasmid pKD46 at 37℃ and 42℃, all transformant cells were amplified using primers designed to amplify the γ-recombinase gene (Table 3.2). The thermocycler conditions used for the amplification of Cam^R gene and γ-recombinase gene are presented in Appendix VIII.

Table 3.2: Internal primers for the detection of Cam^R selective marker gene and γ-recombinase gene in transformant cells

Targeted gene	**Primer**	**Sequence (5'-3')**	**Product size (bp)**
Cam^R selective marker (Long Primers)	Forward	CGCAGTACTGTTGTATTCAT	620
	Reverse	ACTGGATATACCACCGTTGA	
Cam^R selective marker (Short Primers)	Forward	TGAAACTCACCCAGGGATTG	222
	Reverse	GTGAGCTGGTGATATGGGATAG	
γ Recombinase gene	Forward	CGCAACTCTCTACTGTTTCT	386
	Reverse	CAAACGCACGGGTAATGGAT	
YjiJ gene	Forward	GGTTTCATTAGCCAGCAGTTTC	391
	Reverse	GCTGGTTGCCTCTATCTACTTC	

In the present study, a total of 566 specimens were collected from the patients suspected with bacterial infections, visiting four major hospitals in Islamabad/ Rawalpindi within a period of two years (2017 to 2019). Based on the clinical specimen's collection, higher percentage of the specimens were collected from male patients (61%) compared to females. Patients were categorized into 4 age groups with an interval of 20 years, group I comprised of patients with age range from newborn to 20 years, age group II 21-40 years, group III 41 to 60 years while group IV consisted of patients with age greater than 60years. The specimen collection was predominately from age group III as 37% of the patients belonged to this group, followed by age group II. Based on specimen type, urine (42%) samples were most collected followed to blood (19%) and pus (17%). The other specimens were bronchial lavage, wound exudates, catheters, and sputum (Figure 4.1).

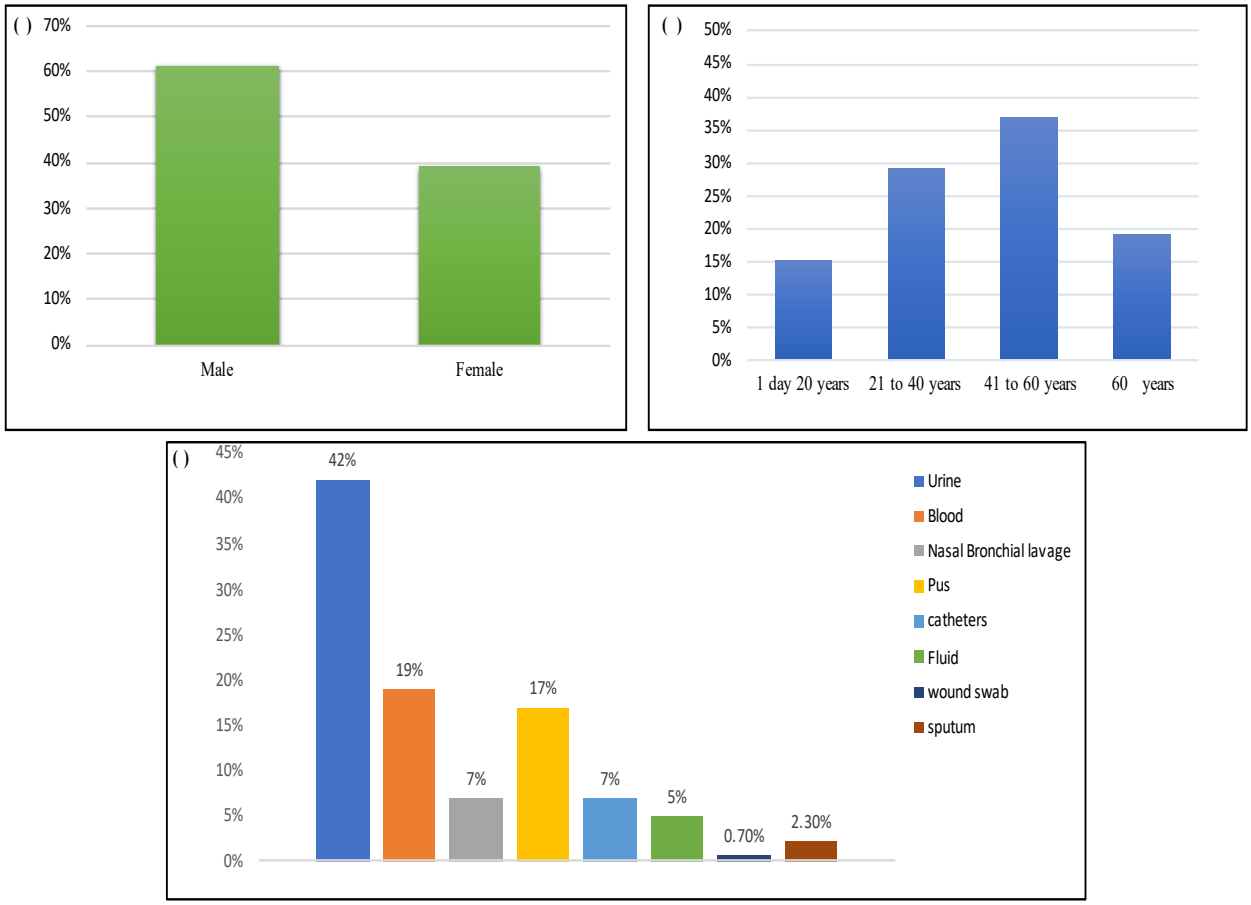

Figure 4.1: Percentage distribution of patient's based on (a) gender (b) age and (c) and type of clinical specimen

From the specimens, bacterial isolation was done, after isolation and purification of single colonies from clinical specimens. Overall, 566 bacterial isolates were recovered, among them 528 isolates appeared pink under the microscope and were confirmed as Gram-negative while 38 isolates which stained dark purple were Gram positives, hence were excluded from the study. Among 528 Gram-negative isolates, 446 isolates appeared rod shaped under bright field microscope while rest of the 82 appeared as coccobacillus. These isolates were selected and subjected to biochemical identification.

4.1. Biochemical Identification of Gram-Negative Bacterial Isolates

4.1.1. Growth on MacConkey

From a total 528 recovered Gram-negative bacteria, 355 isolates showed lactose fermentation as colonies appeared pink on the agar plate. While 81 isolates appeared non-lactose fermenters as formed colourless and opaque colonies on agar plates. The colonies of remaining 92 isolates appeared light pink and were late fermenters.

4.1.2. Oxidase Test

From 528 isolates, 61 non-lactose fermenting isolates displayed a positive oxidase test and were confirmed as *P. aeruginosa*. All the remaining 467 displayed a negative oxidase test and were further processed.

4.1.3. Triple Sugar Iron Test

Among the suspected Gram-negative, some isolates produced yellow(acidic) slant and yellow butt along with gas production were considered *K. pneumoniae*, *E. coli*, *Enterobacter species* and *C. koseri* while 8 of the suspected *C. freundi* produced acidic slant/acidic butt gas and H_2S production. All the suspected 61*P. aeruginosa* isolates further gave alkaline slant and alkaline butt. While alkaline slant and no change in butt colour was observed in all the suspected *A. baumannii.* The suspected *S. maltophilia* and *S. marcescens* produced acidic slant/acidic butt. Only suspected *Providencia* isolates displayed alkaline slant acidic butt.

4.1.5. Indole Test

A total 206 Gram-negative isolates gave positive Indole test. Among these, 182 were lactose fermenters (suspected as *E. coli*, *K. oxytoca*), 4 were suspected as *C. koseri* (late lactose fermenters) while 20 were lactose non-fermenters (suspected as *S. maltophilia* and *Providencia*). The remaining 322 Gram-negative isolates were tested negative for indole production and hence were considered to be potential member of *non-Enterobacteriaceae*.

4.1.6. Citrate Test

From all the 528 Gram-negative isolates, 171 suspected *E. coli* were negative for citrate test while among non-fermenters, 18 suspected *S. maltophilia* isolates were negative for citrate utilization test. The remaining 339 isolates gave positive citrate test.

4.1.7. API 10S Based Testing for *Enterobacteriaceae* and Non-Fermenters

Confirmation of all the suspected Gram-negative bacteria (except *P. aeruginosa*) was carried out using 10S API kit. From a total 528 isolates, 367 isolates were confirmed as member of *Enterobacteriaceae* family while the remaining 161 were member of *non-Enterobacteriaceae*. Among *Enterobacteriaceae*, 159 isolates were *K. pneumoniae*, 11 *K. oxytoca*, 3 *K. aerogens*, 171 *E. coli*, 10 *Enterobacter*, 6 *C. freundii*, 4 *C. Koseri*, 2 *Provedenica*, and 1 was *S. marcescens.* The 161 *non-Enterobacteriaceae* isolates were identified as *A. baumannii* 82, *P. aeruginosa* 61 and *S. maltophilia* 18 (Figure 4.2).

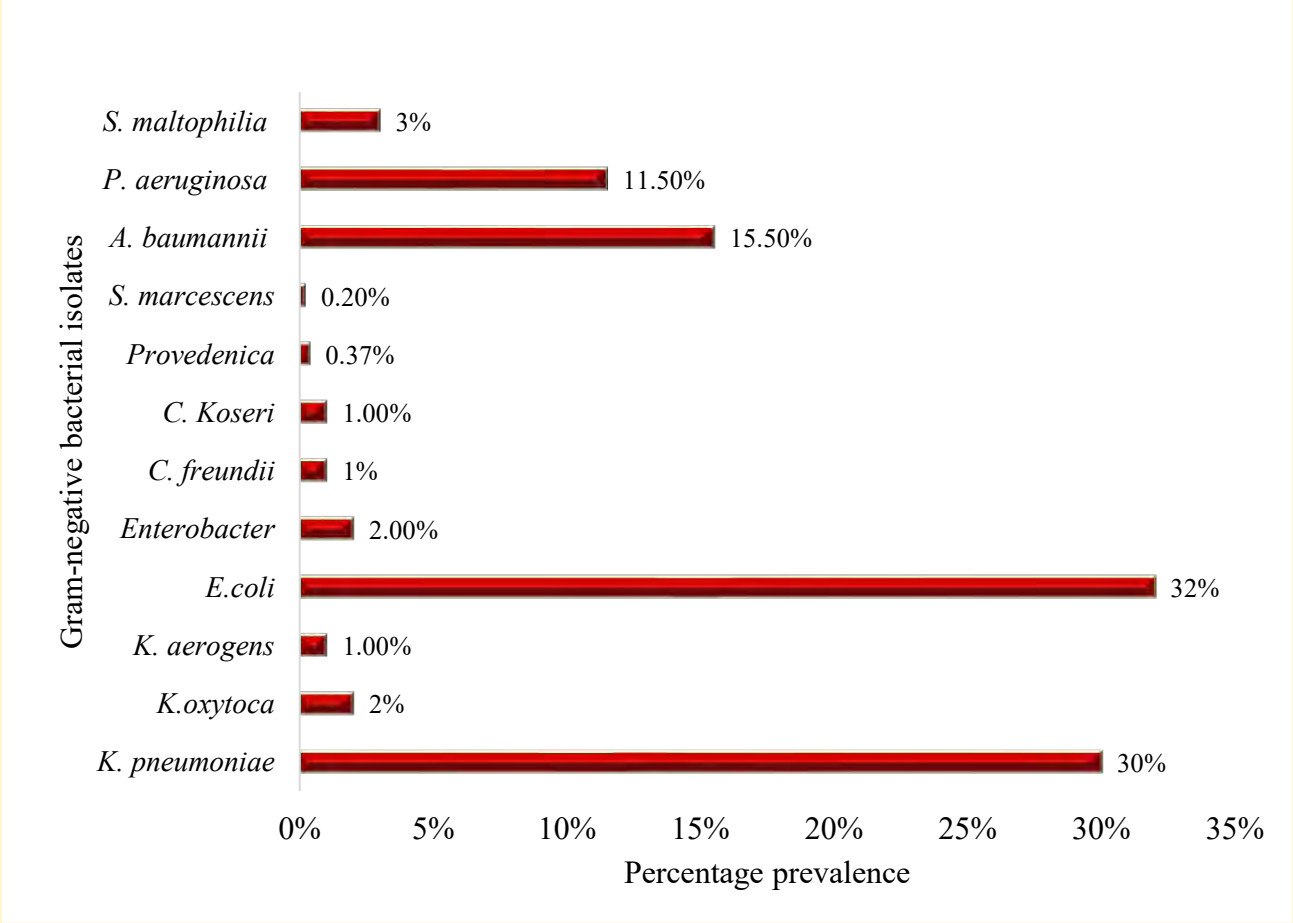

Figure 4.2: Percentage positivity of identified Gram-negatives recovered in various clinical specimens, collected from four Tertiary Care hospitals in Rawalpindi/ Islamabad

The percentage positivity of Gram-negatives was higher in male patient compared to females as 56% of the *Enterobacteriaceae* and 72% of *non-Enterobacteriaceae* isolates were recovered from male patient specimens. Among the confirmed Gram-negatives, percentage positivity of majority of the isolates were higher in males except for *E. coli,* which was higher in females (Figure 4.3).

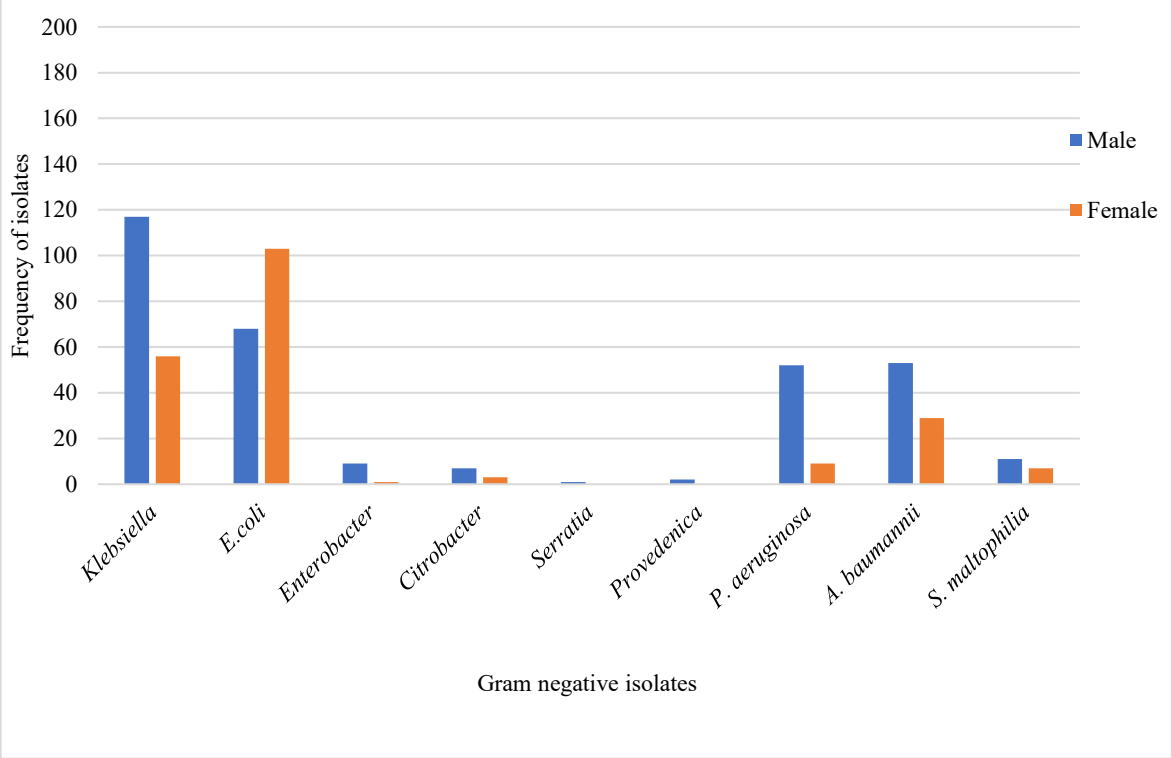

Figure 4.3: Frequency based distribution of isolated Gram negatives (*n*=528) based on patient's gender

In terms of patients age, the most susceptible age group was group-III as majority of *Enterobacteriaceae* (35%) and *non-Enterobacteriaceae* (36%) isolates were from this group. Followed by group II from which 31% of *Enterobacteriaceae* and 25% of *non-Enterobacteriaceae* isolates were recovered. The percentage of *Klebsiella*, *Enterobacter*, *Citrobacter*, *A. baumannii*, and *P. aeruginosa* was higher in group III, in contrast with *E. coli* and *S. maltophilia* which were more common in group II and group I respectively (Figure 4.4).

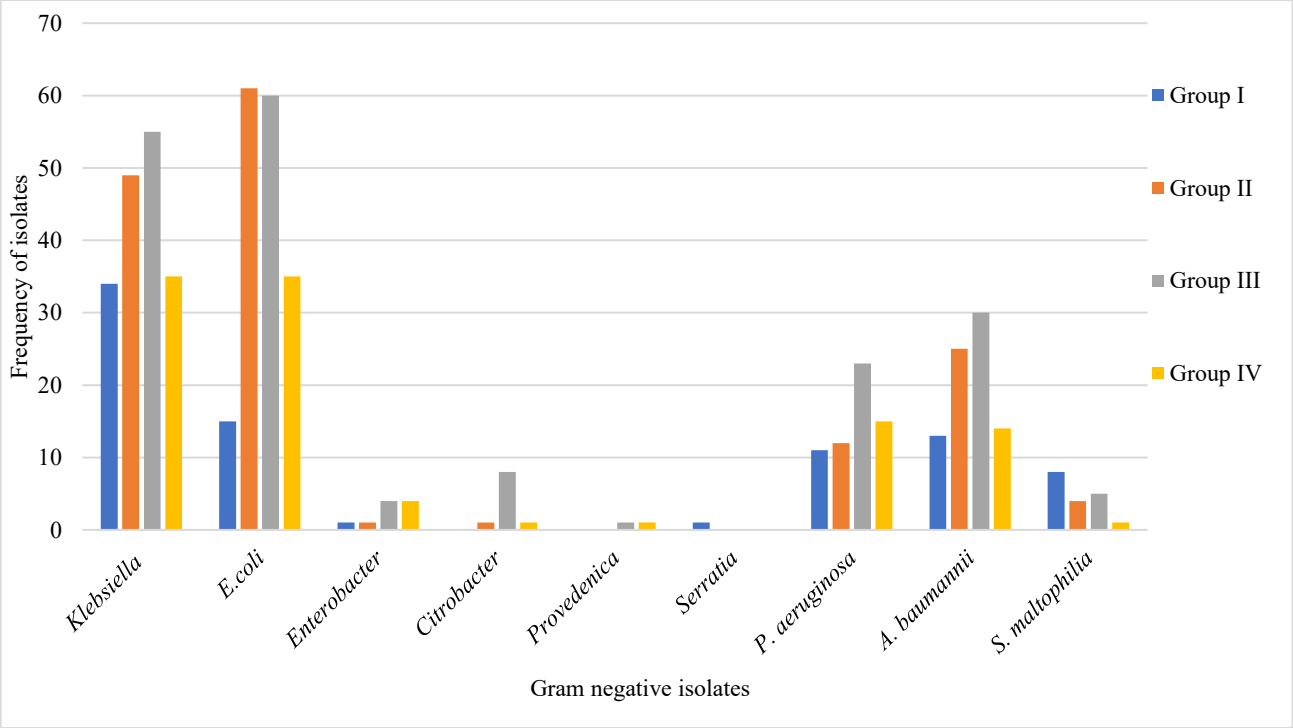

Figure 4.4: Frequency distribution of Gram-negative bacterial isolates recovered from clinical specimens among different patients age-groups Group I neonates-20 years, Group II 21to 40 years, Group III 41 -60 years and Group IV 60+ years.

Isolates based on specimen type for their source of recovery were found to be highest number of *Enterobacteriaceae* isolates recovered from urine (59.4%), followed by blood (14%) while the least number of isolates were from wound swab. Among *non-Enterobacteriaceae*, majority of the isolates were recovered from pus (30%) and blood (24%). The overall frequency distribution of Gram-negatives based on specimen source is provided in Appendix IX.

4.2. Minimum Inhibitory Concentration (MIC) Against Colistin:

For phenotypic colistin resistance, *Provedenica*, and *S. marcescens* isolates were excluded from the study because of their intrinsic resistance against colistin. From total, 525 Gram-negative isolates were subjected to modified broth microdilution test for the assessment of MIC against colistin. Among 525 isolates, 161(31%) Gram-negative isolates were detected with resistance to colistin as MIC value was ≥4 mg/L (as per EUCAST guidelines). In these CR isolates, 70.2% were members of *Enterobacteriaceae* family while 29.8% were *non-Enterobacteriaceae* members. Among *Enterobacteriaceae*, highest resistance against colistin was observed in *K. pneumoniae* followed by *E. coli* while in case of *non-Enterobacteriaceae*, majority of the *P. aeruginosa* showed resistance against colistin followed by *A. baumannii* isolates (Figure 4.5).

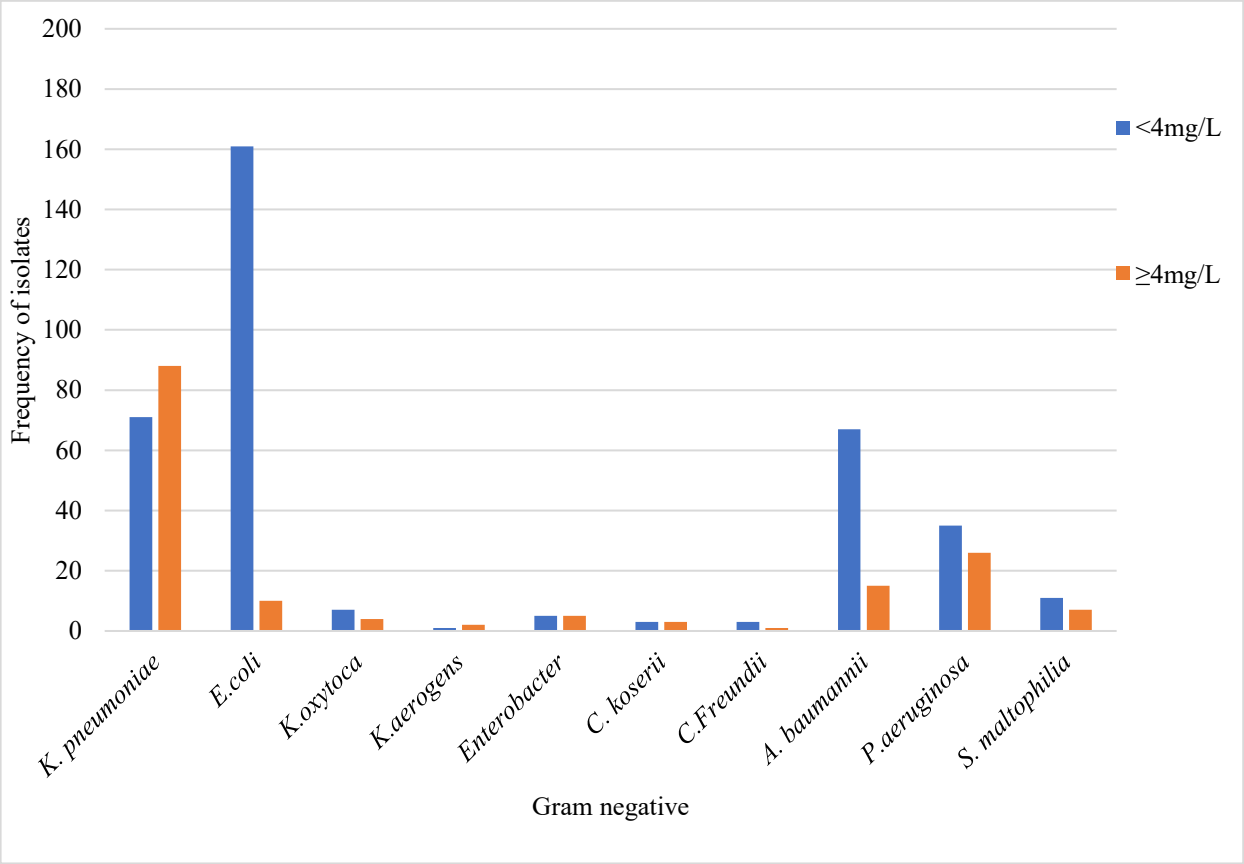

Figure 4.5: Frequency distribution of Gram-negative bacterial isolates based on MIC values among *Enterobacteriaceae* and *non-Enterobacteriaceae* isolates. MIC<4mg/L was considered susceptibility and MIC≥4mg/L resistance to colistin.

Although CR Gram-negative isolates showed varied range of MIC values from 4 to 256 mg/L against colistin, highest number of the isolates (*n*=44) had MIC 4 mg/L followed 28 isolates displaying MIC value of 256mg/L. Based on distribution pattern of Gram-negative isolates with respect to their MIC ranges, majority of the *Enterobacteriaceae* members had MIC range of 4mg/L-16 mg/L while among *non-Enterobacteriaceae*, the highest number of isolates had MIC value of 256mg/L followed by MIC of 4 mg/L (Table 4.1).

Table 4.1: Frequency distribution of phenotypic CR Gram-negative bacterial isolates based on MIC values

Serial number	**CR Gram-negative isolates (*n*=161)**		**Resistant Gram-negatives (*n*), at various colistin concentrations (mg/L)**						
			>4	**8**	**16**	**32**	**64**	**128**	**256**
1	***Enterobacteriaceae* (113)**	*K. pneumoniae* (*n*=88)	23	21	13	11	8	5	7
		K. oxytoca (*n*=4)	4	-	1	1	1	-	1
		K. aerogens (*n*=2)	2	-	-	1	1	-	-
		E. coli (*n*=10)	3	-	1	1	-	1	4
		Enterobacter (*n*=5)	3	-	1	-	-	1	-
		C. koserii (*n*=3)	1	-	1	-	1	-	-
		C. freundii (*n*=1)	1	-	-	-	-	-	-
2	***Non-Enterobacteriaceae* (48)**	*P. aeruginosa* (*n*=26)	6	3	6	-	1	1	9
		A. baumannii (*n*=15)	7	1	3	1	1	-	2
		S. maltophilia (*n*=7)	-	1	-	1	-	-	5
3	Overall CR resistant isolates		44	26	26	16	13	8	28

Majority (71.4%) of the Gram-negative isolates showed phenotypic colistin resistance were recovered from male patients while highest percentage of isolates (30.4%) were from patients of age group II years followed by age group III (28%). The most common specimens' type for CR Gram-negatives isolation was urine (26.7%) followed by blood (22.4%) and pus (19.9%) as shown in Figure 4.6.

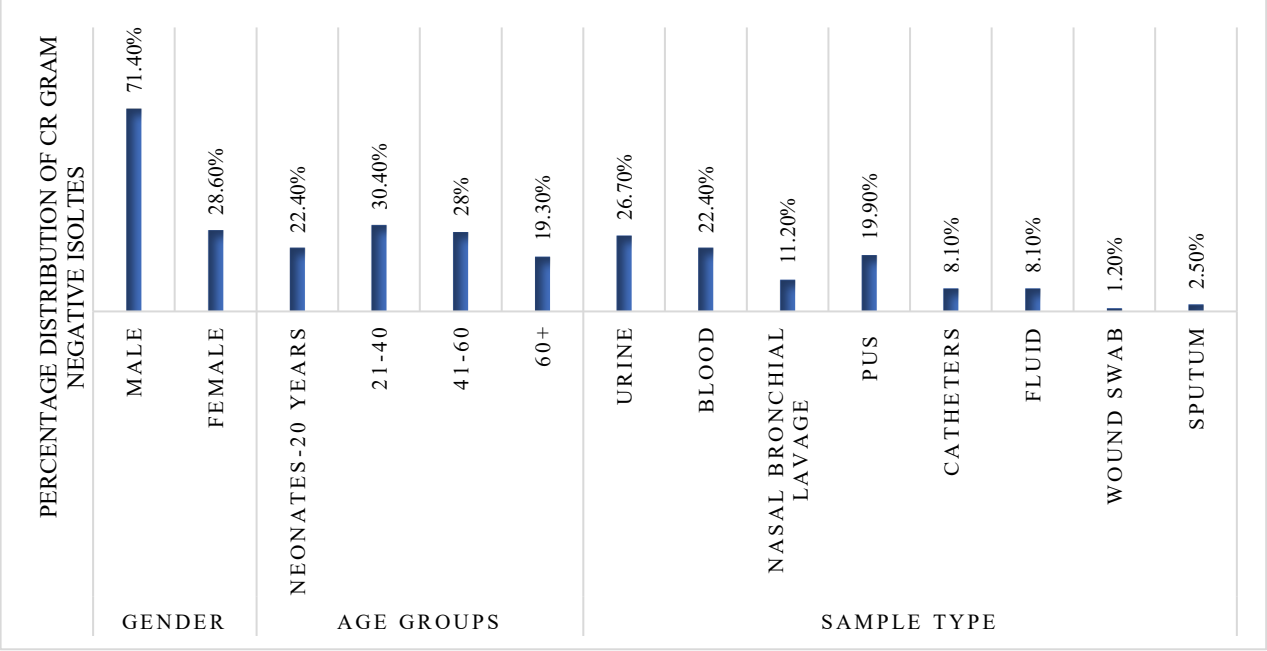

Figure 4.6: Overall distribution of CR Gram-negatives based on patient's gender, age groups, and specimen types.

Among *Enterobacteriaceae* members, highest number of isolates were *K. pneumoniae* (63/88) where other like *K. oxytoca* (3/4)*, Enterobacter* (4/5) and *Citrobacter* (4/4) were in less number recovered from male patients. CR *E. coli* were majorly (6/10) recovered from female patients. Furthermore, *Enterobacteriaceae* isolates showed varied distribution pattern when categorized based on patients age group. As for *K. pneumoniae,* 21-40 years of age group was found most susceptible as 39% of CR isolates were recovered from this age group while for *K. oxytoca*, *K. aerogens* and *E. coli* mostly were from 41-60 and 60+ years age group. For *Enterobacter* the most susceptible age group was 60+years while for Citrobacter spp. all the isolates were recovered from age group I.

Based on clinical specimen type, urine was most frequent specimen type and mostly detection of *K. pneumoniae* with majority being CR *K. pneumoniae*. In case of *non-Enterobacteriaceae* members, majority of CR *P. aeruginosa* and *A. baumannii* isolates were recovered from male patients and from age-group 41-60+ while the most common specimen type was pus. But in case of CR *S. maltophilia* isolates, the susceptible age group was neonates to 20years as 6/7 isolates were recovered from blood specimen (Appendix X).

4.5. Antimicrobial Susceptibility Testing of CR Gram-Negative Isolates

All the of the161 CR Gram-negative isolates were tested against a panel of antibiotics for the assessment of drug resistant phenotype. Among CR *Enterobacteriaceae* isolates (*n*=113), more than 90% of CR *K. pneumoniae* isolates showed resistance against cephalosporins, β Lactam combination agents, quinolones and monobactam class of antimicrobial agents. While highest susceptibility was detected against aminoglycoside as 21% isolates were sensitive to amikacin. Resistance against imipenem was detected in 82% of *K. pneumoniae* isolates, 16% isolates showed intermediate profile and only 4% showed sensitivity to the drug.

In case of *K. oxytoca,* all 4 isolates showed 100% resistance against 1st 3rd and 4th generation of cephalosporins while 75% resistance was seen against cefoxitin, Cefoperazone/sulbactam and ciprofloxacin. The *K. oxytoca* isolates, 2/4 isolates were resistant to amikacin and imipenem while one isolate was resistant to aztreonam. Both *K. aerogens* isolates showed

resistance against 9/10 tested antibiotics with only exception was imipenem as 1 isolate was susceptible.

All the CR *E. coli* isolates showed 100% resistance against cefoperazone/sulbactam, cefotaxime, cefepime and aztreonam while 90% resistance was detected against piperacillin/tazobactam, cefazolin, cefoxitin, ciprofloxacin and imipenem. The most affective antimicrobial was amikacin as 40% of *E. coli* isolates showed sensitivity against amikacin. In case of CR *Enterobacter* isolates, all the five were 100% resistance to cefazolin, cefotaxime, cefepime and ciprofloxacin. While there were some sensitive to amikacin. Among Citrobacter species, *C. koseri* (*n*=3) were completely resistant to most of tested antibiotics with exception of imipenem and amikacin as 2/3 isolates showed intermediate phenotype when tested against imipenem while 1/3 isolate was sensitive to amikacin. Only a single isolate of *C. freundii* was colistin resistant which was 100% resistant to all the tested antibiotics except for amikacin (Table 4.2).

Table 4.2: Antibiotic Susceptibility profile of colistin resistant *Enterobacteriaceae* isolates (*n*=113)

Antibiotic class	Antibiotic agent		*K. pneumoniae* n (%)	*K. oxytoca* n (%)	*K. aerogens* n (%)	*E. coli* n (%)	*Enterobacter* n (%)	*C. freundii* n (%)	*C. Koseri* n (%)
β L Combination agents	Piperacillin/Tazobactam	R	85 (97)	2 (50)	2 (100)	9 (90)	4 (80)	1 (100)	3 (100)
		I	1 (1)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		S	2 (2)	1 (25)	0 (0)	1 (10)	1 (20)	0 (0)	0 (0)
	Cefoperazone/sulbactam	R	79 (90)	3 (75)	2 (100)	10 (100)	4 (80)	1 (100)	3 (100)
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		S	9 (10)	1 (25)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)
Cephalosporins	Cefazolin (1st generation)	R	86 (98)	4 (100)	2 (100)	9 (90)	5 (100)	1 (100)	3 (100)
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		S	2 (2)	0 (0)	0 (0)	1 (10)	0 (00	0 (0)	0 (0)
	Cefoxitin (2nd generation)	R	86 (98)	3 (75)	2 (100)	9 (90)	4 (80)	1 (100)	3 (100)
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		S	2 (2)	1 (25)	0 (0)	1 (10)	1 (20)	0 (0)	0 (0)
	Cefotaxime (3rd generation)	R	86 (98)	4 (100)	2 (100)	10 (100)	5 (100)	1 (100)	3 (100)
		I	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		S	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Cefepime (4th generation)	R	85 (97)	4 (100)	2 (100)	10 (100)	5 (100)	1 (100)	3 (100)
		I	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		S	3 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Monobactam	Aztreonam	R	81 (92)	1 (25)	2 (100)	10 (100)	4 (80)	1 (100)	3 (100)
		I	1 (1)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		S	6 (7)	2 (50)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)
Carbapenem	Imipenem	R	72 (82)	2 (50)	2 (100)	9 (90)	3 (60)	0 (0)	1 (33)
		I	12 (14)	1 (25)	0 (0)	1 (10)	1 (20)	1 (100)	2 (67)
		S	4 (4)	1 (25)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)
Aminoglycoside	Amikacin	R	69 (78)	2 (50)	1 (50)	6 (60)	2 (40)	0 (0)	2 (67)
		I	1 (1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)
		S	18 (21)	2 (50)	1 (50)	4 (40)	3(60)	1 (100)	1 (33)
Quinolones	Ciprofloxacin	R	85 (97)	3 (75)	2 (100)	9 (90)	5 (100)	1 (100)	3 (100)
		I	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)
		S	3 (3)	1 (25)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

In case of CR *non-Enterobacteriaceae* isolates (*n*=48), highest resistance (81%) was detected in *P. aeruginosa* isolates to aztreonam, followed by cefepime (77%), amikacin (73%) and ciprofloxacin (73%) while most isolates were sensitive to imipenem followed by piperacillin/tazobactam as sensitivity recorded was 38% and 31% respectively. Among CR *A. baumannii* isolates, highest resistance (87%) was detected against tested β Lactam combination agents, and cephalosporins while least resistance was observed against imipenem as 27% isolates were sensitive. In case of *S. maltophilia* isolates, complete resistance was against cefoxitin as 100% isolates showed resistance to this drug while all isolates were sensitive to piperacillin/tazobactam (Table 4.3).

Table 4.3: Antibiotic Susceptibility profile of colistin resistant *non-Enterobacteriaceae* isolates (*n*=48)

Antibiotic class	Antibiotic agent		*P. aeruginosa* n (%)	*A. baumannii* n (%)	*S. maltophilia* n (%)
β L Combination agents	Piperacillin/Tazobactam	R	18 (69)	13 (87)	0 (0)
		I	3 (12)	0 (0)	0 (0)
		S	5 (19)	2 (13)	7(100)
Cephalosporins	Cefazolin (1st generation)	R	--	13 (87)	3 (43)
		I	--	0 (0)	0 (0)
		S	--	2 (13)	4 (57)
	Cefoxitin (2nd generation)	R	--	13 (87)	7 (100)
		I	--	0 (0)	0 (0)
		S	--	2 (13)	0 (0)
	Cefotaxime (3rd generation)	R	--	13 (87)	3 (43)
		I	--	0 (0)	0(0)
		S	--	2 (13)	4 (57)
	Cefepime (4th generation)	R	20 (77)	13 (87)	3 (43)
		I	0 (0)	0 (0)	0 (0)
		S	6 (23)	2 (13)	4 (57)
Monobactam	Aztreonam	R	21 (81)	12 (80)	3 (43)
		I	1 (4)	0 (0)	0 (0)
		S	4 (15)	3 (20)	4 (57)
Carbapenem	Imipenem	R	16 (62)	11 (73.3)	4 (57)
		I	2 (8)	2 (13.3)	0 (0)
		S	8 (31)	2 (13.3)	3 (43)
Aminoglycoside	Amikacin	R	19 (73)	12 (80)	0 (0)
		I	0 (0)	0 (0)	0 (0)
		S	7 (27)	3 (20)	7 (100)
Quinolones	Ciprofloxacin	R	19 (73)	12 (80)	0(0)
		I	1 (4)	0 (0)	0 (0)
		S	6 (23)	3 (20)	7 (100)

From a total 161 CR Gram-negative isolates, 135 (84%) CR-isolates showed a MDR profile as were resistant to antimicrobials from 6 of the tested antibiotic classes (β Lactam Combination agents, Cephalosporins, Monobactam, Carbapenem, Aminoglycoside, Quinolones & Fluoroquinolones). The highest number of MDR isolates were from *Enterobacteriaceae* family as 96% of the isolates displayed MDR profile. While in case of *non-Enterobacteriaceae*, 56 % isolates had MDR phenotype (Table 4.4).

Table 4.4: Multidrug resistance profiling of CR Gram-negative isolates (*n*=161)

Serial number	**Number of CR- Gram-negative isolates**		**MDR CR-isolates *n* (%)**	**Overall CR MDR *n* (%)**
1	***Enterobacteriaceae* (113)**	*K. pneumoniae* (88)	84 (95)	108 (96)
		K. oxytoca (4)	3 (75)	
		K. aerogens (2)	2 (100)	
		E. coli (10)	10 (100)	
		Enterobacter (5)	5 (100)	
		C. koseri (3)	3 (100)	
		C. freundii (1)	1 (100)	
2	***Non-Enterobacteriaceae* (48)**	*P. aeruginosa* (26)	19 (73)	27(56)
		A. baumannii (15)	8 (53)	
		S. maltophilia (7)	0 (0)	

4.6. PCR Based Identification of *mcr*-1, *mcr*-2, and *mcr*-3 Genes

Plasmid-mediated colistin resistance was determined based on presence of mobile-colistin resistance genes particularly *mcr*-1, *mcr*-2, and *mcr*-3 genes. Extracted plasmids were used as template for the PCR based amplification of these genes. Optimized PCR conditions used for the amplification of *mcr*-1 *mcr*-2 and *mcr*-3 are presented in Appendix IX. The overall carriage of plasmid-mediated *mcr* genes among study CR isolates was 12%. From total 161 CR isolates, *mcr*-1 gene was detected in 1%, *mcr*-2 gene in 9% while *mcr*-3 gene was detected in 5.5% of the CR isolates. The characteristics of *mcr* positive CR Gram negative isolates is presented in Appendix XI.

Majority of the CR Gram-negative isolates of the study were negative for *mcr*-1 gene except for one *Enterobacter* isolate which had MIC value of 4mg/L against colistin. This isolate was not only positive for *mcr*-1 gene but also showed co- carriage of *mcr*-2 and *mcr*-3 genes. Among all the CR Gram-negative isolates, highest percentage of *mcr*-2 (6%) and *mcr*-3 (2%) gene carriage was detected among ten *K. pneumoniae* isolates which had MIC values of 8 mg/L. All the CR *E. coli* isolates were negative for both *mcr*-1 and *mcr*-2 genes while only two isolates were found to be positive for *mcr*-3 gene carriage. In case of *P. aeruginosa* and *A. baumannii* isolates, all were negative for *mcr*-1 gene carriage while *mcr*-2 gene was detected in 4% and 13%, and *mcr*-3 gene carriage was detected in 8% and 7% *P. aeruginosa* and *A. baumannii* isolates, respectively. No *mcr* genes were detected in *Citrobacter*, *S. maltophilia* and *K. aerogens* isolates (Figure 4.7).

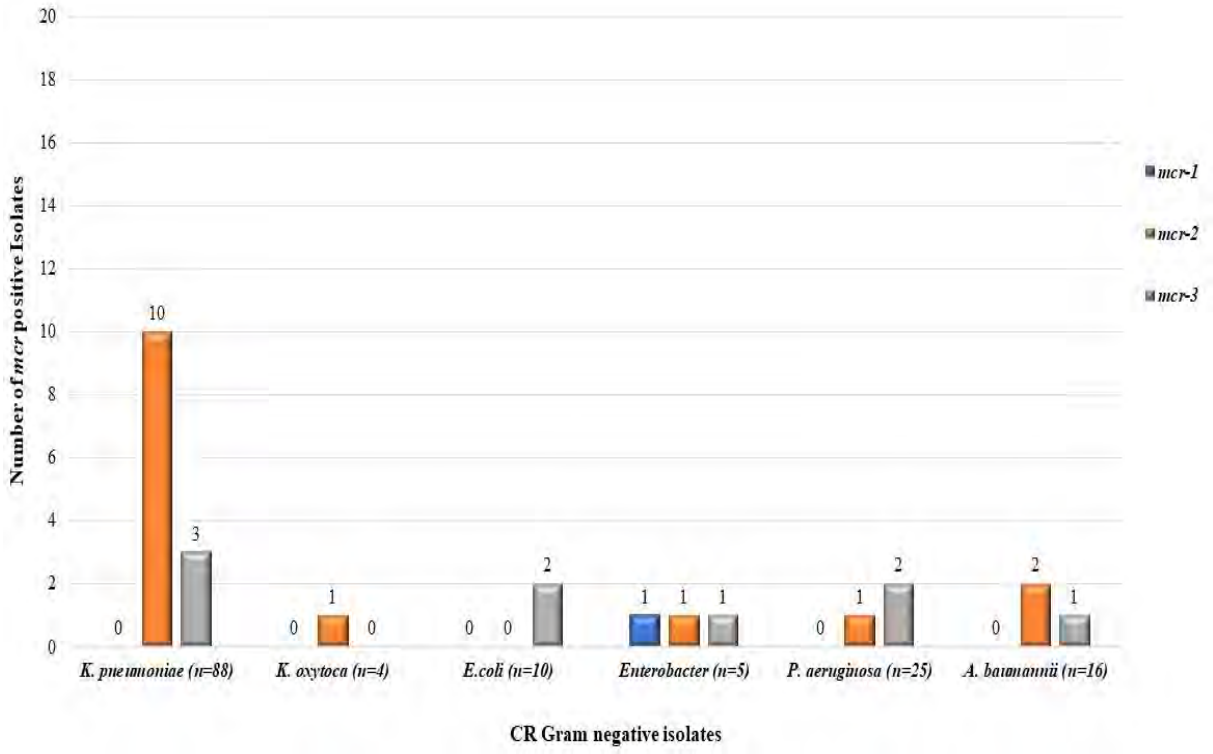

Figure 4.7: Distribution of CR Gram-negative isolates based on the detection of mobile colistin resistance genes (*mcr*-1, *mcr*-2 and *mcr*-3) by using PCR approach.

4.7. WGS of CR Gram-Negative Isolates

From a total 161 CR-isolates, 141 isolates were negative for *mcr* gene carriage. Therefore, to decipher the other possible mechanism of colistin resistance in *mcr* negative CR isolates, 23 isolates were selected for whole genome sequencing based on MIC value against colistin, MDR phenotype, and patient's data (Appendix X). Among *Enterobacteriaceae*, 11 *K. pneumoniae*, 3 *Enterobacter* and 2 *E. coli* genomes were sequenced while in case of *non-Enterobacteriaceae*, whole genome of 7 isolates (6 *A. baumannii* and 1 *S. maltophilia* isolate) were sequenced and characterized. The details of selected *mcr* negative Gram-negative isolates are presented in Appendix XII.

After quality assessment, assembly, and annotation, genomic information of selected CR isolate was extracted. Among *Enterobacteriaceae* members, mean genome size was ±5.5 Mb (minimum=4.5; maximum=6.0) with an average 55.7% GC content. In case of non-*Enterobacteriaceae* members, mean genome size was ±4.1 Mb (minimum=3.9 and maximum=4.5), while the average %GC content was 42.85, all detail regarding number of contigs, coding sequences (CDS) etc. are presented in Appendix XIII.

4.7.1. Detection of Chromosomal Colistin Resistance Gene Mutations

A total 28 non-synonymous mutations were detected in 9 of the chromosomal Lipid A modification genes among CR *K. pneumoniae* isolates. Among Lipid A modification genes, highest number of mutations were detected in *OpgE* (25%) followed by *EptA* (21%), *ArnT* 7.8%) and *ArnD* (14.3%). Two mutations, 53 M>l, 486 T>A accounts for the majority of *OpgE* mutations that were co-carried by more than 90% of the isolates. In case of *EptA*, two sets of mutations which were co-carried (27 C>F, 138 I>V, 319 Q>R and 138 I>V, 257 S>L) were predicted and appear frequently among the isolates. In *ArnT* three mutations were together (114 M>L, 117 V>I, 372 R>K) and were majorly detected mutations. In case of *ArnD* gene, co-carriage of set of three mutations 53 V>I, 94 I>L, and 300 I>V was predicted in four *K. pneumoniae* isolates while 164 S>P mutation was detected in the remaining other four isolates (Figure 4.8).

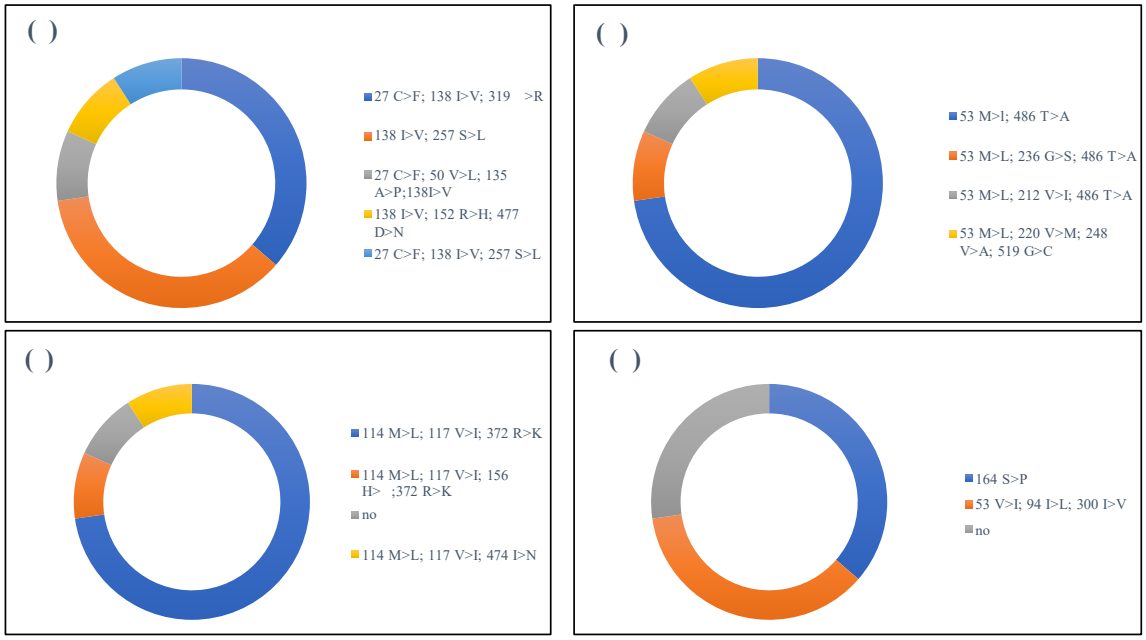

Figure 4.8: Distribution of mutations in 4 Lipid A modification genes (a) *EptA*, (b) *OpgE*, (c) *ArnT*, (d) *ArnD*, that depicted highest nucleotide diversity with majority of mutation were detected.

From total 28 non-synonymous mutations in *K. pneumoniae*, 39% mutations were predicted to be deleterious and were detected in five protein encoding gene which were *EptA, OpgE, PhoQ, MgrB and PmrA* using online bioinformatic tools (Section **3.10.4.)**. Among these deleterious mutations, highest number of mutations were detected in Lipid A modification enzymes, EptA and OpgE. In case of *EptA*, one non-preticted synonymous variation was in cytoplasmic domain (R152H) while the remaining 3 were in sulfatase domain (S257L, 319R, D477N).

In *OpgE*, two deleterious mutations were detected in sulfatase domain of OpgE enzyme (V212I, V220M) while one was detected in its non-cytoplasmic domain. A single deleterious mutation E246D was predicted in HAMP domain of PhoQ Histidine kinase while a deleterious mutation T189P was detected in DNA binding domain of PmrA response regulator. Deletion of 10 amino acids in signal peptide domain was detected in MgrB (Negative response regulator) in two *K. pneumoniae* genomes (Table 4.5).

Table 4.5: Non-synonymous mutations in chromosomal lipid A modification genes with their predicted and annotated impact in CR *K. pneumoniae (n*=11*)*

Genes	**mutation**	**Isolates with mutation *n*=11(%)**	**Annotated Protein**	**Predicted impact**
PhoQ	E246D*	4 (36.4)	HAMP domain	Deleterious
MgrB	△ 1-10	2 (18)	Signal peptide	Deleterious
PmrA	T189P	1 (9)	DNA Binding Domain	Deleterious
PmrB	T246A	7 (64)	Histidine Kinase domain	Neutral
	R256G	5 (45.5)	Histidine Kinase domain	Deleterious
EptA	C27F	6 (54.5)	Transmembrane Domain	Neutral
	I138V	11 (100)	Transmembrane Domain	Neutral
	R152H	1 (9)	Cytoplasmic domain	Deleterious
	S257L	5 (45.5)	Sulfatase domain	Deleterious
	Q319R	4 (36.4)		Deleterious
	D477N	1 (9)		Deleterious
OpgE	M53L	11(100)	Transmembrane Domain	Neutral
	V212I	1 (9)	Sulfatase domain	Deleterious
	V220M	1 (9)		Deleterious
	G236S	1 (9)	Sulfatase domain	Neutral
	V248A	1 (9)		Neutral
	T486A	10 (91)		Neutral
	G519C*	1 (9)	Non-cytoplasmic domain	Deleterious
ArnT/PmrK	M114L	10 (91)	Glycosyl transferase domain	Neutral
	V117I	10 (91)	Glycosyl transferase domain	Neutral
	H156Q	1 (9)		Neutral
	R372K	8 (73)	Transmembrane Domain	Neutral
	I474N*	1 (9)	Transmembrane Domain	Neutral
ArnD	V53I	4 (36.4)	Glycoside hydrolase/deacetylase domain	Neutral
	I94L	4 (36.4)		Neutral
	S164P	4 (36.4)		Neutral
	I300V	4 (36.4)		Neutral
PagP	F170I	8 (73)	Site	Neutral
RamA	D71E*	3 (27)	Homeo like domain	
*indicates novel mutations				

The two CR *E. coli* genomes after WGS, using Blast analysis against reference sequence revealed a total 38 non-synonymous mutations in 11 Lipid A modification genes among CR *E. coli* while other Lipid A modification genes had wildtype genotypes with no mutations (*PhoQ, OpgE, ArnE, PagP and MgrB* gene). From total 38 non-synonymous mutations, only two mutations S29G in PmrA and N232D in ArnT) were common in both sequenced *E. coli* isolates while 15 were detected in isolate exclusively in MML-7 and 21 mutations were predicted only in MML-11229 isolate. Based on prediction, among the non-synonymous mutations highest number of mutations were observed in *ArnT* (26%) followed by *ArnD* (16%), *EptC* (13%), and *PmrD* (13%). Among the detected mutations, 2 mutations C27Y (*EptA*) and K82T (*PmrD)* were predicted to be deleterious having impact on transmembrane helix region and regulatory domain of EptA and PmrD, respectively (Table 4.6).

Table 4.6: Non-synonymous mutations in chromosomal lipid A modification genes with their predicted and annotated impact in CR *E. coli (n*=2*)*

chromosomal determinants	Amino acid variation	Site of mutation in protein	Impact of variation
PhoP	I44L	Signal transduction receiver domain	Neutral
EptA	C27Y	Transmembrane region	Deleterious
	D348G, T413S	Sulfatase domain	Neutral
EptB	I46V	Transmembrane region	Neutral
EptC	V134M, V154I	Transmembrane region	Neutral
	E374D, N419S, N451D	Alkaline phosphatase core domain	
PmrA (BasR)	S29G	Signal transduction receiver domain	Neutral
PmrB (BasS)	A70T	Transmembrane domain	Neutral
	D286G, Y361N	Histidine Kinase domain	
PmrD	N11D, M20K, A27T, V83A	PmrD regulatory domain	Neutral
	K82T		Deleterious
PmrF	T32K	Glycosyltransferase domain	Neutral
PmrK/ArnT	T116A, T197S, A227S, N232D	Glycosyltransferase domain	Neutral
	L248M, D522N	Cytoplasmic domain	
	V261L	Transmembrane region	
	T281A	Non-cytoplasmic domain	
	S322P; P408S	Transmembrane region	
ArnD	R116H, M125L, I126V, K157Q, T227M, E268G	Glycoside hydrolase /deacetylase domain	Neutral
ArnF	M4I, V15A	Signal peptide region	Neutral

In CR- *E. hormaechei,* a total of 21 non-synonymous mutations were detected in 9 Lipid A modification genes, rest of the five genes retained their wildtype genotype. From 21 mutations, 11 mutations were common as were observed in all three *E. hormaechei* isolates which are A31T(*PmrA*), A121T, V134I, T344A (*PmrB*), C90G and S278A (*ArnT*), D31N, S35T, I36L, F59Y and D73N (*ArnD*). The two mutations I128F (*PhoP*) and R423C (*PhoQ*) were detected in two isolates, while 5 mutations (G334S in *PmrB*; S246A in *OpgE*; T94M in *ArnT*; F13V in *PagP*; D27E in *PagP2*) were predicted in single CR- *E. hormaechei* isolate. Based on carriage of mutations, highest mutated gene was *ArnD* (33%) followed by *PmrB* (19), and *ArnT* (14%). Among these non-synonymous mutations, 3 mutations were found to be deleterious and were in I128F (*PhoP*), R423C (*PhoQ*) and T94M (*ArnT*). The annotation of these mutations predicted changes in the functional and regulatory regions of the respective proteins as shown in Table 4.7.

Table 4.7: Non-synonymous mutations in chromosomal lipid A modification genes with their predicted and annotated impact on CR *E. hormaechei (n*=3*)*

Colistin resistant determinants	Amino acid variation	Site of amino acid variation in protein	Impact of variation
PhoP	I128F	PhoB-type DNA binding domain	Deleterious (37%)
PhoQ	R423C	Signal transduction histidine kinase like C terminal domain 2 a.a near to ATP binding site (421)	Deleterious (40%)
PmrA (BasR)	A31T	Response regulator receiver domain	Neutral
PmrB (BasS)	A121T, V134I	HAMP domain	Neutral
	G334S	Sensor protein C-termini signature	
	T344A	HATpase C-terminal domain	
ArnT	C90G,	Glycosyltransferase domain	Neutral
	T94M		Deleterious (40%)
	S278A	Transmembrane helix region	Neutral
ArnD	D31N, S35T, I36L	Glycoside hydrolase/deacetylase	Neutral
	F59Y, D73N, Q258K, D268E	Polysaccharide deacetylase	
OpgE	A121S	Transmembrane helix region	Neutral
	S246A	Sulfatase domain	
PagP-1	F13V	Hydrophobic region of signal peptide	Neutral
PagP-2	D27E	Signal peptide	Neutral

In case of CR *A. baumannii*, a total of 13 non-synonymous mutations were detected in all 5 tested Lipid A modification genes. Among these mutations, 6 amino acid changes were common as were predicted in all the isolates which are F90Y (*LpxF*), Q216K, H218G, S219E, I228V and L329V (*LpsB*) gene, while 3 mutations (Y131H in *LpxA*, C120R; N287D in *LpxC*) as detected in 4/ 5 isolates. Based on mutational diversity, *LpsB* gene was found to be the highly mutated as 8 amino-acid changes were detected followed by *LpxC* and *LpxF*. Apart from the non-synonymous mutations, deletion of 123 amino acids at C-terminal was also predicted in MML-9 due to creation of an early stop codon in *LpxD* sequence. Furthermore, deletion of first 33 amino acids in LpxF present in 4 out of 5 *A. baumannii* isolates. Majority of amino acid deletions and possible protein truncation was detected in MML-16 genome (Table 4.8).

Table 4.8: Non-synonymous mutations in chromosomal lipid A modification genes with their predicted and annotated impact on CR *A. baumannii* isolates (*n*=5)

Amino acid variation in chromosomal determinants		**Site of mutation in protein**	**Impact of variation**
LpxA	Y131H	Hexa-peptide region	Neutral
LpxC	C120R	N-acetylase domain	Neutral
	N287D	Ribosomal D2 type domain	
LpxD	E117K	Hexa-peptide region	Neutral
	Deletion of 123 amino acid	End of Hexa-peptide and all C terminal region	Truncated protein
LpxF	Deletion of 1-33 amino acid	Non-cytoplasmic region	Truncated protein
	F90Y	Halo peroxidase domain	Neutral
LpsB	A166T	Interdomain region	Neutral
	Q216K, H218G, S219E, I228V, L329V, T331I, H334N	Glycosyltransferase-1 domain	Neutral

4.7.2. Detection of Acquisition of Putative PEA and Glycosyltransferase Gene in CR Gram-Negative Genomes

The acquisition of putative PEA and glucosyltransferase genes was identified in CR Gram negative genomes. Six of the putative PEA transferase genes were found to be acquired via phage related integrase mediated insertion in the genome while the mechanism of acquisition of remaining PEA and glycosyl transferase genes was not predicted. Among *Enterobacteriaceae*, carriage of putative transferases was detected in one CR *K. pneumoniae* and two CR *E. coli* genomes. In case of non-*Enterobacteriaceae,* all the *A. baumannii* genomes showed the carriage of putative transferases while a single CR *S. maltophilia* genome was identified to carry the two putative glycosyl transferase genes on the same contig. The details of the identified putative transferases are presented in Table 4.9.

Table 4.9. Predicted acquisition of putative phosphoethanolamine and glycosyl transferase gene in CR Gram-negative isolates

s.#	CR Gram-negative isolates	genes	Putative gene	Contig	Size (bases)	acquisition via
1	*K. pneumoniae* (MML-4771)	2	YhbX	55	243	Phage related integrase
			YjgX	55	432	Not identified
2	*E. coli* (MML-10269)	3	YhbX	7	1626	Not identified
			YjgX	30	291	Phage related integrase
			YjgX	58	852	Not identified
3	*E. coli* (MML11229)	2	YhbX	5	1518	Phage related integrase
			YjgX	14	1500	Not identified
4	*A. baumannii* (MML 19)	2	Putative PEA	7	1650	Not identified
				31	1647	Phage related integrase
5	*A. baumannii* (MML 16)	2	Putative PEA	3	1602	Not identified
				49	1647	Phage related integrase
6	*A. baumannii* (MML9)	1	Putative PEA	15	1602	Not identified
7	*A. baumannii* (MML10)	2	Putative PEA	1	1602	Not identified
				15	1647	Phage related integrase
8	*A. baumannii* (MML11)	1	Putative PEA	9	1650	Not identified
9	*A. baumannii* (MML13)	1	Putative PEA	15	1602	Not identified
10	*S. maltophilia* (MML18)	2	Putative glucosyltransferase	19	1650	Not identified

4.7.3. Whole Genome Based Multi Locus Sequence Typing of CR Isolates

Genomic analysis of 11 CR- *K. pneumoniae* isolates revealed that there 5 STs based on 7 house- keeping genes (*gapA, infB, mdh, pgi, phoE, rpoB, tonB*). After annotation, two STs namely ST-11 (36%) and ST-2096 (36%) were dominant sequence type while for other STs (ST-147, 985 and 21) one isolate each. Capsular determinants play important role in bacterial virulence and resistance, were also predicted in these sequenced isolates. Determinants responsible for capsule synthesis and regulation were harboured by all *K. pneumoniae* isolates. Mucoid phenotype regulatory genes (*RmpA/RmpA2*) were detected in 54.5% of the isolates while the most common capsular polysaccharides (K-serotype) were K12 and K64. Highest percentage (54.5%) of *K. pneumoniae* isolates were predicted to have LPS O1-serotype (Table 4.10).

Table 4.10: MLST typing of CR-*K. pneumoniae* isolates (*n*=11) based on Pasteur and capsular characteristics based on Kaptive tool

Serial number	Isolate ID	MLST	K-loci	Predicted O-type	Detected regulator of mucoid phenotype	Capsule synthesis loci
1	MML-1	ST-11	K-14	03b	No	rcsAB, wzi
2	MML-2	ST-11	K-14	03b	No	rcsAB, wzi
3	MML-3	ST-2096	K-64	01	RmpA/RmpA2 (with frameshift mutation)	rcsAB, wza, wzi
4	MML-4	ST-985	K-39	01	RmpA/RmpA2 (with frameshift mutation)	rcsAB, wzi,
5	MML-5	ST-2096	K-64	01	RmpA/RmpA2 (with frameshift mutation)	rcsAB, wza, wzi,
6	MML-6	ST-11	K-14	03b	Not identified	rcsAB, wzi
7	MML-10718	ST-2096	K-64	01	RmpA/RmpA2 (with frameshift mutation)	rcsAB, wza, wzi
8	MML-4771	ST-147	K-64	02a	Not identified	rcsAB, wza, wzi
9	MML-12	ST-231	K-51	01	RmpA/rmpA2 (with frameshift mutation)	rcsAB, wza, wzi,
10	MML-14	ST-11	K-14	03b	Not identified	rcsAB, wzi
11	MML-17	ST-2096	K-64	01	RmpA/rmpA2 (with frameshift mutation)	rcsAB, wza, wzi,

In case of *E. coli,* Achtman MLST scheme was followed, which was based on 7 house-keeping gene loci (*adk, FumC, gyrB, icd, mdh, purA, recA*). One isolate belonged to ST-448 which is originating from clonal complex ST448 while the other ST was ST-617 belonging to clonal complex ST10. Both *E. coli* isolates depicted the same LPS-serotype that is O-type but different H flagellar serotypes. CH-typing was based on *FumC* (housekeeping gene) and *FimH* (Type-1 fimbrial-adhesin-encoding gene) was also carried out, the isolate MML-7 was predicted to have C6-H35 genotype while isolate MML-11229 C11-H0 genotype (Table 4.11).

Table 4.11: MLST of *E. coli* genomes (*n*=2) based on Achtman Scheme with polysaccharide antigen plus flagellar serotyping using different *in silico* tools

Serial number	Isolate ID	MLST	H-type (*fli*C)	O-type (*wzm* gene)	CH-type	
					Fum	Fim
1	MML-7	ST-448	32	0101	FumC6	H35
2	MML-11229	ST-617	9	0101	FumC11	No

Based on WGS data, all the three CR-*Enterobacter* isolates were confirmed as *Enterobacter hormaechei.* Upon their MLST using 7 house-keeping genes (*dnaA, fusA, gyrB, leuS, pyrG, rplB, rpoB*), 2/3 E. *hormaechei* isolates were predicted to be ST-93 while remaining was ST-2078.

All 6 sequenced genomes of *A. baumannii* were subjected to MLST based on 7 house-keeping genes (*gltA, gyrB, gdhB, recA, cpn60, gpi, rpoD*). Among these, three isolates belonged to ST-1503, while rest of the isolates were ST-2062, ST-1289 and ST-208. MLST typing of *S. maltophilia* based on 7 house-keeping genes (*atpD, gapA, guaA mutM, nuoD, ppsA, recA*) was carried out and it did not match to any existing ST in the databases. This isolate harbored a novel allele 341 at *guaA,* despite other six genes (*atpD* (3)*, gapA* (104)*, nuoD* (112)*, ppsA* (82), *mutM* (3) and *recA* (174) were present (Table 4.12).

Table 4.12: Predicted MLST of CR- *A. baumannii* and *S. maltophilia* isolates using Oxford MLST scheme, and capsular polysaccharide serotyping using online *in silico* tools

Serial number	Isolate ID	Organism	MLST profile	K-loci	OC loci
1	MML-9	*A. baumannii*	ST-1503	KL-12	OCL-1
2	MML-10		ST-2062	KL-235	OCL-1
3	MML-11		ST-1503	KL-12	OCL-3
4	MML-13		ST-1503	KL-12	OCL-3
5	MML-16		ST-1289	-	-
6	MML-19		ST-208	KL-1	OCL-1
7	MML-18	*S. maltophilia*	Novel	-	-

4.7.4. Predication of Antimicrobial Resistant Determinants via Whole Genome Sequencing of CR-Gram-Negative Isolates

A wide array of antimicrobial resistant determinants was detected in the genomes of CR Gram-negative genomes. Both enzymatic and AMR determinants specially efflux pumps were analysed. The details of identified efflux pumps associated with AMR are provided in Appendix XIV.

Among β-lactamase genes, the carriage of *bla*CTX-M-15 and *bla*SHV variants was highest (91%) followed by *bla*TEM (73%) and *bla*CMY (36%). While, in case of carbapenemase encoding genes, *bla*OXA gene variants were predicted in 73% of the *K. pneumoniae* genomes with OXA-1 (55%) and OXA-232 (55%) were being the most common which were co-carried with the β-lactamase gene in 45% of the sequenced genomes. Other antimicrobial determinants predicted were involved in conferring resistance to quinolones (*oqxA, oqxB, qnrS1),* aminoglycosides (*aac(6')-Ib3, aph(3')-VI, rmtC)*, Fosfomycin (*FosA)*, tetracycline (*tet*(A) and others including *mphA, msr(E), dfrA1,dfrA12,dfrA14 and sul1* (Table 4.13).

Table 4.13: Predicted antimicrobial resistant determinants among sequenced CR *K. pneumoniae* genomes using online *in silico* tools

Isolate ID	Beta lactam	Carbapenem	Aminoglycoside	Quinolone	Fosfomycin	Other resistance conferring genes
MML-1	*blaCMY-6, blaSHV-182, blaCTX-M-15*	*blaNDM-1*	*aac(6')-Ib3, aph(3')-VI, rmtC*	*oqxA B, qnrS1*	*FosA*	*Tet(A), mph(A), sul1*
MML-2	*blaCMY-6, blaSHV-182, blaCTX-M-15*	*blaNDM-1,*	*aac(6')-Ib3, aph(3')-VI, rmtC*	*oqxA B, qnrS1*	*FosA*	*Tet(A), mph(A), sul1*
MML-3	*blaSHV-106, blaTEM-1A blaCTX-M-15,*	*blaOXA-1, ,blaOXA-232*	*aac(6')-Ib-cr, aadA2, armA*	*oqxA B*	*FosA*	*Tet(A), mphA, msr(E), dfrA1, dfrA12,dfrA14,sul1*
MML-4	*blaSHV-187, blaTEM-1B blaCTX-M-15,*	*blaOXA-1*	*aac(3)-IIa, aac(6')-Ib-cr, aph(6)-Id*	*oqxA B, qnrB 1*	*FosA*	*tet(A), dfrA14*
MML-5	*blaSHV-106, blaTEM-1A blaCTX-M-15*	*blaOXA-1, blaOXA-232*	*aac(6')-Ib-cr, aadA2, armA,*	*oqxA B*	*FosA*	*tet(D), mphA, msr(E), dfrA1, dfrA12, dfrA14, sul1*
MML-6	*blaCMY-6, blaSHV-182, blaTEM-1D*	*blaNDM-1, blaOXA-232*	*ARR-3, aac(6')-Ib, aadA16, aph(3")-Ib,aph(3')-VI, aph(6)-Id, armA, rmtC*	*oqxA B, qnrB 6,*		*tet(A),tet(B),mph(A), mph(E), msrA,dfrA27,sul1,sul2*
MML-10718	*blaSHV-106, blaTEM-1A, blaCTX-M-15, blaSHV-28*	*blaOXA-232, blaOXA-1*	*aadA2, aac(6')-Ib-cr, armA*	*oqxA B,*	*FosA*	*catB3, mphE, msrE; sul1, tet(D), dfrA1, dfrA12, dfrA14*
MML-4771	*bla-SHV-11, bla-TEM-1, bla-CTX-M-15, blaSHV-67*	*bla-OXA-48*	*aph(3")-Ib, aph(6)-Id, rmtF, aac(6')-Ib-cr, aac(6')-Ib-Hangzhou*	*qnrS1 , oqxA*	*FosA*	*Tet(A), sul1dfrA1*
MML-12	*blaCTX-M-15, blaTEM-1B*	*blaOXA-1, blaOXA-232*	*aadA2, ant(6)-Ia, aph(3')-III, armA, rmtF*	*oqxA*	*FosA*	*tet(B), tet(D), poxtA, mph(E), msrE, erm(B), dfrA1, dfrA12, dfrA14, sul1, optrA, fexA, catA1*
MML-14	*blaCMY-6, blaCTX-M-15, blaSHV-182*	*blaNDM-1*	*aac(6')-Ib, aph(3')-VI, rmtC*	*oqxA B, qnrS1*	*FosA*	*tet(A), mph(A), sul1*
MML-17	*blaSHV-106, blaTEM-1A blaCTX-M-15*	*blaOXA-1, blaOXA-232*	*aac(6')-Ib-cr, aadA2, armA,*	*oqxA B*	*FosA*	*tet(D), mph(E), msr(E), dfrA1, dfrA1, dfrA14, sul1*

Both of the CR *E. coli* sequenced isolates were predicted to carry various antimicrobial resistant determinants predicting a MDR genotype. Both isolates shared common genes associated to β-lactam (*bla*CTX-M-15), carbapenem (*bla*OXA-1) with co-carriage of determinants for aminoglycoside resistance. One of the *E. coli* isolates showed co-carriage of *bla*NDM-1 gene with other carbapenemase encoding genes (Table 4.14).

Table 4.14: Predicted antimicrobial resistance determinants among sequenced CR *E. coli* isolates using online *in silico* tools

Isolate ID	Beta lactam	Carbapenem	Aminoglycoside	Others
MML-7	*blaCTX-M-15, blaTEM-1b*	*blaOXA-10, blaOXA-1, blaNDM-7*	*aac(3)-Iid, aadA1, aadA5, aac(6')-Ib-cr, armA*	*qnrS1, dfrA, sul1, mphA, msrE, catB3, catA1, cmlA1*
MML-11229	*blaCTX-M-15*	*blaOXA-1*	*"aac(3)-IIa, aadA5, aac(6')-Ib-cr*	*--*

A variety of antimicrobial resistant markers were detected in genome of *E. hormaechei* isolates. Co-carriage of β-lactamase encoding gene *bla*ACT variant and Fosfomycin resistant determinant was detected in genomes of all the *E. hormaechei* isolates, while the carriage of carbapenemase encoding gene *bla*NDM variant was identified in two of the *E. hormaechei* belonging to ST-93. Table 4.15 give details of carriage of multiple antimicrobial resistant determinants by CR *E. hormaechei* genomes.

Table 4.15: Predicted antimicrobial resistance determinants among sequenced CR *E. hormaechei* isolates using online *in silico* tools

Isolate ID	β	Carbapenem	Aminoglycoside	Fosfomycin	Others
MML-8	*blaACT-7*	*blaNDM-5*	--	*FosA*	--
MML-8519	*blaACT-7, blaSFO-1, blaTEM-1B, blaLAP-2*	*blaOXA-1, blaNDM-7*	*aac(6')-Ib-cr, aph(3')-Ia, aac(3)-Iid, aadA5;armA*	*FosA*	*catB3, tet(A), aadA5, mphE, msrE, sul1, dfrA1, qnrS1*
MML-15	*blaACT-15*	--	--	*FosA*	--

Co-carriage of multiple antimicrobial resistant genes was predicted among *A. baumannii* genomes. β-lactamase encoding gene *bla*ADC-25, *bla*OXA-23 and *bla*OXA-66 were the most common gene among all isolates in all sequenced genomes. Resistance determinants responsible for aminoglycoside macrolide were detected among the 80% of the isolates too. Apart from ESBL and carbapenemase genes, various other AMR encoding genes were carried by these genomes responsible the MDR genotype (Table 4.16).

Table 4.16: Predicted antimicrobial resistance determinants among selected CR *non-Enterobacteriaceae* members using *in silico* tools

Antimicrobial determinants in *A. baumannii*					
Isolate ID	**Beta lactam**	**Carbapenem**	**Aminoglycoside**	**Macrolide**	**Others**
MML-9	*bla*ADC-25	*blaOXA-23, blaOXA-69*	*aph(3')-VI*	*mph(E), msr(E)*	
MML-10	*blaADC-25*	*blaOXA-23, blaOXA-66*	*aph(3'')-Ib, aph(3')-VIa, aph(6)-Id, armA*	*mph(E), msr(E),*	*sul2,tet(B)*
MML-11	*blaADC-25*	*blaOXA-23, blaOXA-69*	*No*	*mph(E), msr(E)*	
MML-13	*blaADC-25*	*blaOXA-23, blaOXA-69*	*aph(3')-VI*	*mph(E), msr(E)*	
MML-16	*blaADC-25, blaGES-11, blaPER-1*	*blaOXA-114g, blaOXA-23, blaOXA-66*	*aac(3)-Ia, aac(6')-Ib3, aph(3'')-Ib*	No	*dfrA7,sul1,sul2*
MML-19	*blaADC-25, blaTEM-1D*	*blaOXA-23, blaOXA-66*	*aph(3'')-Ib, aph(3')-VI, aph(6)-Id, armA*	*mph(E), msr(E)*	*dfrA27, sul2, tetB*
Antimicrobial determinants in *S. maltophilia*					
MML-18	*blaAmpC*	*blaL1 blaL2*	*aph(3')-IIc*	--	--

4.7.5. Predicted Virulence Determinants via Whole Genome Sequencing of CR-Gram-Negative Isolates

All 11 CR-*K. pneumoniae* isolates were predicted to have hyper-virulent genotypes as these carried multiple virulence factors. These strains had three siderophore systems namely yersiniabactin (*ybt*), enterobactin (*entB)* and salmochelin(*iroN)* while 45.5% of the isolates additionally harboured another system that was aerobactin(*iuc*) with aerobactin ST (AbST)-1. Based on ST profiling of *ybt* loci, *K. pneumoniae* isolates of ST-11 carried *yb*ST-376 while isolates with ST-2096 were predicted to have *yb*ST-14. All isolates had co-carriage of *ybt* loci with integrative and conjugative element *K. pneumoniae* (ICEkp-5). All *K. pneumoniae* were additionally equipped with adhesion and invasion machinery having type-I & type III adhesins, type IV and type-VI secretion system genes (T6SS) etc. as presented in Table 4.17.

Table 4.17: Predicted virulent determinants in CR *K. pneumoniae* genomes (*n*=11) using in online *in silico* tools

Isolate ID	Iron Scavenging systems				Adherence		Invasion
	Yersiniabactin	Aerobactin	Enterobactin	Salmochelin	Type I	Type III	T6SS
MML-1	*irp1,2, ybtAEPQSTUX, fyuA*	*No*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-2	*irp1,2, ybtAEPQSTUX, fyuA*	*No*	*entABCEF, fepABCDG, fes*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-3	*irp1,2, ybtAEPQSTUX, fyuA*	*iucABCD , iutA*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-4	*irp1,2, ybtAEPQSTUX, fyuA*	*No*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-5	*irp1,2, ybtAEPQSTU, fyuA*	*iucABCD , iutA,*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-6	*irp1,2, ybtAEPQSTUX, fyuA*	*No*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-10718	*irp1,2, ybtAEPQSTUX, fyuA*	*iucABCD , iutA*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-4771	*irp1,2, ybtAEPQSTUX, fyuA*	*No*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-12	*irp1,2, ybtAEPQSTUX, fyuA*	*iucABCD , iutA*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-14	*irp1,2, ybtAEPSTU, fyuA*	*No*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*
MML-17	*irp1,2, ybtAEPQSTUX, fyuA*	*iucABCD , iutA*	*entABCEF, fepABCDG, fes, ybdA*	*IroE*	*fimABCD EFGHIK*	*mrkABC DFHIJ*	*Yes*

Determinants of virulence were also predicted in the genomes of CR-*E. coli* isolates. Enterobactin iron scavenging system was predicted in genomes of both isolates while *E. coli* MML-11229 was additionally harbouring *iuc* and *ybt* siderophore systems. Various important adherence and invasion determinants in pathogenesis were also predicted in *E. coli* including curli encoding genes, Type III and type IV secretion system where isolate MML-7 was found to have additionally Type-1 fimbrial genes (Table 4.18).

Table 4.18: Predicted virulent determinants in CR *E. coli* genomes (*n*=2), using online *in silico* tools

Serial Number	**Virulence factors**		**Virulent determinants in CR *E. coli* isolates**	
			MML-7	**MML-11229**
1	**Iron Scavenging systems**	Yersiniabactin	*No*	*irp1,2, ybtAEPQSTUX, fyuA*
		Aerobactin	*No*	*intA, iucABCD*
		Enterobactin	*entABCEF, fepABCDG, fes, ybdA*	*entABCEF, fepABCDG, fes*
2	**Adherence**	Type I Fimbrie	*fimABCDEFGHI*	*No*
		Adhesins	*fdeC*	*afaACD, nfaE, draD, E2, fdeC*
		Curli gene	*csgB*	*csgABCDEFG*
3	**Invasion**	TypeT3SS	*espL1, R1, X1, X4, X5, Y1*	*espL1, R1, X1, X4, X5, Y1*
4	**Serum resistant gene**		*No*	*Css*
5	**Complement resistant gene**		*No*	*traT*
6	**Acriflavine resistance**		*acrB*	*acrAB*

Genomes of all the *E. hormaechei* isolates showed carriage of enterobactin and salmochelin siderophore, catalase peroxidase and flagellar assembly genes. Furthermore, 2/3 isolates were additionally carrying aerobactin siderophore, type 1 fimbri encoding genes and outer-membrane protein. While one of the isolate's genomes showed the carriage of curli encoding genes too (Table 4.19).

Table 4.19: Predicted virulent determinants in CR *E. hormaechei* genomes (*n*=3), using online *in silico* tools

Serial number	**Virulence factors**		**Virulent determinants in CR *E. hormaechei* isolates**		
			MML-8	**MML-8519**	**MML-15**
1	Iron Scavenging systems	Aerobactin	*IutA, iucBC*	--	*IutA, iucBC*
		Enterobactin	*entB, fepCD, ybdA*	*entAES, fepBCDEG*	*entAES, fepBCDEG*
		Salmochelin	*IroBCDEN*	*IroBCDN*	*IroBCDN*
2	Adherence	Type I	*fimCDH*	--	*fimCDH*
3	Catalase peroxidase gene		*katG*	*katG*	*katG*
4	Outer membrane protein		*ompA*	--	*ompA*
5	Curli encoding gene		---	*csgABDEFG*	--
6	Flagellar Assembly gene loci		*present*	*Present*	*Present*
7	Acriflavine resistance		*acrAB*	--	*acrAB*

All the sequenced genomes of *A. baumannii* harboured a wide range of pathogenic determinants which were siderophores, biofilm, pilus, and phospholipase associated genes, depicting highly virulent genotype (Table 4.20). Various virulent determinants were also predicted in CR. *S maltophilia* genome, where heat shock protein-60 (*HtpB*), catalase (*kat*A), isocitrate lyase (*IcI*), Twitching motility protein (*PilGUTR*) and chemotaxis regulatory protein (*cheY*) encoding genes were seen.

Table 4.20: Predicted virulent determinants in CR *A. Baumannii* genome (*n*=6) using online *in silico* tools

Serial number	**Virulence factors**		**Associated genes**	**Presence in *A. baumannii* genome**
1	Iron Scavenging systems	ABC super family	*bauBCDEF*	Carried by All *A baumannii* sequenced genomes
		Acinetobacter biosynthesis gene	*basABCDFGHIJ*	
		Ferric ABC transporter genes	*barAB*	
2	Adherence	CSU pilus	*csuABCDE*	
3	Acetyl-D-glucosamine system genes		*pgaABCD*	
4	Phospholipase		*plc, plcD*	
5	Biofilm controlling two-component system genes		*bfmR, bfmS*	
6	MD cation Efflux pump		*adeF, adeG, adeH*	
7	N-acyl homoserine lactone synthase		*abaI, abaR*	

4.7.6. Predicted Mobile Genetic Elements in CR Gram-Negative Sequenced Genomes:

All the genomes were assessed for the possible acquisition of mobile genetic elements like Insertional sequences (IS), transposons, plasmid replicons, and CRISPR-Cas system. Overall, 157 total IS elements were predicted in *K. pneumoniae* genomes, majority of the IS elements were from IS3 and IS5 family. Carriage of multiple plasmid replicons and CRISPR-Cas regions were predicted in CR *K. pneumoniae* genomes (Table 4.21).

Table 4.21: Predicted IS elements, Plasmid replicon types and CRISPR-Cas regions in CR *K. pneumoniae* genomes using online *in silico* tools

Isolate ID	Number of IS elements	Plasmid replicon types	CRISPR-Cas region (bp)
MML-1	13	IncC, IncFIB(K), IncFIB(pNDM-Mar), IncFII(K), IncHI1B(pNDM-MAR)	IV-A3(819)
MML-2	13	IncC, IncFIB(K), IncFIB(pNDM-Mar), IncFII(K), IncHI1B(pNDM-MAR)	IV-A3 (821)
MML-3	12	ColKP3, ColRNAI, IncFIB(K), IncFIB(pNDM-Mar), ncHI1B(pNDM-MAR)	IE (767*;698) IV-A3 (578*)
MML-4	10	IncFIB(K), IncFIB(pNDM-Mar), IncFII(K)	No
MML-5	12	ColKP3, ColRNAI, IncFIB(K), IncFIB(pNDM-Mar), IncHI1B(pNDM-MAR)	IE (767*;698) IV-A3 (578*)
MML-6	15	ColKP3, ColRNAI, IncC, IncFIB(K), IncFII(K), IncN	No
MML-10718	17	ColKP3, ColRNAI, IncFIB(K), IncFIB(pNDM-Mar), IncHI1B(pNDM-MAR)	IE (767*;698) IV-A3 (578*)
MML-4771	12	Col440I, ColRNAI, IncFIB(K)(pCAV1099-114), IncL, IncR	No
MML-12	24	Col440I, Col440I, ColKP3, ColRNAI, IncFIA, IncFIB(AP001918), IncFIB(K), IncFIB(pNDM-Mar), IncFIB(pQil), IncFII(K), IncFII(pAMA1167-NDM-5), IncFII(pRSB107), IncHI1B(pNDM-MAR)	IV-A3 (199; 698)
MML-14	17	IncC, IncFIB(K), IncFIB(pNDM-Mar), IncFII(K), IncHI1B(pNDM-MAR)	IV-A3 (819)
MML-17	12	ColKP3, ColRNAI, IncFIB(K), IncFIB(pNDM-Mar), IncHI1B(pNDM-MAR)	IE (767*;698) IV-A3 (578*)
*C4X9J1_KLEPN CRISPR-associated Cas3 family helicase			

A total of 17 confirmed CRISPR-Cas regions were predicted in genomes of 73% of the *K. pneumoniae* isolates' genomic sequences. From the predicted 17 CRISPR regions, 8 showed 99% similarity with C4X9J1_KLEPN CRISPR-associated Cas3 family helicase while no significant blast hits were found in local Cas database against the remaining 9 regions (Figure 4.9).

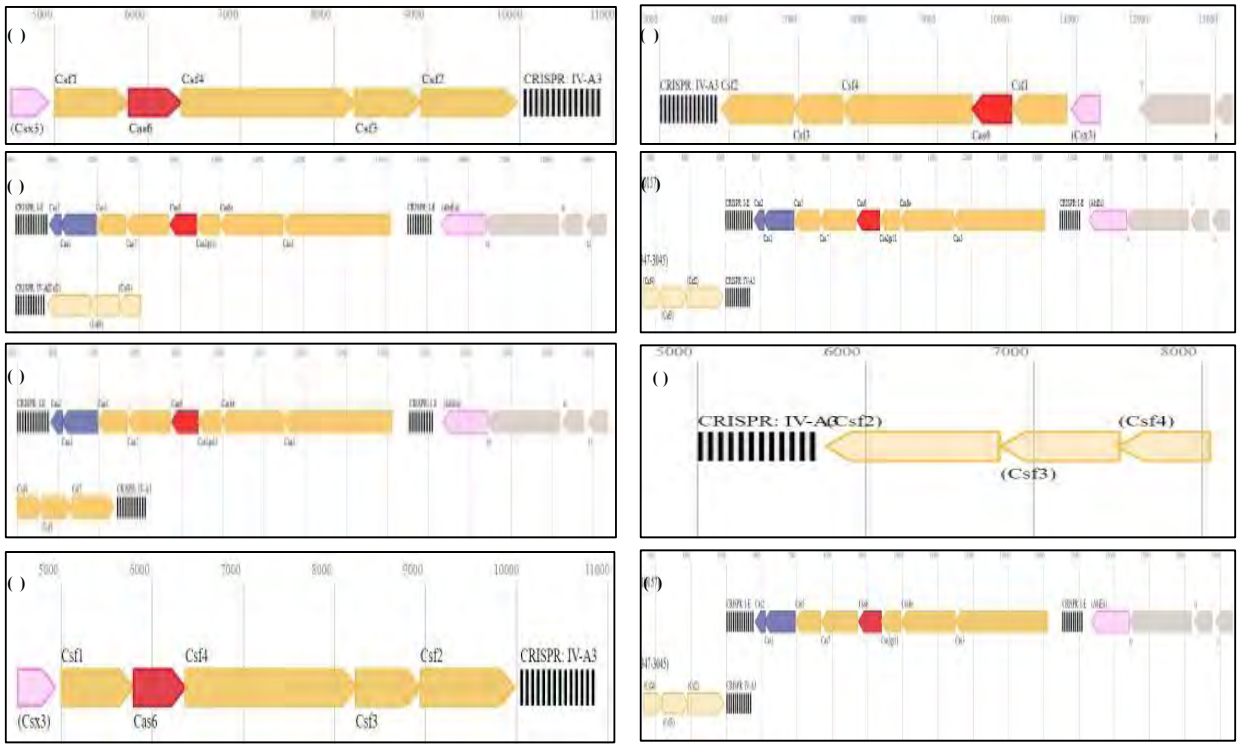

Figure 4.9: Predicted CRISPR-Cas encoding genes among CR *K. pneumoniae* genomes. Part (a) represents the CRISPR-Cas gene array in MML-1 (b) MML-2 (c) MML-3 (d) MML-5 (e)MML-10718 (f) MML-12 (g)MML-14 (h) MML-17

The carriage of conjugative elements was detected in 10/11 CR-*K. pneumoniae* genomes. The genomic environment of these conjugative elements depicted presence of multiple AMR and virulent determinants along with secretion systems (Figure 4.10 to 4.19)

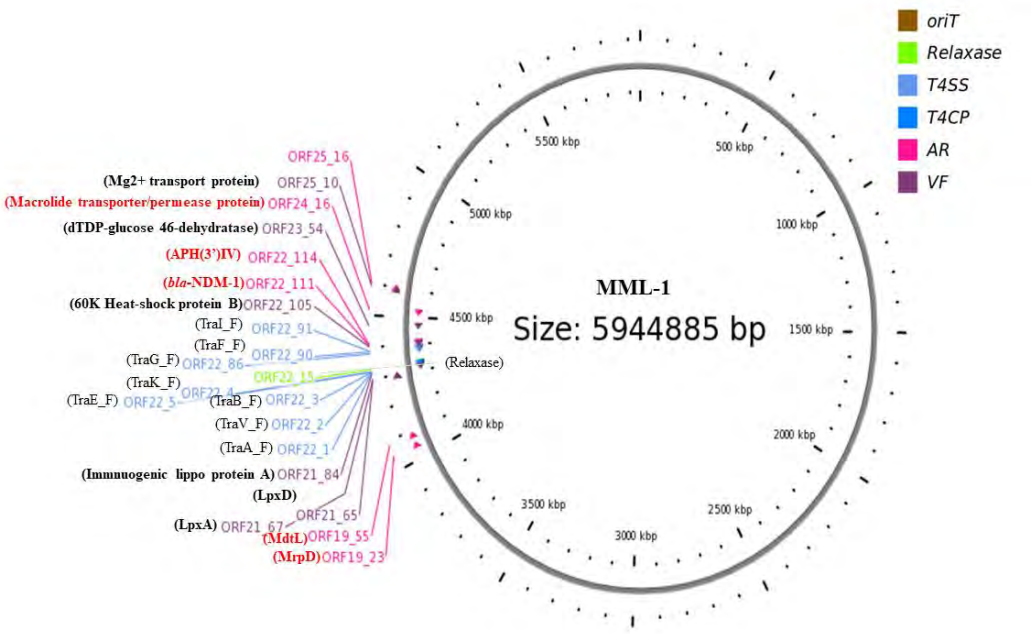

Figure 4.10: Genomic environment associated with OriT sites and potential carriage of conjugative element in CR *K. pneumoniae* (MML-1) genome. Red colour indicates the carriage of AMR determinants while black bold colour description indicates virulence genes.

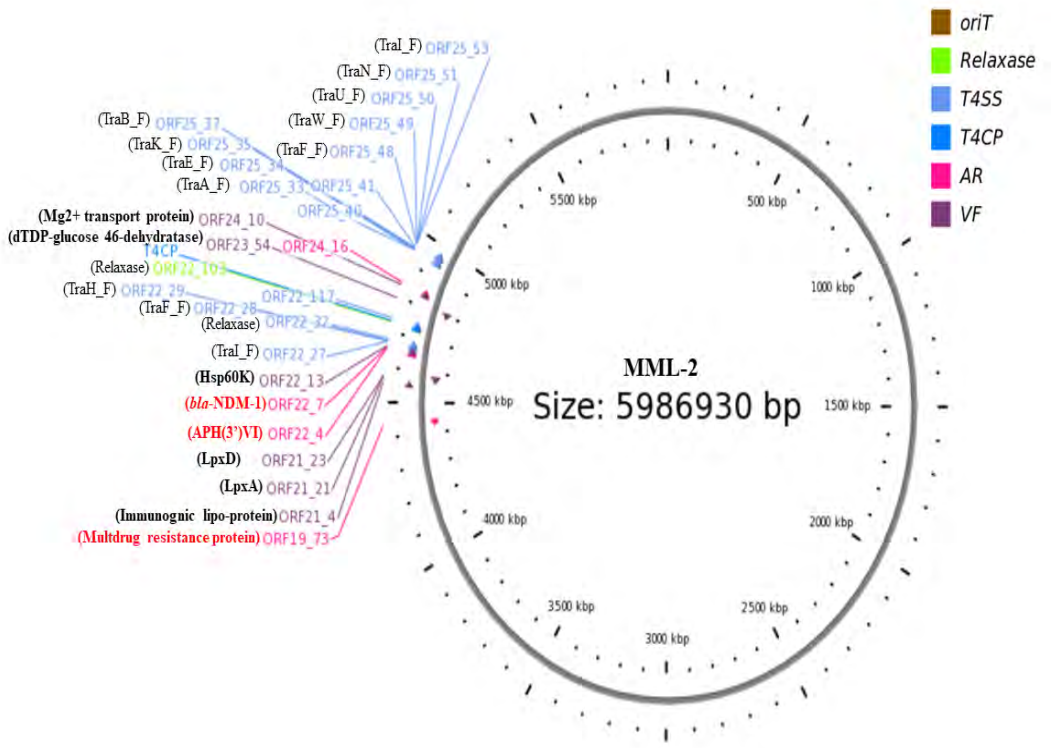

Figure 4.11: Carriage of NDM-1, other AMR and virulent associated genes on a conjugative element in CR *K. pneumoniae* (MML-2) genome.

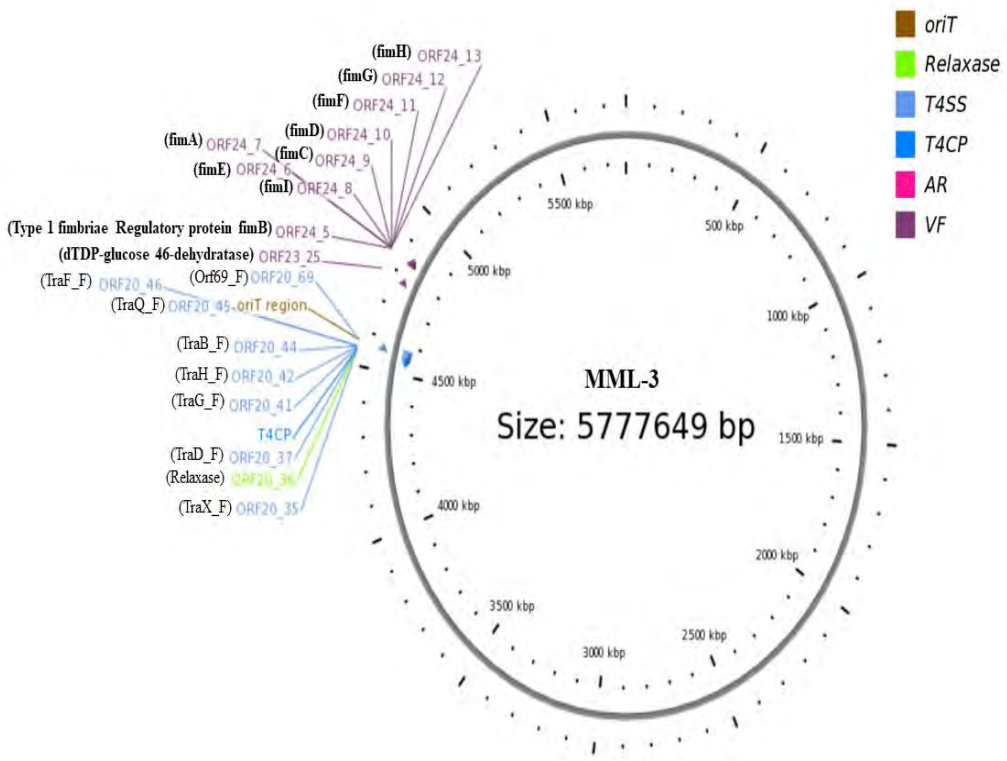

Figure 4.12: Carriage of conjugative element by CR *K. pneumoniae* (MML-3) genome. Black colour indicates carriage of type1 Fimbrie genes.

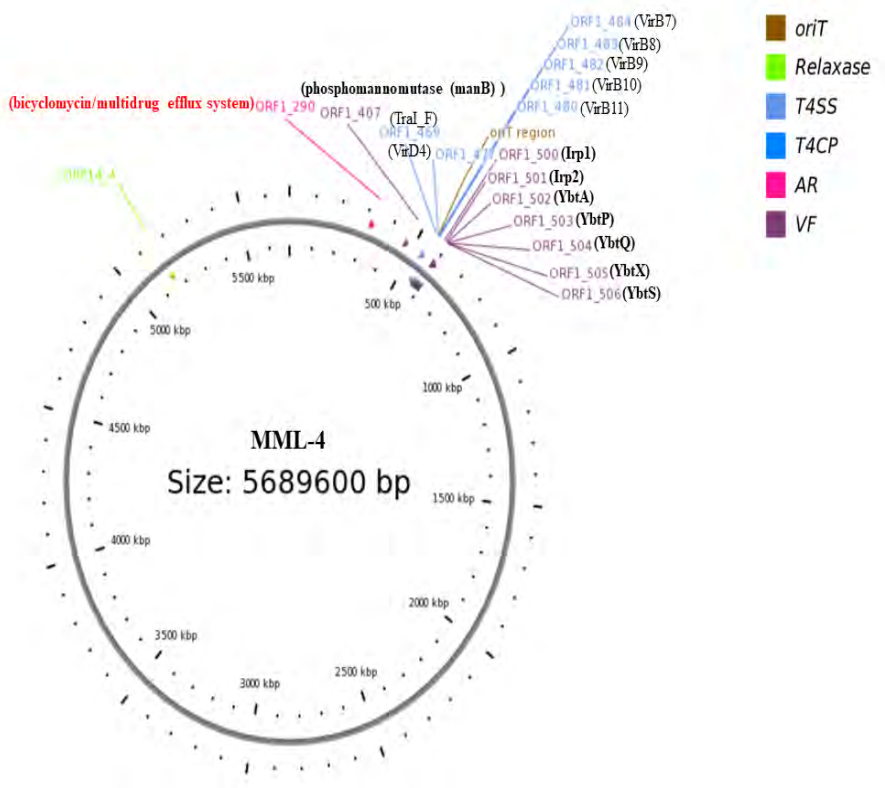

Figure 4.13: Acquisition of yersiniabactin siderophore and MDR efflux system gene associated with conjugative element in CR *K. pneumoniae* (MML-4) genome. Black bold colour indicates yersiniabactin genes while red colour indicates MDR efflux gene.

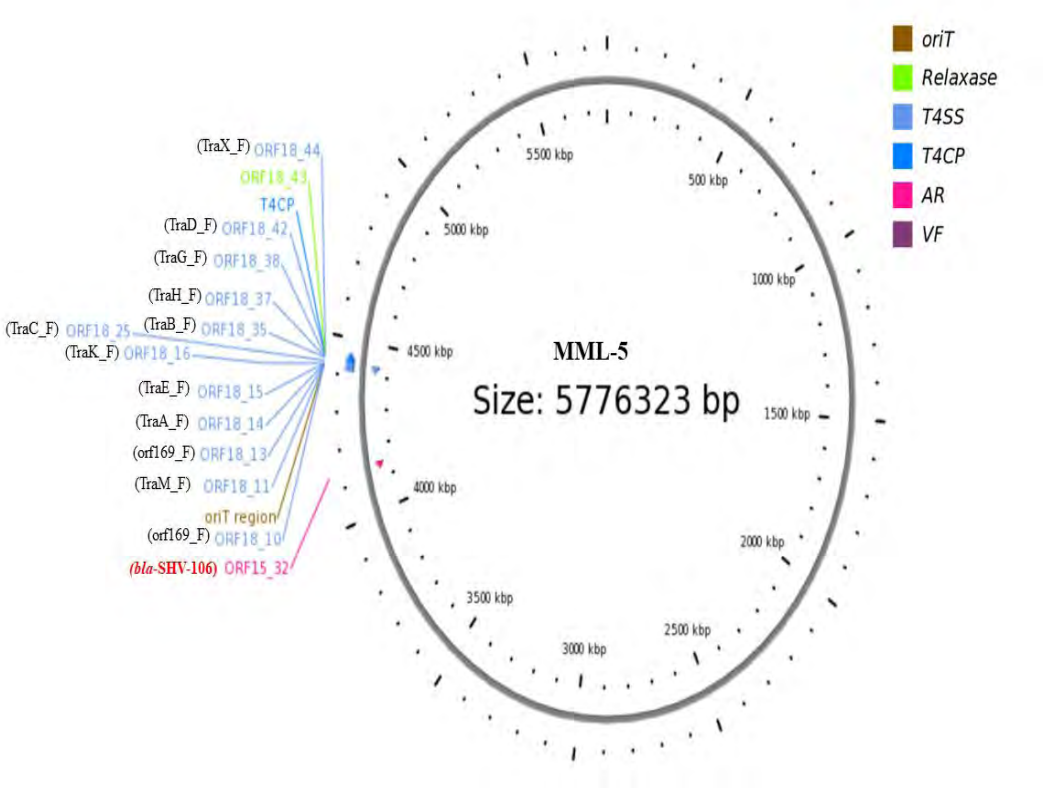

Figure 4.14: Potential carriage of conjugative elements in CR *K. pneumoniae* (MML-5) genome. Red colour indicates presence of *bla*SHV-106 while black colour indicates the presence of T4SS determinants associated with the conjugative element.

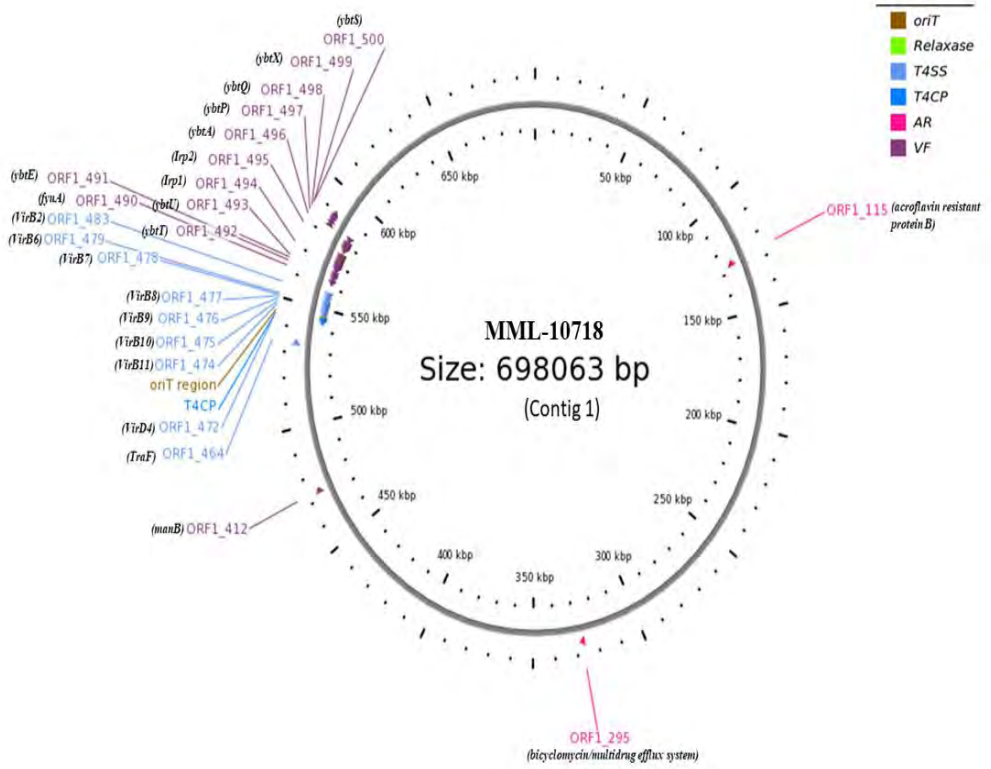

Figure 4.15: Acquisition of yersiniabactin siderophore by CR *K. pneumoniae* genome (MML-10718) via conjugative element.

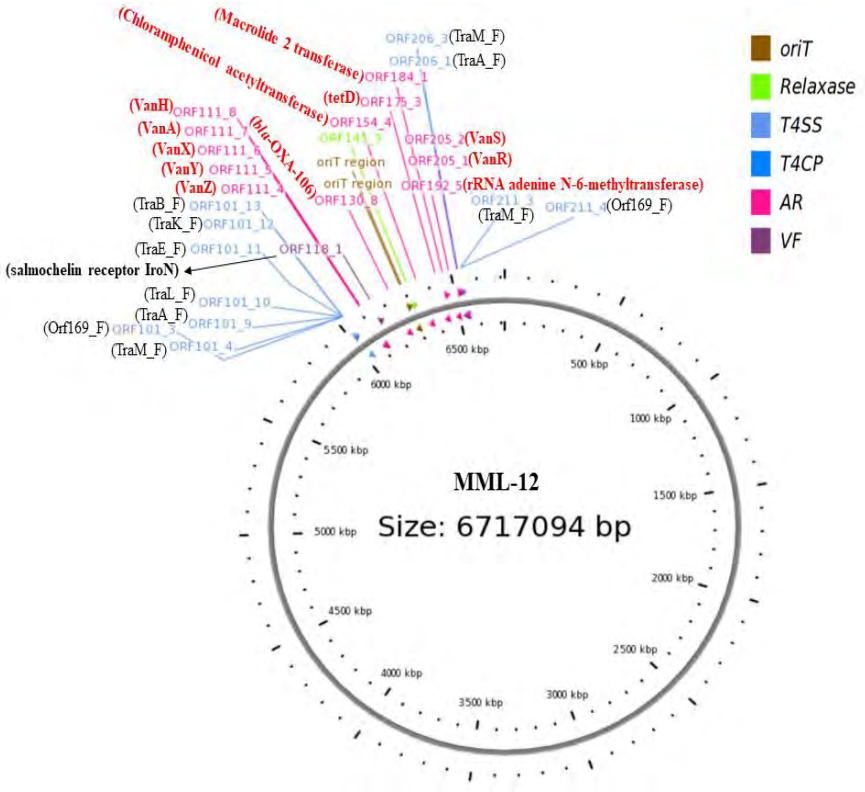

Figure 4.16: The carriage of *bla*OXA-106, vancomycin resistant gene cluster, Tetracycline resistance gene along with multiple other AMR genes associated with conjugative elements in CR *K. pneumoniae* (MML-12), indicated with bold red letters. The presence of enterobactin siderophores is indicated by black letters.

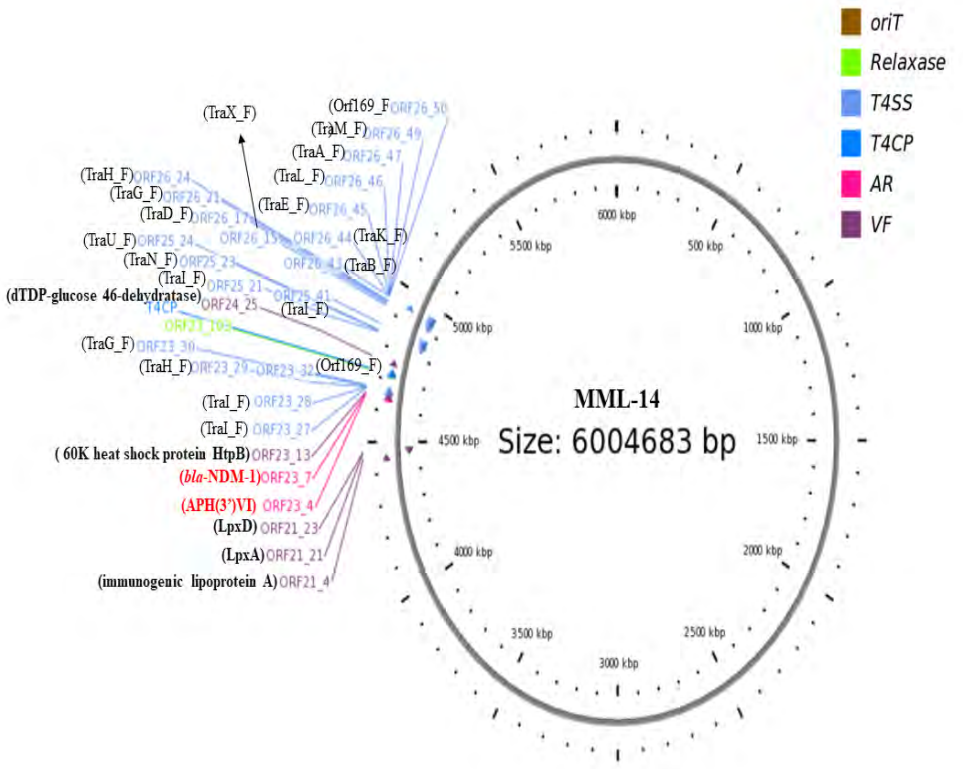

Figure 4.17: Potential acquisition of *blaNDM-1* together with multiple AMR and virulent determinants associated with the conjugative element in CR *K. pneumoniae* (MML-14) genome.

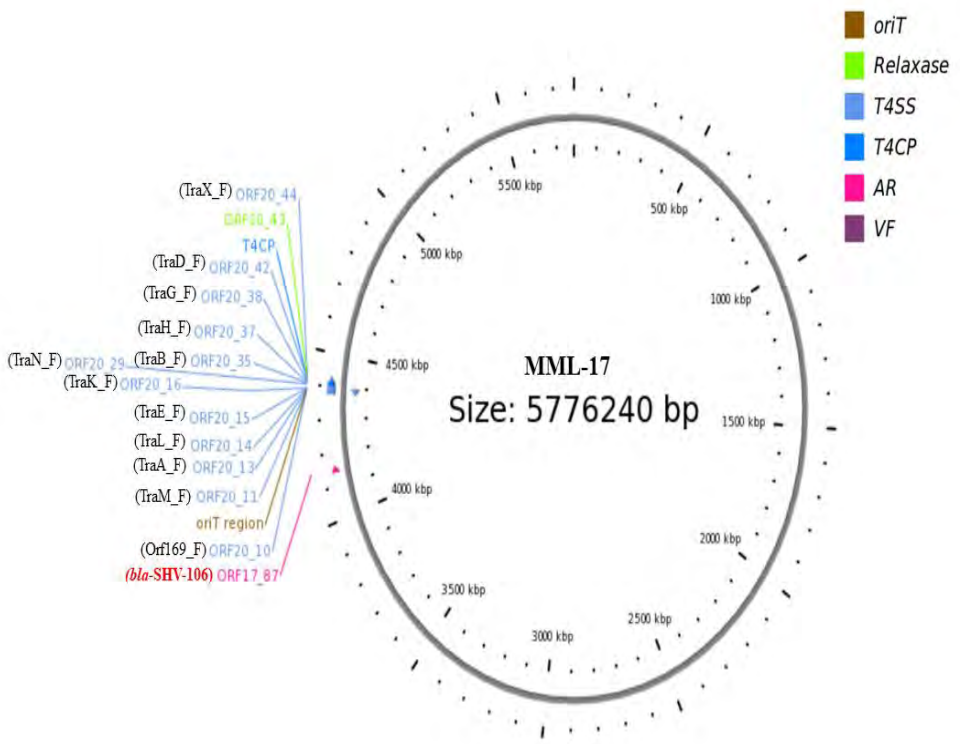

Figure 4.18: The carriage of *bla*SHV-106 and T4SS genes on a conjugative element in CR *K. pneumoniae* (MML-17) genome.

In the sequenced genomes of CR-*E. coli* isolates, predicted acquisition of mobile genomic elements were majorly 38 IS elements while the most common IS family was IS3 and IS5. In sequenced isolate MML-7, a total 9 plasmid replicon types were predicted and isolate MML11229 showed significant alignment with 6 plasmid replicon types. Both the *E. coli* genomes had acquired conjugative plasmid carrying array of antimicrobial and virulent determinants (Figure 4.20-4.21).

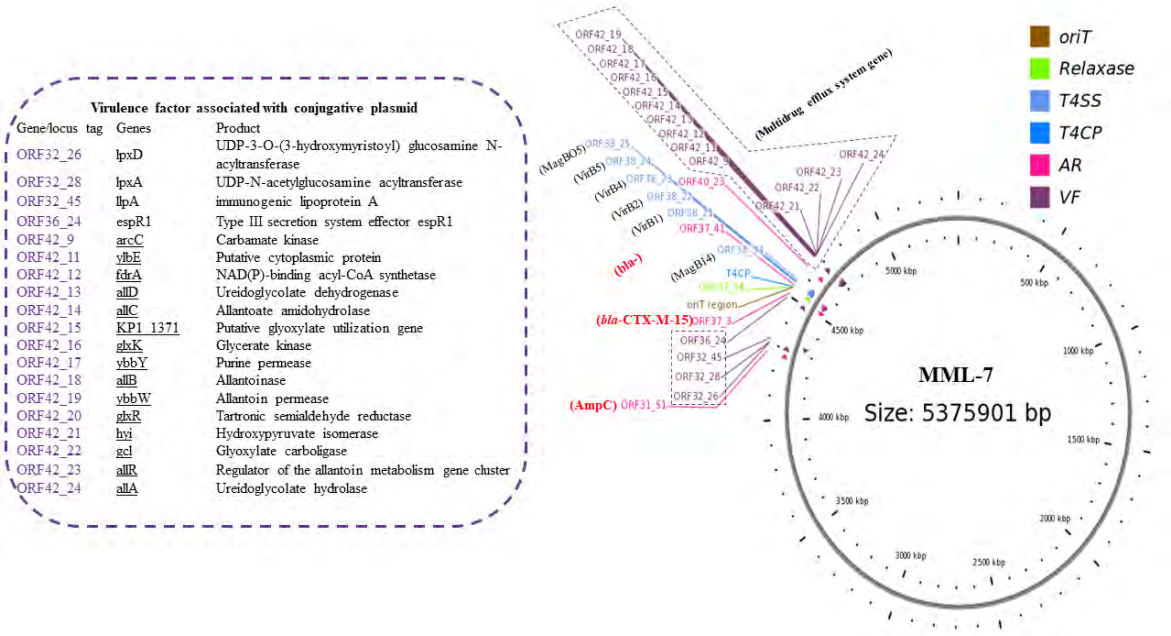

Figure 4.19: Predicted conjugative element and its associated AMR and virulent determinants in CR *E. coli* genome (MML-7).

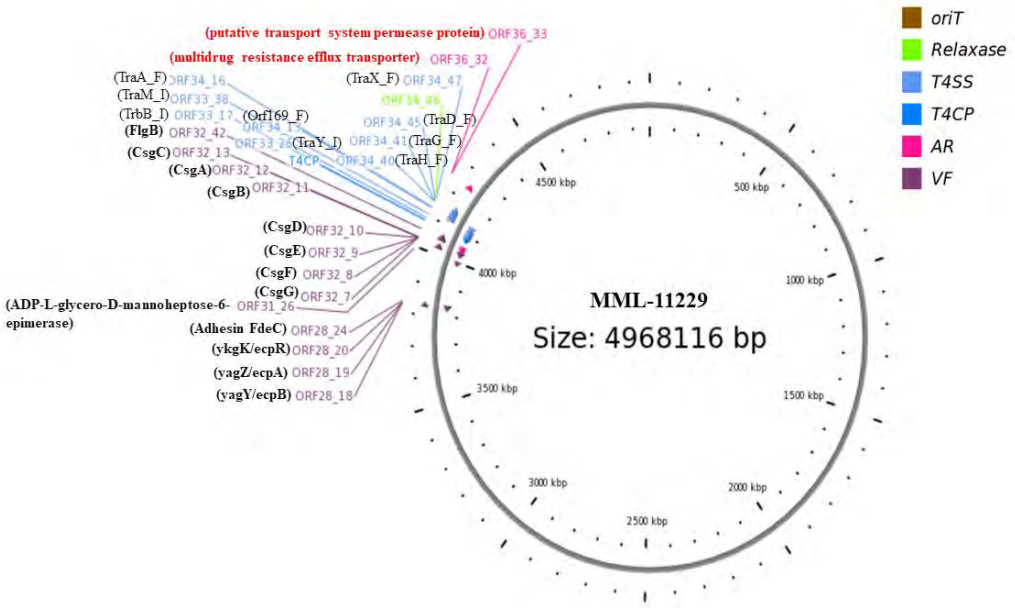

Figure 4.20: The carriage of various AMR and virulent determinants associated with conjugative element in CR *E. coli* genome (MML-11229).

Four CRISPR-Cas regions were also detected in genome of *E. coli* isolate (MML-7), two of CRISPR-Cas regions showed 94% sequence similarity with CRISPR-associated helicase Cas3 (B1LQ84_ECOSM) while the other two showed 61% sequence similarity with A4W8Q3_ENT38 CRISPR-associated helicase Cas3 (Table 4.22).

Table 4.22: Predicted IS elements, Plasmid replicon, CRISPR-Cas regions among sequenced CR *E. coli* isolates using online *in silico* tools

Isolate ID	**Number of IS elements**	**Plasmid replicon types**	**Confirmed CRISPR-Cas region (bp)**
MML-7	17	ColpVC, IncFIA, IncFIB(AP001918), IncFIB(H89-PhagePlasmid), IncFII(pAMA1167-NDM-5), IncHI1A(NDM-CIT), IncHI1B(pNDM-CIT), IncI(Gamma), IncX3	IF (577*, 701*, 807^) IE (1227^)
MML-11229	21	IncFIA, IncFIB(AP001918), IncFII, IncI1-I(Alpha)	None
*B1LQ84_ECOSM CRISPR-associated helicase Cas3 tax=*Enterobacteriaceae* ^A4W8Q3_ENT38 CRISPR-associated helicase Cas3 family tax=*Enterobacteriaceae*			

Based on acquisition of mobile genetic elements in *E. hormaechei*, 2 plasmid replicon types (IncFII (Yp); IncR) showed ≥99%% sequence similarity against MML8 genome, while three plasmid replicons (Col440I; IncR; IncX3) share ≥99% sequence similarity with MML-8159 genome. A total of 5 IS elements were detected in MML-8 while 7 IS elements were predicted in MML-8159 genome. The most common IS family in case of MML-8 was IS3 while in case of MML8159 majority of the IS elements belonged to IS4 family. While no plasmid replicon or IS element was predicted in MML-15 genome (Table 4.23).

Table 4.23: Predicted MLST, Plasmid replicon, IS elements, and CRISPR-Cas among sequenced CR *E. hormaechei* genomes using online *in silico* tools

Isolate ID	MLST profile	Plasmid replicon types	Number of IS elements	CRISPR-Cas region
MML-8	ST-93	IncFII(Yp), IncR	5	No
MML-8519	ST-93	Col440I, IncR, IncX3	7	No
MML-15	ST-2078	no	0	No

The carriage of conjugative element was also predicted in one of the *E. hormaechei* genome (MML-8519) as represented in Figure 4.21

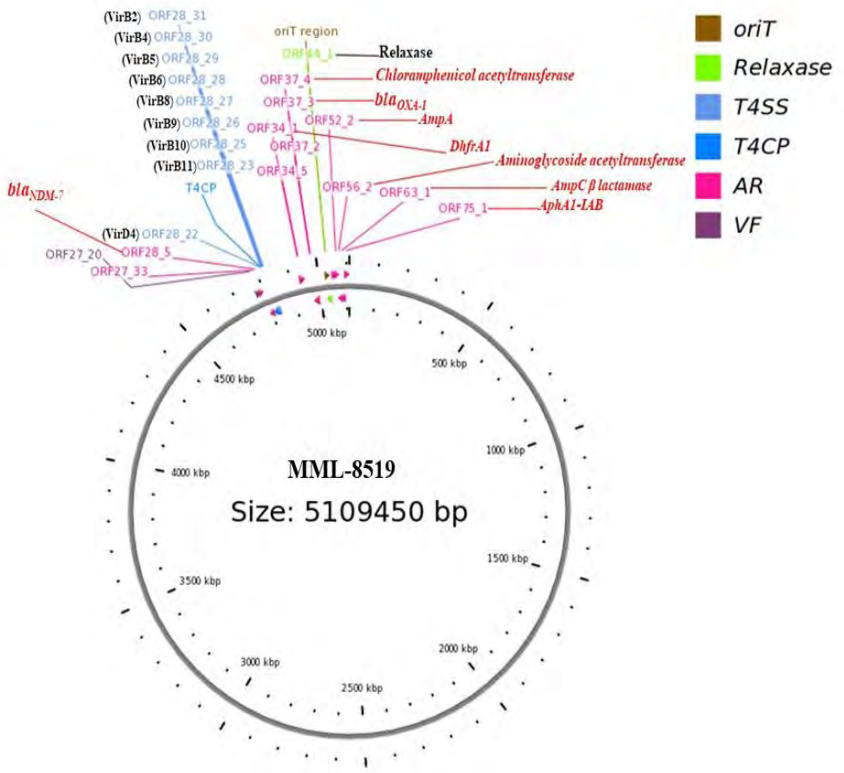

Figure 4.21: Carriage of conjugative element and its associated determinants in CR *E. hormaechei* (MML-8519) genome.

In case of *A. baumannii,* a total 22 total IS elements were predicted in the genomes, three IS families IS5, IS256 and IS39 were dominant. The three *A. baumannii* isolates were predicted to carry same CRISPR-Cas regions which showed 100% sequence similarity with B7GYY5_ACIB3 CRISPR-associated helicase Cas3 based on local cas database blastX results. Based on CRISPR-Cas typing results, three detected regions were typed CRISPR-Cas 3149 and are presented in Figure 4.22

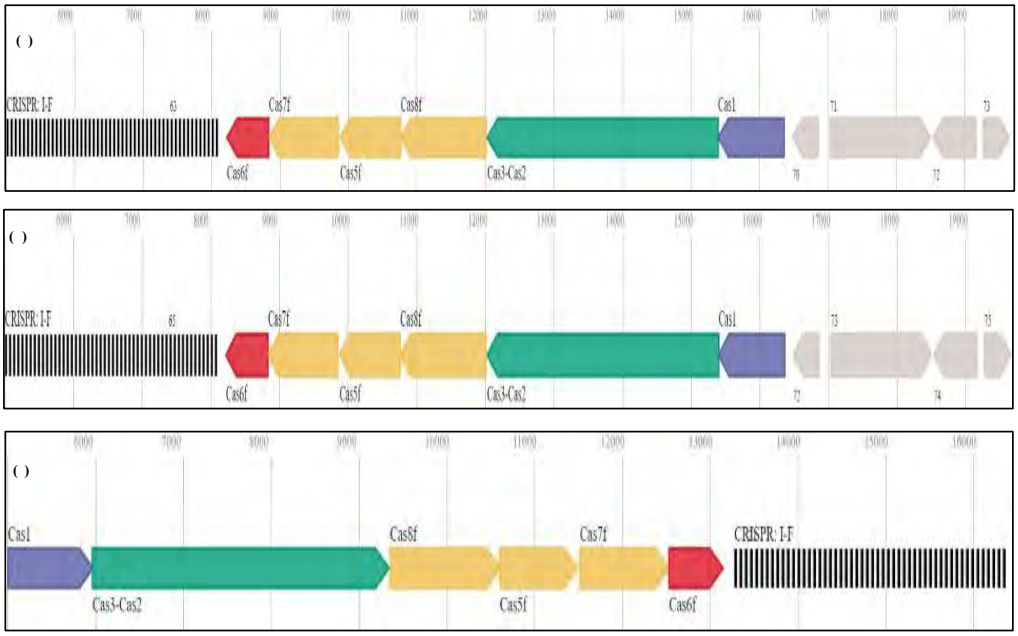

Figure 4.22: The CRISPR-Cas encoding genes in CR *A. baumannii* isolates (a) MML-9 (b) MML-11 and (c) MML-13.

Apart from IS elements and CRISPR-Cas regions, no plasmid replicon type or conjugative elements were detected in *A. baumannii* genome except in MML-19, which showed the carriage of ColKP3 plasmid replicon on contig 128. In case of *S. maltophilia* genome, two IS elements were detected, both were from IS481 family while a single composite transposon (Cn28075_ISStma1) was also predicted in the genome. No CRISPR-Cas associated region was detected in *S. maltophilia* genome. However, acquisition of T4SS and various virulent plus antimicrobial encoding genes were predicted in *S. maltophilia* genome (Figure 4.24).

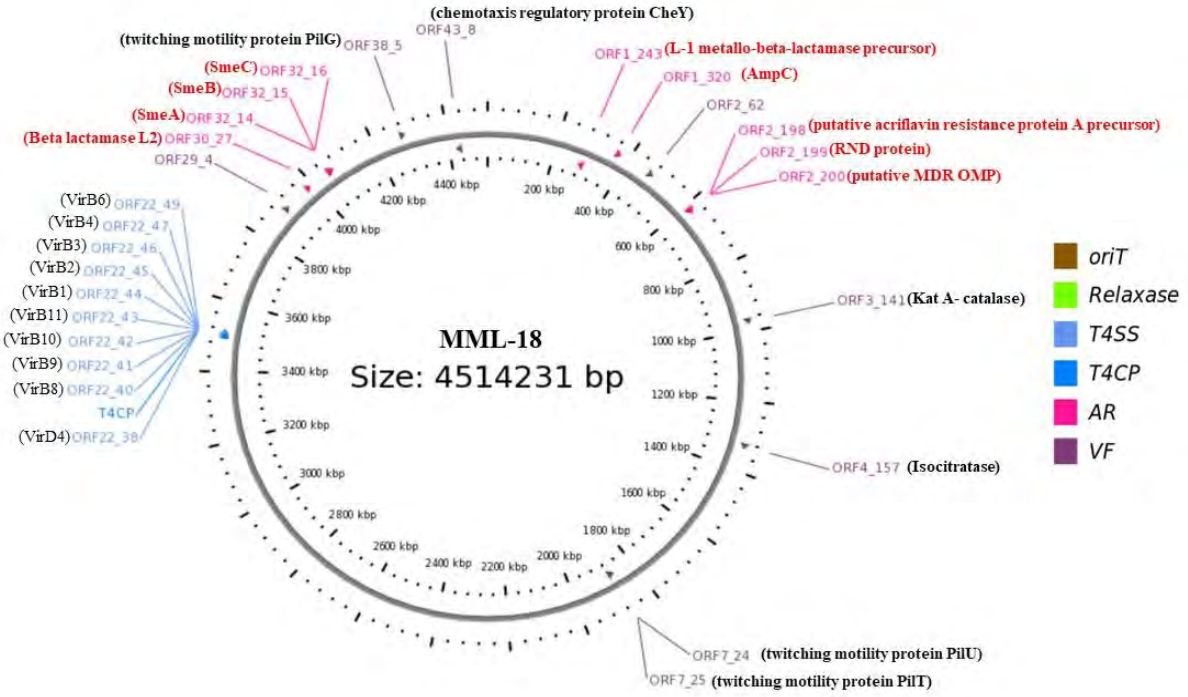

Figure 4.23: Carriage of multiple AMR and virulence determinants by CR *S. maltophilia* genome.

4.8. Generation of YjiJ gene Knock-out *E.coli* for anti-infective Therapy

For Gene knock-out, *YjiJ* gene was targeted by using single step PCR approach followed by transformation and homology-based recombination using ʎ red recombinase system. The 50bp overhang containing Cam^R gene region was amplified using single-step PCR (Figure 4.24). The Optimised thermocycler conditions used for the amplification are presented in Appendix VII.

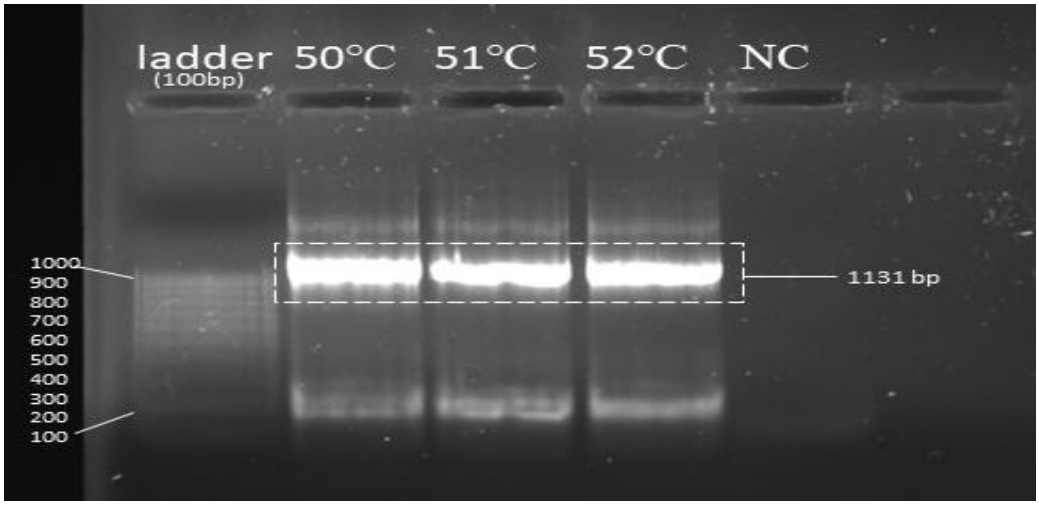

Figure 4.24: Represents the amplified PCR product (1131bp) on 1% agarose gel electrophoresis using 50bp overhang primers using pKD3 as a template. Lane 1 indicates 100bp ladder (ThermoFisher), Lane 2-3 indicates amplified product at 50℃, 51℃, 52℃ while Lane 4 indicates negative control.

The electrocompetent UTI89 cells were successfully transformed using pKD46 plasmid (1µL, 2µL and 3 µL). The electroporation of UTI89 cells with 1µL resulted in ≥50 transformants colonies on Amp LB agar plates while about ≥20 colonies were obtained when electroporation was done using 2µL and 3µL of pKD46 plasmid (Figure 4.25).

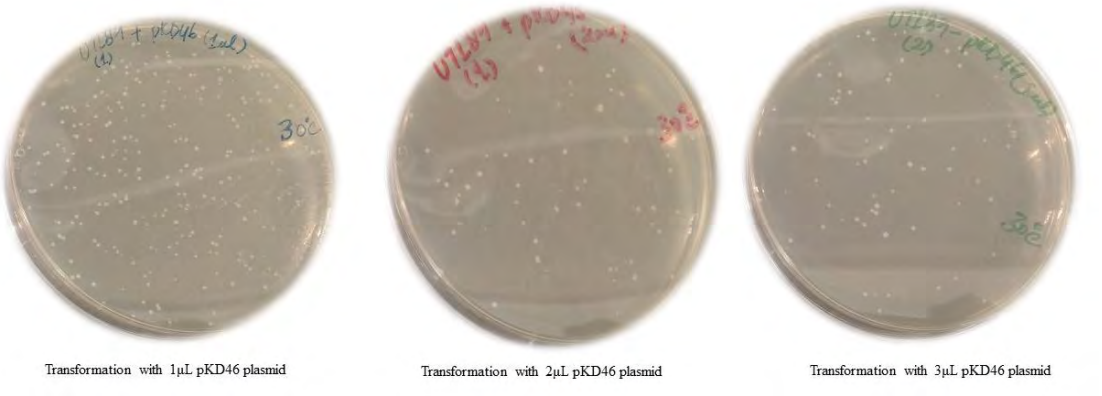

Figure 4.25: Growth of UTI89pKD46 transformants on Amp LB plates at 30°C after electroporation with 1µL, 2 µL, and 3 µL of pKD46 extracted plasmid.

The UTI89pKD46 transformants were again transformed with purified amplified products, which only gave single transformant colony on Cam LB agar plates at 37℃. Extracted DNA from these transformant cells was confirmed by using PCR. A 519 bp band was observed in the transformants on 1% agarose gel electrophoresis instead of 1131 bp. As the transformants were selected on Cam containing LB plates so resistance was confirmed by amplification of Cam^R gene with two internal primers. Transformants were carrying Cam^R genes as presented in Figure 4.26 (a).

The curing for the pKD46 plasmid carriage in transformants was done at 42℃ and later growth at Amp containing media, which also confirmed by PCR based detection of γ recombinase enzyme gene (Figure 4.26 (b). The colony developed on the Cam containing media was checked for *YjiJ* gene deletion and both mutant and wild type strains were assessed for presence of *YjiJ* gene (Figure 4.26 (c). The amplification of both Cam^R marker gene and *YjiJ* gene in single transformed bacterial colony indicated that this gene might be a multicopy genes.

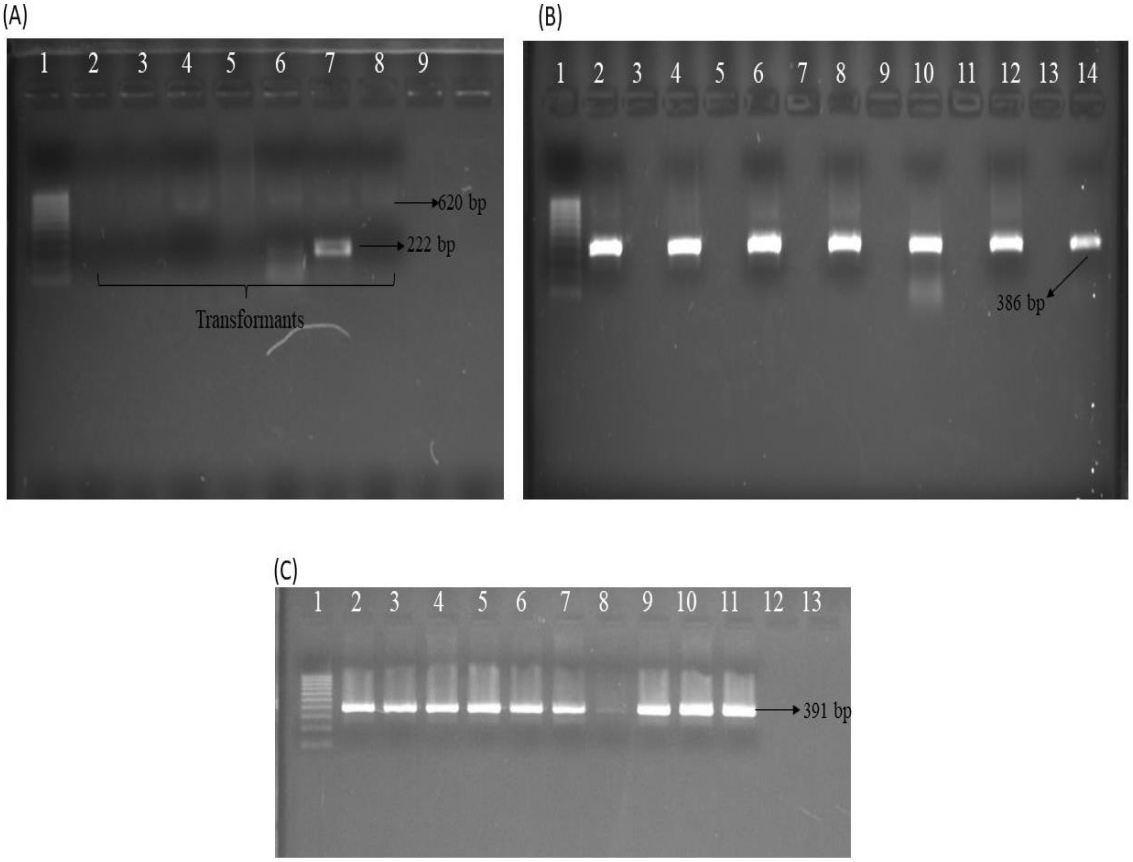

Figure 4.26: Amplified products of Cam^R, γ recombinase gene and *YjiJ* gene in deletion mutants on 1% agarose gel electrophoresis (a) Amplified Cam^R products (620 bp and 222bp), Lane 1 indicates 100 bp Ladder, 2 to 9 for *YjiJ* deletion mutant, 10 for UTI89 as negative control. (b) Amplified γ recombinase gene, lane 1 for 100 bp ladder, 2,4,6,8,10,12,14 for transformant grown at 37°C, lane 3, 5,7,9,11,13 for pKD46 plasmid cured transformant. (c) *YjiJ* gene amplified products (391 bp), lane 1 indicates 100 bp ladder, 2-7 transformant and 8-11 UTI89 wildtype as positive control, 12 and 13 for pKD3 and pKD46 as negative control.

Antimicrobial resistance being a global health concern, considered to be catastrophe for developing countries (Karakonstantis *et al.,* 2020) with failing economies (Sulis *et al.,* 2022). Bacterial pathogens are becoming more and more resilient by adopting strategies to survive the antibiotic stressed environment. The genomic evolution is a ongoing process either due to horizontal transmission of genetic elements or by vertical transmission of genomic traits among the bacterial species, leading to the emergence of bacterial pathogens resistant to majority of the available antimicrobials. With the scarcity of available antimicrobials against Gram-negatives for the treatment of resistant Gram-negative bacterial pathogens, older agents like polymyxins were re-introduced as the priority antimicrobial agents for the treatment of carbapenem resistant Gram-negatives. Soon after its re-introduction, resistance against last-resort antibiotic 'colistin' started to emerged (Meletis *et al.,* 2011). This emergence of resistance to this last line drug was also due to its excessive use in clinical and veterinary settings, which caused selective pressure on MDR pathogens especially in low middle income countries where antimicrobial stewardship is not stringent (de Carvalho *et al.,* 2022).

In this study, an overall 31% phenotypic colistin resistance was observed in Gram-negatives. The resistance level detected in the isolated strains is similar to high colistin resistance (32%) reported from Tamil Nadu, India by Manohar *et al.* (2017). A study on Gram-negatives from ocular infection cases from India, also observed 42% CR prevalence (Mitra *et al.,* 2020). A higher colistin resistance was also recorded in a hospital-based study from Greece where CR rate was also 42% among Gram-negatives (Karvouniaris *et al.,* 2022). An Egyptian study by Ajlan *et al.,* (2022) reported 27.7% colistin resistance among CPR Gram-negatives clinical isolates. On contrary to current findings, lower colistin resistance rates among clinical Gram-negatives were reported from Thailand which was 6.4% (Santimaleeworagun *et al.,* 2020), and <6% from Nepal. A multihospital study from Tunisia, found lower colistin resistance (5.02%) among Gram-negatives (Ferjani *et al.,* 2022). The rate of colistin resistance in this study is much higher as compared to recent studies from Pakistan on clinical isolates where colistin resistance was 15.9% in Faisalabad (Qamar *et al.,* 2017), 10.4% Rawalpindi (Furqan *et al.,* 2022b) and 22.8% in Peshawar (Arif *et al.,*

2022). The difference in prevalence rate between current and cited literature might be due to the difference in study design and sample size as all these studies were focused on carbapenem resistant *Enterobacteriaceae.* These studies from Pakistan, indicate that colistin resistance is emerging in our region., might be due to excessive use of colistin as monotherapy or in combination, incorrect drug administration protocols, inability to conduct tests for antibiotic resistance before giving medications to patients, insufficient staff education, and a lack of hospital drug resistance prevention strategies.

In this era of escalating antimicrobial resistance throughout the globe, an attempt to contain the drug resistance which is emerging at a faster rate is an issue (Ramaloko & Sekyere, 2022). The increasing rates of colistin resistance is reported from members of *Enterobacteriaceae* (ECDC 2020; Homeier-Bachmann *et al.* 2021) and non-fermenter Gram-negatives. However, the resistance rates vary among these Gram-negative bacterial isolates (Li *et al.,* 2019b) (Khuntayaporn *et al.,* 2022). In current study, highest colistin resistance (55%) was detected among *K. pneumoniae* isolates. These results are supported by studies from various countries where high resistance rate against last-line drug is reported from *K. pneumoniae* including Philippines 50% (Turnidge *et al.,* 2007), Turkey 75.6% (Sari *et al.,* 2017), Thailand 67.1% (Eiamphungporn *et al.,* 2018), Iran 75% (Aris *et al.,* 2020), Russia 45% (Shamina *et al.,* 2020), Jordan 49.7% (Gharaibeh *et al.,* 2022) and Egypt 40.4% (Elaskary and Badawy, 2023). The same trend was also seen in various recent studies from Pakistan, where a higher colistin resistance was observed in carbapenem resistant *K. pneumoniae* isolates (Qamar *et al.,* 2017; Imtiaz *et al.,* 2021; Syed *et al.,* 2022).

Resistance against colistin was 6% in *E. coli* isolates in the current study. A study by Zafer *et al.* (2019) from Egypt also detected a 4% colistin resistance in *E. coli* isolates recovered from cancer patients. A study by Santimaleeworagun *et al.* (2020) reported 2.9% CR-*E. coli*. However, colistin resistance among *E. coli* isolates in recent study by Syed *et al.*(2022) from Pakistan was higher as 11.6%. and 19.9% in study by Arif *et al.* (2022).

A higher colistin resistance rates were also observed in non-fermenters Gram-negative isolates in the current study, where phenotypically resistance was 43% in *P. aeruginosa* and

16% in *A. baumannii*. A study from India (2022) reported comparable colistin resistance rates in *P. aeruginosa* (33.3%) and *A. baumannii* (20.37%)(Chauhan *et al.,* 2022). Another study in 2018 from India, reported higher colistin resistance rate among CR *P. aeruginosa* from UTI patients (Jain, 2018). However, in contrast to finding of this research work in SENTRY antimicrobial report on CR non-fermenters published in 2019, found a much lower CR rates in these pathogens as 1% in *P. aeruginosa* and 3% in *A. baumannii* (Diekema *et al.* 2019). In a Canadian report, a comparatively lower resistance rates where were 5% in *P. aeruginosa* and 2.5% in *A. baumannii* were recorded (Zhanel *et al*., 2019). A recent work on non-fermenter from Pakistan had lower rate of colistin resistance in *P. aeruginosa* (5.4%) and *A. baumannii* (4.3%)(Syed *et al.,* 2022). A multiple hospitals study was done by Ahsan *et al.* (2022), in which recorded an overall 3% colistin resistance among *A. baumannii* . A study on *P. aeruginosa* by Arif *et al.* (2022) conducted in Khyber Medical College(Peshawar), 1.3% colistin resistance was reported.

Overall, there is variation in colistin resistance prevalence rates not only in different parts of Pakistan but also on other geographical regions. The change in drug resistance is also affected by patients' general health, immune status, sample types, implementation of antibiotic stewardship programs, emperical antibiotic therapy and environmental factors. The emergence of resistance to colistin might be associated either with its frequent use in food producing animals as feed additive or prophylactic therapy. Such application of antibiotics might be responsible for the selection and transmission of resistant strains to human directly or via food chain eventually causing difficult to treat infections in humans. In Pakistan, there is extensive use of colistin in the poultry farming the inexpensive source of meat used in the country by masses might be an indirect source of resistance transmission. Another explanation for the emergence of colistin resistant pathogens in clinical settings of Pakistan might be due to the over the counter sell and use of these last-line drugs like other LMIC (Marshall & Levy, 2011; de Carvalho *et al.,* 2022; Mustafa *et al.,* 2022).

Emergence of colistin resistance in MDR bacterial pathogens is another major issue in the treatment of resistant infection, these are often interconnected, due to their ability to resist

multiple classes of antibiotics these classified as XDR (Magiorakos *et al.,* 2012). In this study, MDR phenotype was displayed by 84% of CR Gram-negative isolates, these findings are concurrent with a recent study by Ara *et al.* (2021) from Bangladesh in which 85% of CR Gram-negative isolates displayed MDR phenotype. A Chinese hospital-based study, also showed higher percentage (95.2%) of MDR phenotype in CR Gram-negatives (Xu *et al.,* 2022b).

The most prevalent CR *K. pneumoniae* isolates were also having 95%MDR profile in current study. Highest resistance (≥90%) was detected against cephalosporins, β-lactam combination agents, quinolones, and monobactam classes of drugs. Moreover, >80% isolates were resistant to carbapenem. This is line with research work from Egypt where a higher resistance rates in CR *K. pneumoniae* against the above mentioned antibiotics (Zafer *et al.,* 2019). High resistance rates against these drugs were also reported in uropathogenic CR *K. pneumoniae* from Pakistan (Arif *et al.,* 2022). Another recent study from Egypt by Elaskary & Badawy, (2023) also reported high drug resistance pattern in carbapenem and colistin resistant *K. pneumoniae* isolates. In present study, the susceptibility rate of amikacin was 21% in CR *K. pneumoniae,* these findings are consistent with a previous study by Arjun *et al.* (2017) from India, where similar amikacin susceptibility rate (29.17%) among CR *K. pneumoniae* isolated from tertiary care hospital was reported. Amikacin sensitivity was also detected 28% in CR *K. pneumoniae* from Taiwan (Yang *et al.,* 2020).

All the CR *E. coli* isolates in current study, displayed MDR phenotype as were found resistant to cefoperazone/sulbactam, cefotaxime, cefepime and aztreonam. Similar high resistance rates to the tested antibiotics have also been observed among CR *E.coli* isolates from China (Wang *et al.,* 2017) and Taiwan (Wang *et al.,* 2020a). A Egyptian study on hospitalized cancer patients, indicated a higher resistance rate to various tested antibiotic in CR *E. coli* (El-Mahallawy *et al.,* 2022). A hospital-based study from Pakistan on UTI patient also indicated high AMR among CR *E. coli* from Pakistan (Arif *et al.,* 2022).

Among predominant CR non-fermenter study isolates, antimicrobial susceptibility testing found a higher resistance against various tested antimicrobials. As 73% *P. aeruginosa* and

53% *A. baumannii* isolates displayed MDR phenotype. Higher resistance against various antibiotic were also observed in *A. baumannii* in a Malaysian study, where all identified CR A. *baumannii* isolates were found to MDR (Lean *et al.,* 2014). A cross-sectional hospital-based study from India, also recorded high AMR against tested antibiotics among *P. aeruginosa* and *A. baumannii* isolates (Biswas *et al.,* 2022). A hospital-based study from Pakistan also recorded high resistance against various tested antimicrobial among CR *A. baumannii* isolates depicting MDR phenotype (Ahsan *et al.,* 2022).

Although phenotypic resistance detection methods are considered as gold standard, in-expensive and are well suited for clinical diagnosis. However, they are unable to provide the underlying mechanism and dissemination potential of resistance among bacterial species (Jerke *et al.,* 2016). To prevent the spread of colistin resistance, genotypic approaches would be beneficial as they are fast, efficient and have a much higher accuracy compared to phenotypic approaches. Moreover, these approaches have great potential to provide better understanding of underlying mechanism of resistance (Ghandour *et al.,* 2021). The mechanism of colistin resistance among Gram-negatives is not completely elucidated yet. However, one of the most known and frequent mechanism used is addition of the cationic groups, LAra4N and/or PEA to the lipid A by these resistant bacteria. This is achieved either via acquisition of *mcr* genes on plasmid or genetic variation like mutations in various genes (Sharma *et al.,* 2022a).

For detection of plasmid mediated colistin resistance, PCR based approach was used for genotypic identification of *mcr* genes carriage among Gram-negatives (Cavaco *et al.,* 2016; Al-Kadmy *et al.,* 2020; D'Onofrio *et al.,* 2020; Ai *et al.,* 2021; Ajlan *et al.,* 2022) In current study, carriage of *mcr* genes were observed to be 12% among all of tested CR Gram-negatives. These results are in accordance with a report from Bangladesh, where carriage of plasmid mediated CR genes (*mcr*-1 and *mcr*-2) was 11.4% among CR Gram-negatives but were from UTI patients only (Ara *et al.,* 2021). The *mcr*-1 gene is considered as one of the easily disseminating gene and have been detected in much higher rate in *E. coli* especially in food producing animals but it is less prevalent in clinical samples (Giani *et al.,*

2018; Mmatli *et al.,* 2022) However in this study, a low detection of *mcr*-1 gene in CR MDR Gram-negative isolate (<1%) was seen. Concurrent to this research findings, a hospital based study from China also had only one *mcr*-1 gene positive isolate (Li *et al.,* 2018). In 2022, a Chinese study recorded higher prevalence rate of 3.9% *mcr*-1 gene in MDR CR *E. coli* isolated from clinical samples (Xie *et al.,* 2022). The low prevalence in this work could be due to different types of samples or due to less carriage of *mcr* gene by local strains.

Only one *Enterobacter* isolate was detected positive for *mcr*-1 gene which was recovered from urine of a male patient, this finding is concurrent with a study from China in which *mcr*-1 gene in CR *E. cloacae* was detected in male patient's urine (Zeng *et al.,* 2016). There is a trend for reduction in *mcr*-1 gene carriage in different Gram negatives seen in these recent studies, point towards the fact that the overexpression of *mcr*-1 gene can cause decrease in bacterial fitness, slow growth and altered plasma membrane structural integrity (Yang *et al.,* 2021; Feng *et al.,* 2022).

In current findings, *mcr*-2 gene carriage was detected in 11% CR *K. pneumoniae* (10/88), 4% *P. aeruginosa* (1/26) and 12.5% *A. baumannii* (2/16). These findings are in line with the study by Imtiaz *et al.* (2021) in which *mcr*-2 gene carriage was 9% in *K. pneumoniae* uropathogenic isolates. A study by Mitra *et al.* (2020) reported the carriage of *mcr*-2 gene in one *P. aeruginosa* recovered from ocular infection. A Turkish study on carriage of *mcr*-2 gene also found one *A. baumannii* from intensive care unit (Duman *et al.,* 2022). All these studies are documenting low carriage of *mcr*-2.

In current study *mcr*-3 gene carriage was detected as 8% in *P. aeruginosa,* 7% *A. baumannii,* 3.4% CR *K. pneumoniae* and 1% in CR *E. coli*. A study by Hadjadj *et al.* (2019) reported *mcr*-3 gene carriage in *K. pneumoniae* isolated from stool samples of healthy individuals from Laos, contrary to this work. A study from Denmark, detected *mcr*-3 gene in a clinical *E. coli* isolated from patient with blood stream infection (Roer *et al*., 2017). The carriage of *mcr*-3 gene among *P. aeroginosa* isolates was reported from India too. In contrast to these findings, up till now carriage of *mcr*-3 genes in *E. coli, K. pneumoniae, P. aeruginosa* and *A. baumannii* is not reported from Pakistan, hence it is first report of its

carriage from our country. This might be due to the fact that all studies there is different dissemination of *mcr* variants globally (Hameed *et al.,* 2019; Hameed *et al.,*2020; Li *et al.,* 2021). A much higher rate of *mcr* 2 and *mcr*-3 genes detection in *A. baumannii* Iraqi strains was reported (Al-Kadmy *et al.,* 2020; Hafudh *et al.,* 2020). To the best of our knowledge, there is no study on *mcr*-2 and *mcr*-3 detection from our country reported in the published data.

In current study, a higher phenotypic resistance among Gram-negatives and lower prevalence of *mcr* genes among these resistant isolates, revealed a gap and need to decipher other possible causes of such a high resistance among these isolates. Xiaomin *et al.,* (2020), has also suggested the low prevalence of *mcr* gene in clinical samples but high phenotypic colistin resistance, also pointing towards exitance of other mechanisms. Although colistin resistance mechanism is complicated and has not yet elucidated comprehensively so to understand these complex interactions, genome based high throughput sequencing approaches are beneficial and necessary.

In current study, genome of 23 *mcr* negative CR isolates, which includes 11 *K. pneumoniae*, 2 *E. coli* and 3 *E. hormaechei* isolates while 7 were from *non-Enterobacteriaceae* members which includes 6 *A. baumannii* and 1 *S. maltophilia* were sequenced and characterize using WGS approach. Various other studies have also used WGS to study possible genetic alterations associated with colistin resistant among CR pathogens. A Tunisian hospital based study used WGS to understand the molecular basis of colistin resistance among 13 MDR *K. pneumoniae* (Jaidane *et al.,* 2018). A retrospective study from Italian hospital, characterized CR *K. pneumoniae* nosocomial isolates using WGS, and revealed novel genetic alterations in two component regulatory genes associated with lipid A modification (Gentile *et al.,* 2020). An Egyptian study by Fam *et al.,* (2020), on 17 CR *A. baumannii,* also identify various genomic alteration associated with colistin resistance via genome sequencing. A Korean hospital based study also conducted WGS based characterization of 27 CR *A. baumannii* isolates to examine genomic alteration in various potential genes (Hahm *et al.,* 2022). All these WGS studies from various countries have found different mutations,

mobile and genetic elements, showing requirement of WGS from our country to understand mechanism of CR. These can further provide considerable information regarding the source of ARGs and their dynamics for initial step in active AMR surveillance (Irfan *et al.,* 2022).

The main findings of the WGS of the local CR strain unfolded many genetic alterations. Such genomic alterations particularly in genes encoding two component regulatory systems, such as *PhoPQ* and *PmrAB/BasRS*, were found which are associated with colistin resistance in MDR Gram-negatives lacking plasmid-encoded *mcr* genes (Pragasam *et al.,* 2017) (Mathur *et al.,* 2018a). These identified regulatory genes play crucial role in Lipid A modification by regulating the expression of chromosomally encoded PEA transferases which includes *EptA* and the *Arn* operon. Therefore, any mutation in regulatory gene or its associated gene can be cause of constitutive expression of *EptA* and the *Arn* operon, ultimately modifying LPS (Humphrey *et al.,* 2021; Janssen & van Schaik, 2021).

In present the study, a higher number of non-synonymous mutations in 16 sequenced genomes of *Enterobacteriaceae* members (87/100) were detected compared to 7 non-fermenters genomes (13/100), showing more evolution of *Enterobacteriaceae* under colistin pressure. Among all the fermenters (11 *K. pneumoniae*, 2 *E. coli* and 3 *E. hormaechei*) the common genes were *Arn* operon encoding genes (*ArnT & ArnD*), followed by two component encoding genes (*PmrAB*). Such genomic alterations has been reported where over expression of Arn-operon among CR *Enterobacter* (Kang *et al.,* 2019) *E. coli* (Gallardo *et al.,* 2021), and *K. pneumoniae* is detected earlier (Hao *et al.,* 2022). The prevalence of *Arn* operon showed that there is addition of LAra4N in the outer membrane lipid A moiety making these strains colistin resistance as supported by the transcriptomic analysis study by Baron *et al.,* (2018), on genes from the *Arn* operon. Apart from *Arn* operon, *PmrAB* mutational variability were also reported among CR *Enterobacteriaceae* isolates by Jaidane *et al.,* (2018), and Peter *et al.,* (2018). An Italian study reported, genomic variability in *PmrB* gene in CR *K. pneumoniae* too (Gentile *et al.,* 2020). A study by Snyman *et al.,* (2021), also observed mutations in *PmrB* gene in *mcr* negative *E. coli* from South Africa. Thus, the current study is in line with these findings, where higher mutational diversity was observed

in these genes and might be associated with colistin resistance among these *Enterobacteriaceae* isolates in our region too.

In current study, genomes of CR *K. Pneumoniae* were predicted to carry 28 non-synonymous mutations in Lipid-A modification genes (*PhoQ, PmrA, PmrB, EptA, OpgE, PagP, RamA, ArnD, ArnT*). Among the identified mutations, four non-synonymous mutations were novel which were E246D (P*hoQ*), I474N (*ArnT*), G519C (*OpgE)* and D71E *(RamA).* Moreover, deletion of 10 amino acids in *MgrB* gene was also identified and have not previously reported elsewhere. Two amino acids substitutions T246A and R256G in current study, have been also been reported in CR *K. pneumoniae* from Croatia (D'Onofrio *et al.,* 2020). In five *EptA* mutations of this study, three mutations I138V, S257L and Q319R were previously identified in *K. pneumoniae* from Tunisia (Jaidane *et al.,* 2018) rest were identified elsewhere. Mutations in *EptA* along with various other identified variations in current study in *ArnT* (M114L; V117I; R372K; H156Q), *PagP* gene (F170I), *MgrB* (M1V)*,* and *OpgE* (M53L) were concurrent with previous studies from India (Pragasam *et al.,* 2017; Mathur *et al.,* 2018b). These detected mutations in *MgrB* (M1V) and *EptA* (C27F; S257L) were also recorded by Boszczowski *et al.,* (2019) in CR *K. pneumoniae* from Brazilian tertiary care hospital. Amino acid changes in *ArnT* (M114L, V117I, R372K), and *EptA* (D477N and R152H) present in current study, were also identified among *K. pneumoniae* from Portugal (Elias *et al.,* 2022b).

In the above mentioned 11 mutations in genes *EptA, OpgE, PhoQ, PmrA,* and *MgrB* were predicted to have deleterious impact as they were identified in crucial protein coding domains (Table 4.5). The variations in *EptA* (S257L, 319R, D477N) and *OpgE* (V212I, V220M) were in sulfatase domain. In case of regulatory genes, mutation in *PhoQ* (E246D) which encodes histidine kinase was predicted to be deleterious as it was identified in HAMP domain while T189P mutation in PmrA response regulator was in DNA binding domain. A large deletion of nucleotides which was annotated to cause deletion of 10 amino acids in MgrB (Negative response regulator) signal peptide domain among *K. pneumoniae* genomes. As these identified mutations are in important domains, these mutations might affect the

signal transduction ultimately leads to the overexpression of PEA or LAraN transferases which are crucial role in Lipid A modifications.

In this research work on 2 CR *E. coli* genomes, 38 amino acid variations were identified of which 32 mutations were novel. Only two mutations S29G (*PmrA*) and N232D (*ArnT*) were shared between these CR *E. coli* genomes. A mutation S29G in *PmrA* in this study has already mentioned by Quesada *et al.,* (2015) from *E. coli* recovered in Spain from swine faeces. Amino acids variations in *PmrD* gene (N11D, M20K, A27T, K82T) are reported for the first time in clinical *E. coli* isolate in current study, although a study by Kim *et al.,* (2019) identify the same set of variation in *E. coli* from animal samples. In line with current findings, two mutations S29G (*PmrA*) and 144L (*PhoP*) have been detected in clinical *E.coli* from Japan (Sato *et al.,* 2018), and Taiwan too (Wang *et al.,* 2020b). Although, a recent study by Choi *et al.,* (2020), found *EptA* mutation and its over expression in CR *E. coli* isolates but didn't provide the complete details regarding the location and amino acid change. The paucity of data regarding chromosomal alterations in colistin resistance among *E. coli* as compared to other member of *Enterobacteriaceae* could be due to much lower prevalence of these resistant pathogens in clinical settings compared to other members. Despite the low prevalence of CR *E. coli* in current study, a high acquisition of novel chromosomal mutations was detected in local CR *E.coli* genomes and need to be further expressional investigation.

Colistin mechanism being complex has not been completely elucidated especially in *Enterobacter* as few studies reported the possible genomic alterations that might be associated with resistance phenotype (Wand & Mark Sutton, 2020; Laidoudi *et al.,* 2022). In present study, 21 novel non-synonymous mutations were identified in three of CR *E. hormaechei* genomes. The impact of three mutations I128F (*PhoP*), R423C (*PhoQ*) and T94M (*ArnT*) were deleterious as these were predicted to be in the PhoB-DNA binding domain, signal transduction histidine kinase like C terminal domain and glycosyltransferase domain, respectively. In current study, two variants in palmitoylation associated gene *PagP* were identified (F13V and D27E). A recent study by Mushtaq *et al.,* (2020) revealed that the

modification of lipid-A carried out by LpxO and PagP enzyme which mediate 2-hydroxylation and palmitoylation, was present among resistant and hetero resistant *Enterobacter* from UK and Ireland, but did not find any association with CR . The carriage of *PagP* genes along with acquisition of mutations points that the study isolates might have role in lipid A modification as these genes encode lipid A synthesis. Overall, there is complex structural lipid A modification either via conventional PEA or LAraN or with the addition of palmitoyl group which might be possibly cause of colistin resistance in these *E. hormaechei* isolates. As deleterious effect was predicted, these might be involvement in colistin resistance.

The changes in LPS encoding genes could be also responsible for the modification is one of the mechanism but even complete loss of the LPS which is only seen *A. baumannii* (Sun *et al.,* 2020). In present study , 13 non-synonymous mutations were identified in genes for lipid biosynthesis (*LpxA, LpxC, LpxD, LpxF*) and glycosyltransferase (*LpsB*) among 6 local sequenced genomes of CR *A . baumannii*. A study from India, also identified non-synonymous mutations in *LpxA*, *LpxD*, and *LpsB* genes in 8 CR *A. baumannii* Indian isolates but exact location of mutation was missing in their genome report (Vijayakumar *et al.,* 2018). In this work, the non-synonymous mutation Y131H in *LpxA*, was found inside the UDP-binding Pocket (His131) encoding region near the crucial residue Ile133 in the pocket which can have possible effect on UDP combination and interfere with its function (Badger *et al.,* 2012). This mutation was recently identified in Chinese *A. junii* recovered from patient with blood stream infection (Wang *et al.,* 2022). Many mutations in glycosyltransferase encoding gene (A166T, Q216K, H218G, S219E) in current study were also identified from South African isolates (Nogbou et al., 2022). A total of 8 novel variations identified among *A. baumannii* genomes in present work were C120R *(LpxC),*N287D *(LpxC)*, E117K (*LpxD)*, F90Y *(LpxF*), I228V, L329V, T331I, and H334N (*LpsB)*. Apart from these mutations, two other mutations in the lipid biosynthesis genes *LpxD* and *LpxF* were also predicted and were found to make truncated proteins because insertion of early stop codon in the former and deletion of 22 amino acids at C-terminal end of the other protein. These findings suggest that colistin resistance genotypes might be resultant of truncated gene products. The novel

genetic alterations identified in present study and the predicted deleterious effect of these mutations need to be experimentally tested in *in vitro* as well *in vivo* mice model for elucidating the possible effect of these alteration on lipid A modification among Gram-negatives.

Beside acquisition of plasmid mediated genes (*mcr*), chromosomal alterations in Lipid A synthesis and modification genes have been also found to possible cause of colistin resistance among Gram-negatives. The carriage of chromosomal potential determinants might be more crucial in facilitating altered Lipid A modification among these high priority pathogens (Binsker *et al.,* 2022a). In current study, potential acquisition of putative PEA and glycosyl transferases genes found in sequenced genomes are above the already existing chromosomal resistance conferring genes and alterations. This acquisition was via phage integrase mediated insertion of multiple copies of putative PEA transferases genes in CR *K. pneumoniae* (MML-4771), CR *E Coli*, and CR *A. baumannii* as identified *in silico*.

Moreover, similarly potential acquisition of putative glycosyl transferases and lipid biosynthesis genes was found in CR *S. maltophilia* genome, while the acquisition mechanism could not be predicted. Putative PEA transferase gene *yjgX* was initially identified in *E. coli* K-12 strain (Blattner *et al.,* 1997). Later , a study by Saile *et al.,* (2018) reported integration of foreign DNA segments via transposases and phage integrases ,which carried putative PEA transferase gene (*yjgX*) in a food-borne Shiga toxin-producing *E. coli*. The integration of *yjgX* gene was found to be associated with pathogenicity in Shiga toxin-producing *E. coli* isolates (Saile *et al.,* 2018). However, the exact role of *yjgX* gene encoding putative PEA transferase is not fully elucidated and it needs further revaluation for its role in colistin resistance *mcr*-negative isolates. In contrast to previous findings where intra species spread of *yjgX* was reported, this is the first study to report the integration of the putative PEA transferase gene via mobile genetic elements, a possible mechanism of acquisition and inter specie spread of this putative transferase in these isolates.

An important component of public health surveillance and response activities to stop the spread of antibiotic resistant bacteria is the rapid and accurate detection with characterization

of existing and emergent resistance determinants. This is where whole-genome sequencing can play a significant role (Lomonaco *et al.,* 2018). In numerous studies, genomic profiling of CR Gram-negatives revealed the association of various antimicrobial and virulent determinants with various clonal lineages. One of the striking benefits of sequencing analysis provision of identification and classification of pathogens into clonal groups with their lineages based on genomic similarities and differences. By using bioinformatics analysis, genetic relationships between various pathogens and pathogenic transmissions can also be predicted (Zong *et al.,* 2021). WGS based characterization revealed that the majority of the current study isolate's genomes belonged to high-risk clonal lineages.

In present study, predominant CR *K. pneumoniae* genomes were ST-11 (n 4/11) and ST-2096 (4/11), while ST-147, ST-231 and ST-985 were found to be singleton. *K. pneumoniae* ST-11 clones belonged to clonal group (CG)-258, which has been found to be associated with carbapenem resistant *K. pneumoniae* infections particularly in European region (Zhang *et al*., 2019; Wyres *et al*., 2020) ST-2096 and ST-147 are globally disseminated high-risk MDR *K. pneumoniae* lineages while ST-147 has been found to be endemic in Greece, Italy, and India (Liao *et al.,* 2020). ST231 might be also high-risk, carbapenemase producing *K. pneumoniae* clone as is actively spreading throughout Southeast Asia (Momin *et al.,* 2017; Mancini *et al.,* 2018; Shankar *et al.,* 2021) Only one ST-985 was seen in current study, this sequence type of *K. pneumoniae* is found in Institute Pasteur's *Klebsiella* database , as this ST of *K. pneumoniae* is reported from South Africa from spinach only (Richter *et al.,* 2021). In this study, CR *E. coli* (*n*=2) genomes belonged to ST-448 and ST-617 each. ST-448 is less frequently reported as potential high risk MDR *E. coli* clone (Li *et al.,* 2017). While ST-617 belongs to clonal complex (CC)-10, which is globally distributed among commensal *E. coli* isolates(Denamur *et al.,* 2021)and also detected from community acquired CPR *E. coli* from Pakistan (Habib *et al.,* 2022). Among the CR *E. hormaechei* (*n*=3), ST-93 was predominant in current study, a high-risk epidemic clone distributed globally (X *et al.,* 2018). The ST-2078 is recently identified and deposited in Pasture database from UK.

In this research work, diverse ST types of CR *A. baumannii* (n=6) were observed. The most predominant ST was 1503 (*n*=3) while ST-208, ST-2062 and ST-1289 were identified in

singleton. Apart from, ST-208 which belongs to a high risk clonal complex (CC-208) from International clone 2 and is endemic in Korea (Jeon *et al.,* 2018), the other identified ST's (ST-1503, ST-2062 and ST-1289) matched with a single genome entry submitted in Pasteur Database. ST-1503 genome was submitted from India in 2016, genome with ST-2062 was submitted from Russia in 2019 while ST-1289 genome was submitted in 2022 but no data was available regarding isolation source and country. ST-208, ST-2062, and ST-1503 are more recently observed in India (Rose *et al.,* 2021). *S. maltophilia* was novel ST's with no other isolate with same MLST pattern in any database. Overall, it is evident that the indigenous Gram negative belong to high risk lineages and much attention is need to contain such spread.

These Gram negative were also analysed for detection of genes responsible for MDR phenotype as strains co-carrying ESBL's and carbapenemase genes has significantly increased in recent years and are top global public health threat (Temkin *et al.,* 2014). In current study, the most predominant ESBL encoding gene was *bla*CTX-M-15 while the most predominant carbapenemase encoding gene was *bla*OXA-1 in *K. pneumoniae* genomes. In terms of extended-spectrum -lactamases (ESBL's), CTX-M enzymes are considered most significant where CTX-M-15 being the highly prevalent (Younes *et al.,* 2011). Many Gram-negative bacteria have been identified to contain the *bla*OXA-1 β lactamase gene, which hydrolyses broad-spectrum cephalosporins and lowers sensitivity to cefepime and cefpirome (Sugumar *et al.,* 2014). The co-carriage of more than one carbapenemase gene has been reported previously in MDR *K. pneumoniae* from Oman (Dortet *et al.,* 2012), Switzerland (Seiffert *et al.,* 2014), Brazil (Lee *et al.,* 2016), Pakistan (Sattar *et al.,* 2016), China (Xie *et al.,* 2017), and Morocco (Duman *et al.,* 2020). In this study, all the ST-11 genomes showed the co-carriage of *bla*NDM-1 gene with ESBL encoding genes (*bla*SHV-182 and *bla*CMY-6). Interestingly, three ST-11 *K. pneumoniae* genomes (MML-1, 2 & 14) showed the carriage of same AMR determinants except for MML-6 which showed additional carriage of ESBL (*bla*TEM-1 & *bla*ADC-25), and carbapenemase (*bla*OXA-232, *bla*OXA-23 & *bla*OXA-66). The results of study from Portugal on ST-11 *K. Pneumoniae* clones are in line with this work which found potential outbreak in hospital setting due to strains having co-carriage of

*bla*NDM-1, *bla*OXA-1, and CTX-M-15 genes (Mendes *et al.,* 2022). NDM-1 gene carriage was also found to be associated with ST-11 *K. pneumoniae* clinical isolates co-harbouring OXA carbapenemase determinants was detected from Karachi (Lomonaco *et al.,* 2018), and Peshawar (Bilal *et al.,* 2021).

Being globally disseminated MDR high risk lineage, ST11 is frequently linked to the development of multidrug resistance, therefore, it can be assumed from these findings that the co-carriage of diverse antimicrobial resistance determinants patterns in ST-11 *K. pneumoniae* genomes might be due to the capacity of this lineage to acquire several plasmids (Zhao *et al.,* 2020). A single *K. pneumoniae* ST-147 harbouring *bla*OXA-48 carbapenemase was observed in this study. This MDR ST-147 clone was also observed in an Indian study with *bla*NDM-7 gene carriage instead of *bla*OXA-48 (Shankar *et al.,* 2019). Clinical strains from a tertiary care hospital in Lahore showed carriage of *bla*OXA-48 along with carbapenemase genes in ST-147 clone too (Gondal *et al.,* 2020). In this research study, the carriage of *bla*SHV-106, and *bla*TEM-1A ESBL determinants were identified in ST-2096 genomes while *bla*OXA-1and *bla*OXA-232 carbapenemase encoding genes were common in ST-2096 and ST-231 CR *K. pneumoniae*. A study from Switzerland also reported the co-carriage of *bla*OXA-232 and *bla*CTX-M-15 in ST-231 *K. pneumoniae* clone (Mancini *et al.,* 2018). Studies from India (Shankar *et al.,* 2019) and Thailand (Boonyasiri *et al.,* 2021) also reported the carriage of *bla*OXA-232 carbapenemase gene in ST-2096 and 231 in CR *K. pneumoniae.* Another study from Singapore found co-carriage of *bla*OXA-232 with other carbapenemase gene associated with ST-231(Teo *et al.,* 2022).

In this study ST-985 was found to be co-harbouring array of β lactamases, *bla*OXA-1, *bla*CTX-M-15, *bla*SHV187 and *bla*TEM1A along with other AMR determinants displaying a MDR genotype. Carriage of diverse AMR determinants have not been previously seen to be associated with this ST (Richter *et al.,* 2021). There is diverse co-carriage of AMR determinants especially ESBL and carbapenemase genes combinations in study isolates confirms their phenotypic MDR profile and moreover, points toward potential emergence of XDR genotype. Furthermore, the distribution of multiple high-risk MDR ST's among *K.*

pneumoniae isolates in current study is worrisome. The potential MDR ST-985 *K. pneumoniae* identified in current study might be due to the acquisition and dissemination of AMR determinants from the circulating high risk MDR lineages in clinical settings.

The production of ESBL and carbapenemases by *E. coli* is on the rise, which is a global public health concern and a significant number of study *E. coli* isolates had co-carriage of *bla*CTX-M-15 and *bla*OXA-1 in their genomes. Interestingly, one of the CR *E. coli* (ST-448), was co-harbouring *bla*NDM-7, *bla*OXA-10 and *bla*TEM-1 genes. The carriage of *bla*CTX-M-15 have been identified in ST-617 *E. coli* lineages in community settings from Tanzania too (Mshana *et al.,* 2016). The carriage of *bla*NDM-7 in ST-448 *E. coli* lineage has only observed in a single comprehensive genome-based study from Arabian Peninsula, in which *bla*NDM-7 gene was identified among patients that have previous travel history to India (Pál *et al.,* 2017). Apart from that study, carriage of *bla*NDM-7 gene in *E. coli* isolate was identified in several other ST lineages among *E. coli* were observed in studies like ST-167 from France (Cuzon *et al.,* 2013), ST131, and ST410 from China (Wang *et al*., 2016; Xu & He, 2019). A Spanish study observed the co-carriage of *bla*NDM-7 and *bla*CTX-M-15 in a new ST-679 *E.coli* lineage, isolated from a patient with a travelling history to Pakistan (Espinal *et al.,* 2018).

In contrast to current study, different studies from Pakistan have found carriage of ESBL and carbapenemase genes associated with various *E. coli* lineages in clinical samples, these includes ST-405 (Qamar *et al.,* 2018), ST-38 (Rafaque *et al.,* 2019), and ST-131(Ali *et al.,* 2019; Sarwar *et al.,* 2022). A recent study from Pakistan identified ST-448 *E. coli* lineage harbouring *bla*NDM-1 gene on conjugative plasmid (Mohsin *et al.,* 2022a). Interestingly, the ST-448 *E. coli* in this research study showed the co-carriage of *bla*NDM-7, *bla*OXA-10 and *bla*OXA-1 which has not been previously reported elsewhere in the literature. Moreover, the co-carriage of ESBL, carbapenemase and several other AMR determinants in ST-448 and ST-617 highlights a possible evolution of these ST's into the potential high risk MDR lineages. Further studies, with large sample size are therefore necessary for epidemiological surveillance of this emerging threat in clinical settings.

Based on *in silico* analysis, the carriage of various AMR determinants was predicted in *E. hormaechei.* Although two of the isolates belonged to same ST-93 but interestingly, showed a quite diverse pattern of AMR gene carriage. MML-8519 (ST-93) showed the co-carriage of multiple ESBL encoding genes which includes *bla*SFO-1, *bla*TEM-1, and *bla*LAP-2. Additionally, showed carriage of *bla*NDM-7 and *bla*OXA-1 carbapenemase determinant. Other *E. hormaechei* also belonged to ST-93 in current study, showed carriage of *bla*NDM-5 which is in contrast to a study from China, in which a ST-93 clinical *E. hormaechei* clone harboured *bla*NDM-4 gene (X *et al.,* 2018). *bla*SFO-1, an important ESBL capable of hydrolysing cefotaxime very effectively, but not ceftazidime was detected in *E. hormaechei*. However, has no activity against cephamycin's and carbapenems and was recently identified in ST-93 *E. hormaechei* from China (Zhou *et al.,* 2020). NDM-5 gene was also identified from tertiary care hospital in ST-93 *E. hormaechei* from China (Chen *et al.,* 2021). While carriage of *bla*NDM-7 was observed in ST131 from Canada(Chen *et al.,* 2016), and in a high risk international ST-78 *E. hormaechei* from Spain (Villa *et al.,* 2019). A study Mohsin *et al.* (2022) from Faisalabad, identified a *E. hormaechei* isolate carrying various MDR determinants, but AMR pattern in that study was quite different compared to current study. This study further highlights the presence of high-risk ST-93 carrying important carbapenemase and ESBL genes in clinical settings, which might be crucial in further spread of these determinants in clinical settings.

Among CR *A. baumannii* (*n*=6), *bla*ADC-25, and *bla*OXA-23 determinants were predominantly distributed among all the characterized genomes. Among carbapenemase gene, *bla*OXA-23 is considered as the most commonly distributed carbapenemase gene in *A. baumannii* (Schultz *et al.,* 2016). A study from India also observed *bla*OXA-23 predominantly associated with *A. baumannii* isolates belonging from ST-848 (Vijayakumar *et al.,* 2020). The same study also observed ST-1503 *A. baumannii,* harboring *bla*PER gene which is contradictory to current study findings where *bla*PER gene carriage was detected in ST-1289 *A. baumannii* genome. *A. baumannii* isolate with ST-2062 showed co-carriage of another carbapenemase gene, *bla*OXA-66. While isolate displaying ST-1289 coharbored various β lactamase (*bla*GES-11 & *bla*PER-1) and carbapenemase determinants (*bla*OXA-

114g, *bla*OXA-66). The carriage of *bla*TEM-1D, *bla*OXA-66 and *bla*OXA-232 was identified in ST-208 isolate along with various other AMR determinants. A study from India by Rose *et al.* (2021), observed the carriage of *bla*OXA-66 and *bla*OXA-69 in *A. baumannii* genomes displaying multiple ST's.

ST-208 is also a newly emerging carbapenem resistant *A. baumannii* lineage facilitating the transmission of carbapenem resistance through *bla*OXA-23 especially in China (Fang *et al.,* 2016) and India (Rose *et al.,* 2021) while in Pakistan, a multiple hospitals based study, found *bla*OXA-23 carriage in ST-589 (Khurshid *et al.,* 2020). Although a recent study identified *A. baumannii* isolates from hospital settings belonging to International clonal lineage (IC) I and II but the isolates were not further segregated based on their ST's (Khurshid *et al.,* 2017) (Karah *et al.,* 2020). International travel and tourism might be the possible reason for the dissemination of these emerging clonal lineages in Pakistan. The ST-1503 *A. baumannii* clone have not been identified in any other country except for India. The identification of ST-1503 in CR *A. baumannii* from Pakistan is quite concerning. Apart from ST-1503, ST-208 which is newly emerging CPR *A. baumannii* lineage has not been reported previously from Pakistan.

Interestingly, the carriage of an important ESBL encoding gene *bla*AmpC was identified in current study in CR *S. maltophilia* genome. Furthermore, this isolate was co-harbouring two carbapenemase determinants *bla*L1 and *bla*L2 is alarming. Same set of genes were also observed in *S. maltophilia* in a hospital based study from China (Ma *et al.,* 2020). A higher resistance pattern against various antimicrobial agents have been observed in a study from Karachi (Jabeen *et al.,* 2013), Rawalpindi (Ali *et al.,* 2016) and Lahore (Umar *et al.,* 2022). However, not a single study provides genetic basis for the resistance in this emerging opportunistic pathogen.

Although virulent and hypervirulent lineages were thought to be associated with community clones while MDR clones were mostly linked to hospital associated infection. However, recent studies have pointed toward the carriage of virulent gene in MDR clones. This convergence from classical genotype is quite evident in priority pathogens as various recent

studies have identified emergence of MDR plus virulent strains in clinical settings (Nuhu *et al.,* 2020; Mende *et al.,* 2022; Schultze *et al.,* 2023). Being widely distributed, this lineage was previously thought to be a high risk MDR lineage but in recent years, there is increase in virulence potential in these high-risk MDR lineages depicted the possible convergence to the classical genotype (Zhang *et al.,* 2020; Xie *et al.,* 2021).

K. pneumoniae's hypervirulence was attributed to the presence of a virulence plasmid that contains the genes for regulators of capsular polysaccharides (*RmpA*/*RmpA2*) as well as certain siderophore determinants for the hypermucoviscous phenotype(Alcántar-Curiel & Girón, 2015). All the CR *K. pneumoniae* genomes showed co-carriage of array of virulence associated determinants which were yersiniabactin, aerobactin, enterobactin, salmochelin siderophores, type 1 and 2 adhesins depicting a MDR, and hypervirulent genotype. ST-2096, ST-231 and ST-985 genomes were further found to be carrying *RmpA/RmpA2* regulatory genes responsible for hyper-mucoid phenotypes. Interestingly, all the strains displaying ST-2096 were also additionally co-equipped with aerobactin siderophores system. The merging of resistant and virulent genotypes in a single lineage gave rise to a convergent ST's -2096, which have been reported recently coharboring wide range of antimicrobial and virulent determinants depicting a MDR hyper virulent genotype from India (Shankar *et al.,* 2020) and China (Tang *et al.,* 2020a). In this study, isolate with ST-147 showed co-carriage of wide range of virulent determinants like yersiniabactin, enterobactin, adhesion and invasion systems but carriage of aerobactin and *RmpA/RmpA2* genes was not identified. A large scale multicentered study from UK have identified the ST-147 isolates co-harbouring aerobactin, and *RmpA/RmpA2* genes on a large virulent plasmid (Turton *et al.,* 2018). An observational study from Italy also reported the outbreak associated with hypervirulent MDR ST-147 *K. pneumoniae* lineage in COVID-19 patients (Falcone *et al.,* 2022). Another multi hospitals based study from Italy also highlighted unique co-carriage of AMR and virulent determinants in ST-147 *K. pneumoniae* clones (Pilato *et al.,* 2022). ST-147 *K. pneumoniae* displaying a hypervirulent and MDR genotype have been identified from Iran too (Davoudabadi *et al.,* 2022). A Portuguese study also identified increased virulence potential among MDR ST-147 *K. pneumoniae* (Mendes *et al.,* 2022).

K. pneumoniae capsular polysaccharide is considered as one the crucial factor for its virulence (Paczosa & Mecsas, 2016). Based on capsular serotype, K-64 serotype was the most common and found predominantly associated with all ST-2096 (n=4) and ST-147 *K. pneumoniae* in current study. The KL-64 capsular loci were previously found to be associated with virulence among *K. pneumoniae* strains (de Campos *et al.,* 2018). A Portuguese study identified increased virulence in ST-147 CPR *K. pneumoniae* displaying KL-64 capsular genotype (Mendes *et al.,* 2022). In current study, all the ST-11 *K. pneumoniae* were identified to have K-14 capsular locus. This capsular locus has been identified in MDR hypervirulent *K. pneumoniae* (ST-1559) associated with outbreak in a study from Nepal (The *et al.,* 2015). Similar to this research study, K14 capsular loci was identified in ST-11 *K. pneumoniae* isolates from a South Korean study(Cho *et al.,* 2022), which further associated K14 type to more hypervirulent phenotype compared to their other K-type carrying counterparts. Each of the ST-985 and ST-231 *K. pneumoniae* genomes carried K-39 and K-51 capsular serotype. In contrast to this study findings, a study from China, identified KL-64 to be a major capsular loci associated with an MDR hypervirulent ST-11 lineage (Huang *et al.,* 2022). A study from Thailand (Boonyasiri *et al.,* 2021), and Singapore (Teo *et al.,* 2022), found KL51 associated with ST-231 *K. pneumoniae* clones along with co-carriage of multiple virulent as well as AMR associated determinants.

Co-carriage of virulent determinants was previously observed in MDR high risk *E. coli* lineages from Mexico(Silva *et al.,* 2022), Nigeria (Orole *et al.,* 2022),Egypt (El-baz *et al.,* 2022), India (Radera *et al.,* 2022) and Pakistan (Ali *et al*., 2019; Rafaque *et al*., 2019). In this research work, CR *E. coli* MML-11229 (ST-617) was found to be carrying more virulent factors associated genes compared to the MML-7 (ST-448), displaying a high virulent genotype. In support to these results, a Tunisian study also observed a much higher virulent profile among ST-617 MDR *E. coli* isolates (Mhaya *et al.,* 2022). Higher virulent potential was also found associated with ST-617 displaying *E. coli* isolated from animal source in a study from USA (Haley *et al.,* 2022). A study from Pakistan identified MDR *E. coli* isolates from ST-448 lineage, although few virulence genes were observed in those isolates (Mohsin *et al.,* 2022b). However, ST-448 clone from this research work possessed a much extensive

range of virulent determinants and additionally had NDM, OXA and TEM and CTX-M genes displaying a hypervirulent MDR genotype. Up till now, no study from Pakistan identified virulent and MDR ST-617 *E.coli* isolate representing convergence. The presence of potentially high risk hypervirulent MDR clones need to be monitored for better epidemiological surveillance. Further studies are required to experimentally determine the potential virulent nature of these isolates.

All the 3 *E. hormaechei* genomes showed carriage of wide range of virulent determinants including multiple siderophores, adhesins, invasion, curli system encoding gene and others. Interestingly, one MML-8 (ST-93) and MML-15 (novel ST) coharbored similar set of virulent determinants compared to MML-8519 (ST-93). Carriage of only three virulent associated genes were observed in ST-93 *E. hormaechei* from China (X *et al.,* 2018) and Sri Lanka (Perera *et al.,* 2022). A wide range of virulent associated determinants potentially convergent ST *E. hormaechei* genomes in this study needs expressional studies to prove their virulence and AMR.

Due to the rapid emergence of severe infections and the rising antibiotic resistance, patients are frequently given incorrect empirical medication (Espinal *et al.,* 2019). It has been observed that *A. baumannii* virulence can be increased by changing from a generally low virulent organism to a highly virulent one in response wrongly prescribed or low efficacy antibiotics (Kumar *et al.,* 2021). In current study, the carriage of wide array of virulent determinants were also observed in *A. baumannii* and *S. maltophilia* genomes. interestingly, all the *A. baumannii* genomes were carrying similar set of virulent determinants despite belonging from different ST's. A study from Saudi Arabia also observed carriage of virulent determinants in potentially emerging MDR Clonal complex II (Ali *et al.,* 2017). Increased virulence potential was also observed in MDR *A. baumannii* isolates belonging to IC-I and IC-II in Iranian study (Zeighami *et al.,* 2019). In comparison to these cited studies, a much diverse range of virulent determinants points towards potentially emerging high risk MDR lineages in local isolates. The high incidence of virulent (iron siderophores, adhesion, invasion) and MDR genes is alarming.(Mendes *et al.,* 2022). A large scale genome based

studies are required to understand the potential relation of clonality and convergence phenomena in these CR priority pathogens.

Mobile genetic elements (MGE), which include components that encourage both intracellular and intercellular DNA mobility, play a significant role in the capture, accumulation, and spread of resistance as well as virulent genes. ColKP3 was identified in all the four ST-2096 CR *K. pneumoniae* isolate in current study. This plasmid was previously observed to carry *bla*OXA-232 gene in ST-2096 *K. pneumoniae* from India (Shankar *et al.,* 2022). Another Indian study by Sundaresan *et al.,* (2022), identified ColKP3 plasmid replicon associated *bla*OXA-232 and *bla*OXA-181 in *K. pneumoniae*. ColpVc plasmid replicon associated with *K. pneumoniae* isolates, in contrast to their results, this study identified the carriage of ColpVc in CR *E.coli* belonging form ST-448 instead of *K. pneumoniae* ST-147 isolate. In this study, IncF plasmid replicon was observed in 14/16 CR *Enterobacteriaceae* genomes. It has been reported that IncF-type plasmids are associated with multiple resistant gene carriage which includes ESBL, carbapenemase, aminoglycoside modifying enzyme genes, and quinolone resistance gene (Rozwandowicz *et al.,* 2018a). MDR and virulence pathotypes can coexist in a single isolate either through the uptake of a virulence plasmid by an MDR isolate or through the uptake of plasmids carrying ARGs by the virulent isolates (Tang *et al.,* 2020b). The convergence is due to the emergence of mosaic and hybrid plasmids which have been identified previously in *K. pneumoniae* (Yang *et al.,* 2021). Two mosaic plasmid replicons IncFIB(pNDM-Mar) and IncHI1B(pNDM-MAR) were predominantly identified among CR *K. pneumoniae* in this study. These mosaic plasmids were also identified in CPR *K. pneumoniae* strains from India (Shankar *et al.,* 2020). In current study, all the ST-11 isolates were additionally carrying virulent plasmids IncFII(K) and IncFIB(K), which have been reported previously in ST-101 *K. pneumoniae* isolated from septicemia patient from England associated with various virulent and AMR determinants (Turton *et al.,* 2019). Virulent hybrid plasmid containing IncFII(K) was identified in European regions (Lam *et al.,* 2019). The carriage of hybrid IncHI1B/IncFIB was also seen in *K. pneumoniae* from Egypt associated with *bla*CTX-M-15 (Edward *et al.,* 2022). In this study diverse plasmid replicons, were identified in ST-231 among these

Col440I IncFII (pAMA1167-NDM-5), IncFIB(pQil), IncFII(K) not identified in other sequenced genomes. while an Indian study identified carriage of these plasmid replicons associated with ST-231 *K. pneumoniae* (Shukla *et al.,* 2023). However, carriage of other mosaic plasmids by ST-231 *K. pneumoniae* in current study were not identified in their strains. The hybrid plasmids are formed by co-integration of two plasmid backbones (Turton *et al.,* 2019), whereas mosaic plasmids are typically composed of two or more different plasmid backbones which create a scenario where AMR and virulence determinants are encoded on a single large plasmid with a mosaic arrangement of resistance plus virulence genes (Lam *et al.,* 2019). As a result of this bidirectional convergence, MDR-hypervirulent *K. pneumoniae* isolates have begun to appear within nosocomial clones. The spread of nosocomial clones with virulence plasmids is therefore a serious public health concern (Liu *et al.,* 2020). Because reports of hybrid plasmids come from clinical isolates, it is clear that the antimicrobial pressure in this niche not only favours the development of these plasmids but also helps them persist and spread.

Most of IncI plasmids reports are from Europe, which is primarily found in *E. coli.* Moreover, ESBL and plasmid-mediated (p)AmpC genes have been described to be associated with this plasmid replicon type (Rozwandowicz *et al.,* 2018b). In this study, IncI(Gamma) and IncI1-I(Alpha) plasmid replicons were identified in CR *E. coli* genomes. IncI plasmid replicons were also identified in clinical MDR *E. coli* from India and thought to be associated with the transmission of AMR and virulence determinants(Mukherjee & Mukherjee, 2019).

CRISPR is a family of immune systems that can be found in many prokaryotic genomes and that attack foreign DNA (Marraffini & Sontheimer, 2010). In current study, all the 8/11 CR *K. pneumoniae* isolates were carrying type IV-A3 CRISPR-Cas system. This CRISPR system was previously identified in CPR *K. pneumoniae* isolates associated with hybrid plasmid from India (Shankar *et al.,* 2022). Although, limited research has been done on the CRISPR-Cas systems in MDR plasmids of *K. pneumoniae*. But it has been observed that, in order to reduce the fitness cost associated with carrying multiple AMR plasmids, *K.*

pneumoniae attain specific plasmid CRISPR spacers. Co-integrate plasmids carrying IncFIB and IncHI1B replicons make up the majority of the plasmids carrying these spacers (Kamruzzaman & Iredell, 2020; Newire *et al.,* 2020).

AMR is a global health emergency which need serious attention. The introduction of novel resistance mechanisms, the failure of conventional antibiotics in clinical settings, and the industry's reduced focus on antibiotic development necessitate for development of other approaches for the management of infectious diseases (Cantón *et al.,* 2012). There are many targets for bacterial lysis and elimination but one of the most important target is specifically transporters in the membrane acting as efflux pumps for extrusion of the antibiotics, which normally play a crucial role in the development of AMR (Brindangnanam *et al.,* 2022). These transporters and their associated gene can be a potential therapeutic target (Compagne *et al.,* 2023). Recent studies by Arshad and Seed group, also provided a novel connection between the regulation of the multidrug efflux pump and the expression of polysaccharide capsules by sensitizing *E. coli* in a murine sepsis model (M. Arshad *et al.,* 2016, 2021). Based on these studies, this research work attempted to target a multifunctional transporter gene, uropathogenic *E. coli* lab strain UTI89 (from Seed Lab), was used as a model to disrupt transporter gene "*YjiJ*", to assess the potential role of this putative transporter gene as anti-infective therapy. The gene knock out system used in this research work has also been employed previously for disrupting other potential genes in bacteria (Lesic & Rahme, 2008; Peng *et al*., 2022). After knock out of transporter gene, bacterial colony on Cam LB agar was indicative of *YjiJ* gene deletion in mutant carrying Cam^R selective marker gene. However, PCR based detection of *YjiJ* gene in these mutant strains indicated that there was no complete knock out of transporter gene. The amplification the *YjiJ* gene point that there were multiple copies of this gene in the bacterial genome. *YjiJ* gene, being a member of Major Facilitator Superfamily (MFS) transport system, play crucial role in various regulatory process linked with bacterial virulence and resistance (Saidijam *et al*., 2006; Krizsan *et al*., 2015). Deep understanding of the structural makeup and operation of these important multifunctional transporters will provide prospects for the creation of novel inhibitors that may be employed in combination with antimicrobials to combat the

increasing AMR epidemic. Hence, there is need for further investigation to develop efficient anti-infective therapy.

This research work is a first comprehensive genome-based study elucidating the potential mechanism of colistin resistance among high-risk priority pathogens from Pakistan.

- A high phenotypic colistin resistance (31%) was detected among Gram-negatives especially in *K. pneumoniae* (55%) compared to other Gram-negative isolates.
- MDR phenotype was detected in 84% CR Gram-negative isolates against commonly tested antibiotics.
- Prevalence of plasmid mediated colistin resistance was low (12%) in CR Gram-negative isolates where *mcr*-2 was found in 8% followed by *mcr*-3 in 5.59% isolates.
- *mcr*-2 and *mcr*-3 detected in CR *A. baumannii* while *mcr*-3 was seen in *E. coli, K. pneumoniae,* and *P. aeruginosa* has not been previously reported from Pakistan.
- Four novel non-synonymous mutations were identified (E246D in *PhoQ*, I474N in *ArnT*, G519C in *OpgE*, D71E in *RamA*), in CR *K. pneumoniae* sequenced genomes, moreover, ten amino acids encoding sequence deletion in *mgrB* gene was predicted and has not previously reported elsewhere.
- Based on MLST, two high-risk globally distributed ST's which included ST-11 (*n*=4) and ST-2096 (*n*=4) were predominant in CR *K. pneumoniae*.
- CR *E. coli* had 32 novel mutations in which high genetic diversity was observed in *ArnT* (26%), *ArnD* (16%), *EptC* (13%) and *PmrD* (13%) genes. Furthermore, 2 mutations C27Y (*EptA*) and K82T (*PmrD*) were predicted to be deleterious as these were detected in transmembrane helix region and regulatory domain.
- Acquisition of putative PEA and glycosyl transferases were predicted in CR *K. pneumoniae* (MML-4771), CR-*E. coli*, CR *A. Baumannii* and *S. maltophilia* genomes.
- Co-carriage of *bla*NDM-7, *bla*OXA-10, *bla*TEM gene in ST-448 and *bla*CTX-M-15 and *bla*OXA-1 in ST-617 in CR *E. coli* have reported.
- A total of 21 novel non-synonymous mutations were identified in CR *E. hormaechei* genomes. Mutation I128F in *PhoP* (PhoB-DNA binding domain), and R423C in *PhoQ* (Signal transduction histidine kinase like domain), and T94M in ArnT (glycosyltransferase domain) were predicted to be deleterious.

- ST-93 was predominant lineage among CR *E. hormaechei*. Carriage of *bla*NDM-7, *bla*OXA-1, *bla*SFO-1, *bla*TEM-1 and *bla*LAP-2 in ST-93 have not been previously reported.
- A total 8 novel mutations were identified in CR *A. baumannii*. These were C120R & N287D *(LpxC)*, E117K (*LpxD)*, F90Y *(LpxF*), I228V, L329V, T331I, and H334N (*LpsB)*. Apart from these, deletion in two lipid biosynthesis genes (*LpxD* and *LpxF*) were predicted to make *LpxD* and *LpxF* truncated.
- *M*ost predominant ST in CR *A. baumannii* was ST-1503 which has not been identified in any other country except from India. ST-208 was found to be newly emerging CPR *A. baumannii* lineage identified in this study, has also has not been reported previously from Pakistan.
- Novel ST of *E. hormaechei* and *S. maltophilia* with multiple AMR and virulent determinants were detected too.
- This study attempted to knock out and generate single gene deletion mutant (*YjiJ* transporter gene) using *E. coli* as model organism to assess the therapeutic potential of knock out MFS transporters by targeting their gene. However, further investigations are required to assess the possible cause of partial gene disruption in these mutant strains.

Current study is the 1st study on Whole genome-based characterization of colistin resistant Gram negatives isolates from Pakistan. Identified many novel Chromosomal and other mobile mutations with predicted deleterious impact with carriage of putative transferases in *mcr* negative Gram-negatives responsible for higher colistin resistance rate. Overall, chromosomal mediated resistance was playing major role in imparting CR among local Gram-negative. Furthermore, co-carriage of carbapenemase, ESBL's, AMR, virulent determinants, and mobile genetic elements both mosaic plus hybrid were predominately making CR isolate genomes more resilient toward resistance. It also points toward potential emergence of convergent MDR hypervirulent lineages that have not been reported previously from this region.

The findings of this research work identified multiple novel mutations and potential putative transferases as a possible cause of colistin resistance among *mcr* negative Gram-negatives which has paved the door for additional research that will increase our understanding of the genotypic mechanisms underlying high phenotypic colistin resistance.

- Transcriptomic and proteomic based large-scale studies are required to validate the potential impact of novel variations, putative PEA and glycosyl transferase gene in Lipid A modification in these CR isolates.
- Detection of high-risk convergent clonal lineages carrying both AMR and virulent genes is worrisome. Further, highlights the need for comprehensive genomic based epidemiological surveillance for understanding their acquisition potential and spread especially in hospital settings and community.
- Further work is needed to re-evaluate this phage λ based gene knock out system for development of new approach to inactivate resistance and make *E. coli* drug sensitive.
- Other important regulatory genes and PEA transferase involved in AMR should be studied as potential therapeutic target for alternate treatment strategy.

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Appendix I: Recipe for the preparation of Media

Serial number	Reagent	Weight	Final Volume (L)
1	Bacteriological peptone (Sigma)	15 g	1L
2	Blood Agar (Oxoid)	40 g*	1L
3	Chocolate Agar (Oxoid)	45.5 g*	1L
4	CLED Agar (Oxoid)	36.2 g	1L
5	Luria-Bertani Agar plates (Sigma)	25g LB +14g Technical agar	1L
6	Luria-Bertani Broth (LB) (Sigma)	25 g	1L
7	MacConkey Agar (Sigma)	52 g	1L
8	Muller Hinton Agar (sigma)	38 g	1L
9	Muller Hinton Broth (sigma)	21 g	1L
10	Simon Citrate agar (sigma)	23 g	1L
11	Triple Sugar Iron Agar (Sigma)	65 g	1L
*After autoclaving, blood agar media was allowed to be cooled till its temperature reaches 45-50°C, 50 mL of sterile sheep blood was added in blood agar. For Chocolate agar, the media was heated at 70°C, after addition of 50 mL sheep blood.			

Appendix II: Preparation of Mg^{2+} and Ca^{2+} stock solution for MHB cation adjustment

Serial number	Chemicals	Weight (g)	Volume (mL)	Stock concentration
1	$MgCl_2 \cdot 6H_2O$	8.63	100	10mg/L (Mg^{2+})
2	$CaCl_2$	3.68	100	20mg/L (Ca^{2+})
*Filter sterilized using 0.2 mm pore size cellulose acetate filter				

Appendix III: Recommended zone diameter breakpoints for various antimicrobial agents as per CLSI (M100-29th Edition)

Serial number	Antimicrobials	*Enterobacteriaceae*			*Non-Enterobacteriaceae*					
					Pseudomonas			***Acinetobacter***		
		S	I	R	S	I	R	S	I	R
1	Piperacillin/Tazobactam (100/10µg)	≥21	18-20	≤17	≥21	15-20	≤14	≥21	18-20	≤17
2	Cefazolin (30µg)	≥23	20-22	≤19			--			--
3	Cefepime (30µg)	≥25	--	≤18	≥18	15-17	≤14	≥18	15-17	≤14
4	Cefotaxime (30µg)	≥26	23-25	≤22			--	≥23	15-22	≤14
5	Cefoxitin (30µg)	≥18	15-17	≤14						--
6	Aztreonam (30µg)	≥21	18-20	≤17	≥22	16-21	≤15			--
7	Imipenem (10µg)	≥23	20-22	≤19	≥19	16-18	≤15	≥22	19-21	≤18
8	Amikacin (30µg)	≥17	15-16	≤14	≥17	15-16	≤14	≥17	15-16	≤14
9	Ciprofloxacin (5µg)	≥26	22-25	≤21	≥25	19-24	≤18	≥21	16-20	≤15
10	Cefoperazone/sulbactam (75/30µg)						--			--
S=Sensitive, I=Intermediate, R=resistant										

Appendix IV: Sequence of primers used for the amplification of *mcr*-1, *mcr*-2 and *mcr*-3 gene

Gene	Primer (F)	Primer (R)	Band size (bp)
***mcr* 1**	5′-CGG TCA GTC CGT TTG TTC-3′	5′-CTT GGT CGG TCT GTA GGG-3′	309
***mcr* 2**	5’-TGT TGC TTG TGC CGA TTG GA-3’	5’-AGA TGG TAT TGT TGG TTG CTG-3’	567
***mcr* 3**	5’-TTG GCA CTG TAT TTT GCA TTT-3	5’-TTA ACG AAA TTG GCT GGA ACA-3’	542

Appendix V: Thermocycler conditions used for amplification of *mcr*-1, *mcr*-2 and *mcr*-3 gene

Serial number	Stages		T (℃)	Time	Cycles
1	Initial Denaturation		95	3 minutes	1X
2	Final denaturation		95	45 seconds	35X
3	Annealing	*mcr*-1	42-59	30 seconds -1 minute	
		mcr-2	50-59		
		mcr-3	50-59		
4	Initial Extension		72	45 seconds	
5	Final extension		72	7 Minutes	1X

Appendix VI: concentrations and volume of PCR reagents used for amplification of 50bp overhangs with Cam^R selective marker, internal Cam^R gene and γ-recombinase gene

Serial number	Reagents	V (µL)	Concentration	Final volume of reaction mixture
1	2x Apex Taq DNA polymerase Master mix	25	2x	50 µL
2	Forward Primer	1	1mM	
3	Reverse Primer	1	1mM	
4	Template DNA	1	--	
5	DNase free Water	22	--	

Appendix VII: Thermocycler conditions used for amplification of 50bp overhangs with Cam^R selective marker

Serial number	Steps	Temperature (°C)	Time	Number of cycles
1	Initial Denaturation	95	15 minutes	1X
2	Final Denaturation	95	45 seconds	35 X
3	Initial Annealing	50-53	45 seconds	
4	Initial Extension	72	45 seconds	
5	Final Extension	72	10 minutes	1X

Appendix VIII: Thermocycler conditions used for amplification of Cam^R and γ-recombinase gene

Serial number	Steps		Temperature (°C)	Time	Number of cycles
1	Initial Denaturation		94	10 minutes	1
2	Final Denaturation		94	30 seconds	35 X
3	Initial Annealing	**Cm^R**	51	30 seconds	
		γ-recombinase	53		
4	Initial Extension		72	30 seconds	
5	Final Extension		72	7 minutes	1

Appendix IX: Frequency distribution of Gram-negative isolates from overall collected clinical specimens among patient's (*n*=528)

Clinical specimens	***Enterobacteriaceae* (*n*=367)**						***Non-Enterobacteriaceae* (*n*=161)**		
	Klebsiella (n=173)	*E. coli (n=171)*	*Enterobacter (n=10)*	*Citrobacter (n=10)*	*Provedenica (n=2)*	*Serratia (n=1)*	*Pseudomonas (n=61)*	*Acinetobacter (n=82)*	*S. maltophilia (n=18)*
Urine	52	157	1	6	2	0	10	1	1
Blood	46	2	4	0	0	0	6	19	14
NBL	16	0	0	2	0	1	15	3	1
Pus	29	5	2	2	0	0	18	30	0
Catheters	19	3	2	0	0	0	5	9	1
Fluid	5	3	0	0	0	0	4	14	1
Wound swab	3	0	0	0	0	0	0	1	0
Sputum	3	1	1	0	0	0	3	5	0

Appendix X: Frequency distribution of CR-Gram-negatives for isolation based on patient's age, gender and specimen type

CR Gram-negative isolates (*n*=161)		**Gender**		**Age group (years)**				**Specimen type**				
		Male	Female	I	II	III	IV	Urine	Blood	pus	NBL	Others
Enterobacteriaceae (113)	*K. pneumoniae* (88)	63	25	23	34	19	12	21	24	16	10	17
	K. oxytoca (4)	3	1	--	--	2	2	4	--	--	--	--
	K. aerogens (2)	--	2	--	--	1	1	--	--	2	--	--
	E. coli (10)	4	6	1	2	4	3	6	1	1	--	2
	Enterobacter (5)	4	1	--	2	--	3	2	1	--	--	3
	C. koserii (3)	3	-	2	-	1	-	2	-	-	1	-
	C. freundii (1)	1	-	1	-	-	-	1	-	-	-	-
Non-Enterobacteriaceae (48)	*P. aeruginosa* (26)	23	3	5	4	10	7	5	-	8	7	6
	A. baumannii (15)	9	6	3	5	5	2	1	4	5	-	6
	S. maltophilia (7)	5	2	5	2	-	-	1	6	-	-	-

Appendix XI: Characteristics of *mcr* positive CR Gram-negative isolates

Gram negatives	Isolates	MIC	MDR	Sample	Age (years)	Gender	*mcr*-1	*mcr*-2	*mcr*-3
***Enterobacteriaceae* isolates**	***K. pneumoniae (n=10)***	32	Yes	NBL	77	M	-ve	+ve	-ve
		8	Yes	Fluid	Newborn	F	-ve	+ve	-ve
		128	Yes	Urine	28	M	-ve	+ve	-ve
		8	Yes	pus	60	F	-ve	+ve	+ve
		16	Yes	Urine	34	M	-ve	+ve	-ve
		8	Yes	Blood	45	M	-ve	+ve	+ve
		64	Yes	Blood	34	F	-ve	+ve	-ve
		64	Yes	Urine	28	F	-ve	+ve	-ve
		64	Yes	Blood	76	F	-ve	+ve	-ve
		8	Yes	Blood	Newborn	M	-ve	+ve	+ve
	K. oxytoca (n=1)	32	Yes	Urine	45	M	-ve	+ve	-ve
	E. coli (n=2)	16	Yes	Urine	33	M	-ve	-ve	+ve
		256	Yes	Urine	46	F	-ve	-ve	+ve
	Enterobacter (n=1)	4	Yes	Urine	34	M	+ve	+ve	+ve
Non-Enterobacteriaceae	***P. aeruginosa (n=3)***	16	no	Urine	16	M	-ve	-ve	+ve
		4	no	NBL	52	M	-ve	-ve	+ve
		4	Yes	Pus	55	M	-ve	+ve	-ve
	A. baumannii (n=3)	4	Yes	Pus	31	F	-ve	+ve	-ve
		32	Yes	Fluid	12	F	-ve	+ve	-ve
		4	no	Blood	50	M	-ve	-ve	+ve

Appendix XII: List of selected *mcr* negative CR Gram-negative isolates processed for Whole genome sequencing

Organism	Isolate ID	MIC	Gender	Age(years)	Specimen	MDR
K. pneumoniae (n=11)	MML-1	64	F	34	Blood	Yes
	MML-2	64	M	4-month	Urine	Yes
	MML-3	64	F	28	Urine	Yes
	MML-4	4	M	43	Urine	Yes
	MML-5	64	M	23	Pus	Yes
	MML-6	256	M	78	Pus	Yes
	MML-10718	8	F	60	Pus	Yes
	MML-4771	8	M	Newborn	Blood	Yes
	MML-12	256	F	59	Pus	Yes
	MML-14	64	M	35	Fluid	Yes
	MML-17	8	F	76	Pus	Yes
E. coli (n=2)	MML-7	256	M	70	Urine	Yes
	MML-11229	4	M	34	Urine	Yes
Enterobacter spp. (n=3)	MML-18	16	M	80	Blood	Yes
	MML-8159	128	M	67	Urine	Yes
	MML-15	4	M	44	Fluid	Yes
A. baumannii (n=5)	MML-9	64	F	58	pus	Yes
	MML-10	4	F	31	Pus	Yes
	MML-11	16	F	26	Pus	Yes
	MML-13	4	M	50	Blood	Yes
	MML-16	32	F	12	Fluid	No
	MML-19	256	M	78	Pus	Yes
S. maltophilia (n=1)	MML-18	256	M	5	Urine	Yes

Appendix XIII: Whole Genome characteristics of selected mcr negative CR Gram-negative isolates (*n*=23)

S#	Organism	Isolate ID	Contigs	Genome size(Mb)	GC content(%)	CDS	tRNA	rRNA
1	*K. pneumoniae*	MML-1	116	5.9	56.63	6015	78	4
2		MML-2	101	5.98	56.59	6067	78	4
3		MML-3	91	5.7	56.72	5723	71	3
4		MML-4	55	5.68	56.8	5631	78	3
5		MML-5	94	5.7	56.72	5727	72	3
6		MML-6	99	5.5	57.2	5458	77	1
7		MML-10718	182	5.8	56.74	5805	92	7
8		MML-4771	118	5.7	56.89	5768	78	3
9		MML-12	97	5.7	56.7	7090	90	7
10		MML-14	105	6.0	56.57	6116	78	4
11		MML-17	97	5.7	56.72	5730	73	3
12	*E. coli*	MML-7	164	5.3	50.4	5493	85	3
13		MML-11229	131	4.9	50.8	4957	78	5
14	*Enterobacter*	MML-8	98	4.9	55.25	4876	76	3
15		MML-8159	88	5.1	55.12	5070	73	2
16		MML-15	22	4.5	55.79	4232	73	4
17	*A. baumannii*	MML-9	88	4.1	38.8	3963	64	3
18		MML-10	76	3.9	38.82	3833	64	2
19		MML-11	177	4.2	38.73	4177	66	6
20		MML-13	99	4.1	38.8	3977	64	3
21		MML-16	122	3.98	38.8	3916	61	0
22		MML-19	200	4.0	39.4	3923	58	0
23	*S. maltophilia*	MML-18	60	4.5	66.6	4226	68	2

Appendix XIV: Predicted Efflux pump genes associated with drug resistance among CR Gram-negative genomes

CR Gram-negatives	Efflux pump
MML-1	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(A), TolC/OpmH*
MML-2	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(A), TolC/OpmH*
MML-3	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(D), TolC/OpmH*
MML-4	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, SugE, Tet(A), TolC/OpmH*
MML-5	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(D), TolC/OpmH*
MML-6	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(A), Tet(B), TolC/OpmH*
MML-10718	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(D), TolC/OpmH*
MML-4771	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(A), TolC/OpmH*
MML-12	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, FexA family, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(B), Tet(D), TolC/OpmH*
MML-14	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(A), TolC/OpmH*
MML-17	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, QacE, SugE, Tet(D), TolC/OpmH*
MML-7	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, CmlA family, EmrAB-TolC, EmrD, EmrKY-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtEF-TolC, MdtL, MdtM, QacE, SugE, TolC/OpmH*
MML-11229	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC, AcrZ, EmrAB-TolC, EmrD, EmrKY-TolC, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtEF-TolC, MdtL, MdtM, QacE, SugE, Tet(B), TolC/OpmH*
MML-8	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, SugE, TolC/OpmH*
MML-8519	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, QacE, SugE, Tet(A), TolC/OpmH*
MML-15	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC , AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, SugE, TolC/OpmH*
MML-9	*EmrAB-TolC, MacA, MacB, MdfA/Cmr, TolC/OpmH*
MML-10	*EmrAB-TolC, MacA, MacB, MdfA/Cmr, Tet(B), TolC/OpmH*
MML-11	*EmrAB-TolC, MacA, MacB, MdfA/Cmr, TolC/OpmH*
MML-13	*EmrAB-TolC, MacA, MacB, MdfA/Cmr, TolC/OpmH*
MML-16	*EmrAB-OMF, EmrAB-TolC, MacA, MacB, MdfA/Cmr, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexHI-OpmD, MexHI-OpmD system, MexPQ-OpmE, MexXY-OMP, OprM/OprM family, QacE, TolC/OpmH*
MML18	*SmeABCDE, EmrAB-OMF, EmrAB-TolC, MacA, MacB, MdtABC-TolC, TolC/OpmH*

Journal of Infection and Chemotherapy 28 (2022) 602–609

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: www.elsevier.com/locate/jic

Original Article

Elucidation of molecular mechanism for colistin resistance among Gram-negative isolates from tertiary care hospitals

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ARTICLE INFO

Keywords:
Colistin resistance
Gram-negatives
Carbapenem-resistance
Molecular determinants
mcr gene
Non-synonymous mutation

ABSTRACT

Antimicrobial resistance is a growing concern of global public health. The emergence of colistin-resistance among carbapenem-resistant (CPR) Gram-negative bacteria causing fear of pan-resistance, treatment failure, and high mortality across the globe.

Aim: To determine the genotypic colistin-resistance mechanisms among colistin-resistant (CR)Gram-negative clinical isolates along with genomic insight into hypermucoviscous(*hv*)-CR-*Klebsiella pneumoniae*.

Methods: Phenotypic colistin-resistance via broth-microdilution method. PCR-based detection of plasmid-mediated colistin resistance genes(*mcr*-1,2,3). Characterization of selected *hv*CR-*K. pneumoniae* via Whole-genome sequencing.

Results: Phenotypic colistin-resistance was 28% among CPR-Gram-negative isolates of which 90% of CR-isolates displayed MDR profile with overall low plasmid-mediated colistin resistance (*mcr*-2 = 9.4%;*mcr*-3 = 6%). Although *K. pneumoniae* isolates showed the highest phenotypic colistin-resistance (51%) however, relatively low plasmid-mediated gene-carriage (*mcr*-2 = 11.5%;*mcr*-3 = 3.4%) pointed toward other mechanisms of colistin-resistance. *mcr*-negative CR-*K. pneumoniae* displaying *hv*-phenotype were subjected to WGS. *In-silico* analysis detected 7-novel mutations in lipid-A modification genes includes *ept*A(I38V; V50L; A135P), *opg*B(M53L; T486A; G2365), and *arn*D(S164P) in addition to several non-synonymous mutations in lipid-A modification genes conferring resistance to colistin. Insertion of 6.6-kb region harboring putative-PEA-encoding gene(*yjgX*) was detected for the first time in *K. pneumoniae* (*hv*CRKP4771). *In-silico* analysis further confirmed the acquisition of not only MDR determinants but several hypervirulent-determinants displaying a convergent phenotype.

Conclusion: overall high prevalence of phenotypic colistin resistance but low *mcr*-gene carriage suggested complex chromosomal mediated resistance mechanism especially in *K. pneumoniae* isolates. The presence of novel mutations in lipid-A modification genes and the acquisition of putative-PEA-encoding gene by *hv*CR-*K. pneumoniae* points toward the role of chromosomal determinants conferring resistance to colistin in the absence of *mcr*-genes.

1. Introduction

Antimicrobial resistance (AMR) is a growing concern of public health globally, as infections caused by Multidrug-resistant (MDR) Gram-negative bacteria restrict treatment choices and has led to the reuse of ancient drug-like Polymyxins [1]. After the development of carbapenem resistance by MDR Gram-negative bacteria revival of Colistin (polymyxin-E) was considered as the last drug in the available antibiotic armament for the treatment of CPR-Gram-negative infections [2]. Despite being potent against the MDR pathogens, the non-judicious use of colistin in agriculture, poultry, veterinary fields, and even in clinical settings has resulted in the development of colistin resistance among pathogens [3]. This widespread use of colistin also contributed to resistance in CPR Gram-negative pathogens via diverse chromosomal

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https://doi.org/10.1016/j.jiac.2022.01.002
Received 30 September 2021; Received in revised form 4 January 2022; Accepted 6 January 2022
Available online 17 January 2022

Short Communication

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Emergence of hypermucoviscous colistin-resistant high-risk convergent *Klebsiella pneumoniae* ST-2096 clone from Pakistan

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Klebsiella pneumoniae convergent clones are considered a threat to healthcare settings. Here we report a comprehensive genomic profiling of an emerging colistin-resistant *K. pneumoniae* ST-2096 convergent clone from Pakistan. **Methods:** Whole-genome sequencing was performed and raw reads were assembled. antimicrobial resistance and virulence genes were predicted using various online tools. **Results & conclusion:** The phenotypically multidrug-resistant (MDR) and hypermucoviscous (hv) colistin-resistant *K. pneumoniae* (hvCRKP-10718), which, intriguingly, possessed a wide range of antimicrobial resistance (bla_{TEM-1A}, bla_{OXA-1}, $bla_{OXA-232}$, $bla_{CTX-M-15}$, $bla_{SHV-106}$, *oqxA*, *oqxB*, *aac(6')-Ib-cr*, *aadA2*, *aac(6')-Ib-cr*, *armA*, *tetD*, *mphE*, *msrE*, *fosA*, *dfrA1*, *dfrA12*, *dfrA14*, *catB3*, *sul1*) and virulence determinants (*RmpA*/*RmpA2*, yersiniabactin [*ybt*], aerobactin [*iuc*/*iut*], enterobactin). Furthermore, the acquisition of various mobile genetic elements (MDR/virulent plasmids, type II integron gene cassette, insertional sequences, transposases) and associated hv capsular type made this MDR/hv isolate a convergent clone belonging to a high-risk lineage (ST-2096). Based on core-genome multilocus sequence typing and single-nucleotide polymorphism analysis, this isolate showed ≥99% nucleotide identity with MDR *K. pneumoniae* isolates from India, depicting its evolutionary background. This study provides a comprehensive genomic profiling of this high-risk convergent *K. pneumoniae* ST-2096 clone from Pakistan. Comparative genomics of MDR/hv colistin-resistant *K. pneumoniae* isolates with other MDR convergent strains from the Indian subcontinent indicated the emergence of this evolving superbug.

First draft submitted: 3 December 2021; Accepted for publication: 14 June 2022; Published online: 00 July 2022

Keywords: colistin resistance • convergent ST-2096 clone • hypervirulent • *Klebsiella pneumoniae*

Klebsiella pneumoniae is a well-known opportunistic human pathogen and one of the most common causative agents of nosocomial infections worldwide. A wide spectrum of infections have been associated with *K. pneumoniae*, including pneumonia, bacteremia, urinary tract infections, meningitis, sepsis, and skin and soft-tissue infections [1,2] Being notorious for multidrug-resistant (MDR) or hypervirulent (hv) traits, it has been added to the critical priority pathogens list by WHO [3]. Multidrug resistance, and especially the carbapenem resistance trait, in *K. pneumoniae* is due to the acquisition of extended-spectrum β-lactamase and carbapenemase determinants and is mostly associated with hospital-acquired infection [4] while the hv or hypermucoviscous trait is associated with the positive string test, presence of *RmpA*/*A2* gene and aerobactin siderophore system genes (*iucA*/*iutA*), restricted to community acquired infections and consider to be susceptible to antibiotics [5]. MDR and hypervirulence were considered as two separate evolutionary lineages but in recent years, the integration of both MDR and hv determinants by *K. pneumoniae* resulted in the emergence of a new and more potent lineage of *K. pneumoniae* with a convergent phenotype (i.e., co-harboring *iucA*/*iutA* plus extended-spectrum β-lactamase and/or carbapenemase gene) [6,7].

Carbapenem-resistant hv *K. pneumoniae* clones are mostly prevalent in Asia, especially in China [8], but have also been reported from other countries, including Argentina [9], UK [10], Italy [11], Canada [12], Russia [13], Germany [14],

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