A genomic approach to understanding the molecular epidemiology and clinical burden of multi-drug resistant Enterobacterale Infections in Bangladesh

by

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Summary

This PhD was the first comprehensive study in South-Asia, investigating epidemiology of AMR in a Bangladeshi health setting by aligning demographic, clinical, and genomic data. Carbapenem-resistant Enterobacterales (CRE) from clinical specimens were 11.1% (210/1893) and carbapenem-sensitive Enterobacterales (CSE) were 22.8% (433/1893). CRE was associated with age (6-25 years), gender, burn unit and ICU patients. Additionally, with patients given levofloxacin, amikacin, clindamycin, and meropenem during hospital stay ($p<0.05$). CRE cases were associated with all-cause in-hospital 30-days mortality (27.8%) than CSE (13.5%) ($p<0.05$). Clinical CRE clustered in particular clonal types compared to CSE e.g. ST167, ST448, ST8346, ST405, and ST648 in *E. coli*, ST16; and ST231, ST11, ST515, and ST23 in *K. pneumoniae* ($p<0.05$). CRE clades were associated with direct clonal transmission in putative outbreak clusters (contained isolates of 0-2 SNPs differences), designated as KP5 (*K. pneumoniae* ST23), KP1 (*K. pneumoniae* ST15), EC9 (*E. coli* ST648), and Eco1 (*E. cloacae* ST113). Plasmid-mediated horizontal transfer of CRE was linked mostly linked to IncFII and IncX3.

CRE faecal carriage was 34.8% (244/700) and significantly higher among inpatients (53.8%) than the outpatients (12%) ($p<0.05$). The clinical and colonisation studies were undertaken about a year apart; however, clusters were found across clinical and faecal isolates (≤20 SNP); these were, EC4 (*E. coli* ST8346), EC6 (*E. coli* ST405), EC7 (*E. coli* ST5954), KP1 (*K. pneumoniae* ST15), and Eco1 (*E. cloacae* ST113).

Additionally, this PhD describes outbreaks at Dhaka Medical College Hospital e.g. an MDR Klebsiella variicola clone (ST771) in neonatal unit from October 2016 to January 2017, associated with high mortality (54.5%), and by *Burkholderia cepacia* ST1578 from burn sepsis cases. This study reported the first human-associated mobile colistin resistance in Bangladesh (*mcr-1* in faecal colonisation and *mcr-8 i*n clinical infections).

Data derived from this study indicate an urgent need of antibiotic stewardship program and standard infection control policy in Bangladeshi hospitals.

Publications and Presentations

Publications resulting from data presented in this thesis:

1. Farzana R, Jones LS, Rahman MA, Andrey DO, Sands K, Portal E, Watkins WJ, Pervin M, Banerjee M, Walsh TR. Outbreak of Hypervirulent Multidrug-resistant *Klebsiella variicola* Causing High Mortality in Neonates in Bangladesh. Clin Infect Dis. 2019;68(7):1225-1227. doi: 10.1093/cid/ciy778.
2. Farzana R, Jones LS, Rahman MA, Toleman MA, Sands K, Portal E, Boostrom I, Kalam MA, Hassan B, Uddin AN, Walsh TR. Emergence of *mcr-1* mediated colistin resistant *Escherichia coli* from a hospitalized patient in Bangladesh. J Infect Dev Ctries. 2019;13(8):773-776. doi: 10.3855/jidc.11541.
3. Farzana R, Jones LS, Rahman MA, Sands K, Portal E, Boostrom I, Kalam MA, Hasan B, Khan A, Walsh TR. Molecular and epidemiological analysis of a *Burkholderia cepacia* sepsis outbreak from a tertiary care hospital in Bangladesh. PLoS Negl Trop Dis. 2020;14(4):e0008200. doi: 10.1371/journal.pntd.0008200.
4. Farzana R, Jones LS, Barratt A, Rahman MA, Sands K, Portal E, Boostrom I, Espina L, Pervin M, Uddin AKMN, Walsh TR. Emergence of Mobile Colistin Resistance (*mcr-8*) in a Highly Successful *Klebsiella pneumoniae* Sequence Type 15 Clone from Clinical Infections in Bangladesh. mSphere. 2020;5(2):e00023-20. doi: 10.1128/mSphere.00023-20.
5. Farzana R, Jones LS, Rahman MA, Sands K, Portal E, Criollo JM, Martyn Guest M, Van-Tonder AJ, Parkhill J, Uddin AN, Hasan B, Ian Boostrom, Walsh TR. Determining the burden of carbapenem resistance in a tertiary public heath setting in Bangladesh: an epidemiological, clinical, and molecular study (manuscript in preparation).
6. Farzana R, Jones LS, Rahman MA, Sands K, Portal E, Criollo JM, Martyn Guest M, Van-Tonder AJ, Parkhill J, Jove T, Walsh TR. Horizontal spread of carbapenem resistance in a Bangladeshi health setting (manuscript in preparation).

Presentations resulting from data presented in this thesis:

1. Farzana R et al. Outbreak of Hypervirulent Multi-Drug Resistant *Klebsiella variicola* Causing High Mortality in Neonates in Bangladesh. Paper poster presentation at ECCMID 2018.
2. Farzana R et al. Epidemiology and impact of carbapenem-resistant *Escherichia coli* in a Bangladeshi clinical setting. Paper poster presentation at ECCMID 2019
3. Farzana R et al. Double blinded study evaluating the GenePOC™ Carba assay and revogene™ instrument for rapid detection of carbapenemase-producing organisms in rectal swab samples. Paper poster presentation at ECCMID 2019.
4. Farzana R et al. The emergence of *bla*$_{\text{NDM-5}}$ positive *Escherichia coli* in a Bangladeshi hospital: linkage between clinical infections and faecal carriage. Paper poster presentation at ICPIC 2019.
5. Farzana R et al. Molecular and epidemiological analysis of a *Burkholderia cepacia* sepsis outbreak from a tertiary care hospital in Bangladesh. Oral presentation at I&I Annual meeting 2019.
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7. Farzana R et al. Emergence of mobile *mcr-8* colistin resistance in *Klebsiella pneumoniae* from clinical infections of hospitalised patients in Bangladesh. Accepted for paper poster presentation at ECCMID 2020 (the meeting was cancelled due to pandemic COVID 19).

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AA	Amino acid
ACT	Artemis comparison tool
AMR	Antimicrobial resistance
ANC	Antenatal care
API	Active pharmaceutical ingredient
ARGs	Antimicrobial resistance genes
AST	Antimicrobial susceptibility testing
Bcc	Burkholderia cepacia complex
BDT	Bangladeshi Taka
BEAST	Bayesian Evolutionary Analysis by Sampling Trees
BFs	Bayes factors
bla	β-lactamase
BSIs	Bloodstream infections
CAI	Community acquired infections
CARD	Comprehensive Antibiotic Resistance Database
CF	Conversion Factor
cfu	Colony forming unit
CGE	Center for Genomic Epidemiology
CLRW	Clinical Laboratory Reagent Water
CPI	Consumer price index
CRE	Carbapenem-resistant Enterobacterales
CSE	Carbapenem-sensitive Enterobacterales
CU	Cardiff University
DAMA	Discharge against medical advice
DHP	Dehydropeptidase
DLS	Department of Livestock Services
DMCH	Dhaka Medical College Hospital
ECOFF	Epidemiological cut-off
EEA	European Economic Area
EOS	Early-onset neonatal sepsis
ERC	Ethical Review Committee
ESBL	Extended-spectrum β-lactamase

EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FF	Fleming Fund
GDP	Gross domestic product
GLASS	Global Antimicrobial Resistance Surveillance System
GTR	General time reversible
GWASs	Genome-wide association studies
HDU	High dependency unit
HGT	Horizontal gene transfer
HICs	High-income countries
HKG	Housekeeping gene
HMM	High molecular mass
IAIs	Intra-abdominal infections
ICU	Intensive care unit
IEDCR	Institute of Epidemiology Disease Control and Research
INR	Indian Rupee
IPC	Infection prevention and control
IR	Inverted repeat
IS	Insertion sequence
LBW	Low-birth weight
LMICs	Low- and middle-income countries
LMM	Low molecular mass
LoS	Length of hospital stay
LOS	Late-onset neonatal sepsis
MBL	Metallo beta-lactamase
MCC	Maximum clade credibility
MCH	Medical College Hospital
MCMC	Markov Chain Monte Carlo
MCRPEC	MCR-1.1-positive E. coli
MCRPKP	MCR-8.1-positive K. pneumoniae
MDR	Multi-drug resistant
MIC	Minimum inhibitory concentration
ML	Maximum likelihood
MLST	Multi-locus sequence typing

MODS	Multiple organ dysfunction syndrome
MOHFW	Ministry of Health and Family Welfare
MRSA	Methicillin-resistant Staphylococcus aureus
n.d.	no date
NAP	National Action Plan
NCC	National Coordinating Centre
NDMs	New Delhi metallo-β-lactamases
NICU	Neonatal intensive care unit
OD	Optical density
OMPs	Outer membrane proteins
OPD	Out-patient department
OXA	Oxacillinase
PBPs	Penicillin-binding proteins
PBRT	PCR-based replicon typing
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PROM	prolonged rupture of the chorioamniotic membrane
RND	Resistance nodulation cell division
RSs	Rectal swabs
SA	South-Asia
SDG	Sustainable development goal
SDS	Sodium dodecyl sulfate
SEA	Southeast Asia
SEAR	SEA region
SNP	Single nucleotide polymorphism
TBSA	Total body surface area
TMRCA	The most recent common ancestor
UNICEF	United Nations Children's Fund
US	United States
UTIs	Urinary tract infections
VAP	Ventilator-associated pneumoniae
VF	Virulence factor
VFDB	Virulence Factor Database
WGS	Whole Genome Sequencing

WHO	World Health Organization
XDR	Extensively drug-resistant

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Section One

General Introduction

1.1 Antimicrobial resistance: A global public health concern

The magnitude of antimicrobial resistance (AMR) as a foremost public health threat in the 21st century has been established on a global scale. The continual evolution of resistance is unexpectedly ominous; propelling us into catastrophic 'post-antibiotic era' as antibiotics underpin modern medicine to dealing not only with simple bacterial sore throat but to performing all aspects of medical procedures from gut surgery to organ transplantation (Hwang and Gums, 2016). World Health Organization (WHO)'s report on 2014 has demonstrated the manifestation of AMR worldwide, even to 'last reserve antibiotics', endorsing no country or region is exempt from the consequences of AMR; however, there is scarcity of data on epidemiology of AMR in WHO African and Southeast Asian (SEA) region and systemic approaches to collect data are yet to be developed. This data reflects the gap in our understanding of the current situation of AMR across developing countries. High prevalence of resistance to 3rd generation cephalosporins indicates the necessity of carbapenem usage in clinical practice which is likely to drive the emergence and dissemination of carbapenem resistance in the health setting, limiting options to manage multi-drug resistant (MDR) infections. Carbapenem-resistant Enterobacterales (CRE) has been highlighted as one of the critical WHO priority pathogens. Rapid emergence of carbapenem resistance leads to invariable usage of tigecycline and colistin - either singularly or in combination with other antibiotics in human infections. Hence, the discovery of plasmid mediated colistin resistance, *mcr-1* in China in 2015 poses a significant public health concern. The MCR-like mechanisms have been shown to be widespread across five continents (WHO, 2017; Liu *et al.,* 2016; Wang *et al.,* 2018a; Durante-Mangoni *et al.,* 2019).

Forecasts predict that deaths due to AMR will reach 10 million lives each year by 2050, which specifies the death of one person in every three seconds; however, it is more likely to be an underestimate due to significant lack of surveillance data from developing countries (O'Neill J, 2016; WHO, 2020a). A recent report estimates that about 33000 people die each year in the European Union (EU) and European Economic Area (EEA) as a direct consequence of MDR infections (Cassini *et al.,* 2019). According to report by Centers for Disease Control and Prevention (CDC), MDR organisms are accountable for the 35,000 annual deaths in the United States (US) (CDC, 2019).

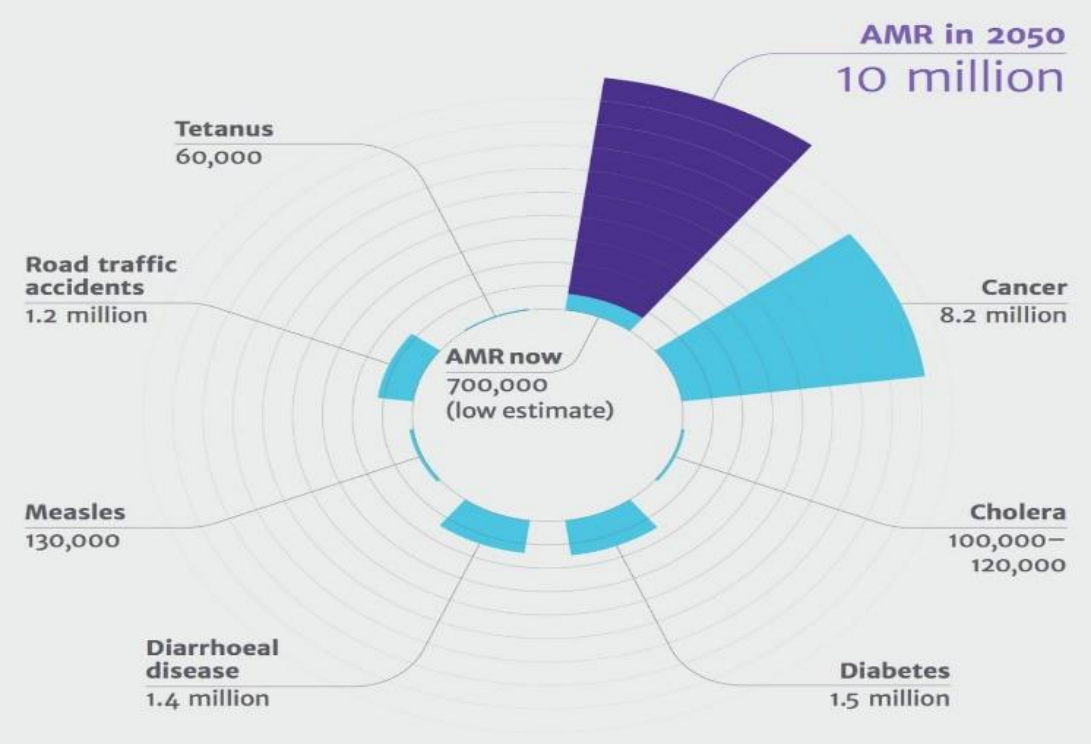


Figure 1.1 Death attributable to AMR by 2050 compared to other disease condition (O'Neill J, 2016).

Further to health risks, AMR would significantly impact on the global economy and is a threat to attaining global sustainable development goals (SDGs). AMR would consume 1.1–3.8% of its gross domestic product (GDP) annually by 2050 which would affect more the Low- and middle-income countries (LMICs). A reduction of 0.1–2.5% in GDP has been anticipated in the sub-Saharan Africa region (The World Bank, 2016). Economic assessment in high-income countries (HICs) calculates the expenditure of excess $2.9 billion only for five pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* in the US (Shrestha *et al,* 2018), and €1.1–1.5 billion for extra healthcare costs and lost productivity in the EU/EEA each year due to AMR (European Commission, 2020). An approximation of 24 million people in the world would face extreme poverty by 2030, particularly in LMICs, if there is no action to combat AMR (The World Bank, 2016).

The recent pandemic COVID-19 caused by severe respiratory syndrome coronavirus 2 (SARSCoV-2) has drawn massive attention globally due to very rapid spread, drastic economic breakdown, and death toll caused by the disease. Given that

pandemic COVID-19 contracts 5.2% of global GDP in 2020. Gross growth downgrades to 7.2% in Latin America; 4.7% Europe and Central Asia, 4.2% in Middle East and North Africa, 2.8% in Sub-Saharan Africa, 2.7% in South-Asia (SA), and 0.5% in East Asia and the Pacific, and tip tens of millions of people back into extreme poverty (The World Bank, 2020a). The number of deaths globally from COVID-19 is 1,099,586 by October 2020, estimated by WHO (WHO, 2020b). The health and economic impact due to this pandemic in nine months period is extremely abrupt and would take years to reverse towards the developmental goals. Nevertheless, the number of deaths from AMR would be 10 million, and the costs would be $300 billion to more than $1 trillion per annum by 2050 which has been predicted to be still higher than the COVID shock (Dadgostar, 2019; Nieuwlaat *et al.,* 2020). Moreover, the vast majority of COVID-19 patients receiving intensive care unit (ICU) care have been prescribed with antibiotics because of difficulties of ruling out bacterial co-infection or secondary bacterial infections which perhaps exacerbates the burden of pre-existing pandemic AMR (Langford *et al.,* 2020).

1.2 Antibiotics experience in the pre-antibiotic era and evolution of AMR

The proof of antibiotic or antibiotic-like properties have been revealed in the ancient practices where naturally available substances such as herbs, honey, animal faeces and mouldy bread were used for the treatment of infections (Gould, 2016). Tetracyclines were traced in the skeleton of ancient Sudanese Nubia dating back to 350–550 CE and Dakhleh Oasis, postulating intake of tetracycline-like materials in their diet which had been documented to be protective against infections. However, only archaic anecdotal or cultural facts provide few evidence of using antibiotic-like properties, for example, use of antibiotic-like properties of red soils for skin infections in Jordan or *Artemisia* plants in Chinese traditional medicine. Given the circumstances, actinomycete bacteria was isolated from these soils, producing actinomycin C2 and actinomycin C3 which are polypeptide antibiotics and anti-malarial drug, qinghaosu (artemisinin) was discovered from *Artemisia* plants in the 1970s. Genomic evolutionary analysis proposed that most of the resistance genes originated billion years ago (Aminov, 2010). Presumably, the primordial resistant gene pool originated from environmental bacterial communities and therefore, diversified and mobilized into genetically distinct bacterial populations including pathogens. The existence of AMR in nature is, therefore, not an event that happened by chance as selective antibiotic pressure and other factors contribute to the spread of AMR (Aminov and Mackie, 2007).

1.3 Discovery of antibiotics and subsequent resistance

Pyocyanase (liberates from *P. aeruginosa*) was the first antibacterial agent which was introduced for human infections based on the observation by Rudolf Emmerich (1856–1914) and Oscar Löw (1844–1941); however, Salvarsan is an arsenic-based chemical, first truly modern antimicrobial agent, discovered by Paul Ehrlich and his team in 1909. Alexander Fleming discovered penicillin in 1928 and Howard Florey (1898–1968) and Ernst Chain (1906–1979) illustrated a purification method, resulting penicillin available for clinical use in the 1940s. During the same time, Klarer (1898–1953) and Mietzsch (1896–1958), synthesized Prontosil in 1932 by combing sulphonamide (was synthetized on 1909) with a dye which was a prodrug and metabolized to sulphonamide and was proved to be effective in treating streptococcal infections. Later the dye component was removed in the commercial sulphonamide. The introduction of penicillin and sulphonamide in medicine was the breakthrough in establishing golden era of antibiotic, saved thousands of lives during World War II (Zaffiri *et al.*, 2012; Gould, 2016; Wikipedia, 2020a; Wikipedia, 2020b). Following the discovery early three antibiotics, the period between 1950s and 1970s was the milestone in the development of new antibiotic classes. Simultaneously, resistance to antimicrobials was becoming apparent following the discovery of each class of drug (Gould, 2016).

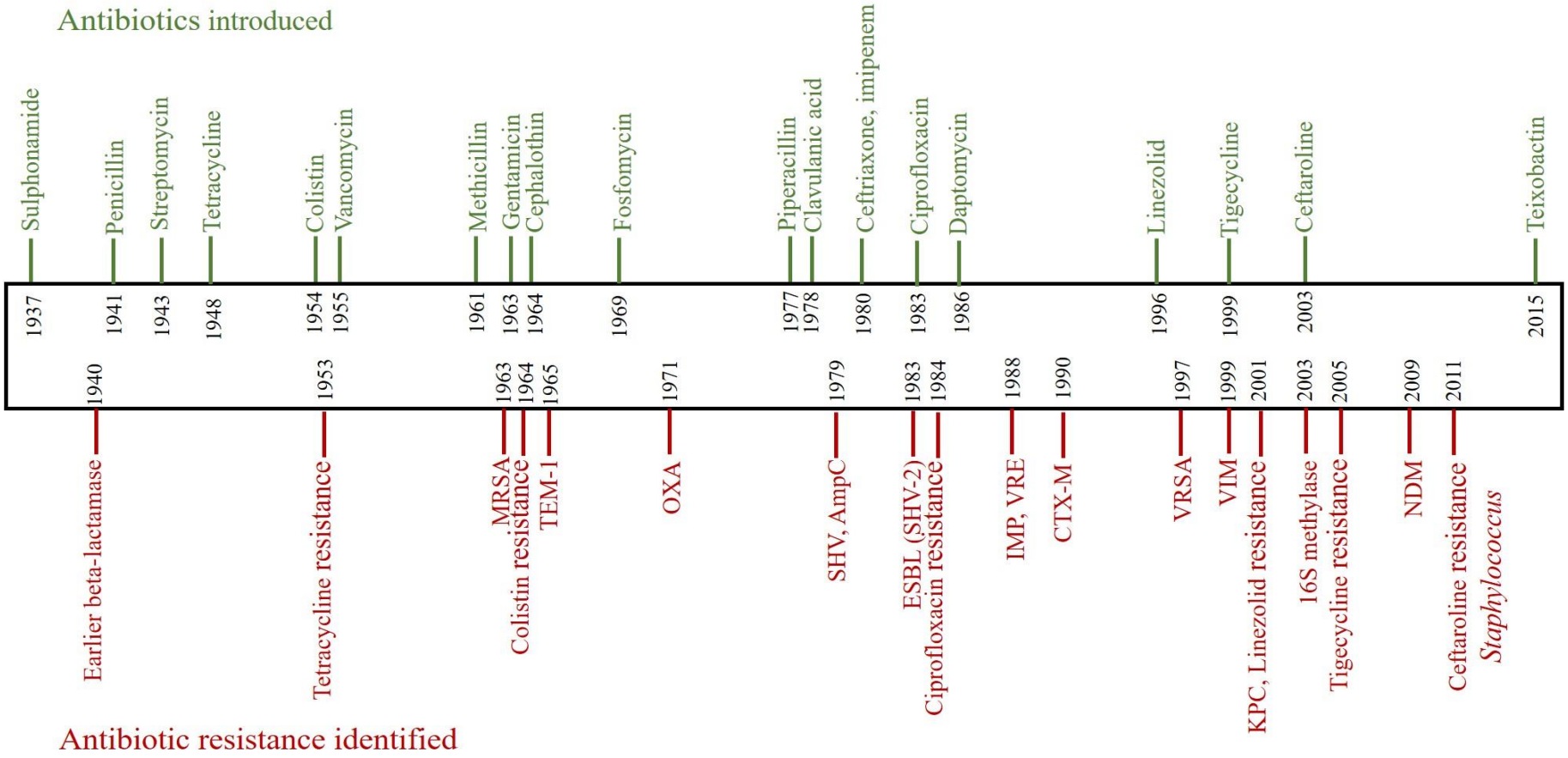


Figure 1.2 The timeline of discovery and resistance of antimicrobials (Zakeri and Wright, 2008; Zaffiri *et al.*, 2012; Gould, 2016; Iredell *et al.*, 2016).

In response to the decline of novel drugs discovery in the 1970s, the mainstream approach to overcome the situation of AMR was to the modification of older classes for the management of infections; however, bacteria have a natural tendency to confer resistance to new drugs through rapid modification of existing resistance mechanisms or the lateral transfer of acquired resistance mechanisms. AMR has now become a complex problem, resulting significant health and economic burden for a nation. Policy makers, national stakeholders, constitutional bodies including clinical microbiologists and the public need to be involved to tackle the situation (Aminov and Mackie, 2007; Aminov, 2010; WHO, 2015).

1.4 Mechanism of AMR

1.4.1 Genetic basis of AMR

Bacteria have remarkable genetic plasticity, allowing them to alter gene and/or protein expression because of exposure to an environmental trigger, e.g., stress, nutrient conditions, growth state, and subinhibitory levels of the antibiotics themselves. Bacteria have two major genetic strategies to adapt in adverse conditions: 1. mutation 2. horizontal gene transfer (HGT). Once a resistant mutant emerges, the antibiotic eliminates the susceptible population and the resistant bacteria predominate, though often leading to decreased bacterial fitness. Mutation results in AMR due to either modifications of the antimicrobials' target or transport system in the cell membrane controlling drug uptake, or resistance due to cell adaptation. HGT is the most significant driver of evolution and dissemination AMR genes among bacterial populations. The main three strategies of HGT include transformation (incorporation of naked DNA), transduction (phage mediated), and conjugation (by "sex" pili) (Munita and Arias, 2016; Kapoor *et al.*, 2017).

1.4.2 Mechanistic basis of AMR

There are four major mechanisms that mediate resistance to antimicrobial agents: including 1. bacterial production of drug inactivating enzymes 2. modification of drug binding sites 3. reduce permeability of drug to bacterium (e.g. porin loss) or export of drug by 'efflux pump' or 'MDR pump' 4. global cell adaptations. AMR due to efflux of antibiotics either due to mutations in chromosomally encoded regulatory genes or the acquisition of plasmid mediated genes causing overexpression of 'MDR' efflux pump and/or drug-specific efflux pumps encoded by plasmids (Poole, 2007; Li *et al.*, 2015; Munita and Arias, 2016; Kapoor *et al.*, 2017).

1.5 AMR and bacterial production of β-lactamase

β-lactam antibiotics are the most widely prescribed antimicrobials classes worldwide. β-lactamases are the enzymes that inactivate the action of β-lactams by hydrolysing the β-lactam ring. Based on the sequence analysis, β-lactamases, and the penicillin-binding proteins (PBPs) are believed to diverge from a common ancestor. All PBPs possess β-lactam catalysing capability to a smaller extent. To date, four classes of β-lactamases are recognized (A-D), correlating with the functional classification. Classes A, C, and D act by a serine based mechanism, whereas class B or metallo-β-lactamases (MBLs) need zinc for their action (Kong *et al.*, 2010; Bush, 2018). Classifications of antibiotics and β-lactamase are described in appendices (appendix A, and appendix B).

The first β-lactamase, capable to destroy antibiotics of penicillin family was described in 1940 by Edward Abraham and Ernst Chain (Davies and Davies, 2010). Many genera of bacteria possessing chromosomal β-lactamase, supposed to be owned by selective pressure of environmental β-lactam producing soil organisms. The earliest transferrable β-lactamase in Gram-negatives was the TEM-1 in *E. coli*, named after the patient Temoniera in Greece from whom it was recovered in 1965. Hitherto, this enzyme was found to be distributed worldwide also into the other species. Another β-lactamase, SHV-1 (for sulphydryl variable) was instigated as a chromosomal gene in *Klebsiella* spp. and was later incorporated into plasmid and therefore disseminated into other species. These enzymes did not confer resistance to oxyimino-cephalosporins (Bradford, 2001; Shaikh *et al.*, 2015).

1.6 Penicillin-binding proteins

PBPs are transpeptidases or carboxypeptidases, involved in peptidoglycan biosynthesis (Figure 1.3). PBPs have been divided into two main categories, the high molecular mass PBPs and the low molecular mass PBPs. Generally, HMM PBPs have transpeptidase and glycosyltransferase activity. To illustrate the function LMM PBPs, the PBPs of *E. coli* have been used as an example. LMM PBPs of *E. coli* are involved in cell separation, peptidoglycan maturation or recycling. PBP4 and PBP7 have endopeptidases activity, cleaving cross-bridges between two glycan strands. PBP5, PBP6, and PBP6b have carboxypeptidase activities, of which PBP5 are the most abundant, cleaving the terminal d-Ala-d-Ala bond, and making the stem peptide unavailable for transpeptidation (Sauvage *et al.*, 2008).

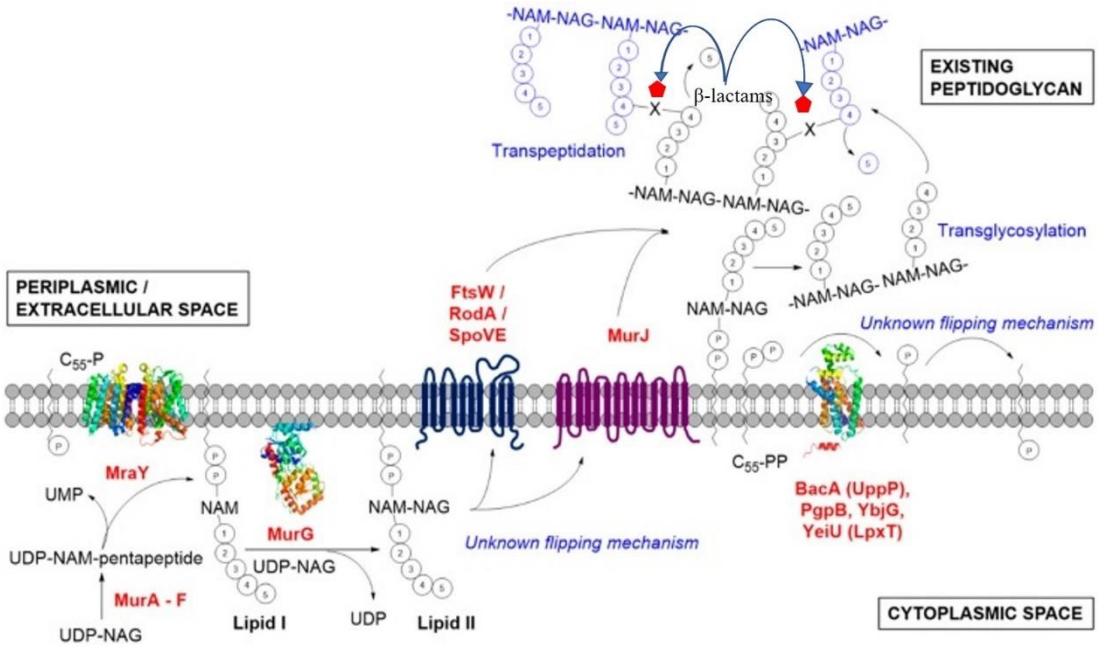


Figure 1.3 Peptidoglycan biosynthesis. Reproduced from (Teo *et al.*, 2015) with permission. i. Conversion of UDP-N-acetylglucosamine (UDP-GlcNAc) to UDP-N-acetylmuramic acid (UDP-MurNAc) by MurA and MurB ii. Conversion of UDP-MurNAc to UDP-MurNAc-pentapeptide by MurC to MurF iii. Adding of soluble precursor to the membrane-bound undecaprenyl phosphate (C55-P) carrier lipid MraY to form Lipid I. iv. Lipid I is then glycosaminylated by a membrane-associated glycosyltransferase, MurG, with UDP-GlcNAc to yield Lipid II v. Lipid II is translocated through the cytoplasmic membrane vi. Lipid II is polymerised by glycosyltransferase enzymes vii. Peptide cross linking by transpeptidases.

1.7 Extended-spectrum-β-lactamases

Generally extended-spectrum β-lactamases (ESBLs) are the β-lactamases, capable to hydrolyse penicillin, first-, second- and third-generation cephalosporins and monobactams (but not cephamycins or carbapenems) and inhibited by β-lactamase inhibitors, has been attained attention in the scientific community and clinicians since the first report of SHV-2, derived from point mutation of SHV-1, found in a single strain of *Klebsiella ozaenae* in Germany in1983. TEM-3, variants of TEM-1 has reported as ESBL phenotype in 1989, settled by substitution of one amino acid (AA), though various subtle alterations of AAs have been observed in other TEM derivatives belongs to ESBLs. As of August 2020, there are 92 TEM-type and 45 SHV-type ESBLs have reported (Naas *et al.*, 2017). Another growing family of ESBL are OXAs, having high hydrolysing capacity against oxacillin and cloxacillin which were devised due to mutations of OXA-10 and OXA-2. Whilst OXAs are mostly found in *P. aeruginosa*, other sorts of ESBLs are predominant among the *E. coli, K. pneumoniae* and other Enterobacterales (Bradford, 2001; Shah *et al.*, 2004). Over the last decades, CTX-M being the most prevalent ESBL worldwide (Bevan *et al.*, 2017).

Owing to preferential hydrolysing capacity against cefotaxime than ceftazidime, the enzyme was named as CTX-M, and 'M' was derived from Munich from where $bla_{\text{CTX-M}}$ was first reported. This resistance mechanism has been reported simultaneously in Europe and South America at the early 1989 and by 2000, worldwide dissemination of the members of this phenotype have been recognised. Despite the initial evidence among *E. coli, K. pneumoniae* and *Salmonella* spp., rapid emergence of this enzyme into other *Enterobacteriaceae* and rarely to non-fermenters has been reported. The gene, $bla_{\text{CTX-M}}$ originated from chromosomal *bla* gene of *Kluyvera* species mainly by insertion sequence (IS) element and therefore evolved into five clusters of CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 and diverted into new variants. Different IS elements (IS*Ecp*1, IS*CR*1, IS*10*, IS*26*) have been described associated with $bla_{\text{CTX-M}}$ genes and contributing to their over-expression, selection and further sequestration and dissemination of the genes. The most intensely diffused member of this family, CTX-M-15 that belongs to CTX-M-1 cluster, is an ESBLs has been found to be associated with IS*Ecp*1. Further to incorporation into incompatibility group FII plasmids and dispersion into high-risk

international clone ST131, *bla*$_{\text{CTX-M-15}}$ have been distributed globally (Cantón *et al.*, 2012; Lartigue *et al.*, 2004; Toleman *et al.*, 2006; Bevan *et al.*, 2017).

1.8 Carbapenemases

1.8.1 Discovery and introduction of carbapenem in clinical practice

Emergence of β-lactamase in the late 1960s limited the therapeutic use of earliest penicillin. The first β-lactam possessing a "carbapenem backbone" was olivanic acids, discovered in 1970s from *Streptomyces clavuligerus*. Within a few years' interval, series of carbapenems were discovered, of which a naturally derived product of *Streptomyces cattleya*, thienamycin was the most potent. However, both olivanic acids and thienamycin were not adopted for medical application because of their chemical instability. Series of clinically effective carbapenems were devised during 1970s and 1980s (Birnbaum *et al.,* 1985; Bush and Bradford, 2016). Imipenem/cilastatin was the first carbapenem, approved for medical use on 1985 (Lyon, 1985).

1.8.2 Antibacterial spectrum and mechanism of action of carbapenem

β-lactam antibiotics contain a β-lactam ring in their structure. A β-lactam ring is a four-membered cyclic amide (Tahlan and Jensen, 2013). Carbapenems have four-membered β-lactam structure fused to a novel five-membered ring in which carbon rather than sulphur was present at the C-1 position, a double bond between C-2 and C-3 and hydroxyethyl side chain. The structural complexity of carbapenems compared to penicillins and cephalosporins results in increased antimicrobial spectrum of these drugs. The stability of earlier carbapenem (thienamycin) was achieved by adding the *N*-formimidoyl group to the 2-position, resulting in imipenem. Imipenem is broad spectrum antibiotic, having effects on Gram-positive, Gram-negative, non-fermentative, and anaerobic bacteria, however, is required to administer with human renal dehydropeptidase (DHP) inhibitor such as cilastatin because of its liability by DHP. Later carbapenems (meropenem, biapenem, ertapenem, and doripenem) DHP stable due to 1-β-methyl group in their structure. Several modifications of carbapenems were made in subsequent two decades which introduced novel carbapenems. The novel carbapenems included antipseudomonal carbapenems, anti-MRSA carbapenems (i.e., cationic and dithiocarbamate carbapenems), orally available carbapenems, trinem carbapenems, a dual quinolonyl-carbapenem, and others (Birnbaum *et al.,* 1985; Bush and Bradford, 2016). Carbapenems act on peptidoglycan

biosynthesis like other β-lactam antibiotics. The mechanism of action of β-lactams are depicted in Figure 1.4.

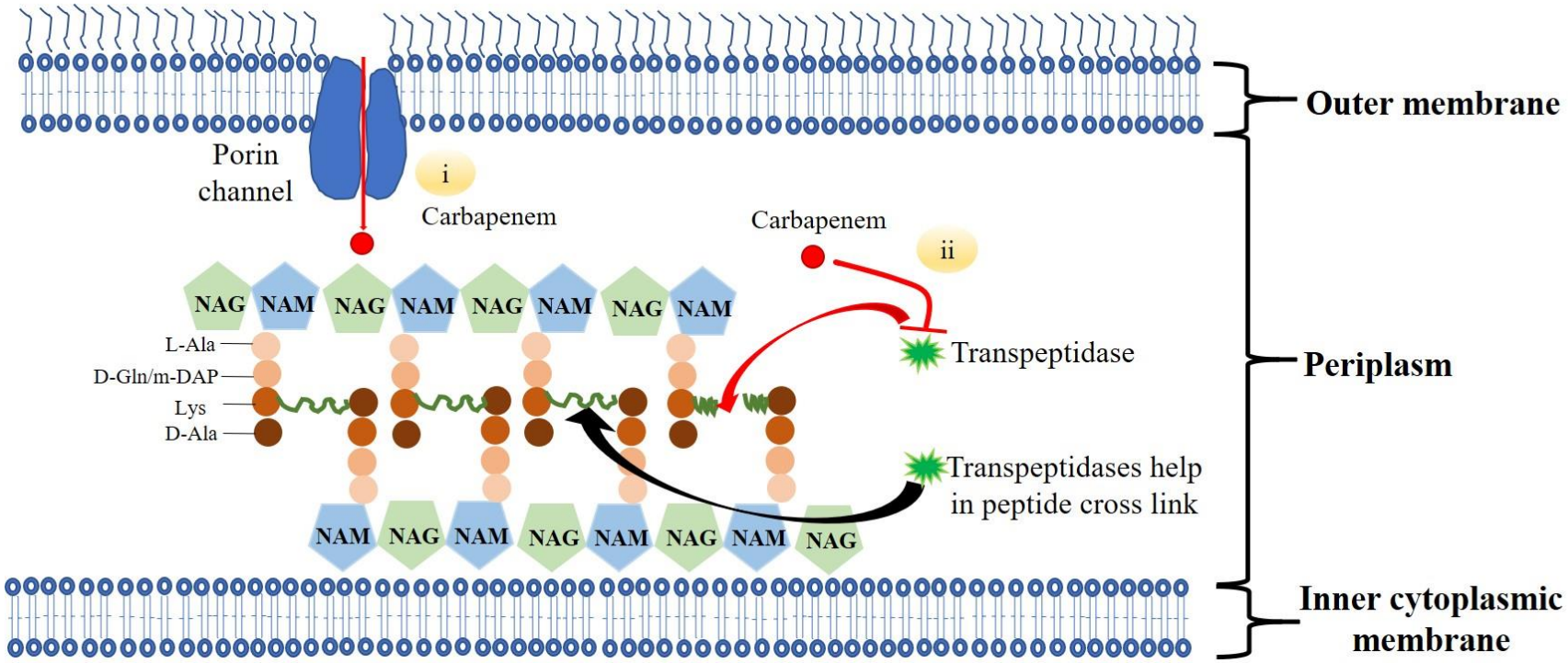


Figure 1.4 Mechanism of action of carbapenems (Birnbaum *et al.*, 1985; Bush and Bradford, 2016). i. Carbapenems enter into the Gram-negative bacteria through outer membrane proteins (OMPs), called porins ii. In the periplasmic space, carbapenems inhibit transpeptidases and therefore, prevent peptide cross-linking.

1.8.3 Carbapenem Resistance: An Overview

Carbapenemases denote as member of β-lactamases, having capacity to degrade carbapenems. Although termed as "carbapenemases," many of these enzymes hydrolyse almost all β-lactams, and most are not inhibited by all commercially viable β-lactamase inhibitors. The substrate specificity of individual carbapenemase is described in Table 1.1. The first carbapenemases were characterized in the 1980s from the environmental strains, *Bacillus cereus* (BCII), *Bacteroides fragilis* (CfiA) and *Stenotrophomonas maltophilia* (L1). Carbapenemases were initially reported as chromosomally encoded β-lactamases. The magnitude of carbapenem resistance was exaggerated in medicine when the horizontal spread took place (Queenan and Bush, 2007; Walsh, 2010; Codjoe and Donkor, 2017). The carbapenemases, New Delhi metallo-β-lactamases (NDMs) and the oxacillinase, OXA-48-like have been addressed here in details as these resistance mechanisms are contextually vital in the regions of SA (Hsu *et al.*, 2017) and especially pertinent to this PhD.

Table 1.1 Classification of carbapenemases.

Molecular class	Functional group	Enzymes	Penicillins	Early cephalosporins	Extended-spectrum cephalosporins	Aztreonam	Carbapenems
A	2f	NMC	+	+	+	+	+
		IMI	+	+	+	+	+
		SME	+	+	±	+	+
		KPC	+	+	+	+	+
		GES	+	+	+	-	±
B	3	IMP	+	+	+	-	+
		VIM	+	+	+	-	+
		GIM	+	+	+	-	+
		SPM	+	+	+	-	+
		NDM	+	+	+	-	+
D	2d	OXA	+	+	±	-	±

1.8.4 Metallo-β-lactamases

MBLs include diverse range of enzymes with less than 25% sequence homology between some enzymes, can be subdivided into subclasses B1, B2 and B3. B1 (appendix B). MBLs have a characteristic αβ/βα fold which supports up to six residues at the active site which coordinate either one or two zinc ions that are central to the catalytic mechanism. The zinc binding motifs around the active site can distinguish the subclasses of MBLs. B1 has two zinc binding sites, Zn1 and Zn2, contains H116, H118, and H196 at Zn1 and D120-C221-H263 at Zn2, respectively, while the zinc ligands for the other subclasses are N116-H118-H196 and D120-C221-H263 for B2 and H/G116-H118-H196 and D120-H121-H262 for B3 (Figure 1.5). B1 and B3 are most active with binding of two zinc ions at Zn1 and Zn2 but binding of the second zinc in the B2 enzymes inhibits catalysis. The degrading effect of B1 and B3 is broad-spectrum such as penicillins, cephalosporins, and carbapenems while the B2 enzymes can only degrade carbapenems (Palzkill, 2013; Sawa *et al.*, 2020).

β-lactam ring is cleaved by B1 and B2 enzymes via attack of a hydroxide ion on the carbonyl carbon. The hydroxide ion is stabilized by Zn1 and Zn2 and resides between the metal ions to attack the carbonyl carbon. Enzyme catalysis initiates with the binding of carbonyl oxygen of β-lactam ring with Zn1 and the carboxyl group on the 5- or 6-membered fused ring of β-lactam with Zn2 (Figure 1.5) (Palzkill, 2013; Sawa *et al.*, 2020).

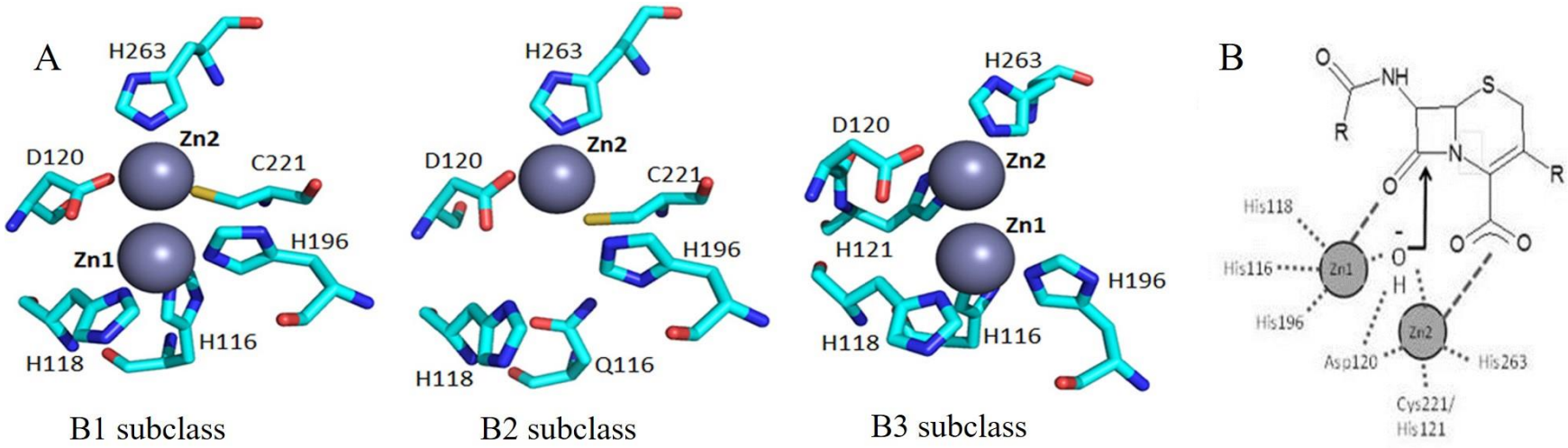


Figure 1.5 Structure of metallo β-lactamases and their binding sites with β-lactam antibiotics. **A.** Schematic layout of the amino acid residues that serve as zinc binders in the active sites of subclass B1, B2, and B3 metallo-β-lactamases. **B.** Schematic layout of binding of β-lactam with the active site of β-lactamase. The Figure has been reproduced (Palzkill, 2013).

1.8.5 New Delhi metallo-β-lactamase

1.8.5.1 Discovery

NDM was first reported in 2009 in a *K. pneumoniae,* and *E. coli* from a Swedish patient of Indian origin who had a history of hospitalisation in India (Yong *et al.,* 2009). Literature reviews following the initial characterization of $bla_{\text{NDM-1}}$ reveal that resistance had a strong link with the Indian subcontinent and $bla_{\text{NDM-1}}$ had been widespread in India, Pakistan, and Bangladesh around the year of its first isolation. SENTRY Antimicrobial Surveillance Program, 2006-2007 indicated that $bla_{\text{NDM-1}}$ had been circulating in India since 2006 (Castanheira *et al.,* 2011). However, NDM-producers were reported from the Balkan states, the Middle East and China without any connection to Indian subcontinent. It was speculated that the Balkan states and the Middle East might the secondary source of global NDM dissemination (Kumarasamy *et al.,* 2010; Nordmann *et al.,* 2011; Farzana *et al.,* 2013; Islam *et al.,* 2013; Khan *et al.,* 2017; Dadashi *et al.,* 2019).

1.8.5.2 Epidemiology and spread of bla_{NDM}

Since the discovery of bla_{NDM}, it has been disseminated worldwide rapidly from the early geographical settings. The global distribution of NDM-producers is depicted in Figure 1.6. The spread of bla_{NDM} is not either associated with a specific clone or specific plasmid background. The gene, bla_{NDM} can be located either on the chromosome or on plasmids. The enzyme, NDM has been predominantly found among the species of Enterobacterales such as *K. pneumoniae*, *E. coli*, *Enterobacter cloacae*; however, wide range of bacterial families, Aeromonadaceae, Alcaligenaceae, Cardiobacteriaceae, Enterobacterales, Moraxellaceae, Morganellaceae, Neisseriaceae, Pseudomonadaceae, Shewanellaceae, Vibrionaceae, and Xanthomonadaceae have been found to be the reservoir for $bla_{\text{NDM-1}}$ and its variants, often in association with wide varieties of plasmid replicon types (IncC, IncB/O/K/Z, IncFIA, IncFIB, IncFIC, IncFIII, IncHI1, IncHI2, IncHI3, IncN, IncN2, IncL/M, IncP, IncR, IncT, IncX1, IncX3, IncX4, IncY, and ColE10), however, the gene mostly belonged to IncFII, IncC, and IncX3. The plasmids having bla_{NDM} usually harbour the determinants of other antimicrobial resistance genes (ARGs), confer resistance to quaternary ammonium compounds, aminoglycosides, sulphonamides, and trimethoprim. The gene, bla_{NDM} are generally located in a plasmid carrying multiple ARGs (*aadA*, *dfrA*, *mphA*, *APH,*

AAC(6')-Ib-cr, *bla*$_{OXA}$, *bla*$_{TEM}$). In the recent years, IncX3 appears to be one of the common types of plasmid of low molecular weight (~46 kb), carrying different variants of *bla*$_{NDM}$ without the association of other ARGs, retrieved from diverse host scale including human, animal and environment. Genetic environment around the *bla*$_{NDM}$ revealed the presence of complete or truncated IS, IS*Aba125* at the 5' end and *ble*$_{MBL}$ gene (bleomycin resistance gene) at the 3' end. A composite transposon, Tn*125* formed by two copies of IS*Aba125* along with *bla*$_{NDM}$ was described in *A. baumannii* and perhaps transferred to the species of Enterobacterales through insertion of truncated Tn*125* (IS*Aba12*5-*bla*$_{NDM}$-*ble*$_{MBL}$). Other IS elements, including IS*Kpn14*, IS*26*, IS*5*, IS*CR1* or Tn*3*-like elements, which have been also identified in Enterobacterales, involving the dissemination of *bla*$_{NDM}$ through a combination of transposition and homologous recombination. Additionally, certain clonal lineages such as *E. coli* ST167, *E. coli* ST617, *K. pneumoniae* ST11, *Enterobacter hormaechei* ST78, *E. hormaechei* ST114, *E. xiangfangensis* ST171, *A. baumannii* ST85 and *P. aeruginosa* ST308 have been emerged as high-risk clones for the spread of *bla*$_{NDM}$. (Dortet *et al.,* 2014; Marquez-Ortiz *et al.,* 2017; Khan *et al.,* 2017; Choudhury *et al.,* 2018; Peirano *et al.,* 2018; Tang *et al.,* 2019; Wu *et al.,* 2019). The geographical distributions of predominant clones of *E. coli,* and *K. pneumoniae* harbouring *bla*$_{NDM}$ are described in Figure 1.7 and Figure 1.8. The distributions have been assessed by analysing genomes harbouring *bla*$_{NDM}$ in NCBI archive by 31 July 2020. The genome attributes are mentioned in appendix C.

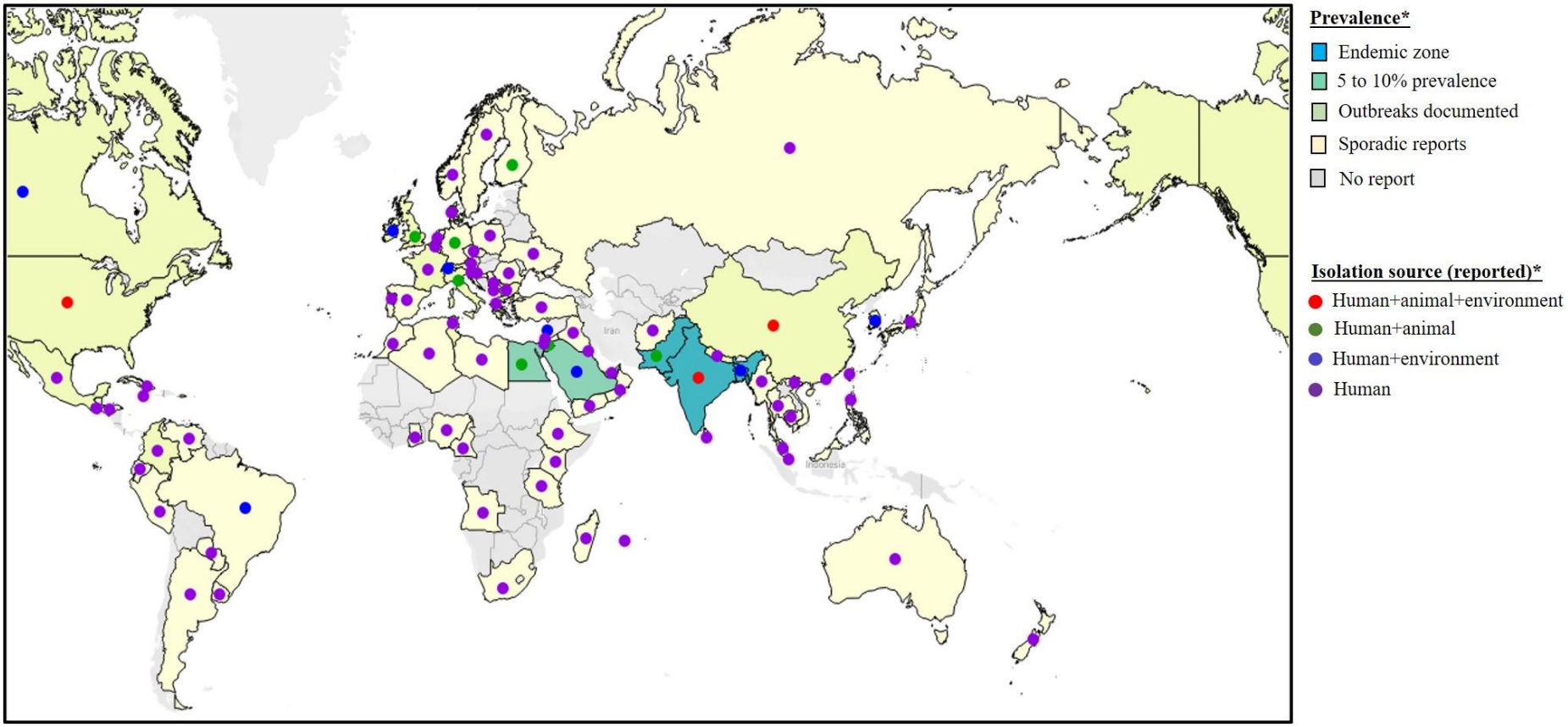


Figure 1.6 Global distribution of NDM producers. *Map has been generated based on published reports and relevant metadata available in NCBI until 31 July 2020.

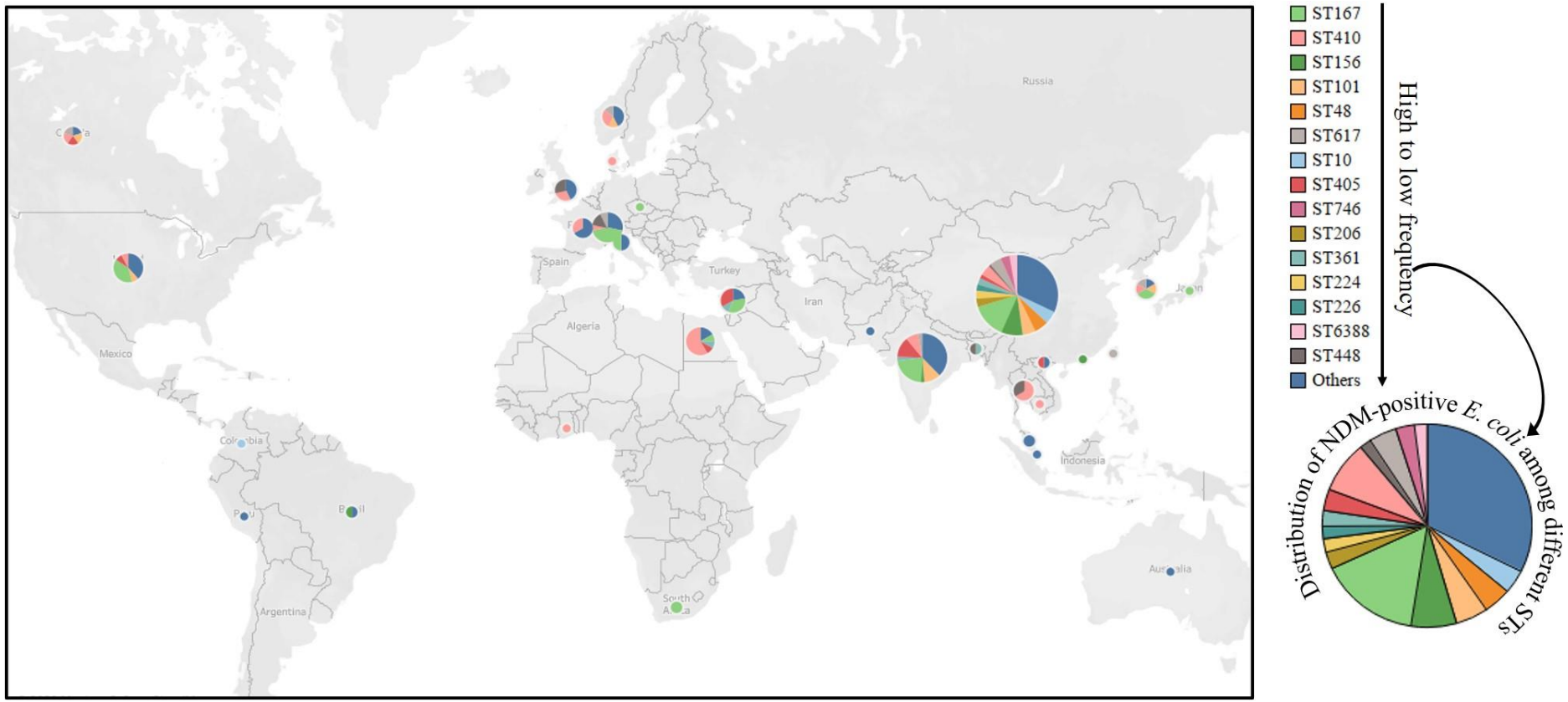


Figure 1.7 Geographical distribution of predominant clones of *E. coli* harbouring bla_{NDM} based on *E. coli* genomes in NCBI archive until 31 July 2020 (n=575). Size of the pie charts is proportional to the number of genomes. The number of whole genome sequence data for NDM-positive *E. coli* in NCBI as per isolation from different geographical locations was: China (n=410), India (n=37), Switzerland (n=14), US (n=13), Egypt (n=12), Lebanon (n=9), Norway (n=7), UK (n=7), France (n=6), South Korea (n=6), Thailand (n=6), Canada (n=5), Italy (n=4), Bangladesh (n=2),

Brazil (n=2), Malaysia (n=2), South Africa (n=2), Vietnam (n=2), Australia (n=1), Cambodia (n=1), Colombia (n=1), Czech Republic (n=1), Denmark (n=1), Ghana (n=1), Hong Kong (n=1), Japan (n=1), Pakistan (n=1), Peru (n=1), Singapore (n=1), Taiwan (n=1). Country data are not available for 17 genomes.

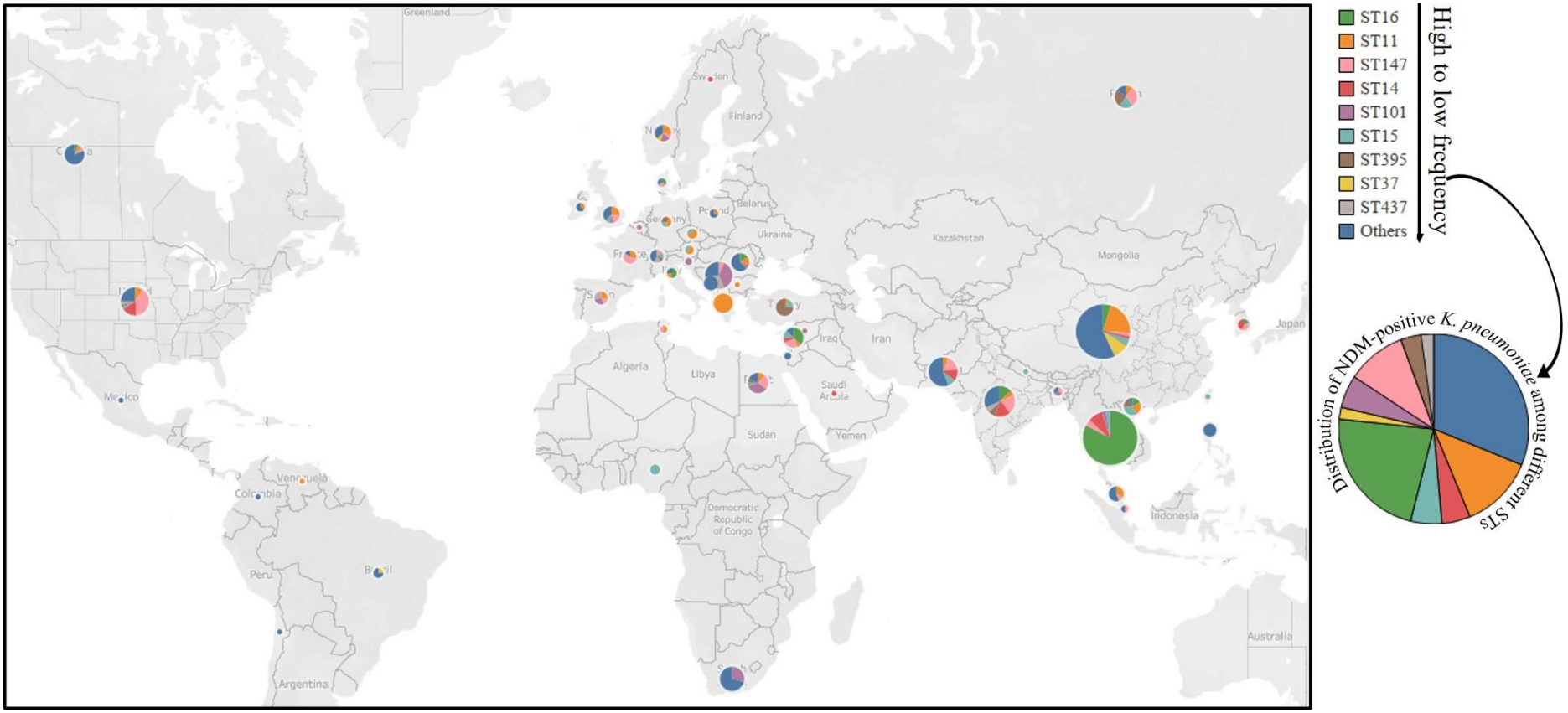


Figure 1.8 Geographical distribution of predominant clones of *K. pneumoniae* harbouring bla_{NDM} based on *K. pneumoniae* genomes in NCBI archive until 31 July 2020 (n=735). Size of the pie charts is proportional to the number of genomes. The number of whole genome sequence data for NDM-positive *K. pneumoniae* in NCBI as per isolation from different geographical locations was: Thailand (n=161), China (n=133), India (n=38), Pakistan (n=34), US (n=32), Serbia (n=30), South Africa (n=24), Russia (n=20), Egypt (n=17), Canada (n=16), Lebanon (n=16), Greece

(n=15), Romania (n=13), Turkey (n=12), Vietnam (n=12), Norway (n=11), UK (n=11), Malaysia (n=9), Montenegro (n=8), France (n=7), Philippines (n=7), Spain (n=7), Switzerland (n=7), South Korea (n=5), Brazil (n=4), Czech Republic (n=4), Germany (n=4), Italy (n=4), Nigeria (n=4), Austria (n=3), Bangladesh (n=3), Denmark (n=3), Ireland (n=3), Poland (n=3), Israel (n=2), Singapore (n=2), Slovenia (n=2), Tunisia (n=2), Belgium (n=1), Bulgaria (n=1), Chile (n=1), Colombia (n=1), Mexico (n=1), Nepal (n=1), Saudi Arabia (n=1), Sweden (n=1), Syria (n=1), Taiwan (n=1), Venezuela (n=1). Country data are not available for 36 genomes.

1.8.5.3 Variants of *bla*$_{NDM}$

NDM enzymes are composed of 813 nucleotides, resulting 270 AAs. Till date, 28 variants of *bla*$_{NDM}$ have been reported which evolved from AA substitutions of *bla*$_{NDM}$. The key properties of *bla*$_{NDM}$ variants with relevant references are summarized in Table 1.2.

Table 1.2 Key properties of variants of *bla*$_{NDM}$.

NDM-1 variants	AA substitutions*	Country & Year of isolation	Geographical distribution	Host species	Location of gene	Plasmid Inc group	Associated mobile element	References
NDM-2	Pro28Ala	Egypt, 2009	Colombia, Egypt, Israel, Switzerland, UAE	*A. baumannii*, *Acinetobacter nosocomialis*, *E. coli*	Cr	-	IS*Aba125*	Espinal *et al.*, 2011; Kaase *et al.*, 2011; Ghazawi *et al.*, 2012; Espinal *et al.*, 2013
NDM-3	Asp95Asn	Japan, 2013	China, Japan	*E. coli*, *K. pneumoniae*	Pl	Un	*tnpA*	Tada *et al.*, 2014a; Hu *et al.*, 2017
NDM-4	Met154Leu	India, 2010	India, China, France, Malaysia, Thailand, Vietnam	*Enterobacter aerogenes*, *E. coli*, *K. pneumoniae*,	Pl	IncF, IncX3, IncFIA	IS*Aba125*, IS*26*	Nordmann *et al*, 2012; Khalifa *et al.*, 2016; Qin *et al.*, 2016; Ahmad *et al.*, 2018; Choudhury *et al.*, 2018; Zhang *et al.*, 2018
NDM-5	Val88Leu Met154Leu	UK, 2011	Algeria, Bangladesh, Canada, Cambodia, Chad, China, Czech Republic, Denmark, Egypt, Finland, France, Japan, Lebanon, Malaysia, Myanmar, Nigeria, Pakistan, Soudi Arabia, South Korea, Switzerland, Thailand, UK, USA	*Citrobacter freundii*, *E. cloacae*, *E. coli*, *E. hormaechei K. pneumoniae*, *Klebsiella quasipneumoniae*, *Proteus mirabilis*, *Salmonella enterica*	Cr, Pl	IncFIA/IncFIB, IncFII, IncX3	IS*Aba125*, IS*5*	Hornsey *et al.*, 2011; Balm *et al.*, 2013a; Nakano *et al.*, 2014; Rahman *et al.*, 2014; Cho *et al.*, 2015; Yousfi *et al.*, 2015; Gamal *et al.*, 2016; Khalifa *et al.*, 2016; Soliman *et al.*, 2016; Zhang *et al.*, 2016; Zhu *et al.*, 2016; Almakki *et al.*, 2017; Rojas *et al.*, 2017; Abd El Ghany *et al.*, 2018; Ahmad *et al.*, 2018; Grönthal *et al.*, 2018;

								Li *et al.*, 2018a; Shen *et al.*, 2018; Uchida *et al.*, 2018; Ahn *et al.*, 2019; Baek *et al.*, 2019; Brinkac *et al.*, 2019; Ouchar Mahamat *et al.*, 2019; Sekizuka *et al.*, 2019; Tang *et al.*, 2019; Cole *et al.*, 2020; Flerlage *et al.*, 2020; Hong *et al.*, 2020; Tian *et al.*, 2020; Zou *et al.*, 2020
NDM-6	Ala233Val	New Zealand, 2012	China, Guatemala, India, Iran, New Zealand, USA	*E. coli*, *E. hormaechei*, *K. pneumoniae*	Pl	IncA/C IncF	NC	Williamson *et al.*, 2012; Rahman *et al.*, 2014; Bahramian *et al.*, 2019
NDM-7	Asp130Asn Met154Leu	France, 2008	Arabian Peninsula, Canada, China, France, Gabon, Germany, India, Norway, Pakistan, South Korea, UK, USA	*E. coli*, *K. pneumoniae*	Pl	IncX3 IncR,	IS*Aba125*	Cuzon *et al.*, 2013; Göttig *et al.*, 2013; Lee *et al.*, 2014; Rahman *et al.*, 2014; Moussounda *et al.*, 2017; Pál *et al.*, 2017; Hao *et al.*, 2018; Qamar *et al.*, 2019; Xu *et al.*, 2019
NDM-8	Asp130Gly Met154Leu	Nepal, 2013	Nepal	*E. coli*	NC	-	-	Tada *et al.*, 2013
NDM-9	Glu152Lys	China, 2013	China, South Korea, Switzerland, Taiwan	*Cronobacter sakazakii*, *E. aerogenes*, *E. coli*, *K. pneumoniae*, *Klebsiella variicola*, *S. enterica*	Pl	IncH IncFII(Y) IncB/O	IS*Aba125*, IS*15*, IS*26*	Wang *et al.*, 2014; Di *et al.*, 2017; Lai *et al.*, 2017; Liu *et al.*, 2017a
NDM-10	Arg32Ser	India, 2013	India	*K. pneumoniae*	Pl	IncFII	NC	Khajuria *et al.*, 2016

	Gly36Asp Gly69Ser Ala74Thr Gly200Arg							
NDM-11	Met154Val	India, 2013	China, India	*E. coli, K. pneumoniae*	Pl	IncF	NC	Rahman *et al.*, 2018
NDM-12	Gly222Asp Met154Leu	Nepal, 2013	Nepal, South Africa	*E. coli*	Pl	IncF	*tnpA*	Tada *et al.*, 2014b
NDM-13	Asp95Asn Met154Leu	Nepal, 2013	China, Nepal, South Korea	*E. coli*	Cr, Pl	IncFIB Inx3	IS*Aba125*	Shrestha *et al.*, 2015; Lv *et al.*, 2016; Kim *et al.*, 2019
NDM-14	Asp130Gly	China, 2011	China	*Acinetobacter lwoffii*	Pl	Un	IS*Aba125*	Zou *et al.*, 2015
NDM-15**	Ala233Val Met154Leu	India, NA	India	*E. coli*	NC	-	-	
NDM-16	Arg264His	Russia, 2013	China, Lebanon, Russia, Thailand, Tunisia	*A. baumannii, E. coli, E. hormaechei, K. pneumoniae*	Pl	IncF	IS*15DIV*	Kazmierczak *et al.*, 2015; Li *et al.*, 2018b
NDM-17	Val88Leu Met154Leu Glu170Lys	China, 2015	China	*E. coli*	Pl	IncX3	IS*Aba125*, IS*5*	Liu *et al.*, 2017b
NDM-18	Identical to NDM-1; tandem repeat of 5 AAs (QRFGD, AA positions 44 to 48)	Germany, 2016	Germany, India, South Africa	*E. coli, Providencia rettgeri*	Pl	NC	NC	Ntshobeni *et al.*, 2019

NDM-19	Asp130Asn Met154Leu Ala233Val	Egypt, 2015	Canada, China, Egypt, Lebanon	*E. coli*, *K. pneumoniae*	Pl	IncX3, IncFII	IS*Aba125*, IS*5*, IS*3000*, *tnpA*	Liu *et al.*, 2019a; Mancini *et al.*, 2019
NDM-20	Gly62Thr Ala460Cys Gly809Ala	USA, 2013	China, USA	*E. coli*, *K. pneumoniae*	Pl	IncX3	IS*Aba125*, IS*5*, IS*3000*	Liu *et al.*, 2018a
NDM-21	Val88Leu Met154Leu Gly69Ser	USA, 2012	China, USA	*E. coli*, *E. hormaechei*, *K. pneumoniae*	Pl	IncX3	IS*Aba125*, IS*5*	Liu *et al.*, 2018b
NDM-22**	Met248Leu	China, 2015	China, India, Philippines, Thailand	*E. cloacae*, *E. coli*, *E. hormaechei*, *K. pneumoniae*	NC	-	-	-
NDM-23**	Ile101Leu	Spain, 2017	Spain	*K. pneumoniae*	NC	-	-	-
NDM-24**	Val88Leu	Georgia, 2017	Georgia	*Providencia stuartii*	NC	-	-	-
NDM-25**	Ala55Ser	India, 2016	India	*K. pneumoniae*, *P. aeruginosa*	NC	-	-	-
NDM-26**	Val88Leu Met154Leu Gly222Ser	China, 2016	China	*E. coli*	NC	-	-	-
NDM-27**	Asp95Asn Ala233Val	USA, 2015	USA	*E. coli*	NC	-	-	-
NDM-28**	Ala266Val	China, 2015	China, India	*K. pneumoniae*	NC	-	-	-

Cr, chromosome; NA, information not available; NC, not characterized; Pl, plasmid; UAE, United Arab Emirates; Un, Not assigned to known plasmid type; UP, unpublished. *AA substitutions compared to $bla_{\text{NDM-1}}$. AAs: Ala, Alanine; Arginine, Arg; Asn, Asparagine; Asp, Aspartic acid;

Cys, Cysteine; Glu, Glutamic acid; Gly, Glycine; His, Histidine; Ile, Isoleucine; Leu, Leucine; Lys, Lysine; Met, Methionine; Pro, Proline; Ser, Serine; Thr, Threonine; Val, Valine. **Data available in NCBI archive (unpublished) are also included.

1.8.6 OXA carbapenemases

The class D β-lactamases are termed as oxacillinases (OXAs) due to their hydrolytic capacity for oxacillin and cloxacillin is much faster than penicillins i.e. benzylpenicillin. This group of enzymes are functionally serine β-lactamases and poorly inhibited by clavulanic acid and EDTA. Early OXAs, OXA-1, -2 & -3 have been described in the late 1970s and early 1980s. The first OXA ESBL, OXA-11 (derivatives of OXA-10) was identified in *P. aeruginosa* from a patient in Turkey in 1991. Eventually other OXA ESBLs, OXA-13, -14, -16, -17, -19, -28, -35, -145, and -147 (derived from OXA-10) and OXA-15, and -36 (derived from OXA-2) have been detected. Although most of OXAs (up to ESBLs) had been originally identified in *P. aeruginosa*, these enzymes have been later described in a wide variety of Gram-negatives. The first OXA β-lactamase with carbapenemase activity was OXA-23, was reported in *A. baumannii* in 1985. The enzyme showed 36% AA identity to OXA-5 and OXA-10. Other major OXA carbapenemases identified afterwards includes OXA-24 and OXA-40, OXA-51, OXA-58, OXA-48 and OXA-48-like. OXA-51 is an intrinsic carbapenemase to *A. baumannii* and usually exhibit carbapenemase activities when the gene is overexpressed. The host species and other properties of OXA carbapenemases are summarised in Table 1.3 (Queenan and Bush, 2007; Evans and Amyes, 2014; Codjoe and Donkor, 2017; Bonomo *et al.*, 2018).

Table 1.3 Key properties of OXA carbapenemases.

Enzyme group	Year of first isolation	Country of isolation	Enzymes	Host species	Location
OXA-23-like	1985	UK	OXA-23, OXA-27, OXA-49, OXA-73, OXA-102, OXA-103, OXA-105, OXA-133, OXA-134, OXA-146, OXA-165–OXA-171, OXA-225, OXA-239	*A. baumannii*, *Acinetobacter junii*, *Acinetobacter radioresistens*, *Acinetobacter pittii*, *P. mirabilis*, *Acinetobacter phenon 5*, *A.* phenon 6/ct 13TU, *A. nosocomialis*, *Acinetobacter* genomic species 10/11, *A. lwoffii*, *Acinetobacter baylyi*, *K. pneumoniae*	Cr, Pl
OXA-40/24-like	1997	Spain	OXA-40, OXA-25, OXA-26, OXA-72, OXA-139, OXA-160, OXA-207	*A. baumannii*, *Acinetobacter haemolyticus*, *A. pittii*, *A. baylyi*, *P. aeruginosa*, *Acinetobacter calcoaceticus*, *K. pneumoniae*	Cr, Pl
OXA-51-like	1996	Argentina	OXA-51, OXA-64–OXA-71, OXA-75–OXA-80, OXA-82–OXA-84, OXA-86–OXA-95, OXA-98–OXA-100, OXA-104, OXA-106–OXA-113, OXA-115–OXA-117, OXA-120–OXA-128, OXA-130–OXA-132, OXA-138, OXA-144, OXA-148–OXA-150, OXA-172–OXA-180, OXA-194–OXA-197, OXA-200–OXA-203, OXA-206, OXA-208, OXA-216, OXA-217, OXA-219, OXA-223, OXA-241, OXA-242, OXA-248–OXA-250, OXA-254	*A. baumannii*, *A. nosocomialis*, *E. cloacae*, *E. coli*, *K. pneumoniae*	Cr, Pl
OXA-58-like	2003	France	OXA-58, OXA-96, OXA-97, OXA-164	*A. baumannii*, *A. pittii*, *A. nosocomialis*, *A.* phenon 6/ct 13TU, *A. junii*, *Acinetobacter* genomic species 9, *Acinetobacter bereziniae*, *A. calcoaceticus*, *A. radioresistens*, *E. cloacae*, *Comamonas testosteroni*, *E. coli*, *K. pneumoniae*, *Delftia acidovorans*	Cr, Pl

OXA-134a-like	2010	France	OXA-134a, OXA-186–OXA-191	*A. lwoffii*	Cr
OXA-143-like	2004	Brazil	OXA-143, OXA-182, OXA-231, OXA-253, OXA-255	*A. baumannii, A. pittii*	Pl
OXA-213-like	2012	France	OXA-213	*A. calcoaceticus*	Cr
OXA-214-like	2012	France	OXA-214, OXA-215	*A. haemolyticus*	Cr
OXA-211-like	2012	France	OXA-211, OXA-212, OXA-309	*Acinetobacter johnsonii*	Cr
OXA-229-like	2012	France	OXA-228–OXA-230, OXA-257	*A. bereziniae*	Cr
OXA-235-like	2007	USA & Mexico	OXA-235–OXA-237, OXA-278	*Acinetobacter schindleri*	Pl
OXA-48-like	2001	Turkey	OXA-48, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247, OXA-894	Enterobacterales*, *Shewanella xiamenensis, A. baumannii*	Cr, Pl

Cr, chromosome; Pl, plasmid. *The distribution of OXA-48-like among the species of Enterobacterales are stated in Table 1.4.

1.8.6.1 OXA-48 β-lactamase

The class D carbapenemases distributed widely among the members of Enterobacterales are OXA-48 and its variants. The group of enzymes are relatively weak carbapenem hydrolyser, having greater activity against imipenem than meropenem. OXA-48 sharing 46%, 36%, 32% and 21% AA identity with OXA-10, OXA-23, OXA-40 and OXA-1, respectively. OXA-48 differs from OXA-10 at the β5–β6 loop where a conformational change is occurred by the interaction of Arg-214 with the Ω loop Asp-159 residue, extending the loop into the active site of the enzyme. Therefore, β5-β6 loop provides a hydrophilic environment for binding water molecules that are required for hydrolysis. The enzyme has minor differences in the active-site cavity with OXA-10/-13. His-109 at the active site interacts with Thr-104 and Thr-113 which modify the hydrogen bonding network. Taken together, the antibiotic can be brought into proximity with a water molecule for deacylation of the antibiotic (Docquier *et al.,* 2009; Poirel *et al.,* 2012; Evans and Amyes, 2014).

1.8.6.2 Discovery and origin of OXA-48 β-lactamase

OXA-48 was identified in a *K. pneumoniae* isolate in 2001 from a patient in Istanbul, Turkey. It has been speculated that the gene was originated from aquatic organism, *Shewanella oneidensis* which have an intrinsic *bla*$_{\text{OXA-54}}$, sharing 92% amino acid identity with *bla*$_{\text{OXA-48}}$. Overall, OXA-48-like are composed of 798 nucleotides, encoding 261 to 265 AAs. Till date, several chromosomally encoded OXA carbapenemases' variants, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-199}}$, *bla*$_{\text{OXA-204}}$, *bla*$_{\text{OXA-416}}$, *bla*$_{\text{OXA-538}}$, *bla*$_{\text{OXA-252}}$, *bla*$_{\text{OXA-514}}$, *bla*$_{\text{OXA-515}}$, *bla*$_{\text{OXA-546}}$, *bla*$_{\text{OXA-547}}$ and *bla*$_{\text{OXA-894}}$ have been reported from environmental source of *S. xiamenensis* which are often linked to IS*1999*. The genetic environment of *bla*$_{\text{OXA-48-like}}$ in Enterobacterales revealed the association of complete or truncated similar insertion elements, IS*1999* (Table 1.4). This mobile element perhaps led to the acquisition of *bla*$_{\text{OXA-48}}$ in Enterobacterales from *Shewanella* species (Poirel *et al.,* 2012; Antonelli *et al.,* 2015; Ceccarelli *et al.,* 2017; Tacão *et al.,* 2018; Tafoukt *et al.,* 2018; Zou *et al.,* 2019).

1.8.6.3 Epidemiology and spread of *bla*$_{\text{OXA-48}}$

The rapid spread and increasing trend of Enterobacterales producing OXA-48-like enzymes in different niches such as in the health facilities, community, and animals (e.g., livestock, companion animals, and wildlife) has become a serious issue recently (Mairi *et al.,* 2018). The genetic context around *bla*$_{\text{OXA-48}}$ demonstrated the connection of IS*1999* at the upstream, involving the mobilisation of this carbapenem resistance gene. The transferable element was often found in plasmid harbouring *bla*$_{\text{OXA-48}}$ as composite transposons (Tn*1999*, Tn*1999.2* or Tn*1999.3*). Tn*1999.2* or Tn*1999.3* have been formed by the disruption of IS*1999* with IS*1R* introduction (Evans and Amyes, 2014; Pitout *et al.,* 2019). IS*10A*-like was also found to be inserted at the upstream of composite transposon (Gijón *et al.,* 2020). Several other insertion elements have also been described in association with *bla*$_{\text{OXA-48-like}}$ in a variety of plasmid backgrounds in a wide range of bacterial species (Table 1.4). Plasmids contribute a vital role in worldwide dissemination *bla*$_{\text{OXA-48-like}}$ (Kasap *et al.,* 2013; Evans and Amyes, 2014; Potron *et al.,* 2016; Shankar *et al.,* 2019). The global epidemiology of *bla*$_{\text{OXA-48-like}}$ is summarized in Figure 1.9, Figure 1.10, and Table 1.4 according to previous literature reviews (Cuzon *et al.,* 2011; Glupczynski *et al.,* 2012; Poirel *et al.,* 2012; Zong *et al.,* 2012; Adler *et al.,* 2013; Balm *et al.,* 2013b; Gomez *et al.,* 2013; Teo *et al.,* 2013; Voulgari *et al.,* 2013; Majewski *et al.,* 2014; Sampaio *et al.,* 2014; Wrenn *et al.,* 2014; Pereira *et al.,* 2015; Zhang *et al.,* 2015; Both *et al.,* 2016; Guo *et al.,* 2016; Jayol *et al.,* 2016; Lahlaoui et al., 2017; Magagnin *et al.,* 2017; Mansour *et al.,* 2017; van Duin *et al.,* 2017; Yin *et al.,* 2017; Al-Baloushi *et al.,* 2018; Guducuoglu *et al.,* 2018; Lu *et al.,* 2018; Mairi *et al.,* 2018; Mancini *et al.,* 2018; Pulss *et al.,* 2018; Solgi *et al.,* 2018; Srijan *et al.,* 2018; Tafoukt *et al.,* 2018; Ahn *et al.,* 2019; Carrasco-Anabalón *et al.,* 2019; Aires-de-Sousa et al., 2019; Haller *et al.,* 2019; Iovleva *et al.,* 2019; Lowe *et al.,* 2019; Nigg *et al.,* 2019; Obeng-Nkrumah *et al.,* 2019; Pitout *et al.,* 2019; Benulič *et al.,* 2020; El-Kholy *et al.,* 2020; Lu *et al.,* 2020; Mukherjee *et al.,* 2020; Sherchan *et al.,* 2020; Villacís *et al.,* 2020; ECDC, 2020).

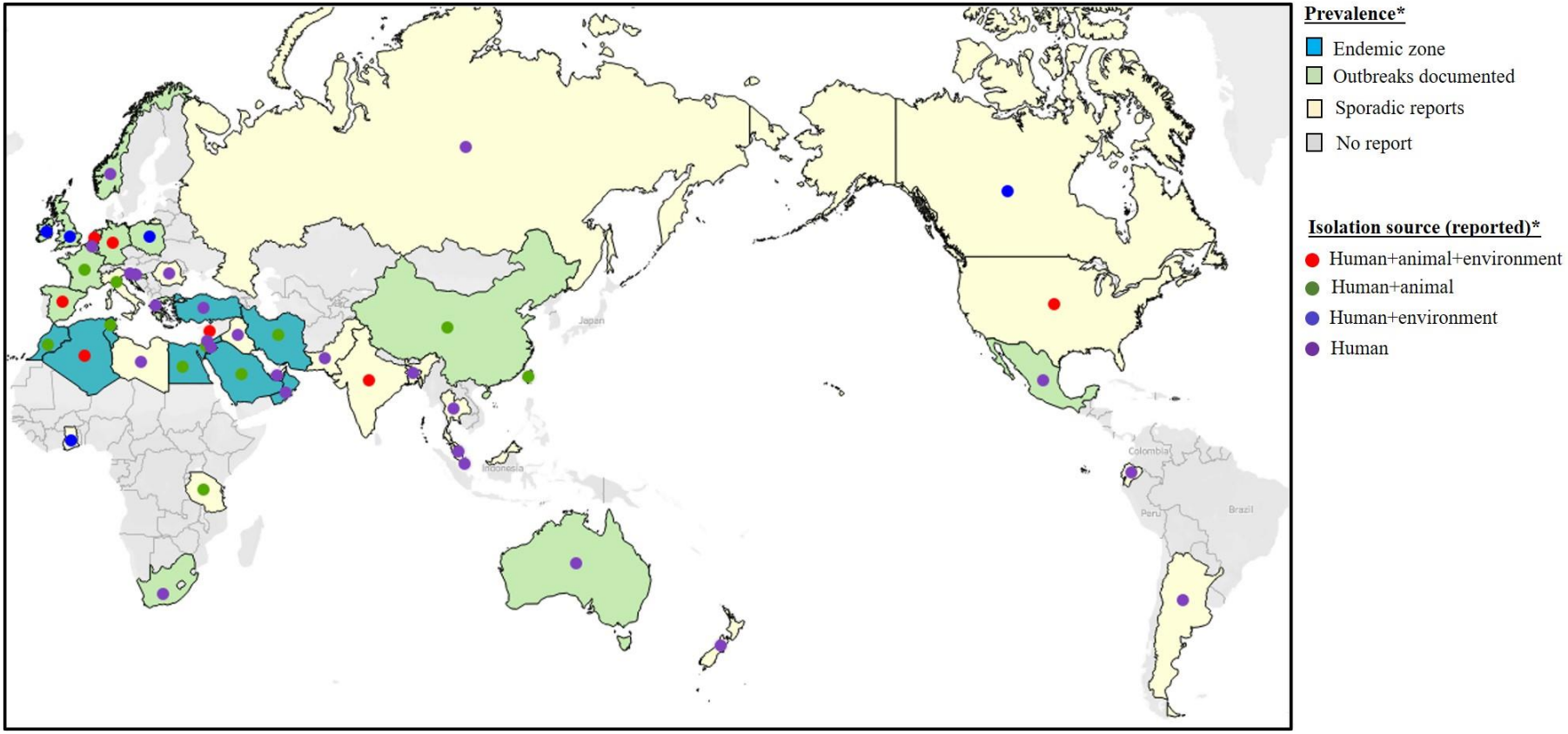


Figure 1.9 Global distribution of OXA-48 producers. *Map has been generated based on published reports and relevant metadata available in NCBI until 31 July 2020.

Figure 1.10 Global distribution of OXA-181 and OXA-232 producers. *Map has been generated based on published reports and relevant metadata available in NCBI until 31 July 2020.

Table 1.4 Key properties of *bla*$_{\text{OXA-48-like}}$.

Enzymes	AA substitutions*	Country & Year of isolation	Host species	STs associated with clonal dissemination		Location of gene	Plasmid Inc group	Associated mobile element
				K. pneumoniae	**Others**			
OXA-48	-	Turkey, 2001	*C. freundii, E. cloacae, C. sakazakii, E. coli, K. pneumoniae, Klebsiella oxytoca, Kluyvera spp., P. mirabilis, P. rettgeri, P. aeruginosa, Salmonella spp., Serratia marcescens*	ST11***, ST101, ST13, ST131, ST147, ST15, ST1853, ST221, ST39, ST307, ST383, ST392, ST395, ST437, ST893	*E. cloacae*, ST89, *E. coli* ST38***	Pl	IncL/M, IncA/C2, IncFII, IncFIA, IncFIB, IncHI1B, IncHI2, IncHI1, ColKP3, IncX3, IncN	IS*1999*, Tn*1999*, Tn*1999.2*, Tn*1999.3*, IS*10A-like*
OXA-181	Thr104Ala Asn110Asp Glu168Gln Ser171Ala	India, 2006	*C. freundii, E. cloacae, E. coli, K. pneumoniae, K. variicola, P. rettgeri*	ST101, ST307	*E. coli* ST410***	Pl	ColKP3, IncX3, ColE2, IncT	IS*Ecp1* (forms a transposon named Tn*2013*), IS*26*
OXA-232	Thr104Ala Asn110Asp Glu168Gln Ser171Ala Arg214Ser	France, 2011	*E. coli, K. pneumoniae Raoultella ornithinolytica*	ST231, ST15, ST23, ST14	-	Pl	ColE, ColKP3	IS*Ecp1*, IS*Kpn26*, IS*Kpn25*, IS*Kpn1*, IS*1*, Tn*As3*, Tn*3*, Tn*2*
OXA-162	Thr213Ala	Turkey, 2008	*K. pneumoniae, E. coli, C. freundii, R. ornithinolytica*	ST11	-	Pl	IncL/M	Tn*1999.2*
OXA-163*	Ser212Asp Deletion: Arg214, Ile215, Glu216, Pro217	Argentina, 2008	*E. cloacae, K. pneumoniae*	-	-	Pl	NC	IS*Ecl4*
OXA-199	His37Tyr Val44Ala Asp153Gly	China, 2012	*S. xiamenensis*	-	-	Cr	-	IS*Shes2*
OXA-204	Gln98His Thr99Arg	Tunisia, 2012	C. *freundii, E. coli, K. pneumoniae, P.*	ST147	*E. coli* ST90	Cr, Pl	IncA/C	IS*Ecp1*

			mirabilis, S. marcescens, S. xiamenensis					
OXA-244*	Arg222Gly	Spain, 2011	*E. aerogenes, E. coli, K. pneumoniae, P. mirabilis*	-	-	Pl	IncL/M	IS*1999*
OXA-245	Glu125Tyr	Spain, 2011	*K. pneumoniae*	-	-		IncL/M	IS*1999*
OXA-247	Tyr219Ser Asp220Asn	Argentina, 2010	*K. pneumoniae*	-	-			IS*4321*, IS*4-like*
OXA-370*	Ser220Glu	Brazil, 2013	*E. aerogenes, E. coli, E. cloacae, E. hormaeche, K. pneumoniae, Morganella morganii, C. freundii P. mirabilis, P. stuartii, Serratia* spp.	ST16	-	Pl	IncF	tnp*A*
OXA-436	Val3Ala Phe10Leu Leu11Met Ala13Thr Ser14Thr Ile15Met Thr36Ser Ser40Thr Lys51Thr Asn58Asp Thr104Ala Asn110Asp Val153Leu Glu168Gln Ser171Ala Gly201Ala Thr213Val Val226Ile Met237Thr Ser244Ala Asp245Glu Ala252Thr Glu256Ala	Denmark, 2017	*Enterobacter asburiae, C. freundii, K. pneumoniae*	-	-	Pl	IncHI2	-

OXA-484	Thr104Ala Asn110Asp Glu168Gln Ser171Ala Arg214Gly	UK, 2017	*K. pneumoniae*	-	-	Pl	-	-
OXA-519	Val120Leu	Belgium, 2018	*K. pneumoniae*	-	-	Pl	IncL	IS*1999*

*AA substitutions compared to *bla*$_{\text{OXA-48}}$. **Weak carbapenemase activity. ***International high-risk clone

1.9 Mobile colistin resistance

1.9.1 Introduction of colistin as an antibacterial agent

Colistin was first extracted in Japan by Koyama and co-workers from the spore-forming soil bacterium Bacillus polymyxa subsp. colistinus in 1947. During the 1950s colistin was used in clinical practice in Japan and Europe. Colistin was approved by the USA FDA and has been available in the USA since 1959 for the treatment of infections caused by Gram negative bacteria, however the antibiotic has been reserved for severe bacterial infections and used topically for eye and ear infections during the succeeding decades. High incidence of colistin nephrotoxicity led to replace new antimicrobials such as gentamicin and carbenicillin in clinical practice. Since the mid-1990s, therapeutic interest towards colistin has been renewed as last resort antibiotic in prevalent MDR Gram negative bacterial infections (Falagas and Kasiakou, 2005; Bialvaei and Samadi, 2015).

1.9.2 Antibacterial spectrum and mode of action of colistin

There are five different chemical compounds of polymyxins (polymyxins A, B, C, D, and E). Mainly colistin A (polymyxin E1) and colistin B (polymyxin E2), which differ only in their fatty acid tails, have been used in clinical practice. Colistin is polycationic at physiological Ph, consisting of a cyclic heptapeptide with a tripeptide side chain acylated at the N terminus by a fatty acid tail (Bialvaei and Samadi, 2015). Colistin confers bactericidal effects against Gram negative bacilli, however, *Proteus, Neisseria, Serratia, Providencia, Burkholderia pseudomallei, M. morganii, Edwardsiella tarda* and anaerobic bacteria are intrinsically resistant to colistin (Falagas and Kasiakou, 2005; Bialvaei and Samadi, 2015). The mechanism of action of colistin is shown in Figure 1.11.

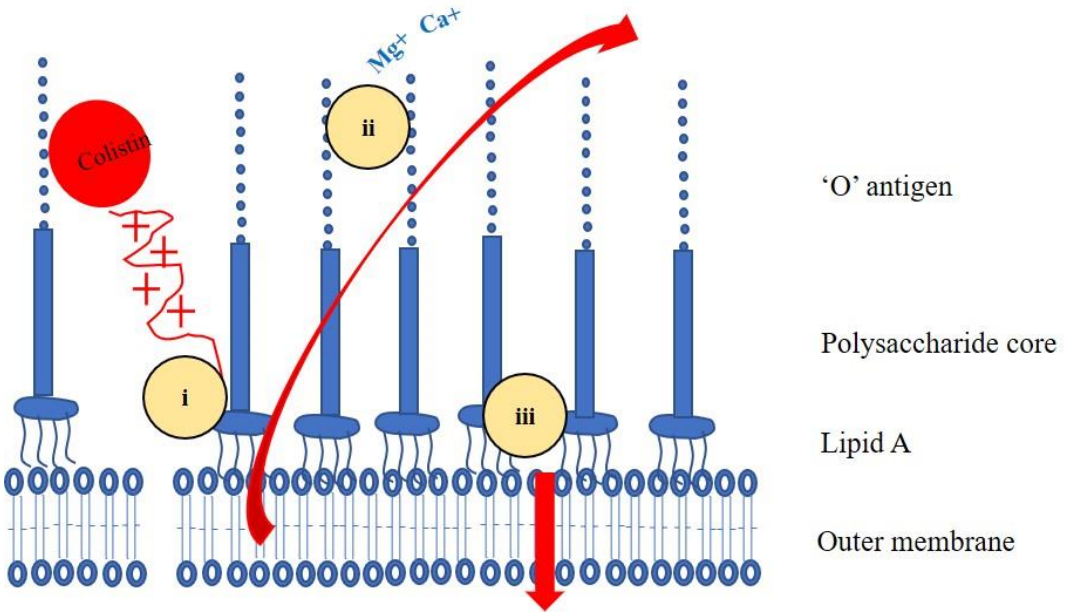


Figure 1.11 Mechanism of action of colistin. i. Polycationic colistin binds with lipid A of lipopolysaccharide (LPS) molecule in the outer membrane of Gram-negative bacteria which is negatively charged. ii. Displacement of magnesium (Mg2+) and calcium (Ca2+). iii. Disruption of outer membrane and subsequent cell death.

Colistin also ensues anti-endotoxin activity due to its binding with endotoxin (lipid A) of Gram-negative bacteria.

1.9.3 Mechanism of colistin resistance

Polymyxin resistance mechanisms are mediated by various lipopolysaccharide (LPS) modifications. These includes: 1. alteration of drug binding site, lipid A (most common), 2. increased drug efflux, 3. overproduction of capsuler polysaccharide. The resistance mechanism of colistin is described in Figure 1.12 (Olaitan *et al.,* 2014; Bialvaei and Samadi, 2015; Mlynarcik and Kolar, 2018).

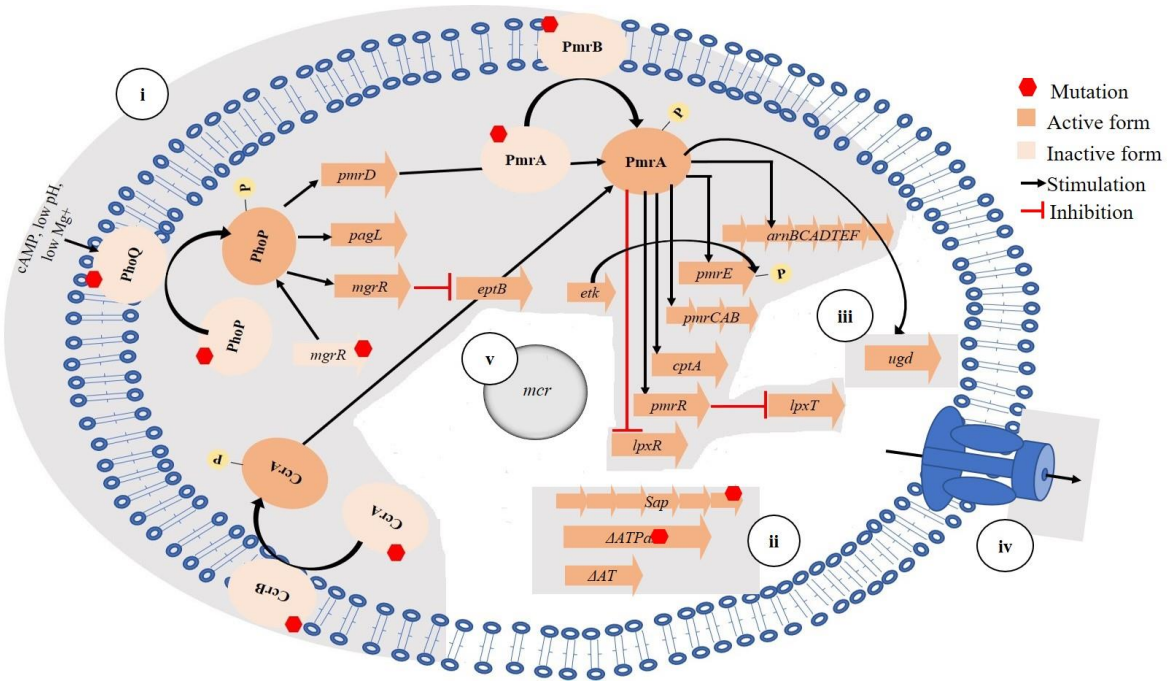


Figure 1.12 Mechanism of colistin resistance. Each mode of resistance is separated by shading.

i. Activation of two component system (TCS): **a)** External stimuli or mutations in PhoP/PhoQ or mutational or insertional inactivation of *mgrB* activate PhoP/PhoQ which in turn activate PmrA/PmrB via PmrD. The PmrA/PmrB TCS is also activated due to mutations in PmrA/PmrB or CcrA/CcrB (in *K. pneumoniae*). ColR/ColS, and CprR/CprS in *P. aeruginosa* and RppA/RppB in *P. mirabilis*, and *M. morganii* have been reported in relation to developing high level of colistin resistance. Phosphorylated PmrA activates *arnBCADTEF*, and *pmrE* which catalyse the addition of 4-amino-4deoxy-L-arabinose (L-Ara4N) to lipid A. Phosphorylated PmrA also activates *pmrCAB* (*eptA*), and *cptA* which catalyse the addition of phosphoethanolamine (PEtN) to lipid A. Phosphorylated PmrA downregulates *lpxT* (via activation of *pmrR*), and *lpxR*. **b)** Activated PhoP/PhoQ activates *pagL*, which deacylates lipid A in *Salmonella*. **c)** Activated PhoP/PhoQ represses *eptB* via the activation of MgrR. This repression prevents the modification of the outer Kdo (3-deoxy-D-manno-octulosonic acid) residues of LPSs with PEtN. **ii. Mutations of genes responsible LPS biosynthesis:** Mutations in *sap* operon (encode a transport protein), ATPase gene, and putative *O*-acetyltransferase (*AT*) (most likely are involved either in the biosynthesis or transfer of aminoarabinose) affect LPS biosynthesis in *P. mirabilis*. Mutation of genes, *galU*, l*ptC*, *wapR*, and *ssg*, identified in *P. aeruginosa* which impedes LPS biosynthesis. Mutation of lpxA, lpxC, and lpxD in *A. baumannii* leads to complete loss of LPS. **iii. Activation of *ugd***: This plays a dual role. **a)** Increases capsular polysaccharides (CPSs) synthesis and mediates colistin resistance due to electrostatic interactions between cationic polymyxin and anionic CPSs **b)** Adds L-Ara4N to lipid A. **iv. Activation of efflux pumps:** KpnEF in *K. pneumoniae*, AcrAB in *E. coli*, and *K. pneumoniae*, Emr in *A. baumannii*, MexAB-OprM in *P. aeruginosa* have been identified in relation

to colistin resistance. Expression of this pump's proteins is dependent on the PhoPQ TCS. **v. Acquired:** Mobile colistin resistance (MCR) is the member of PEtN transferase, causing colistin resistance due to horizontal transfer among bacteria.

1.9.4 Discovery of *mcr*

A transferable colistin resistance gene, *mcr-1* was first reported in *E. coli* from China in 2015, located on an IncI2 conjugative plasmid. The first report suggested the dissemination of *mcr-1* in human and food animal (Liu *et al.,* 2016). However, surprisingly, *mcr* was identified in isolates dating back to 1970s from China when colistin was first used Chinese livestock and there were sporadic occurrence and outbreak of *mcr* over two decades in China which had not been picked up at the time. It has been speculated that increased usage of colistin during 2009 to 2014 in China has contributed the spread of *mcr* (Shen *et al.,* 2016).

1.9.5 Epidemiology and spread of *mcr*

There is strong association between usage of colistin and emergence of *mcr* in a geographical region. Data from several epidemiological surveys indicates that there has been increased prevalence of *mcr* in China since 2009 which coincides with the fact that colistin was used as a growth promoter in higher frequency in the Chinese agriculture sector from 2000 to 2015. Perhaps high prevalence of *mcr* in Vietnam has been related to colistin usage in farming as well. *mcr*-like resistance mechanisms have now been disseminated worldwide, recovered from a wide range of sources such as livestock, pet animal, meat, water, human infections, and carriage. The gene, *mcr* is typically embedded in plasmid and very rarely resides on the chromosome. The global dissemination of *mcr* have been mostly observed along with horizontal gene transfer among intra- and inter-species and genus. Literature review suggested that plasmids of varied sizes and diverse replicon types have been identified in relation to the spread of *mcr*-like resistance (Table 1.6). The global distribution of *mcr* variants is described in Figure 1.13 (Perrin-Guyomard *et al.,* 2016; Lei *et al.,* 2017; Matamoros *et al.,* 2017; Ovejero *et al.,* 2017; Wang *et al.,* 2017a; Wang *et al.,* 2017b, Fukuda *et al.,* 2018; Wang *et al.,* 2018a; Wang *et al.,* 2018b;Wise *et al.,* 2018; Sun *et al.,* 2018; Aeksiri *et al.,* 2019; Kneis *et al.,* 2019; Long *et al.,* 2019; Bich *et al.,* 2019; Gelbíčová *et al.,* 2019; Liu *et al.,* 2019b; Dos Santos *et al.,* 2020; Furlan *et al.,* 2020; Hoa *et al.,* 2020; Luo *et al.,* 2020).

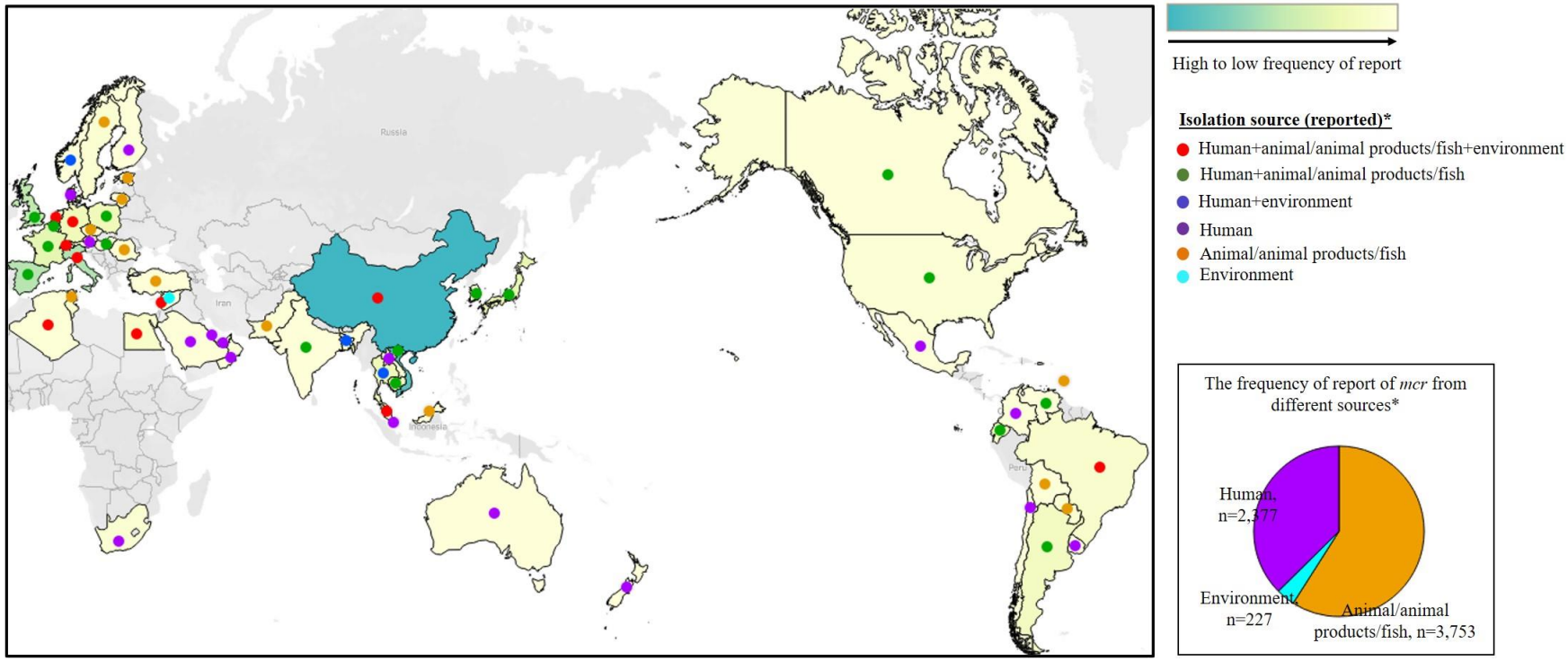


Figure 1.13 Global distribution of *mcr*-like mechanism. *Map has been generated based on published reports and relevant metadata available in NCBI until 31 July 2020.

1.9.6 Variants of *mcr*

Data suggest that *mcr-1* has existed for more than three decades, however, the other known variants have evolved during the last decades (Figure 1.14). At the time of writing, ten variants of *mcr* (*mcr-1* to *mcr-10*) have been identified. The AAs identity among the different variants are stated in Table 1.5. A greater number of minor variants for *mcr-1* (18 variants), *mcr-2* (two variants), *mcr-3* (28 variants), *mcr-4* (five variants), *mcr-5* (two variants), and *mcr-8* (three variants) have so far been identified. The geographical distribution of *mcr-1* and its variants is depicted in Figure 1.15 and the other epidemiological properties are described in Table 1.6 (Tian *et al.,* 2017; Yi *et al.,* 2017; Chavda *et al.,* 2018; Fukuda *et al.,* 2018; Mendes *et al.,* 2018; Bich *et al.,* 2019; Aeksiri *et al.,* 2019; Kneis *et al.,* 2019; Wang *et al.,* 2019; Dos Santos *et al.,* 2020; Filioussis *et al.,* 2020; Furlan *et al.,* 2020; Hoa *et al.,* 2020; Lei *et al.,* 2020; Luo *et al.,* 2020; Wang *et al.,* 2020; Yang *et al.,* 2020).

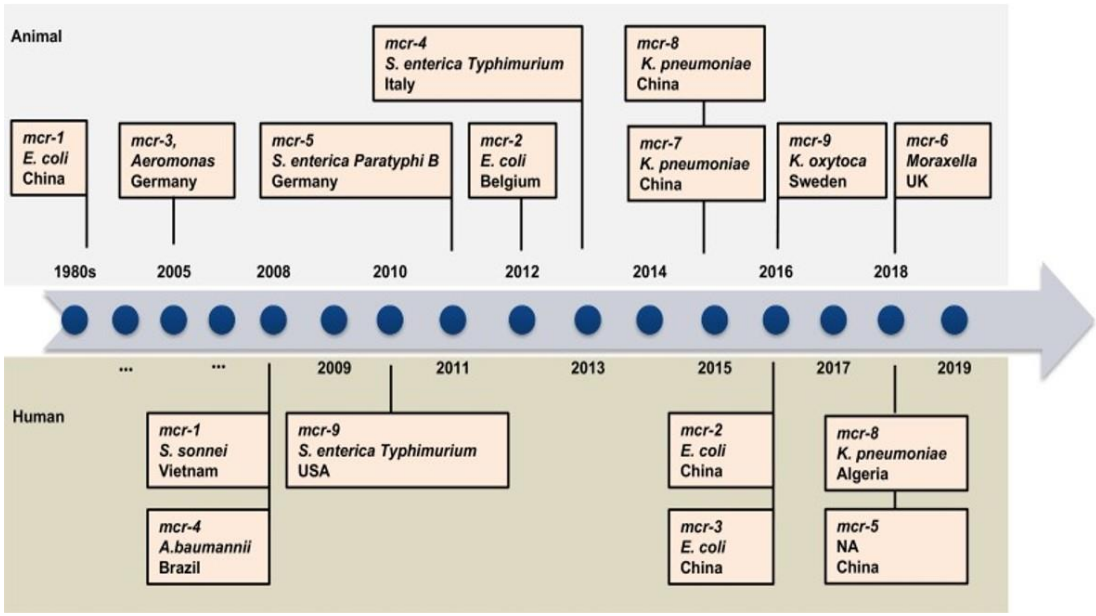


Figure 1.14 Chronological discovery of *mcr* variants from animal and human. Reproduced from (Luo *et al.*, 2020) with permission. Discovery of *mcr-10* has not been included in the Figure. MCR-10 was first identified in 2020 from both human and animal source in China.

Table 1.5 Nucleotide identity among the *mcr* variants.

Variants	**Nucleotide Identity (%)**								
	mcr-1	*mcr-2*	*mcr-3*	*mcr-4*	*mcr-5*	*mcr-6*	*mcr-7*	*mcr-8*	*mcr-9*
mcr-2	76.7	-	-	-	-	-	-	-	
mcr-3	45.0	47.0	-	-	-	-	-	-	
mcr-4	34.0	35.0	49.0	-	-	-	-	-	
mcr-5	36.1	35.3	34.7	33.7	-	-	-	-	
mcr-6	98.7	87.9	34.5	33.0	37.3	-	-	-	
mcr-7	35.0	34.0	70.0	45.0	36.0	33.0	-	-	
mcr-8	31.1	30.3	39.9	37.8	33.5	30.4	37.5	-	
mcr-9	36.3	33.9	64.7	43.4	33.2	33.7	63.4	44.8	
mcr-10	36.2	34.5	62.1	44.7	36.2	34.2	59.9	43.4	82.9

Figure 1.15 Geographical distribution of different *mcr* variants. *Map has been generated based on published reports and relevant metadata available in NCBI until 31 July 2020. Data on *mcr-10* (n=2) were not included in this map. MCR-10 (n=2) was isolated from China.

Table 1.6 Key properties of *mcr* variants.

***mcr* variants**	**Host species**	**STs associated with possible clonal spread**		**Location of *mcr***	**Associated plasmid replicon types**	**Associated mobile elements**
		E. coli	**Others**			
mcr-1	*A lwoffii, Citrobacter amalonaticus, Citrobacter braakii, C. freundii, C. sakazakii, E. aerogenes, Enterobacter agglomerans, E. cloacae, E. coli, Escherichia fergusonii, K. oxytoca, K. pneumoniae, K. quasipneumoniae, K. variicola, Kluyvera ascorbathare, P. mirabilis, Providencia alcalifaciens, P. aeruginosa, Pseudomonas putida, R. ornithinolytica, Raoultella planticola, Salmonella* spp., *Shigella sonnei, Vibrio parahaemolyticus*	ST10, ST131, ST93	*S. typhimurium* ST34, *K. pneumoniae* ST11, *K. pneumoniae* ST45	Cr, Pl	IncFII, IncFIA, IncFIB, IncFIC, IncH, IncI1, IncI2*, IncK, IncL/M, IncN, IncP, IncX4*, IncY, p0111	IS*Apl1*
mcr-2	*E. coli, K. pneumoniae, Moraxella pluranimalium, P. aeruginosa, Salmonella* spp.	-	-	Cr, Pl	IncX4	IS*1595*, IS*Ec69*
mcr-3	*Aeromonas hydrophila, Aeromonas veronii, E. coli, K. pneumoniae, Salmonella* spp.	-	*S. typhimurium* ST34	Cr, Pl	IncF, IncFIA, IncH, IncI2, IncK, IncN, IncP	IS*26*, IS*4321*, IS*Kpn40*, ΔTn*As2*
mcr-4	*A. baumannii, E. cloacae, E. coli, Salmonella* spp., *Shewanella* spp.	ST10	-	Pl	Col8282, ColE10	IS*5*, IS*15DIV*, IS*Aba19*, Tn*5044*
mcr-5	*A. hydrophila, E. coli, P. aeruginosa, Salmonella* spp.	-	-	Cr, Pl	ColE, IncFIB, IncH, IncI1, IncX1	*tnpA*
mcr-6	*M. pluranimalium*	-	-	Cr	-	IS*Apl1*
mcr-7	*K. pneumoniae*	-	-		IncI2	-
mcr-8	*K. pneumoniae, R. ornithinolytica*	-	*K. pneumoniae* ST15	Pl	IncFII, IncFIB(pQil), IncP	IS*903B*
mcr-9	*C. freundii, E. cloacae, E. hormaechei, E. coli, K. oxytoca, Salmonella* spp.	-	-		IncHI2, IncR	IS*1*, IS*5*, IS*6*, IS*903B*
mcr-10	*E. cloacae, Enterobacter roggenkampii*	-	-	Pl	IncFIA	IS*903*

Cr, chromosome; Pl, plasmid. *Most common plasmid types associated with *mcr*.

1.10 Rationale for the study design in this PhD

The WHO SEA region (SEAR) includes 11 countries: Bangladesh, Bhutan, Democratic People's Republic of Korea, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, Thailand, and Timor-Leste (WHO, 2020c); however, topographically, Afghanistan, Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan, and Sri Lanka are the constituent countries of SA (Wikipedia, 2020d). Global Antimicrobial Resistance Surveillance System (GLASS) early implementation reports were reviewed to understand the status of AMR surveillance in each country of SA. The overview of AMR surveillance status for each country of SA by 2020 are described in Table 1.7. The GLASS reports infer the following: **1.** National surveillance in this region has been commenced only since 2017 or onwards in seven countries and has not been operational yet in Bhutan, **2.** Countrywide surveys are yet to be established, **3.** A very limited number of samples have been included in the surveillance, **4.** Lack of data to estimate prevalence and clinical burden of AMR, and **5.** Reports to GLASS did not cover all WHO priority pathogens. The list for WHO priority pathogens is mentioned in Table 1.8. Urgent development of new antibiotics is required against these pathogens (WHO, 2020a).

Table 1.7 The recent update of AMR surveillance in the countries of SA according to GLASS Early Implementation Report 2020.

Country	NAP	NCC	Surveillance site	Data on infection origin	Data on number of tested patients	Carbapenem resistance [NR*/NT]	Colistin resistance [NR/NT]	Current surveillance status
Afghanistan	NP	Established	One	Reported	No data reported	0/10 (IPM)	NA	The country is working to enhance diagnostic capacity and to expand the number of participating surveillance sites and laboratories in coming years.
Bangladesh	P	Established	Eight	No data reported	No data reported	88/469 (IPM)	NA	IEDCR is conducting AMR surveillance implemented as case-based surveillance of clinical syndromes.
Bhutan	P	Established	Three	-	-	NA	NA	No AMR data reported to GLASS.
India	P	Established	130	<70% reported	Reported	5780/13751 (IPM); 6280/13892 (MEM)	0/107	There are 3 AMR surveillance networks participating in the National AMR Surveillance in the country. 1. National AMR Surveillance network, NCDC. 2. Antimicrobial Surveillance and Research network, ICMR; 3. Gonococcal Antimicrobial Resistance surveillance network, Safdarjung Hospital.
Maldives	P	Established	Four	Reported	Reported			All national surveillance sites reported to GLASS
Nepal	P	Established	21	<70% reported	Reported	236/899 (IPM); 269/1675 (MEM)	NA	AMR surveillance started in Nepal since 1999 with six participating laboratories/hospitals. The network has extended and includes 21 hospitals.
Pakistan	P	Established	10	<70% reported	No data reported	629/8406 (IPM); 511/4037 (MEM)	NA	All national surveillance sites reported to GLASS
Sri Lanka	P	Established	17	No data reported	Reported	NA	NA	National AMR Surveillance System for public as well as the private sectors has been established in 2017. Each sector will develop their own surveillance system under One Health approach.

NA, no data available; NAP, National Action Plan; NCC, National Coordinating Centre; NP, not in place; NR, number of resistant strains; NT, number of tested strains by antimicrobial susceptibility testing (AST); IEDCR, Institute of Epidemiology Disease Control and Research; IPM,

imipenem susceptibility; MEM, meropenem susceptibility; P, place. *Data includes strains which were intermediate resistant or resistant to carbapenem.

Table 1.8 WHO listed priority pathogens.

Priority 1: CRITICAL	**Priority 2: HIGH**	**Priority 3: MEDIUM**
• *A. baumannii* (carbapenem-resistant) • *P. aeruginosa* (carbapenem-resistant) • Enterobacterales (carbapenem-resistant, ESBL-producing)	• *Enterococcus faecium* (vancomycin-resistant) • *S. aureus* (methicillin-resistant, vancomycin-intermediate, and resistant) • *Helicobacter pylori* (clarithromycin-resistant) • *Campylobacter* spp. (fluoroquinolone-resistant) • Salmonellae (fluoroquinolone-resistant) • *Neisseria gonorrhoeae* (cephalosporin-resistant, fluoroquinolone-resistant)	• *Streptococcus pneumoniae* (penicillin-non-susceptible) • *Haemophilus influenzae* (ampicillin-resistant) • *Shigella* spp. (fluoroquinolone-resistant)

The scoping exercise for this study included scrutinising the literatures relevant for the countries in SA, if the studies carried out in public institutions, whether related to Enterobacterales infections or colonisations (any organism associated with *mcr* was included), and if published after 2010. Databases searched included Pubmed and The WHO Library in English with the relevant keywords, "carbapenem resistance, and mortality," "carbapenem resistance, and risks," "carbapenem resistance, and faecal carriage", "carbapenem resistance, and clonal dissemination/outbreak", "carbapenem resistance, and plasmid", and "mobile colistin resistance/*mcr*" under each country of SA. Reviews, and metanalysis were excluded. Result searches are listed in Table 1.9. Briefly, scoping searches recognise carbapenem resistance as a predictor of high mortality in SA. In-hospital patients' management such as antibiotic exposure, introduction of artificial devices, and treatment in ICU are found to be major risks of CRE infections/colonisation in the health settings of SA. Outbreaks yield an added concern in the hospitals, suggesting poor implementation of infection prevention and control (IPC) measures. A growing prevalence of *mcr* has been observed in the region. However, none of the studies described the impact or burden of AMR by combining epidemiological, clinical, and genomic data. Only eight clinical studies in SA were conducted with a >300 sample sizes and only four clinical studies were documented in relation to *mcr*. Molecular analysis based on whole genome sequencing (WGS) was only performed for the reported emerging resistance mechanism. Hitherto, basic AMR data, such as prevalence, has not been estimated systematically for Bangladesh; however, geographically, Bangladesh has been considered as an endemic zone for many resistant mechanisms (Kumarasamy *et al.,* 2010; Nordmann *et al.,* 2011; Farzana *et al.,* 2013; Islam *et al.,* 2013; Khan *et al.,* 2017; Hsu *et al.,* 2017).

Table 1.9 Scoping findings relevant to this study from previous literature searches.

Article attributes	Country	Sample size	Species	Keynotes for relevant findings	Relevant findings
Stewardson *et al.* Lancet Infect Dis. 2019;19(6):601-610.	Multinational (LMICs)	297	Enterobacterales	Mortality assessment	Carbapenem resistant bloodstream infections (BSIs) were significantly associated with increased mortality and increased length of hospital stay. Investigation of clonal relatedness based on Multilocus sequence type (MLST).
Snyder *et al.* Epidemiol Infect. 2019;147:e137.	India	213	*K. pneumoniae*	Risks assessment	CVC placement, prior carbapenem use and ICU admission were the risk for the development of BSI with NDM-1 producing and other MDR strains.
Choudhuri *et al*. Saudi J Anaesth. 2018;12(3):389-394.	India	106	MDR bacteria	Mortality and risks assessment	Significant higher mortality was found in MDR group. The independent predictors of MDR bacterial infection were Child-Pugh score >10, prior carbapenem use, antibiotic use for more than 10 days, total parenteral nutrition, and concurrent antifungal administration.
Shankar *et al.* J Assoc Physicians India. 2018;66(12):13-16.	India	86	*K. pneumoniae*	Mortality assessment	Significant higher mortality was observed in carbapenem resistant *K. pneumoniae*. There were also associations between hypermucoviscous *K. pneumoniae* and mortality.
Naim *et al.* J Glob Infect Dis. 2018;10(3):133-139.	India	116	Gram-negative Bacilli	Risks assessment	Major risk factors in patients infected with MBLs were in-dwelling devices, prolonged hospital stay, and prior antibiotic treatment, but the risks were not assessed with any comparator.
Kaur *et al.* Am J Infect Control. 2017;45(11):1289-1291.	India	75	*K. pneumoniae*	Prevalence of mortality in CRE	5-year survey of dual colistin- and carbapenem-resistant BSIs showed high mortality (69.3%).

Mariappan *et al.* Int J Appl Basic Med Res. 2017;7(1):32-39.	India	111	Enterobacterales	Mortality and risks assessment	Carbapenem resistance had significant association with mortality among the patients with mechanical ventilation and indwelling invasive device.
Kumar *et al.* J Clin Diagn Res. 2015;9(11):DC08-DC13.	India	186	*E. coli* and *Klebsiella* Spp.	Risks assessment	Co-morbidity, ICU admission, and administration of artificial device were shown to risks for infections by NDM-1 producers.
Kalam *et al.* J Pak Med Assoc. 2014;64(5):530-536.	Pakistan	243	Gram-negative bacteria	Mortality and risks assessment	MDR bacteraemia is a significant risk for mortality. Risk factors for carbapenem resistant bacteraemia were age > 50 years, septic shock on presentation, ICU stay of > 72 hours, and receiving immunosuppressant medications.
Ramanathan *et al.* Indian J Med Microbiol. 2018;36(4):572-576.	India	102	Enterobacterales	Risks assessment	Data showed that the development of infections following CRE colonisation in critical care unit. Patients exposed to high end antibiotic and past history of surgery had significant association with CRE colonization
Singh *et al.* Am J Infect Control. 2018;46(6):e31-e35.	India	300	Enterobacterales	Risks assessment	Statistically significant risk factors of CRE colonisation of neonates in neonatal intensive care unit (NICU) were found to be nasogastric (NG) tube, breastfeeding, NG feeding, top feeding, expressed breastmilk, ventilation, antibiotic administration, and duration of hospitalization
Bharadwaj *et al.* BMC Infect Dis. 2018;18(1):504.	India	897	Enterobacterales	Risks assessment	CRE colonisation was significantly associated with recent healthcare and ICU admission.
Mohan *et al.* Indian J Med Microbiol. 2017;35(4):555-562.	India	232	Enterobacterales	Risks assessment	ICU admission, administration of indwelling device, and nasogastric tube were the independent risks for CRE colonisation.

Mittal *et al.* BMC Microbiol. 2016;16(1):138.	India	100	Enterobacterales	Risks assessment	The risk factors associated with CRE carriage were duration of ICU stay, use of ventilator and aminoglycosides
Datta *et al.* Indian J Med Microbiol. 2015;33(4):612-613.	India	75	Enterobacterales	Risks assessment	Patients with CRE colonisation in ICU were more likely to be associated with co-morbidity, prior surgery, intrahospital transfer, and prior exposure to carbapenem, cephalosporin, fluoroquinolones, and metronidazole.
Naha *et al.* Int J Antimicrob Agents. 2020;55(3):105903.	India	4	*K. pneumoniae*	Molecular characterization by WGS	Characterization of KPC producers by WGS.
Shankar *et al.* BMC Microbiol. 2019;19(1):137.	India	49	*K. pneumoniae*	Molecular characterization by WGS	Clonal relatedness of isolates harbouring $bla_{\text{OXA-48-like}}$ and genetic background of OXA-48-like using WGS. Genetic distance based on SNP was not revealed.
Shankar *et al.* J Infect Public Health. 2019;12(5):741-743.	India	One	*K. pneumoniae*	Molecular characterization by WGS	Characterization of an isolate of NDM producer by WGS.
Zhu *et al.* Front Microbiol. 2018;9:2044	Sri Lanka	379	*K. pneumoniae*	Molecular characterization by WGS	Transmission dynamics of OXA-181 producers by WGS.
Lomonaco *et al.* PLoS One. 2018;13(6):e0198526.	Pakistan	10	*K. pneumoniae*	Molecular characterization by WGS	Transmission dynamics of 10 carbapenemase producers by WGS.
Shankar *et al.* J Med Microbiol. 2018;67(7):927-930.	India	One	*K. pneumoniae*	Molecular characterization by WGS	Characterization of KPC producers by WGS.
Shrestha *et al.* Antimicrob Agents Chemother. 2017;61(12):e01425-17.	Nepal	250	*E. coli*	Molecular characterization by WGS	Clonal relatedness of isolates harbouring variants of bla_{NDM}, revealed by WGS. Genetic distance based on SNP was not revealed.
Subramanian *et al.* J Glob Antimicrob Resist. 2017;8:121-122.	India	One	*E. coli*	Molecular characterization by WGS	Characterization of an isolate of NDM producer by WGS.

Nahid *et al.* PLoS One. 2017;12(12):e0189438.	Pakistan	One	*K. pneumoniae*	Molecular characterization by WGS	Characterization of an isolate of OXA-181 producer by WGS.
Ranjan *et al.* Antimicrob Agents Chemother. 2016;60(11):6795-6805.	India	510	*E. coli*	Molecular characterization by WGS	Distribution of bla_{NDM} in diverse STs of *E. coli*. Typing was done ERIC-PCR along with WGS of 5 isolates.
Wailan *et al.* Antimicrob Agents Chemother. 2015;59(12):7405-7410.	Pakistan	Four	Gram-negative bacteria	Molecular characterization by WGS	Characterization of 11 NDM-1 producers isolated from stool of four patients. WGS was deployed to characterize the isolates.
Stoesser *et al.* Antimicrob Agents Chemother. 2014;58(12):7347-7357.	Nepal	94	*K. pneumoniae*	Molecular characterization by WGS	Clonal relatedness and transmission dynamic of K. pneumoniae by WGS following an outbreak in a neonatal unit. Genetic distance based on SNP was revealed.
McGann *et al.* Antimicrob Agents Chemother. 2012;56(4):1673-1679.	Afghanistan	One	*Providencia stuartii*	Molecular characterization by WGS	Characterization of an isolate of NDM producer by WGS.
Wangkheimayum *et al.* BMC Infect Dis. 2020;20(1):544.	India	329	*E. coli*	Characterization by molecular approaches other than WGS	Characterization of NDM and OXA-48 producers by MLST and PBRT.
Gondal *et al.* Infect Drug Resist. 2020;13:2105-2115.	Pakistan	227	*K. pneumoniae*	Characterization by molecular approaches other than WGS	Evidence of clonal and non-clonal dissemination of NDM, and OXA-48 producers, demonstrated by MSLT.
Khalid *et al.* Microb Drug Resist. 2020;26(3):284-289.	India	18	Gram-negative bacteria	Characterization by molecular approaches other than WGS	Prevalence of NDM producers in a neonatal unit along with probable spread of carbapenem resistance by plasmid.
Mukherjee *et al.* Infect Genet Evol. 2019;69:166-175.	India	200	*K. pneumoniae*	Characterization by molecular approaches other than WGS	Associations of bla_{NDM} in diverse STs of *K. pneumoniae* from neonatal septicaemia, revealed by REP-PCR, PFGE, and MLST.
Ahmad *et al.* Int J Antimicrob Agents. 2019;53(4):525-529.	India	14	*K. pneumoniae*	Characterization by molecular approaches other than WGS	MLST based clonal relatedness with characterization of genetic context and plasmid of NDM producers from a neonatal unit.

Qamar *et al.* Future Microbiol. 2019;14:691-704.	Pakistan	117	*E. coli*, and *K. pneumoniae*	Characterization by molecular approaches other than WGS	Transmission dynamics of carbapenemase producers by PFGE and DNA hybridization.
Remya *et al.* J Lab Physicians. 2019;11(4):312-316.	India	370	*K. pneumoniae*	Characterization by molecular approaches other than WGS	Clonal relatedness of NDM and KPC producers by PFGE.
Choudhury *et al.* J Infect Public Health. 2018;11(1):111-114.	India	One	*E. coli*	Characterization by molecular approaches other than WGS	Clonal relatedness of NDM-4 producers, revealed by MLST and PFGE along with plasmid characterization.
Rahman *et al.* J Glob Antimicrob Resist. 2018;14:154-157.	India	33	*E. coli*	Characterization by molecular approaches other than WGS	Clonal relatedness of NDM producers.
Ahmad *et al.* Microb Drug Resist. 2018;24(2):161-165.	India	402	Enterobacterales	Characterization by molecular approaches other than WGS	Characterize genetic context of NDM producers and plasmid typing by PBRT.
Gajamer *et al.* J Glob Antimicrob Resist. 2018;14:228-232.	India	973	*E. coli*	Characterization by molecular approaches other than WGS	Characterization of plasmids harbouring bla_{NDM}, isolated from urine specimens.
Ahmad *et al.* Front Microbiol. 2018;9:407.	India	44	Enterobacterales	Characterization by molecular approaches other than WGS	Characterization of plasmids harbouring bla_{NDM}.
Paul *et al.* Microb Drug Resist. 2017;23(7):815-821.	India	900	Carbapenem resistant bacteria	Characterization by molecular approches other than WGS	Evaluated the horizontal transmission of NDM producers.
Paul *et al.* J Infect Chemother. 2017;23(4):206-210.	India	Six	*E. coli*	Characterization by molecular approches other than WGS	Characterization of plasmids harbouring blaNDM-7.
Khan *et al.* J Pak Med Assoc. 2016;66(8):999-1004.	Pakistan	114	Enterobacterales	Characterization by molecular approaches other than WGS	Clonal relatedness of NDM-1 producers by tandem repeat analysis.
Subramanian *et al.* Indian J Med Microbiol. 2016;34(3):286-292.	India	Three	*K. pneumoniae*	Characterization by molecular approaches other than WGS	Characterization of plasmids harbouring bla_{NDM}.

Krishnaraju *et al.* Indian J Med Microbiol. 2015;33(1):30-38.	India	76	Enterobacterales	Characterization by molecular approaches other than WGS	Characterization of NDM producers.
Pesesky *et al.* Emerg Infect Dis. 2015;21(6):1034-1037.	Pakistan	78	Enterobacterales	Characterization by molecular approaches other than WGS	Characterization of plasmid harbouring $bla_{\text{NDM-1}}$ and bla_{KPC} from Pakistan and USA by sequencing of plasmid.
Hall *et al.* J Med Microbiol. 2014;63(Pt 8):1087-1092.	Sri Lanka	22	*K. pneumoniae*	Characterization by molecular approaches other than WGS	Clonal relatedness of carbapenemase producers by PFGE.
Sartor *et al.* Antimicrob Agents Chemother. 2014;58(9):5589-5593.	Pakistan	66	Enterobacterales	Characterization by molecular approaches other than WGS	Clonal spread of NDM producers, revealed by rep-PCR.
Khajuria *et al.* Indian J Pathol Microbiol. 2014;57(1):65-68.	India	Six	*K. pneumoniae*	Characterization by molecular approaches other than WGS	Outbreak caused by NDM producers in a neonatal unit.
Rahman *et al.* Int J Antimicrob Agents. 2014;44(1):30-37.	India	464	Enterobacterales	Characterization by molecular approaches other than WGS	Characterization of plasmid harbouring bla_{NDM}.
Khajuria *et al.* Chemother Res Pract. 2014;2014:972646.	India	130	Enterobacter Species	Characterization by molecular approaches other than WGS	Clonal relatedness and plasmid characterization of NDM producers.
Khajuria *et al.* J Clin Diagn Res. 2014;8(6):DC01-DC4.	India	300	*E. coli*	Characterization by molecular approaches other than WGS	Characterization of co-producing NDM-1 and OXA-48 carbapenemases by ERIC-PCR and PBRT.
Castanheira *et al.* Diagn Microbiol Infect Dis. 2013;75(2):210-213.	India	13	Enterobacterales	Characterization by molecular approaches other than WGS	Characterization of plasmids harbouring bla_{NDM}.
Islam *et al.* Eur J Clin Microbiol Infect Dis. 2012;31(10):2593-2600.	Bangladesh	1,816	Gram-negative bacteria	Characterization by molecular approaches other than WGS	Characterization of NDM producers by PFGE.
Castanheira *et al.* Antimicrob Agents Chemother. 2011;55(3):1274-1278.	India	39	Enterobacterales	Characterization by molecular approaches other than WGS	Clonal dissemination of carbapenemase producing bacteria, revealed by PFGE.

Roy *et al.* J Antimicrob Chemother. 2011;66(12):2773-2780.	India	99	*E. coli*	Characterization by molecular approaches other than WGS	Clonal relatedness NDM producers by PFGE, phenotyping (A, B1, B2 and D), and based on virulence factors (*hly, papC, sfa, iroNE. coli, cnf1, iucC* and *ibeA*).
Akter *et al.* Vet World. 2020 Feb;13(2):266-274.	Bangladesh	140	E. coli	Report of *mcr* in SA	Report of *mcr-3* from houseflies.
Azad *et al.* Pathogens. 2019;8(3):118.	Bangladesh	400	E. coli	Report of colistin resistance in SA	Report of colistin resistant *E. coli* from broiler chickens.
Sobur *et al.* J Adv Vet Anim Res. 2019;6(1):50-53.	Bangladesh	150	*E. coli*	Report of *mcr* in SA	Report of *mcr-3* positive *E. coli* from poultry, house flies, and pond water.
Johura *et al.* Gut Pathog. 2020;12:5.	Bangladesh	65	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* positive *E. coli* from food, water, hand rinse, and healthy human gut.
Islam *et al.* Gut Pathog. 2017;9:77.	Bangladesh	48	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* positive *E. coli* from urban sludge samples.
Sobur *et al.* Future Microbiol. 2019;14:847-858.	Bangladesh	300	*E. coli*	Report of *mcr* in SA	Report of *mcr-3* positive *E. coli* from house flies.
Sarker *et al.* J Adv Vet Anim Res. 2019;6(3):272-277.	Bangladesh	60	*E. coli*	Report of colistin resistance in SA	Report of colistin resistant *E. coli* from broilers sold.
Amin *et al.* J Glob Antimicrob Resist. 2020;22:546-552.	Bangladesh	104	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* positive *E. coli* from poultry environments.
Gogry *et al.* Environ Sci Pollut Res Int. 2019;26(32):33715-33717.	India	253	*Aeromonas* spp.	Report of *mcr* in SA	Report of *mcr-1* from urban sewage water.
Ghafur *et al.* J Glob Antimicrob Resist. 2019;16:48-52.	India	110	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* positive *E. coli* from food.
Singh *et al.* Antimicrob Agents Chemother. 2018;62(2):e01885-17.	India	200	*K. pneumoniae*	Report of *mcr* in SA	Report of *mcr-1* in chromosome.
Marathe *et al.* Water Res. 2017;124:388-397.	India	10*	NR	Report of *mcr* in SA	Report of *mcr-1* from waste.
Roy *et al.* Infect Control Hosp Epidemiol. 2020;41(3):378-380.	India	Two	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* from clinical sample.
Palani *et al.* Diagn Microbiol Infect Dis. 2020;97(1):114998.	India	65	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* from human gut flora.

Hameed *et al.* Rev Soc Bras Med Trop. 2019;52:e20190237.	Pakistan	146	*A. baumannii*, and *P. aeruginosa*	Report of *mcr* in SA	Report of *mcr-1* from clinical samples.
Azam *et al.* J Glob Antimicrob Resist. 2017;11:152-153.	Pakistan	NR	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* in avian pathogenic *E. coli*.
Mohsin *et al.* Pathog Dis. 2019;77(7):ftz064.	Pakistan	One	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* positive *E. coli* from broiler chicken.
Lv *et al.* Virulence. 2018;9(1):994-999.	Pakistan	100	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* positive E. *coli* from broiler chicken.
Mohsin *et al.* Antimicrob Agents Chemother. 2016;61(1):e01945-16.	India	29	*E. coli*	Report of *mcr* in SA	First report of *mcr-1* in Indian subcontinent from clinical isolate.
Rafique *et al.* Front Microbiol. 2020;10:3052.	Pakistan	92	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* from domestic chicken.
Joshi *et al.* FEMS Microbiol Lett. 2019;366(20):fnz237.	Nepal	180	*E. coli*	Report of *mcr* in SA	Report of *mcr-1* from chicken meats.
Joshi *et al.* Microb Drug Resist. 2019;25(6):846-854.	Nepal	324	*E. coli*	Report of *mcr* in SA	First report of *mcr-1* in Nepal from chicken.

NR, not reported. *Sediment samples.

My study has focused on two major aspects of AMR in Bangladesh. I incorporated WHO listed priority pathogens, CRE and *mcr*-producers in my PhD to assess the molecular epidemiology of AMR in the Bangladeshi settings. To the best of my knowledge, this is the first prospective systematic AMR survey in Bangladesh, conducted in the largest public health facilities, Dhaka Medical College Hospital (DMCH) of Bangladesh. WGS was deployed and genomic data was merged with pertinent patient's demographic and clinical data, to investigate the clinical burden, associations, potential outbreaks, and investigate the drivers of AMR. A follow-up carriage AMR survey was conducted one year after the clinical study to explore factors associated with faecal colonisation and to investigate clonal relatedness between clinical and faecal isolates. The specific objectives of this PhD were followings:

1. To identify the Enterobacterales species and to undertake a comprehensive phenotypic antibiogram on Enterobacterales from Bangladeshi hospitals with an aim to relate antibiotic resistance profile with patients' therapy and outcome – please see point #4.

2. Deep sequencing to provide the following information: A) Identify key bacterial clones dominant in causing infections. B) Enable the tracking of outbreaks and transmission routes for infections. C) Facilitate in determining genomic AMR gene clusters to identify recent evolution of strains expressing MDR phenotypes i.e. is the MDR mechanism mobile and spreading from one bacterial strain/species to another? Data from point #1 was supposed to inform as to which bacterial genes would be scrutinize – for example, for colistin resistance I would initially focus on those genes associated with colistin resistance e.g. *mcr-1*.

3. Understand the molecular epidemiology of AMR in the hospital settings of Bangladesh hospitals and plasmid tracking to investigate the dynamics of MDR gene spread. To identify and characterize the population genetic structure and clonal relationships of MDR pathogens.

4. Identify patient risk factors for infections with MDR Enterobacteriaceae clones by merging genomic sequencing with patient clinical and demographic data. To establish a detailed patient profile for investigating key risk factors of Enterobacterale infections associated with prevalent resistance mechanism in

Bangladesh. To verify risk factors with appropriate comparator for the study using standard statistical analysis.

5. To explore the prevalence of CRE in faecal colonisation among the patients hospitalised at DMCH in order to understand better about the transmission dynamics of CRE in the hospital [this objective was formulated after having the preliminary findings on resistance in Enterobacterale infections].

This thesis describes the most comprehensive AMR study in SA in both epidemiological and molecular perspectives which can be integrated into Bangladeshi national AMR data. My findings represent a baseline dataset to implement Fleming Fund (FF) initiatives from Bangladesh for effective interventions in IPC or antibiotic prescriptions/consumption.

Section Two

Materials and Methods

2.1 Collection of samples

2.1.1 Hospital setting

We performed this study at DMCH which is the largest public tertiary care hospital in Bangladesh containing 2600 allocated beds. The hospital setting is generally oversubscribed, regularly provides healthcare to the public 4-5 times than its capacity. To elucidate the extent of the hospital services, data on utilization of services at DMCH based on 'Health Bulletin' by Ministry of Health and Family Welfare (MOHFW), Bangladesh are shown in Table 2.1 (MOHFW, 2016).

Table 2.1 Utilization of services in DMCH (nominal, 2019) (MOHFW, 2016)*.

Indicators	Unit
Admissions (n)	149122
Emergency visits (n)	346580
Out-patient department (OPD) visits (n)	799896
In Patient deaths (n)	13865
Bed Occupancy Rates (%)	131.4
Average Length of Hospital Stay (LoS) (No of days per patient)	8.4
Hospital Death Rate (%)	9.3

*Data for 2019 have been updated in the health bulletin by MOHFW. n, number

2.1.2 Ethical considerations

This study was ethically approved by the Ethical Review Committee (ERC) of DMCH in accordance with the Helsinki Declaration [Memo no: MEU-DMC/ECC/2017/122]. Ethical permission is enclosed in appendix D. Required written consent was taken from all the participants for this study. The consent form is also attached in appendix D. Patient data anonymized with spreadsheets was protected by encryption and passwords to protect patients in accordance with the Helsinki Declaration (General Assembly of the World Medical Association, 2014).

2.1.3 Study design

During Oct 2016 to Sep 2017, a prospective cross-sectional survey on the prevalence of CRE in clinical infections of hospitalised patients was undertaken. We did a prospective case-control study for risk and outcome analysis of CRE infections. Secondly, a prospective cross-sectional carriage study was undertaken from May 2018 to June 2018 to understand the prevalence of CRE in faecal carriage. We estimated risk of CRE carriage through a case-control approach.

2.1.4 Collection of clinical specimens

Clinical specimens (n=1830) referred to the microbiology laboratory of DMCH from inpatients hospitalised for two or more days during October 2016 to September 2017 were included in this study. The clinical cases included in this study were suspected as infections by the physicians of DMCH, and specimens were referred for microbiology according to their decision. This study did not involve with the diagnosis of clinical infections, decision for undertaking microbiology testing, or clinical reporting. The specimens were plated initially at the local laboratory of DMCH and were broadly divided into two groups: 1) positive culture, and 2) no growth. Positive cultures obtained at the local laboratory of DMCH were transferred to Cardiff University (CU) for further analysis. Specimens sent to private laboratory during the study period were not included.

2.1.5 Collection of rectal swabs

During May 2018 to June 2018, 700 non-duplicated rectal swabs (RSs) from both inpatients (n=383) and outpatients (n=317) of DMCH. We estimated risk of CRE carriage through a case-control approach.

2.1.6 Collection of patients' demographic and clinical data

Data collected in the clinical study were patients' name, age, sex, locality, family member, family income, clinical symptoms or reason for hospitalization, admitting wards, type of clinical specimens, outcome [discharge, discharge against medical advice (DAMA), or death], date of admission, date of sample collection, date of outcome, and ongoing antibiotics used during hospitalisation, and the data were taken from the patients with positive cultures only.

Carriage study recorded data from 700 participants enrolled, and the data included patients' demography (as clinical study), admitting wards, date of admission, date of sample collection, and antibiotics used up to sampling.

2.1.6.1 Assessment of socio-economic status

Per capita monthly income (total monthly income of the family/total members of family) was used as parameter to determine the socio-economic condition of the patient in this study (Agarwal, 2008). To calculate the original income scale range of each socio-economic class in Bangladesh in 2017 and 2018, the income range (INR) according to modified proposed classification of the month of December 2004 was converted to Bangladeshi Taka (BDT) (1 INR is equivalent to about 1.25 BDT in 2017, and 2018) and then multiplied by conversion factor (CF) (Ghosh and Ghosh, 2009). CF between 2004 and the current year was determined by the following formula:

$$\text{CF between 2004 and 2017 in Bangladesh} = \frac{\text{Consumer Price Index (CPI) in 2017}}{\text{CPI in 2004}}$$

In Bangladesh, the average CPIs were 235, and 245 in 2017, and 2018, respectively. In 2004, CPI was around 90 (Trading Economics, 2020). So, the conversion factor was 2·61 and 2·72 for 2017, and 2018, accordingly. The modification is illustrated in Table 2.2. Fractions in income were ignored.

Table 2.2 Assessment of socio-economic status.

Social classes	**Per capita monthly family income limit**			
	BG Prasad's Classification of 1961 (INR)	Modified proposed classification of the month of December 2004 (INR)	Modified for 2017 Bangladesh (on (BDT)	Modified for 2018 in Bangladesh (on (BDT)
I. Upper-high (UH)	100 and above	10000 and above	32,625, and above	34,000, and above
II. High	50-99	5000-9999	16,312-32,624	17,000-33,996
III. Upper-middle (UM)	30-49	3000-4999	9,787-16,311	10,200-16,996
IV. Lower-middle (LM)	15-29	1500-2999	4,893-9,786	5,100-10,196
V. Poor	Below 15	500-1499	1,631-4,892	1,700-5,096
VI. Below poverty level (BPL)		Below 500	Below 1,630	Below 1,700

2.2 Transfer of biological specimens

Clinical isolates and RSs were collected in amines transport swabs with charcoal (Deltalab, Barcelona, Spain) and were transferred from Bangladesh to the UK in UN3373 containers (UN3373, Lelystad, the Netherlands) with proper documentation.

2.3 Cultivation of bacteria

Positive cultures from clinical specimens obtained at the local laboratory of DMCH were transferred to Cardiff University (CU) for further analysis. Blood specimens included in this study were cultured using BacT/ALERT 3D (bioMerieux, North Carolina, USA) at DMCH. Clinical isolates were sub-cultured onto chromogenic UTI agar (E&O Laboratories Ltd, Scotland, UK) without any antibiotic selection and faecal samples onto chromogenic UTI agar with vancomycin (10 mg/L) and ertapenem (2 mg/L) (Liofilchem, Roseto, Italy). Unless otherwise stated, the growth conditions used for all strains was overnight incubation aerobically at 37^{o} C. Bacteria were initially isolated by colony colour and morphology on chromogenic UTI. The species were identified by Matrix-Assisted Laser Desorption/Ionization-Time of Flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany).

2.4 Immortalization

All the bacterial isolates involved in this work were frozen down for future use with cryopreservation Storage Beads (Technical Service Consultants Ltd, Lancashire, UK) at -80^{o} C.

2.5 Determination of phenotypic resistance patterns

Minimum inhibitory concentration (MIC) was undertaken according to the latest guidelines outlined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) (v10.0) (EUCAST, 2020). Where appropriate, latest recommendations by Clinical Laboratory Standard Institute (CLSI) were implemented (CLSI, 2020). Agar dilution method was implemented to determine the MIC of clinically relevant antimicrobials (Andrews, 2001). Fifteen antimicrobials (amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftriaxone, ceftazidime, cefotaxime, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, amikacin, gentamicin, sulfamethoxazole-trimethoprim, fosfomycin, and colistin) underwent MIC testing in this study (Table 2.3). Although EUCAST does not recommend agar dilution for the determination of colistin MIC, we deployed the procedure for colistin contemplating it as more sensitive and reproducible than microbroth dilution (Turlej-Rogacka *et al.*, 2018).

Mueller Hinton agar plate containing antibiotic of interest ranging from 0.06 µg/ml to 256 µg/ml were prepared and 10^4 cfu/spot of each strain of up to 80 bacterial colonies per plate were applied by multi-point inoculator. The plates were then incubated overnight at 37º C. The lowest concentration of plate showing no growth was considered as the MIC of the strain. Antimicrobial susceptibility patterns were interpreted according to EUCAST clinical breakpoints or epidemiological cut-off (ECOFF) value, where appropriate (v10.0) (EUCAST, 2020). All antibiotics were prepared to a concentration of 2560 mg/L (stock A), with serial dilution to reach the desired concentration (calculation shown in Table 2.4).

Table 2.3 Details of antimicrobials used in this study for susceptibility testing.

Antibiotics	Manufacturer	Solvent	Diluent
Amikacin	Sigma-Aldrich, Missouri, USA	Water	Water
Amoxicillin:clavulanate (4:1)	Sigma-Aldrich, Missouri, USA	Saturated NaHCO3	Saturated NaHCO3
Cefepime	Sigma-Aldrich, Missouri, USA	Water	Water
Cefotaxime	Sigma-Aldrich, Missouri, USA	Water	Water
Ceftazidime	Sigma-Aldrich, Missouri, USA	Saturated NaHCO3	Water
Ceftriaxone	Bowmed Ibisqus Ltd, Clwyd, UK	Water	Water
Ciprofloxacin	Sigma-Aldrich Missouri, USA	Water	Water
Colistin	Sigma-Aldrich, Missouri, USA	Water	Water
Fosfomycin*	Mercury Pharmaceuticals Ltd, London, UK	Water	Water
Gentamicin**	Sigma-Aldrich, Missouri, USA	-	Water
Imipenem	Sigma-Aldrich, Missouri, USA	Phosphate buffer	Phosphate buffer
Levofloxacin	Sigma-Aldrich, Missouri, USA	Water	Water
Meropenem	Ranbaxy (UK) Ltd, Hayes, UK	Water	Water
Piperacillin	Sigma-Aldrich, Missouri, USA	Water	Water
Sulphamethoxazole	Sigma-Aldrich, Missouri, USA	DMSO	Water
Tazobactam	Sigma-Aldrich, Missouri, USA	Saturated NaHCO3	Water
Trimethoprim	Sigma-Aldrich, Missouri, USA	DMSO	Water

*Granular form; **In solution.

Table 2.4 Serial dilution of antimicrobials to reach desired concentration.

Stock solution	Serial dilution	Desired concentration
Stock A (2560 mg/L)	3.5 ml of stock A + 31.5 ml of media	256 µg/ml
	1.75 ml of stock A + add up to 35 ml of media	128 µg/ml
	900 µl of stock A + add up to 35 ml of media	64 µg/ml
	450 µl of stock A + add up to 35 ml of media	32 µg/ml
	225 µl of stock A + add up to 35 ml of media	16 µg/ml
	112.5 µl of stock A + add up to 35 ml of media	8 µg/ml
Stock B (80 mg/L: 500 µl of stock A+ 15.5 ml solvent)	1.75 ml of stock B + add up to 35 ml of media	4 µg/ml
	900 µl of stock B + add up to 35 ml of media	2 µg/ml
	450 µl of stock B + add up to 35 ml of media	1 µg/ml
	225 µl of stock B + add up to 35 ml of media	0.5 µg/ml
	112.5 µl of stock B + add up to 35 ml of media	0.25 µg/ml
Stock C (2.5 mg/L: 500 µl of 80 mg/L + 15.5 ml solvent)	1.75 ml of stock B + add up to 35 ml of media	0.125 µg/ml
	900 µl of stock B + add up to 35 ml of media	0.06µg/ml

2.6 Operational definition of carbapenem-resistant Enterobacterales

Whether an isolate was resistant or increased exposure to imipenem or meropenem or both was defined as CRE. However, *Proteus*, *Providencia* and *Morganella* were not considered as carbapenem resistant, if they were sensitive to meropenem, but resistant to imipenem, because reduced susceptibility to imipenem is considered intrinsic for those species (van Duin, 2017). If the isolate was sensitive to both imipenem and meropenem was considered as carbapenem-sensitive Enterobacterales (CSE). The participant, positive for at least one positive culture of CRE isolated from clinical and faecal specimens, was considered as a CRE clinical case and carriage, respectively. Whether any patient had positive culture with Enterobacterales from clinical and faecal specimens, but the respective Enterobacterales was sensitive to carbapenem, was regarded as CSE clinical case and carriage, accordingly.

2.7 Polymerase chain reaction

Polymerase chain reaction (PCR) was deployed, where appropriate. Overnight growth of bacterial colonies was suspended in 200 µl of water. For each reaction, master mix containing 18 µl of Clinical Laboratory Reagent Water (CLRW), 1 µl of bacterial suspension, and 1 µl of loading dye was added to puReTaq Ready-To-Go PCR Beads (Merck Life Science, New Jersey, USA). PCR reaction was performed using Bioer GeneTouch™ Thermal Cycler (Alpha Laboratories Ltd, Eastleigh, UK). The parameters applied in PCR reaction were: initial denaturation at 94° C for five minutes followed by 35 cycles of denaturation at 94° C for 45 seconds, annealing of primer (temperatures varied according to primer sequences, Table 2.5) for 45 seconds, extension at 72° C for one minute, and a final extension at 72° C for five minutes. The PCR amplicons were loaded into a set 1.5% agarose gel (Lonza Pharma & Biotech, Basel, Switzerland), and run in electrophoresis tank (Thermo Fisher Scientific, Waltham, USA) with 1% Tris-Borate-EDTA (TBE) buffer (appendix E) using 260 Volts for 30 minutes. Primers sequences used in this study with specific targets and conditions are illustrated in Table 2.5.

Table 2.5 Primers used in this study.

Targets	Primers	Sequences	Annealing Tm	Amplicon size	Reference
bla$_{\text{CTX-M-15}}$	CTXM15- F	ATGCGCAAACGGCGGACGTA	55° C	600 bp	Hasan, 2017
	CTXM15- R	CCCGTTGGCTGTCGCCCAAT			
bla$_{\text{NDM}}$	NDM-M-F	AGCTGAGCACCGCATT	60° C	648 bp	Hasan, 2017
mcr-1	NDM-M-R	CTCAGTGTCGGCATCAC			
	MCR-1-F	GCTACTGATCACCACGCTGT	55° C	953 bp	Yang *et al.*, 2017
	MCR-1-R	TGGCAGCGACAAAGTCATCT			
mcr-8	MCR-8-F	TATTCGCGGGTAACCAACCC	57° C	643 bp	This study
	MCR-8-R	ATATTCCGCTTTCCCCCAGC			

Tm, temperature

2.8 Whole genome sequencing

2.8.1 DNA extraction

A pure isolated bacterial colony was sub-cultured into an eppendorf containing 2 ml of fresh Luria-Bertani (LB) broth and incubated overnight at 37^{o} C in a shaking incubator. Overnight culture was centrifuged at 14,000 rpm for 10 minutes and supernatant was discarded. An automated extraction of DNA was undertaken using QIAcube (Qiagen, Hilden, Germany) according to manufacturer's protocol. The extracted DNA was preserved at -20^{o} C until use.

2.8.2 DNA quantitation

For each standard and DNA sample, 200 µl of working solution was prepared by diluting the Qubit reagent 1:200 in Qubit buffer. Assay tubes for standards were prepared by mixing 10 µl standard and 190 µl of working solution. From each DNA, 2 µl of DNA was added into 198 µl working solution to measure the DNA concentration. Following 2 minutes incubation at room temperature, assay tubes were inserted in the Invitrogen Qubit Fluorometer (Thermo Fisher Scientific, Waltham, USA) to take the readings.

2.8.3 Illumina MiSeq sequencing

2.8.3.1 Preparation of genomic libraries

Whole genome sequencing (WGS) of all isolates of interest was performed at illumina MiSeq platform (Illumina Inc., San Diego, USA). Nextera XT library preparation was performed according to manufacturer's instructions. The first step is the tagmentation of genomic DNA (gDNA) which was done by adding reagents [Tagment DNA Buffer (TD), Amplicon Tagment Mix (ATM), Neutralize Tagment Buffer (NT)] with 1 ng of input DNA followed by a limited cycle PCR with the mixture of tagment DNA, Nextera PCR Master Mix (NPM) and index adapters (initial 72^{o} C for 3 minutes, and 95^{o} C for 30 seconds, and then 12 cycles of 95^{o} C for 10 seconds, 55^{o} C for 30 seconds, and 72^{o} C for 30 seconds, and a final extension of 72^{o} C for 5 minutes). Libraries were cleaned up using Agencourt AMPure XP beads (AMPure XP beads) and freshly prepared 80% ethanol (EtOH) and the gDNA were eluded with Resuspension Buffer (RSB). To ensure more equal library representation in the pooled library, each library was mixed with Library Normalization Additives 1 (LNA1) and Library Normalization Beads 1 (LNB1) by shaking at 1800 rpm for 30 minutes

followed by a wash with Library Normalization Wash 1 (LNW1), and elution by 0.1 N NaOH. Then Library Normalization Storage Buffer 1 (LNS1) was added to each library. All the libraries were pooled in a tube and then diluted with Hybridization Buffer (HT1). PhiX was added as sequencing control into diluted pooled libraries. Paired end sequencing (2x301 cycles) was performed in Illumina MiSeq system.

2.8.3.2 Bioinformatic analysis of raw reads (fastq) from Illumina MiSeq system

Quality control of raw reads included fastqc (v0.11.2) (Wingett and Andrews, 2018) and adaptor trimming was performed using Trimgalore (v0.4.3) (Babraham Bioinformatics, 2019). Reads were assembled into contigs using the de novo assembler SPAdes (v3.9.0) (.fasta) (Bankevich *et al.,* 2012) and were aligned to the original fastq reads using Burrows-Wheeler aligner (BWA) (v0.7.15) (Li and Durbin, 2009). Any error was corrected using Pilon (v1.2) (Walker *et al.,* 2014). Assembly metrics were evaluated using Quast (v2.1) (Gurevich *et al.,* 2013). The de novo assembly produced multiple contigs (145-330), was then annotated with Prokka (v1.12) (Seemann, 2014).

2.8.4 MinION sequencing

2.8.4.1 Preparation of genomic libraries

To obtain long reads sequence data, minION sequencing was undertaken (Oxford Nanopore technologies, Oxford, UK). AMPure XP (Beckman Coulter, California, USA) was used to process gDNA to obtain a concentration 53.3 ng/µl. DNA library was prepared by pooling of all barcoded samples. 1 µl of RAD was added to pooled DNA. A final mixture of 75 µl (34 µl sequencing buffer, 25.5 µl loading beads, 4.5 µl water and 11 µl DNA library) was loaded to flow cell. MinION device was connected to MinKNOW GUI to obtain the reads.

2.8.4.2 Bioinformatic analysis of raw Nanopore reads

Demultiplexing of reads was performed using Porechop (0.2.3). Unicycler (0.4.4) was used to yield hybrid assembly using both Illumina short reads and minION long reads.

2.8.5 Phylogenetic analysis

Annotated assemblies generated by Prokka were used for core-genome alignment using Roary (v3.12.0) with default setting (a gene was considered as core if it was present in 99% of isolates and clustering of isolates based on presence of genes showing minimum blastp percentage identity of 95%), where all the genomes were from the same species (Page *et al.*, 2015). Single nucleotide polymorphism (SNP) calling and core-genome SNP-based alignment were performed using Snippy (v4.4.5) (Seemann, 2015). We constructed maximum likelihood (ML) tree with core alignment using RAxML-ng (v0.9.0.git-mpi) with a general time reversible (GTR) evolutionary model and gamma correction with iterations until bootstrapping converged with cut-off value of 3% (by default) (Kozlov *et al.*, 2019). Visualisation of phylogenetic trees and incorporation of metadata with the trees were performed using iTOL (v5) (Letunic and Bork, 2019). Recombination removal was performed using Gubbins (v2.3.4) (Croucher *et al.*, 2015) followed by calculation of pairwise SNP distance matrices using pairsnp (v0.0.7) (GitHub, 2018). SNPs-based network graphs were deployed to plot putative transmission and epidemiological links using Cytoscape (v3.8.0) with the clades having the following criteria: 1) contained isolates differed by ≤20 SNPs, 2) contained clinical isolates, 3) consisted of at least five isolates, and 4) contained carbapenem-resistant isolates (Shannon *et al.*, 2003). Connections between isolates were drawn whether the isolates were differed by ≤20 SNPs.

2.8.6 Phylogeographic analysis

Clonal phylogenetic signal can be obliterated due to recombination (Didelot and Falush, 2007). Hence time-scale molecular evolution was estimated using Bayesian Evolutionary Analysis by Sampling Trees (BEAST) package (v1.10.4) with known sequence sampling time after removal of recombination following whole genome alignment using Snippy (v4.4.5) (Drummond and Rambaut, 2007; Croucher *et al.*, 2015; Seemann, 2015). The following parameters were applied for Markov Chain Monte Carlo (MCMC) runs: 1) HKY substitution model, Gamma plus invariant sites heterogeneity model with estimated base frequency of 4 relative rates of mutations across sites 2) four different models (strict clock and constant population size, strict clock and exponential growth, relaxed clock and constant population size, and relaxed clock and exponential growth) was deployed in each alignment 3) MCMC chains were carried out at least 10^8 generations with sampling at every 1000 steps. The numbers of invariant sites were extracted from the recombination masked whole genome alignment and the numbers of constant sites were added to the document (.xml), generated by BEAUti. Each MCMC run was performed in triplicates. Best fit model was selected by calculation of Bayes factors (BFs). The BF is a ratio of two marginal likelihoods obtained for the two models, was used to compare the models from each other (Baele *et al.*, 2012; Baele *et al.*, 2013). Convergence of MCMC runs were assessed using Tracer (v.1.7.1). MCMC runs were only accepted whether there were estimations of >200 effective sample size (ESS). The maximum clade credibility (MCC) tree was obtained from the trees' posterior distributions, after a 10% burn-in, with the Tree-Annotator (v1.10.4) in BEAST package. The trees were visualized using FigTree (v1.4.4) (Rambaut, 2010).

2.8.7 Genomes retrieved from National Center for Biotechnology Information

Genomes from National Center for Biotechnology Information (NCBI) were included in this study for three purposes: 1) used in constructing phylogenetic trees in order to assess clonal lineages of the isolates in the respective trees, 2) used as reference genome for core-genome SNP-based tree, 3) identification and removal of recombinations from a set of closely related isolates, and 4) phylogeographic analysis. The details on NCBI genomes used in this study have been mentioned along with the relevant findings.

2.8.8 Analysis using publicly available database

Isolates were assigned as a species according to best match species in Kmer database embedded in Center for Genomic Epidemiology (CGE). MLST was delineated based on seven loci MLST databases in CGE, where appropriate (https://cge.cbs.dtu.dk/services/). Antimicrobial resistance genes (ARGs) were retrieved using Comprehensive Antibiotic Resistance Database (CARD) with a cut off ≥99.8% coverage and ≥99.8% identity, and virulence factors (VFs) and plasmid replicon types using Virulence Factor Database (VFDB) and PlasmidFinder, respectively with a cut off ≥99% coverage and ≥99% identity embedded in ABRicate (Liu *et al.,* 2019c; Alcock *et al.,* 2020).

2.9 Pulsed-field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) was performed to investigate the clonal relationship of outbreak isolates of *K. variicola* and to determine the size of plasmids having gene of interest in this study, as described previously (Toleman, 2018).

2.9.1 Making of plugs

A half loop full bacterial colony was mixed with 2 ml of Cell Suspension Buffer (appendix E) and adjusted the concentration of cell suspensions by Jeneway 7315 spectrophotometer (Geneflow limited, Staffordshire, UK) [610 nm wavelength, absorbance (Optical Density) of 1.00 (range of 0.8-1.0)]. A mixture consisted of 400 µl of cell suspension buffer, 20 µl of Proteinase K (20 mg/ml) and 400 µl melted 1% SeaKem Gold agarose (Lonza Pharma & Biotech, Basel, Switzerland) was made by gently pipetting mixture up and down a few times. The mixture was dispensed immediately into the appropriate wells of reusable plug mold. The plugs were kept at room temperature for 10-15 minutes to solidify.

2.9.2 Lysis of Cells and washing in Agarose Plugs

Agarose plugs (3-4) of each strain of interest were dispensed into a mixture of 5 ml cell lysis buffer (appendix E) and 20 µl of proteinase K (20 mg/ml) (Roche, Basel, Switzerland) for incubation in a shaking incubator at 54-55° C for 2 hours. Therefore, series of washing by pre-heated CLRW (2 times) and preheated Tris-EDTA (TE) (10 mM Tris:1 mM EDTA) buffer (appendix E) (4 times) were undertaken.

2.9.3 Digestion of DNA in agarose plugs

2.9.3.1 Digestion by restriction enzyme

A master mix of 180 µl CLRW with 20 µl of FastDigest restriction buffer (10X) (Thermo Fisher Scientific, Waltham, US) was prepared to accomplish pre-restriction incubation of 2.0 to 2.5 mm wide slice of a plug at room temperature for 10-15 minutes. A master mix was prepared which contained 177 µl of CLRW, 22 Tango buffer (10X) premixed with Bovine Serum Albumin (BSA) (Thermo Fisher Scientific, Waltham, USA), and 1 µl of SpeI (10 U/µL) (Thermo Fisher Scientific, Waltham, USA). The plugs were digested with the mixture at 37˚ C for 2 hours.

2.9.3.2 Digestion by S1 enzyme

Each individual plug was washed at room temperature for 20 minutes in 1 ml of TE buffer (10 mM Tris:1 mM EDTA) followed by a second wash at room temperature for 20 minutes in 1 ml of 1X S1 buffer (appendix E). Master mix containing 0.5 ul of S1 (100U/ul) into 6 ml of 1X S1 buffer was prepared. 200ul of the enzyme solution was then used to digest each plug. The digest was incubated at 4^{o} C overnight. Once digested the plug is removed from the digest solution and cut in half for loading into agarose gel.

2.9.4 Electrophoresis

The digested plugs were loaded into a set 1% Pulsed-field gel electrophoresis (PFGE) agarose gel (Sigma-Aldrich, Missouri, USA). A Lamda Ladder DNA Marker (Bio-Labs, Massachusetts, USA) was used as a control to determine the size. Electrophoresis was performed in a PFGE tank (CHEF-DRIII System, Bio-Rad Laboratories, Hemel Hempstead, Hertforshire, UK) by means of conditions of 5 minutes of initial switch time, 45 minutes of final switch time, 21 hours run time, 6V volts, 120^{o} included angle and 4^{o} cooling module. After completion, gel was visualised by UVP Gel-Doc It^{TM} Imaging System (Thermo Fisher Scientific, Waltham, USA) and the images were saved for further analysis.

2.10 In-gel hybridization of gene of interest

2.10.1 Pre-hybridization processing of gel

Gels were dried in a drying cabinet at 50^{o} C overnight between 2 sheets of blotting paper and stored for hybridization. Before hybridization, gels were re-hydrated by 200 ml of deionized DNase free water for 5 minutes to pull away from the blotting paper. Then the gels were treated with 200 ml of denaturing solution (appendix E) at room temperature for 45 minutes followed by 200 ml of neutralising solution (appendix E) for 45 minutes at room temperature. The gels were placed in a hybridisation tube with 20 ml of pre-hybridisation solution (appendix E) and incubated for at least 24 hours at 65^{o} C.

2.10.2 Preparation of radioactive probe

The probe was prepared by the random priming labelling method. The purified PCR product (200ng in 15ul water) was mixed with 8 ul of DNAase free water and 10

ul of random primer in a sterile top eppendorf and boiled for 5 minutes. ^{32}P radioactive probe (2.5 ul), dCTP buffer (10 ul) and Klenow (1 ul) were added and the eppendorf was incubated within a lead jar at 37° C for 15 minutes. A Random Primer Labelling Kit (Strategene, California, USA) was used. The labelled probe was run through a silica gel Nick™ Column (VWR International Ltd, Leicestershire, UK) with following an initial washing by 320 ul of 0.1M Tris (pH 7.5) and a subsequent wash with 430 ul of 0.1M Tris (pH 7.5). The flow through from second wash was collected as the eluded radiolabelled target gene. The labelled probe was then boiled for 6 minutes in a screw cap eppendorf tube.

2.10.3 In gel hybridization step

The labelled probe was added into the pre-hybridized gel and left to hybridize overnight at 65° C. The liquid from hybridization tube was removed and the hybridized gel was washed with 100 ml solution of 2X SSC (appendix E), and 0.1% SDS (Thermo Fisher Scientific, Waltham, USA) for 1 hour at 65° C, and subsequently, with 100 ml solution of 0.1X SSC (appendix E), and 0.1% SDS for 1 hour at 65° C. The gel was wrapped in a single layer of flat, compressed cling film, and then was fixed against a sheet of film in a film cassette overnight at -80° C. The film was treated using standard film development until all bands were visible followed by fixation.

2.11 Investigating the stability of plasmid

Serial passaging of *mcr-8.1*positive *K. pneumoniae* (MCRPKP) was performed in a colistin-free medium up to 12 days to investigate whether the *mcr-8* was stable. Overnight cultures of MCRPKP were diluted 1:1,000 in fresh LB medium without colistin and incubated with vigorous shaking (220 rpm) at 37°C for 24 h. Total gDNA was extracted on day 0, day 3, day 6, day 9 and day 12 using the QIAcube (Qiagen, Hilden, Germany) from the overnight culture with optical density (OD) ranged from 0.08 to 0.1 at 600 nm. OD value was measured using Jeneway 7315 spectrophotometer (Geneflow limited, Staffordshire, UK). The prepared DNA was analysed to quantify *mcr-8.1* (plasmid-borne) and *RcsA*, a housekeeping gene (HKG) in triplicate by real-time quantitative PCR (qPCR). Primers and probes were designed manually using the Geneious prime primer design tool (11.0.2; Biomatters Ltd.) and synthesised by Eurofins (Ebersberg, Germany). The primers and probes used in this study are shown the Table 2.6. The real-time qPCR mixture of 20 µl was prepared using 10 µl SSO advanced master mix (Bio-Rad, USA), 0.4 µl primer mix (mixture of 10 µL of each primer [100 pmol] and 60 µl of molecular grade water), 0.4 µl probe mix (mixture of 10 µL of each probe [100 pmol] and 80 µl of molecular grade water), 4.2 µl of molecular grade water and 5 µl template DNA. qPCR was performed using the CFX96 real-time system (Bio-Rad, USA) with cycling parameters of 1 x (95°C x 5 minutes), 44 x (95°C x 15 seconds, 60°C x 10 second). C_T value was measured by maestro interpretative software installed in the qPCR system. Relative abundance of *mcr-8.1*, compared to HKG were calculating by delta-delta C_T method ($2^{-\Delta\Delta Ct}$). Gene expression on day 0 was used a control.

Table 2.6 Sequences of primers and probes for qPCR designed in this study.

Name	Target	Tm	GC%	Sequence	Product length	Product (Tm)
MCR 8_F	*mcr-8*	58.2	52.0	CTCGCTTGCAGATTCCCTTACAACC	-	-
MCR 8_R	*mcr-8*	58.2	52.2	TCCGTGCCGCATCAGAAGAAAAC	-	-
MCR 8_ Probe	*mcr-8*	61.7	46.7	ACCATTGTTATTGTTGCGCCATAGCACCTC	108 bp	73
RcsA_F	*K. pneumoniae*	58.4	59.1	TCCCAACCGGGTATAGCTGCAC	-	-
RcsA_R	*K. pneumoniae*	59.3	59.1	TATCGCCCGCAAGGACTGCTTC	-	-
RcsA_Probe	*K. pneumoniae*	63.1	41.0	ACATGACCCATCCTCAATCAACACGTAACGATATACACT	117 bp	74.5

Tm, temperature

2.12 Conjugation experiment

Conjugation experiment was performed using isolates carrying the *mcr-1* and *mcr-8* as donors and *Klebsiella variicola* (BD_DM_07) and *E. coli* J53 as recipients, respectively. Donor and recipient strains were grown in luria broth (Sigma-Aldrich, Missouri, USA) until they reached the exponential growth phase (OD at 620 nm [OD_{620}] of 0.6). Conjugation experiments were performed on chromogenic UTI agar (Sigma-Aldrich, Missouri, USA) plates, using a 1:1 donor-recipient mix, and plates were incubated at 37°C for 6 hours. The conjugation mixture was suspended in 1 ml of phosphate-buffered saline, and dilutions were plated on selective media. Selective media was prepared using colistin (4 μg/ml) only to obtain transconjugants harbouring *mcr-1* and colistin (4 μg/ml) with sodium azide (100 μg/ml) to obtain transconjugants harbouring *mcr-8* and transconjugants were picked based on colony colour. Transferability of *mcr* in transconjugants was confirmed by PCR. Transfer frequencies were calculated by colony forming unit (cfu) count of transconjugants against the cfu count of donor (Saavedra *et al.*, 2017).

2.13 *In vitro* time-growth studies

Bacterial suspension of OD ranged from 0.08 to 0.1 at 600 nm was prepared from overnight culture and diluted 1:100 in fresh LB medium. *In vitro* growth rate of *E. coli* J53 and transconjugants (TDM_697b, TDM_782 and TDM_914b) was determined by OD in 30 minutes interval for 24 hours with shaking at 100 rpm using FLUOstar Omega microplate reader (BMG LABTECH Ltd., Aylesbury, UK). The experiment was performed in five replicates. The growth curve of each strain was generated with mean OD at each time point and was fitted to the modified Gompertz model developed by Zwietering *et al.* (1990) using GraphPad Prism (v7.04). The growth rate and lag time were calculated. The growth rate of each transconjugant was compared to that of *E. coli* J53 by unpaired two-tailed t test using GraphPad Prism (v7.04).

2.14 *In vivo* pathogenicity testing in *Galleria mellonella*

In vivo pathogenicity of *K. variicola* was compared to hypervirulent *K. pneumoniae* A58300 in a *Galleria mellonella* infection model (larval stage) (Live Foods UK Ltd., Somerset, UK). *K. pneumoniae* A58300 was provided for this study by Prof. Ana C Gales (Coutinho *et al.*, 2014). Inoculum to be injected into larvae were prepared by washing of bacterial pellets and dilution until standardised at OD_{600nm}= 0.7 followed by serial dilution. Using a Hamilton syringe, 10 µl aliquots of each serially diluted bacterial suspension (10^3 to 10^7 cfu/ml) was injected into a group of 10 larvae (this was repeated three times). Larvae were incubated at 37 °C, and the survival of larvae was monitored daily for 3 days. Death was denoted when larvae no longer responded to touch. Three groups of larvae (first group with 10 µl saline injection, second with mock injection and third without injection) were used as controls.

2.15 Statistical analysis

Statistical analyses were done using IBM SPSS (v26). Chi-square text (x^2) was carried out to analyse the risks with categorical variables and independent-samples t-test was performed if the dependent variable with continuous data and statistical significance (p<0.05) values were adjusted by Benjamini-Hochberg correction. We did univariate statistics to assess the key risk factors. Cox proportional hazards model was applied for analysing heath burden of carbapenem resistance (described in detail with the respective chapters). Kaplan–Meier method was deployed for survival analysis with respect to both carbapenem resistance and *in-vivo* pathogenicity testing in *G. mellonella* (described in detail with the respective chapters). Pearson correlation coefficient (r) was calculated to evaluate correlation between variables.

Section Three

Estimating Prevalence, Risks, and Burden of Carbapenem-Resistant Enterobacterales in Clinical Infections of a Bangladeshi Health Setting

3.1 Introduction

Enterobacterales are a large family of Gram-negative bacteria that includes *Escherichia*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Salmonella*, *Shigella*, *Proteus*, *Serratia* and other species. The members of Enterobacterales are present as normal flora in the human intestinal tract. These pathogens are commonly associated with urinary tract infections (UTIs), intra-abdominal infections (IAIs), nosocomial pneumonia, and BSIs. Carbapenems are drugs used to treat serious MDR infections in the hospital settings worldwide (Wikipedia, 2020e; Martirosov and Lodise, 2016; van Duin and Doi, 2017). The global spread of CRE is regarded as a public health threat resulting in enormous health and economic burdens, particularly in LMICs (O'Neill J, 2016; Walsh, 2010; Martirosov and Lodise, 2016; Pokharel *et al.,* 2019; Stewardson *et al.*, 2019). *E. coli*, *K. pneumoniae* and *Enterobacter* spp. have been recognised as the most frequent reservoir of carbapenemase determinants, causing life threatening infections (Hsu *et al.,* 2017; Jaggi *et at.*, 2019; Wu *et al.*, 2019; Walia *et al.,* 2019). Estimating the risks and outcome of any disease condition is a vital indicator to investigate the intensity of the ailment which facilitates developing effective interventions, and therefore, to evaluate the costs and benefit of the interventions. Tackling AMR in a country mandates periodic high-quality data on both epidemiological, and clinical components (Midega, 2020).

Previous AMR data from Bangladesh directs an overall high frequency of MDR pathogens in the country (Ahmed *et al.,* 2019; Hoque *et al.,* 2020; Mahmud *et al.*, 2020; Parvin *et al.*, 2020; WHO, 2020a). This chapter has achieved following aims of this project [**objectives #1, and #4**]: **1)** the prevalence of CRE in Bangladeshi health settings and a detailed resistance profile of clinical Enterobacterales identified in this study, **2)** predisposing factors for developing carbapenem resistance in the largest tertiary care hospital of Bangladesh, **3)** the impact of CRE infections on human health.

3.2 Results

3.2.1 Prevalence of Enterobacterales infections in a clinical setting of Bangladesh

Study population includes patients admitted at DMCH during 21st October 2016 to 23rd September 2017 whose clinical specimens were referred to clinical microbiology laboratory of DMC. The total number of clinical cases enrolled in this pilot surveillance was 1831, of which 534 (29.2%) patients had Enterobacterales infections. The clinical infections enrolled in this study were confirmed by the physicians at DMCH. Study outline is illustrated in Figure 3.1.

A total of 1893 clinical specimens were included in this study. The frequency of culture-positive clinical samples was 58% (1098/1893) (Table 3.1), and a total of 1583 organisms were isolated (Table 3.2). The prevalence of Enterobacterales among the clinical isolates was 40.6% (643/1583) (Figure 3.1; Table 3.2). The most predominant members of Enterobacterales were *E. coli* (228/1583, 14.4%), and *K. pneumoniae* (228/1583, 14.4%) followed by *P. mirabilis* (64/1583, 4%), *E. cloacae* (37/1583, 2.3%), *P. stuartii* (19/1583, 1.2%), *K. variicola* (14/1583, 0.9%), *S. marcescens* (13/1583, 0.8%) and others (40/1583, 2.5%) (Table 3.2).

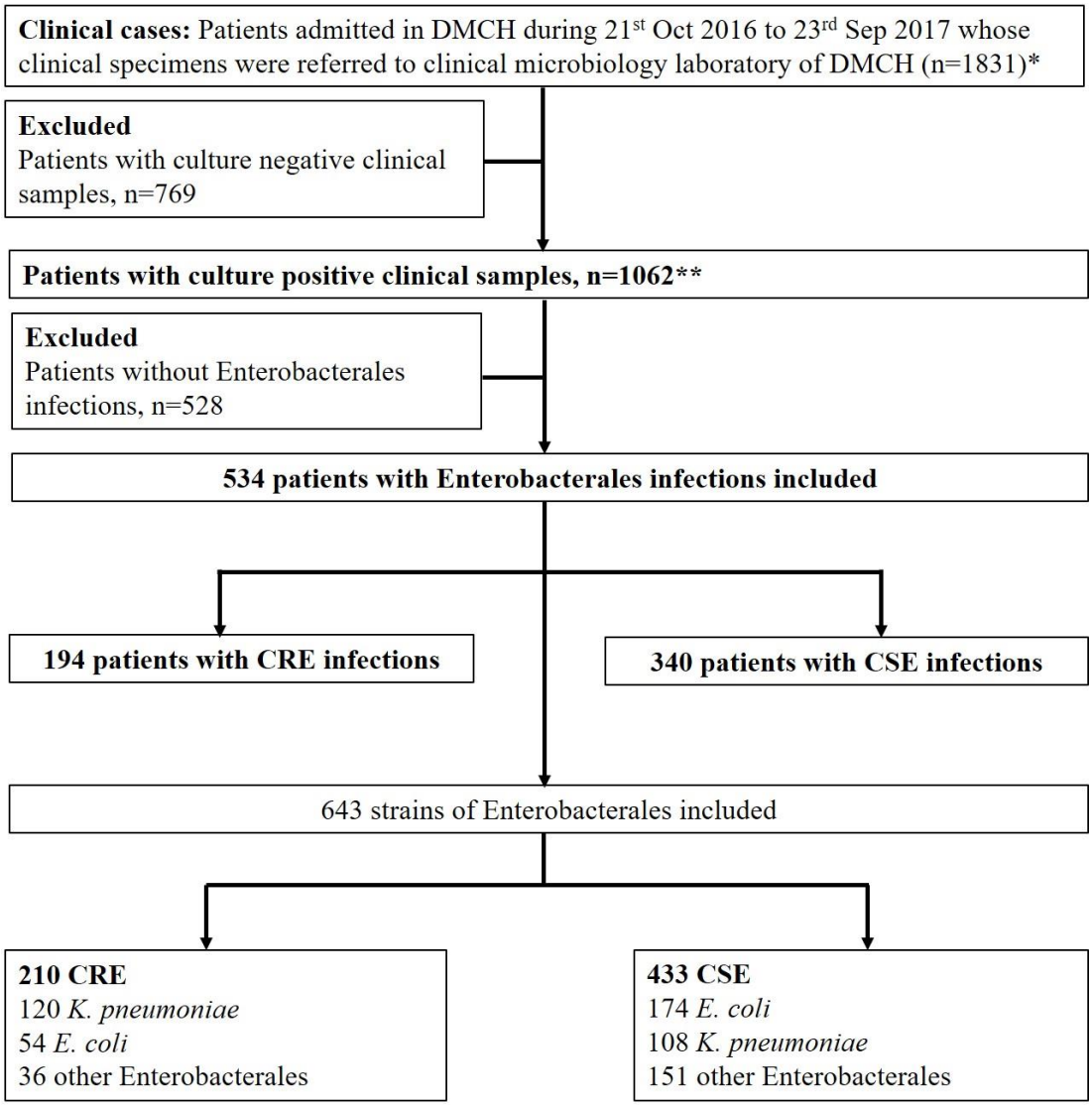


Figure 3.1 Flowchart diagram of clinical sample collection at DMCH.

CRE, carbapenem-resistant Enterobacterales; CSE, carbapenem-sensitive Enterobacterales. *During 4th July to 23rd Sep 2017, specimens were collected only from NICU. Multiple clinical specimens were collected from 61 patients (blood & wound swab, n=51; blood & urine, n=3; blood & tracheal aspirate, n=2; wound swab & urine, n=2; blood & catheter tip, n=1; urine & tracheal aspirates, n=1; blood, urine & catheter tip, n=1).**Patients with multiple culture positive clinical samples was 35 (blood & wound swab, n=26; blood & urine, n=2; blood & tracheal aspirate, n=2; wound swab & urine, n=2; blood & catheter tip, n=1; urine & tracheal aspirates, n=1; blood, urine & catheter tip, n=1).

Table 3.1 Total number of culture-positive clinical specimens from different wards of DMCH in this study (n=1893*).

Ward	Blood (n=527)			CT (n=6)			CV tip (n=7)			TA (n=99)			Urine (n=442)			WS (n=809)			Total
	S	M	NG	S	M	NG	S	M	NG	S	M	NG	S	M	NG	S	M	NG	
Burn	90	45	121	-	3	-	1	-	-	-	-	-	4	3	6	122	110	172	677
Casualty unit	-	-	1	-	-	-	-	-	-	-	-	-	-	-	1	6	4	9	21
CCU	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1
Dialysis Unit	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1
Fistula unit	-	-	-	-	-	-	-	-	-	-	-	-	31	11	7	-	1	-	50
Gynaecology	5	-	2	-	-	-	-	-	-	-	-	-	5	5	4	33	11	18	83
Haematology	6	1	9	-	-	1	-	-	-	-	-	-	5	4	3	1	-	3	33
HDU	1	1	2	-	-	-	-	-	1	3	1	2	1	-	5	2	1	7	27
ICU	8	6	11	-	1	-	2	-	-	32	47	14	6	10	16	1	1	1	156
Medicine	6	-	5	-	-	-	-	-	-	-	-	-	7	-	20	4	1	10	54
Nephrology	1	-	-	-	-	-	-	-	-	-	-	-	8	5	14	-	-	-	28
Neurology	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Neurosurgery	-	-	6	-	-	-	-	-	-	-	-	-	1	-	-	1	2	-	10
NICU	43	12	118	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	173
OCC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
Oncology	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1	2
Orthopaedics	1	-	2	-	-	-	-	-	-	-	-	-	2	2	1	27	12	7	54
PS	3	1	2	-	-	1	-	-	-	-	-	-	12	6	13	5	2	4	49
Paediatrics	2	1	9	-	-	-	-	-	-	-	-	-	-	-	11	1	1	8	33
Postnatal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
Surgery	3	-	3	-	-	-	-	1	1	-	-	-	8	1	8	99	45	70	239
Urology	-	-	-	-	-	-	-	-	-	-	-	-	70	62	62	2	2	-	198
Total	170	67	291	-	4	2	3	2	2	35	48	16	161	109	172	305	193	311	1,891

CT, catheter tip; CV, central venous; TA, tracheal aspirate; WS, wound swab; S, single growth; M, mixed growth; NG, no growth; CCU, coronary care unit; HDU, high dependency unit; ICU, intensive care unit; NICU, neonatal intensive care unit; OCC, one-stop crisis centres; PS, paediatric surgery. *One bile and one urethral discharge were collected which were culture negative and positive, respectively.

Table 3.2 Total number of clinical isolates identified in this study (n=1583).

Organisms	Blood	CT	CV tip	TA	UD	Urine	WS	Total
A. baumannii	36	1	1	32	0	15	84	169
Acinetobacter spp. (other than *A. baumannii*)*	8	0	0	4	0	15	5	32
Burkholderia spp.*	19	0	0	1[a]	0	1	0	21
Candida spp.*	6	0	0	0	0	7	2	15
Citrobacter farmeri	0	0	0	0	0	1	0	1
C. freundii	0	0	0	0	0	1	2	3
Citrobacter koseri	2	0	0	0	0	1	0	3
Citrobacter rodentium	0	0	0	0	0	0	2	2
Citrobacter sedlakii	0	0	0	0	0	0	2	2
Corynebacterium striatum	2	0	0	0	0	1	8	11
Entercoccus spp.*	23	1	2	11	0	59	74	170
E. aerogenes	0	0	0	0	0	0	2	2
E. asburiae	0	0	0	0	0	0	1	1
E. cloacae	8	0	0	1	0	8	20	37
E. coli	15	2	0	10	0	118	83	228
Escherichia hermannii	1	0	0	0	0	0	0	1
K. oxytoca	0	0	0	0	0	3	1	4
K. pneumoniae	44	3	1	30	1	54	95	228
K. quasipneumoniae	0	0	0	0	0	0	1	1
K. variicola	14	0	0	0	0	0	0	14
Leclercia adecarboxylata	0	0	0	0	0	1	0	1
M. morganii	0	0	0	0	0	5	1	6
Pantoea agglomerans	0	0	0	1	0	0	0	1
Proteus hauseri	0	0	0	0	0	2	0	2
P. mirabilis	10	0	0	8	0	7	39	64
Proteus vulgaris	1	0	0	0	0	0	0	1
P. rettgeri	0	0	0	0	0	2	2	4
P. stuartii	6	0	0	1	0	0	12	19
P. aeruginosa	65	1	1	24	1	60	203	355
Pseudomonas spp. (other than *P. aeruginosa*)*	5	1	0	0	0	6	14	26
Salmonella spp.**	5	0	0	0	0	0	0	5
S. marcescens	6	0	0	3	0	3	1	13
S. aureus	3	0	1	0	0	2	39	45
Staphylococcus spp. (other than *S. aureus*)*	22	0	0	0	0	7	10	38
S. maltophilia	1	0	0	10	0	5	2	18
Others***	8	0	1	4	0	16	10	40
Total	310	9	7	140	2	400	715	1583

CT, catheter tip; CV, central venous; TA, tracheal aspirate; UD, urethral discharge; WS, wound swab. *The organisms at the species level identified were: *A. bereziniae* (n=9), *A. junii* (n=6), *A. pittii* (n=5), *A. schindleri* (n=3), *Acinetobacter ursingii* (n=3), *Acinetobacter baylyi* (n=2), *A. radioresistens* (n=2), *Acinetobacter towneri* (n=2), *Burkholderia cepacia* (n=16), *Burkholderia cenocepacia* (n=4), *Burkholderia gladioli* (n=1), *Candida tropicalis* (n=9), *Candida albicans* (n=4), *Candida auris* (n=2), *Enterococcus faecalis* (n=116), *Enterococcus faecium* (n=39), *Enterococcus gallinarum* (n=6), *Enterococcus hirae* (n=3), *Enterococcus avium* (n=2),

Enterococcus casseliflavus (n=2), *Enterococcus raffinosus* (n=1), *Enterococcus thailandicus* (n=1), *Pseudomonas mendocina* (n=11), *P. putida* (n=6), *Pseudomonas mosselii* (n=2), *Pseudomonas oleovorans* (n=2), *Pseudomonas brassicacearum* (n=1), *Pseudomonas composti* (n=1), *Pseudomonas guariconensis* (n=1), *Pseudomonas pseudalcaligenes* (n=1), *Pseudomonas stutzeri* (n=1), *Staphylococcus haemolyticus* (n=19), *Staphylococcus sciuri* (n=6), *Staphylococcus epidermidis* (n=5), *Staphylococcus hominis* (n=3), *Staphylococcus arlettae* (n=2), *Staphylococcus cohnii* (n=2), *Staphylococcus capitis* (n=1), *Staphylococcus saprophyticus* (n=1). **Organism could not be identified at the species level by MALDI. ***Any non-Enterobacterales was considered as 'others', if the frequency of respective species isolation was less than 10.[a]*B. cepacia* from TA.

3.2.2 Characterization of clinical Enterobacterales for carbapenem resistance and susceptibility patterns of other relevant antimicrobials

CRE isolation from the clinical specimens was 11.1% (210/1893), and clinical cases of CRE was 10.6% (194/1831) (Figure 3.1). Carbapenem resistance at the species level were: *K. pneumoniae*, 52.6% (120/228); *E. coli*, 23.7% (54/228); *E. cloacae*, 27% (10/37); *P. mirabilis*, 11% (7/64); *P. stuartii*, 36.8% (7/19); *K. variicola*, 35.7% (5/14); and others 13.2% (7/53) (Table 3.3). Range of MIC values of all antimicrobials against each species of Enterobacterales are listed in Table 3.3. The phenotypic resistance patterns of other antimicrobials of each species are also shown in Table 3.4. Minor species, *Salmonella* spp. (n=5), *P. agglomerans* (n=1), *E. hermannii* (n=1), and *L. adecarboxylata* (n=1) were not assessed for MIC_{50} and MIC_{90}. *Salmonella* spp. and *P. agglomerans* were sensitive to all antibiotics. Susceptibility patterns of minor species are described in Figure 3.2. *K. pneumoniae* was significantly more resistant to carbapenems than other species ($p<0.05$) (Table 3.5).

Table 3.3 MIC ranges of clinical Enterobacterales.

	Antibiotics	MIC_{50} (mg/l)	MIC_{90} (mg/l)	Range of MIC (mg/l)	n (%) Resistance
***E. coli* (n=228)**	AMC	128	256	2 to 256	219 (96.1)
	TZP	32	>256	0.125 to >256	130 (57)
	CRO	>256	>256	0.125 to >256	194 (85.1)
	CAZ	64	256	≤0.06 to >256	194 (85.1)
	CTX	256	>256	≤0.06 to >256	194 (85.1)
	FEP	128	>256	≤0.06 to >256	188 (82.5)
	IPM	0.25	8	≤0.06 to 256	38 (16.7)
	MEM	≤0.06	16	≤0.06 to >256	53 (23.2)
	CIP	128	>256	≤0.06 to >256	204 (89.5)
	LVX	32	64	≤0.06 to 256	204 (89.5)
	AMK	4	>256	1 to >256	65 (28.5)
	GEN	1	>256	≤0.06 to >256	115 (50.4)
	SXT	256	>256	0.5 to >256	160 (70.2)
	FOF	0.5	1	0.25 to 8	0 (0)
	CST	0.125	0.25	≤0.06 to 2	0 (0)
***K. pneumoniae* (n=228)**	AMC	256	256	4 to >256	225 (98.7)
	TZP	256	>256	1 to >256	153 (67.1)
	CRO	256	>256	≤0.06 to >256	211 (92.5)
	CAZ	64	256	≤0.06 to >256	214 (93.9)
	CTX	128	>256	≤0.06 to >256	212 (93)
	FEP	32	128	≤0.06 to >256	206 (90.4)
	IPM	2	16	≤0.06 to >256	94 (41.2)
	MEM	4	32	≤0.06 to >256	119 (52.2)
	CIP	16	256	≤0.06 to >256	216 (94.7)
	LVX	8	64	≤0.06 to >256	180 (78.9)
	AMK	>256	>256	0.5 to >256	152 (66.7)
	GEN	256	>256	0.125 to >256	174 (76.3)
	SXT	>256	>256	1 to >256	225 (98.7)
	FOF	8	32	1 to >256	16 (7)
	CST	0.5	0.5	≤0.06 to 64	4 (1.8)
***Klebsiella* other than *K. pneumoniae* (n=19)**	AMC	128	256	16 to 256	19 (100)
	TZP	8	>256	2 to >256	9 (47.4)
	CRO	128	>256	≤0.06 to >256	15 (78.9)
	CAZ	32	256	≤0.06 to >256	15 (78.9)
	CTX	64	256	≤0.06 to 256	15 (78.9)
	FEP	8	64	≤0.06 to 64	15 (78.9)
	IPM	0.5	8	0.5 to 8	4 (21.1)
	MEM	≤0.06	8	≤0.06 to 8	5 (26.3)
	CIP	2	4	≤0.06 to 8	16 (84.2)
	LVX	0.5	2	≤0.06 to 2	9 (47.4)
	AMK	2	16	1 to 16	5 (26.3)
	GEN	0.25	1	0.25 to 32	1 (5.3)
	SXT	>256	>256	4 to >256	17 (89.5)
	FOF	4	16	2 to 16	0 (0)
	CST	0.25	0.05	≤0.06 to 0.5	0 (0)
***Proteus* spp. (n=67)**	AMC	>256	>256	0.5 to >256	61 (91)
	TZP	0.5	16	≤0.06 to 64	8 (11.9)
	CRO	4	16	≤0.06 to >256	44 (65.7)
	CAZ	64	>256	≤0.06 to >256	50 (74.6)
	CTX	16	64	≤0.06 to >256	49 (73.1)
	FEP	8	32	≤0.06 to 128	48 (71.6)
	IPM	4	8	≤0.06 to 64	66 (98.5)
	MEM	≤0.06	0.5	≤0.06 to 16	3 (4.5)

	CIP	32	256	≤0.06 to >256	60 (89.6)
	LVX	32	64	≤0.06 to >256	60 (89.6)
	AMK	>256	>256	1 to >256	46 (68.7)
	GEN	>256	>256	0.5 to >256	60 (89.5)
	SXT	256	>256	1 to >256	65 (97)
	FOF	8	32	0.25 to >256	6 (8.9)
	CST	>256	>256	>256	67 (100)
***Enterobacter* spp. (n=40)**	AMC	256	256	64 to >256	40 (100)
	TZP	8	>256	1 to >256	17 (42.5)
	CRO	128	>256	0.25 to >256	32 (80)
	CAZ	16	256	≤0.06 to >256	34 (85)
	CTX	128	>256	0.25 to >256	33 (82.5)
	FEP	8	256	≤0.06 to >256	29 (72.5)
	IPM	1	64	0.125 to 128	10 (25)
	MEM	≤0.06	64	≤0.06 to 64	11 (27.5)
	CIP	2	64	≤0.06 to >256	26 (65)
	LVX	1	16	≤0.06 to 128	21 (52.5)
	AMK	4	>256	1 to >256	8 (20)
	GEN	32	>256	0.125 to >256	24 (60)
	SXT	256	>256	1 to >256	32 (80)
	FOF	8	64	0.5 to 128	6 (15)
	CST	0.25	0.5	≤0.06 to 64	1 (2.5)
***Citrobacter* spp. (n=11)**	AMC	>256	>256	32 to >256	11 (100)
	TZP	8	>256	2 to >256	4 (36.4)
	CRO	32	>256	≤0.06 to >256	7 (63.6)
	CAZ	16	>256	≤0.06 to >256	7 (63.6)
	CTX	32	>256	≤0.06 to >256	7 (63.6)
	FEP	8	64	≤0.06 to 64	7 (63.6)
	IPM	0.5	8	0.25 to 16	4 (36.4)
	MEM	≤0.06	16	≤0.06 to 16	3 (27.3)
	CIP	2	8	≤0.06 to 16	7 (63.6)
	LVX	1	8	≤0.06 to 32	6 (54.5)
	AMK	4	>256	1 to >256	4 (36.4)
	GEN	32	>256	0.125 to >256	6 (54.5)
	SXT	8	256	1 to 256	8 (72.7)
	FOF	0.5	4	0.25 to 64	1 (9.1)
	CST	0.25	0.5	0.125 to 0.5	0 (0)
***Providencia* spp. (n=23)**	AMC	>256	>256	2 to >256	22 (95.7)
	TZP	32	>256	2 to >256	17 (73.9)
	CRO	64	>256	≤0.06 to >256	20 (87)
	CAZ	256	>256	0.125 to >256	22 (95.7)
	CTX	64	128	≤0.06 to >256	21 (91.3)
	FEP	16	64	≤0.06 to 64	17 (73.9)
	IPM	2	16	0.125 to 32	23 (100)
	MEM	≤0.06	16	≤0.06 to 16	7 (30.4)
	CIP	32	256	0.25 to >256	22 (95.7)
	LVX	32	128	0.5 to 256	22 (95.7)
	AMK	>256	>256	0.5 to >256	19 (82.6)
	GEN	>256	>256	≤0.06 to >256	19 (82.6)
	SXT	>256	>256	4 to >256	23 (100)
	FOF	16	32	1 to 128	2 (8.7)
	CST	>256	>256	>256	23 (100)
***S. marcescens* (n=13)**	AMC	32	256	8 to >256	12 (92.3)
	TZP	2	256	1 to >256	3 (23.1)
	CRO	0.125	>256	≤0.06 to >256	3 (23.1)
	CAZ	0.5	256	0.25 to >256	2 (15.4)
	CTX	0.125	128	0.125 to >256	3 (23.1)
	FEP	0.06	64	≤0.06 to 256	2 (15.4)

	IPM	0.5	16	0.5 to 128	2 (15.4)
	MEM	≤0.06	8	≤0.06 to 32	2 (15.4)
	CIP	≤0.06	0.25	≤0.06 to 4	1 (7.7)
	LVX	0.125	1	≤0.06 to 8	2 (15.4)
	AMK	2	4	1 to 64	1 (7.7)
	GEN	0.5	4	0.25 to 256	2 (15.4)
	SXT	8	16	2 to >256	12 (92.3)
	FOF	8	16	2 to 16	0 (0)
	CST	>256	>256	256 to >256	13 (100)
***M. morganii* (n=6)**	AMC	64	>256	64 to >256	6 (100)
	TZP	2	32	0.5 to 32	1 (16.7)
	CRO	≤0.06	128	≤0.06 to 128	3 (50)
	CAZ	0.25	64	0.25 to 64	3 (50)
	CTX	≤0.06	128	≤0.06 to 128	3 (50)
	FEP	≤0.06	8	≤0.06 to 8	3 (50)
	IPM	2	4	1 to 4	6 (100)
	MEM	≤0.06	0.5	≤0.06 to 0.5	0 (0)
	CIP	256	>256	1 to >256	6 (100)
	LVX	16	64	2 to 64	6 (100)
	AMK	16	>256	0.5 to >256	4 (66.7)
	GEN	8	>256	0.125 to >256	4 (66.7)
	SXT	256	>256	4 to >256	6 (100)
	FOF	64	>256	64 to >256	6 (100)
	CST	>256	>256	>256	6 (100)

n, number of resistant isolates to respective antibiotic; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin.

Table 3.4 Antimicrobial susceptibility patterns of clinical Enterobacterales.

Species	Resistance to respective antibiotics, n (%)														
	AMC	TZP	CRO	CAZ	CTX	FEP	IPM	MEM	CIP	LVX	AMK	GEN	SXT	FOF	CST
E. coli (n=228)	219 (96.1)	130 (57)	194 (85.1)	194 (85.1)	194 (85.1)	188 (82.5)	38 (16.7)	53 (23.2)	204 (89.5)	204 (89.5)	65 (28.5)	115 (50.4)	160 (70.2)	0 (0)	0 (0)
K. pneumoniae (n=228)	225 (98.7)	153 (67.1)	211 (92.5)	214 (93.9)	212 (93)	206 (90.4)	94 (41.2)	119 (52.2)	216 (94.7)	180 (78.9)	152 (66.7)	174 (76.3)	225 (98.7)	16 (7)	4 (1.8)
Other *Klebsiella* spp. (n=19)*	19 (100)	9 (47.4)	15 (78.9)	15 (78.9)	15 (78.9)	15 (78.9)	4 (21.1)	5 (26.3)	16 (84.2)	9 (47.4)	5 (26.3)	1 (5.3)	17 (89.5)	0 (0)	0 (0)
Proteus spp. (n=67)	61 (91)	8 (11.9)	44 (65.7)	50 (74.6)	49 (73.1)	48 (71.6)	67 (100)	3 (4.5)	60 (89.6)	60 (89.6)	46 (68.7)	60 (89.5)	65 (97)	6 (8.9)	67 (100)
Enterobacter spp. (n=40)	40 (100)	17 (42.5)	32 (80)	34 (85)	33 (82.5)	29 (72.5)	10 (25)	11 (27.5)	26 (65)	21 (52.5)	8 (20)	24 (60)	32 (80)	6 (15)	1 (2.5)
Citrobacter spp. (n=11)	11 (100)	4 (36.4)	7 (63.6)	7 (63.6)	7 (63.6)	7 (63.6)	4 (36.4)	3 (27.3)	7 (63.6)	6 (54.5)	4 (36.4)	6 (54.5)	8 (72.7)	1 (9.1)	0 (0)
Providencia spp. (n=23)	22 (95.7)	17 (73.9)	20 (87)	22 (95.7)	21 (91.3)	17 (73.9)	23 (100)	7 (30.4)	22 (95.7)	22 (95.7)	19 (82.6)	19 (82.6)	23 (100)	2 (8.7)	23 (100)
S. marcescens (n=13)	12 (92.3)	3 (23.1)	3 (23.1)	2 (15.4)	3 (23.1)	2 (15.4)	2 (15.4)	2 (15.4)	1 (7.7)	2 (15.4)	1 (7.7)	2 (15.4)	12 (92.3)	0 (0)	13 (100)
M. morganii (n=6)	6 (100)	1 (16.7)	3 (50)	3 (50)	3 (50)	3 (50)	6 (100)	0 (0)	6 (100)	6 (100)	4 (66.7)	4 (66.7)	6 (100)	6 (100)	6 (100)

Values in parentheses indicate row percentage. AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin. Data on *Salmonella* spp. (n=5), *P. agglomerans* (n=1) and *E. hermannii* (n=1) are not included in this table. **Klebsiella* species other than *K. pneumoniae*. Cells are highlighted according to the proportion of resistance of the species of Enterobacterales against respective antibiotics: ≥90% are represented by red (●); 60% to 89% by amber (●); 30% to 59% by yellow (●); <30% by green (●); intrinsic resistance by grey (●).

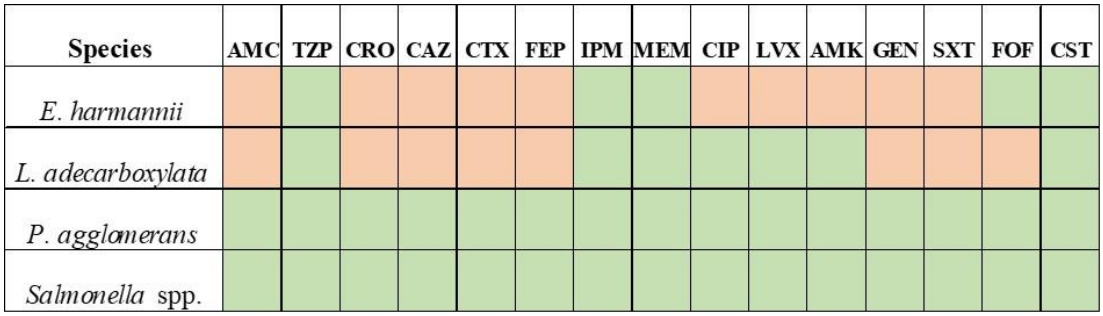


Figure 3.2 Susceptibility patterns of *Salmonella* spp. (n=5), *P. agglomerans* (n=1), *E. hermannii* (n=1), and *L. adecarboxylata* (n=1). Green indicates susceptible and red indicates resistance to respective antibiotics. AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin.

Table 3.5 Comparative analysis for the distribution of carbapenem resistance among the species of Enterobacterales (n=643).

Attributes		CRE (n=210)	CSE (n=433)	*p* value
Carbapenem resistance in *K. pneumoniae*	*K. pneumoniae*	120 (57.1%)	108 (24.9%)	<0.0001
	Other Enterobacterales	90 (42.9%)	325 (75.1%)	
Carbapenem resistance in *E. coli*	*E. coli*	54 (25.7%)	174 (40.2%)	<0.0001
	Other Enterobacterales	156 (74.3%)	259 (59.8%)	
Carbapenem resistance in *Proteus* spp.	*Proteus* spp.	7 (3.3%)	373 (86.1%)	<0.0001
	Other Enterobacterales	203 (96.7%)	60 (13.9%)	
Carbapenem resistance in *Enterobacter* spp.	*Enterobacter* spp.	11 (5.2%)	29 (6.7%)	0.472
	Other Enterobacterales	199 (94.8%)	404 (93.3%)	

Bivariate analysis was performed to measure the associations. Cells are highlighted whether any variable was significantly higher with CRE.

3.2.3 The prevalence of carbapenemases alleles in clinical Enterobacterales

Carbapenemase alleles were characterized according to WGS data. The most prevalent carbapenemase gene among the clinical Enterobacterales was *bla*$_{\text{NDM-5}}$ (97/643, 15.1%) followed by *bla*$_{\text{NDM-1}}$ (62/643, 9.6%), *bla*$_{\text{OXA-232}}$ (26/643, 4%), *bla*$_{\text{OXA-181}}$ (24/643, 3.7%), *bla*$_{\text{NDM-7}}$ (8/643, 1.2%), *bla*$_{\text{KPC-1}}$ (5/643, 0.8%), *bla*$_{\text{NDM-4}}$ (4/643, 0.6%), and novel NDM variants (9/643, 1.4%) (Table 3.6). The combinations of carbapenemase alleles recovered were: *bla*$_{\text{KPC-1}}$+*bla*$_{\text{OXA-181}}$ (5/643, 0.8%), *bla*$_{\text{NDM-1}}$+*bla*$_{\text{OXA-232}}$ (3/643, 0.5%), *bla*$_{\text{NDM-5}}$+*bla*$_{\text{OXA-181}}$ (3/643, 0.5%), *bla*$_{\text{NDM-5}}$+*bla*$_{\text{OXA-232}}$ (3/643, 0.5%), and *bla*$_{\text{NDM-7}}$+*bla*$_{\text{OXA-181}}$ (1/643, 0.2%). The most prevalent carbapenemase in *E. coli* was *bla*$_{\text{NDM-5}}$ (41/228, 17.1%), and *K. pneumoniae* were *bla*$_{\text{NDM-5}}$ (50/228, 21.9%), and *bla*$_{\text{NDM-1}}$ (38/228, 16.7%). There were associations between the prevalence of specific carbapenemase gene, and certain species of clinical Enterobacterales [*bla*$_{\text{NDM-1}}$ in *K. pneumoniae* (38/228, 16.7%) than others (24/415, 5.8%) (p<0.05), *bla*$_{\text{OXA-181}}$ in *K. pneumoniae* (17/228, 7.5%) than others (7/415, 1.7%) (p<0.05), and *bla*$_{\text{NDM-5}}$ in *K. pneumoniae* (50/228 21.9%) than others (47/415, 11.3%) (p<0.05)]. Among the clinical Enterobacterales, *bla*$_{\text{NDM-4}}$ was only found in *E. coli* and *bla*$_{\text{OXA-232}}$ and *bla*$_{\text{KPC-1}}$ were only in *K. pneumoniae* (Figure 3.3).

All the clinical Enterobacterales phenotypically resistant to carbapenem, had at least one carbapenemase, but we found the presence of *bla*$_{\text{OXA-232}}$ (n=9) and *bla*$_{\text{OXA-181}}$ (n=1) in phenotypically carbapenem-susceptible isolates (low clinical breakpoint according to EUCAST). Table 3.7 describes the range of MIC of OXA-232-, and OXA-181-producing Enterobacterales.

We considered the alleles as novel NDM variants whether there were base substitutions in the variants compared to known *bla*$_{\text{NDM}}$ variants (data not shown). Elucidating functional and molecular properties of the novel NDM variants was beyond the scope of this study. Further work has been planned to characterise the novel variants.

Table 3.6 The distribution of carbapenemases alleles among the species of clinical Enterobacterales (n=643).

Carbapenemase alleles	Host species (n)	n (%)
NDM-5	CRo (1), CSe (1), EC (41), EnC (4), KP (50)	97 (15.1)
NDM-1	EC (1), EnC (4), KP (38), KV (5), PM (6), PS (7), SM (1)	62 (9.6)
OXA-232	KP (26)	26 (4)
OXA-181	EC (5), KP (17), PM (1), MM (1)	24 (3.7)
Novel NDM variants	CRo (1), EC (4), EnC (1), KP (3)	9 (1.4)
NDM-7	CFa (1), EC (3), EnA (1), EnC (1), KP (1), SM (1)	8 (1.2)
KPC-1	KP (5)	5 (0.8)
NDM-4	EC (4)	4 (0.6)

n, number of isolates; CFa, *Citrobacter farmeri;* CRo, *C. rodentium;* CSe, *C. sedlakii;* EC, *E. coli;* EnA, *Enterobacter aerogenes;* EnC, *E. cloacae;* KP, *K. pneumoniae;* KV, *K. variicola;* MM, *M. morganii;* PM, *P. mirabilis;* PS, *P. stuartii;* SM, *S. marcescens.*

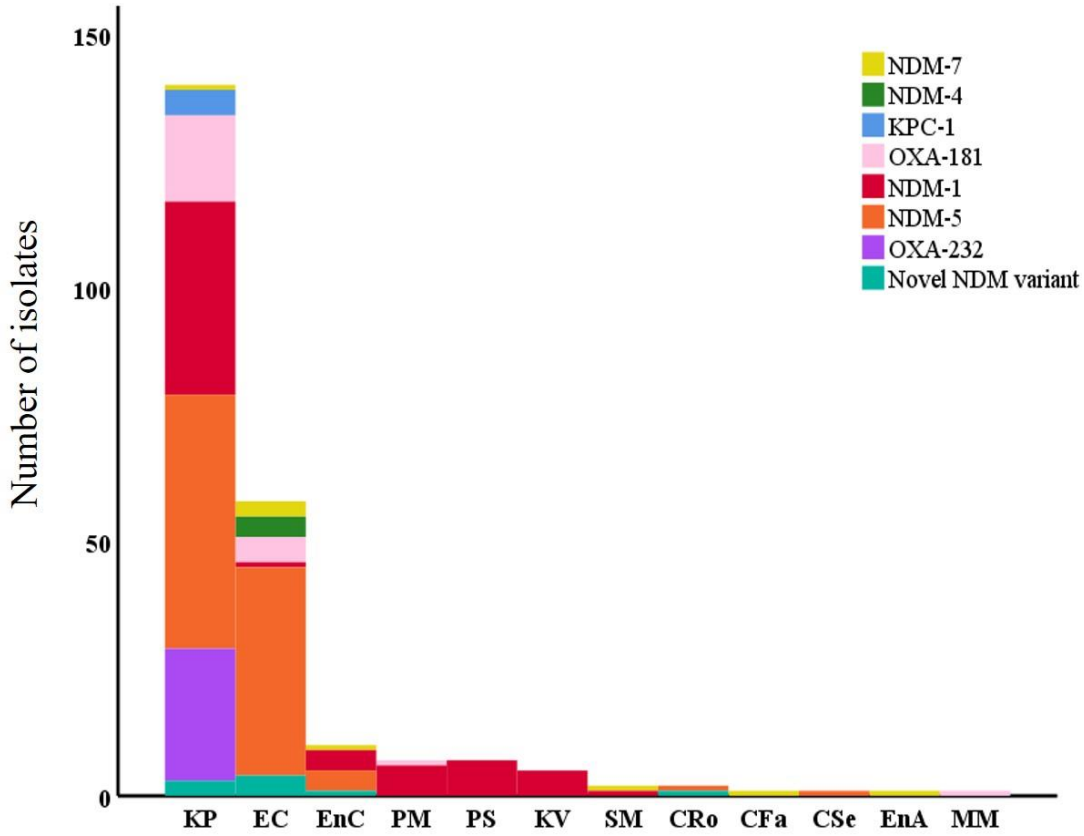


Figure 3.3 Comparative distribution of carbapenemase alleles among different species of clinical Enterobacterales. CFa, *Citrobacter farmeri;* CRo, *C. rodentium;* CSe, *C. sedlakii;* EC, *E. coli;* EnA, *Enterobacter aerogenes;* EnC, *E. cloacae;* KP, *K. pneumoniae;* KV, *K. variicola;* MM, *M. morganii;* PM, *P. mirabilis;* PS, *P. stuartii;* SM, *S. marcescens.* Significant associations were observed in *K. pneumoniae* for $bla_{\text{NDM-1}}$ (38/228, 16.7%) than others (24/414, 5.8%) (p<0.0001), for $bla_{\text{OXA-181}}$ (17/228, 7.5%) than others (7/414, 1.7%) (p=0.0002), and for $bla_{\text{NDM-5}}$ (50/228 21.9%) than others (47/414, 11.4%) (p=0.0003).

Table 3.7 Range of carbapenems' MIC of OXA-232 and OXA-181 producers.

Carbapenemase variants	Carbapenems	Range of MIC (mg/l)
bla$_{NDM-1}$+*bla*$_{OXA-232}$ (n=3)	IPM	8 to 16
	MEM	16 to 32
bla$_{NDM-5}$+*bla*$_{OXA-232}$ (n=3)	IPM	16 to 32
	MEM	64 to 128
bla$_{OXA-232}$ (n=11)	IPM	1 to 8
	MEM	16
bla$_{OXA-232}$ (phenotypically characterized as CSE) (n=9)	IPM	0·5 to 2
	MEM	0·5 to 2
bla$_{KPC-1}$+*bla*$_{OXA-181}$ (n=5)	IPM	16 to 32
	MEM	32 to 64
bla$_{NDM-5}$+*bla*$_{OXA-181}$ (n=3)	IPM	2 to 8
	MEM	8 to 32
bla$_{NDM-7}$+*bla*$_{OXA-181}$ (n=1)	IPM	256
	MEM	≥256
bla$_{OXA-181}$ (n=14)	IPM	4 to 32
	MEM	0·06 to 64
bla$_{OXA-181}$ (phenotypically characterized as CSE) (n=1)	IPM	0·125
	MEM	0·06

IPM, imipenem; MEM, meropenem.

3.2.4 Investigating the associations of non-β-lactam resistance in clinical CRE

The overall phenotypic resistance of the clinical Enterobacterales is depicted in Figure 3.4. Fosfomycin, and colistin were shown to be the most active antimicrobials. CRE exhibited significantly greater resistance rates to ciprofloxacin, levofloxacin, amikacin, gentamicin, sulfamethoxazole-trimethoprim, and fosfomycin than CSE ($p<0.05$) (Table 3.8). The associations between non-β-lactam resistance and carbapenem resistance in *E. coli* and *K. pneumoniae* were evaluated individually which revealed significant associations as to overall analysis ($p<0.05$), however, the relation with fosfomycin resistance was varied. All *E. coli* were sensitive to fosfomycin, but carbapenem-resistant *K. pneumoniae* had a significant association with the fofomycin resistance ($p<0.05$) (Table 3.9; Table 3.10).

The associations of prevalent ARGs with the certain prevalent carbapenemase allele ($bla_{\text{NDM-1}}$, $bla_{\text{NDM-5}}$, $bla_{\text{OXA-181}}$) were examined. ARGs significantly present in association with specific carbapenemase alleles are illustrated in Table 3.11-Table 3.13. We assessed the linkage of any ARGs with CRE whether the frequency of ARG was above 10. Known ARGs were retrieved using CARD with a cut off ≥99.8% coverage, and ≥99.8% identity. The number of efflux pumps was not included in this analysis. *Salmonella* spp. (n=5), *L. adecarboxylata* (n=1), and one strain of *Proteus* spp. (due to low quality sequence data) were not included in the analysis.

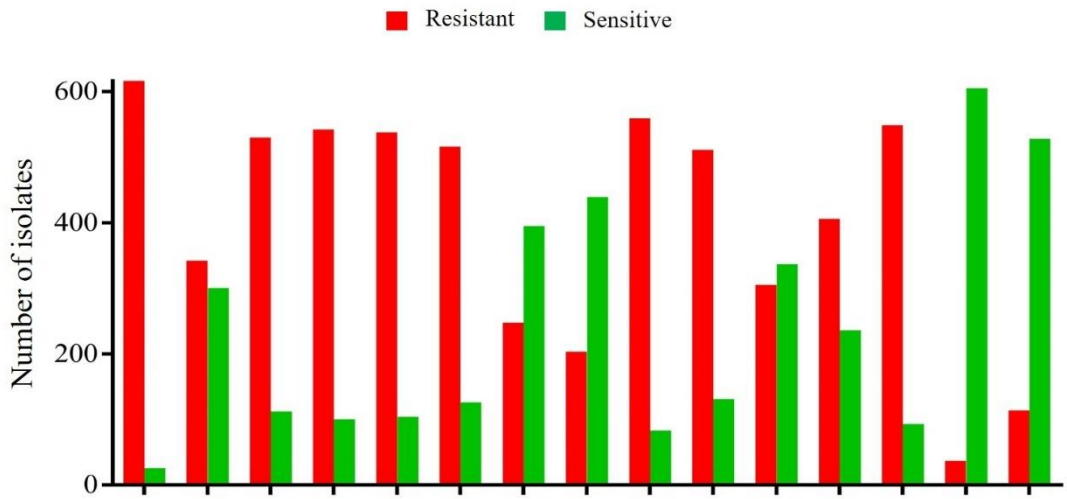


Figure 3.4 Susceptibility patterns of clinical Enterobacterales against the antibiotics tested. All *Proteus* spp., *Providencia* spp., *S. marcescens* and *M. morganii* isolated in this study were resistant to colistin. AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin.

Table 3.8 Comparative resistance profile to non-β-lactam antibiotics between CRE and CSE.

Antibiotics	Resistant to respective antibiotics, n (%)		*p* value
	CRE (n=210)	CSE (n=433)	
CIP	206 (98.1)	353 (81.5)	<0.0001
LVX	191 (91.0)	320 (73.9)	<0.0001
AMK	190 (90.5)	115 (26.6)	<0.0001
GEN	197 (93.8)	210 (48.5)	<0.0001
SXT	200 (95.2)	350 (80.8)	<0.0001
FOF*	18/210 (8.6)	13/426 (3.1)	0.002
CST**	1/194 (0.5)	4/340 (1.2)	0.446

Values in parentheses indicate column percentage. CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin. **M. morganii* (n=6), and *L. adecarboxylata* (n=1) were excluded from the analysis as the species are intrinsically resistant to fosfomycin. ***Proteus* spp. (n=67), *Providencia* spp. (n=23), *S. marcescens* (n=13) and *M. morganii* (n=6) were excluded from the analysis as the species are intrinsically resistant to colistin. Cells are highlighted whether any variable was significantly higher with CRE.

Table 3.9 Comparative resistance profile to non-β-lactam antibiotics between carbapenem-resistant *E. coli* and carbapenem-sensitive *E. coli*.

Antibiotics	**Resistant to respective antibiotics, n (%)**		***p* value**
	Carbapenem-resistant *E. coli* (n=54)	Carbapenem-sensitive *E. coli* (n=174)	
CIP	54 (100)	150 (86.2)	0.004
LVX	54 (100)	150 (86.2)	0.004
AMK	41 (75.9)	24 (13.8)	<0.0001
GEN	51 (94.4)	64 (36.8)	<0.0001
SXT	45 (83.3)	115 (66.1)	0.016

Values in parentheses indicate column percentage. CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim. All *E. coli* were sensitive to fosfomycin and colistin. Cells are highlighted whether any variable was significantly higher with CRE.

Table 3.10 Comparative resistance profile to non-β-lactam antibiotics between carbapenem-resistant *K. pneumoniae* and carbapenem-sensitive *K. pneumoniae*.

Antibiotics	**Resistant to respective antibiotics, n (%)**		***p* value**
	Carbapenem-resistant *K. pneumoniae* (n=120)	Carbapenem-sensitive *K. pneumoniae* (n=108)	
CIP	119 (99.2)	97 (89.8)	0.002
LVX	105 (87.5)	75 (69.4)	0.001
AMK	120 (100)	32 (29.6)	<0.0001
GEN	118 (98.3)	56 (51.9)	<0.0001
SXT	120 (100)	105 (97.2)	0.066
FOF	15 (12.5)	1 (0.9)	0.001
CST	1 (0.8)	3 (2.8)	0.264

Values in parentheses indicate column percentage. CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin. Cells are highlighted whether any variable was significantly higher with CRE.

Table 3.11 The associations of prevalent ARGs with *bla*$_{NDM-1}$-positive clinical isolates compared to *bla*$_{NDM-1}$-negative clinical isolates.

ARGs	Presence of ARG, n (%)		*p* value
	bla$_{NDM-1}$-positive isolates (n=62)	*bla*$_{NDM-1}$-negative isolates (n=574)	
aadA2	25 (40.3)	157 (27.4)	0.032
APH(3")-Ib	4 (6.5)	9 (1.6)	0.012
APH(3')-VI	16 (25.8)	1 (0.2)	<0.0001
armA	24 (38.7)	87 (15.2)	<0.0001
rmtF	14 (22.6)	26 (4.5)	<0.0001
SAT-1	9 (14.5)	46 (8)	0.078
bla$_{OXA-1}$	39 (62.9)	205 (35.7)	<0.0001
bla$_{OXA-9}$	10 (16.1)	19 (3.3)	<0.0001
bla$_{SHV-1}$	7 (11.3)	13 (2.3)	0.0001
bla$_{SHV-106}$	13 (21)	27 (4.7)	<0.0001
bla$_{SHV-182}$	8 (12.9)	19 (3.3)	0.0005
bla$_{SHV-187}$	4 (6.5)	10 (1.7)	0.018
arr2	20 (32.3)	52 (9.1)	<0.0001
arr-3	6 (9.7)	17 (3)	0.009
catB3	6 (9.7)	17 (3)	0.008
mphE	29 (46.8)	127 (21.1)	<0.0001
qnrA1	6 (9.7)	16 (2.8)	0.007
qrrD1	7 (11.3)	28 (4.9)	0.035
sul1	47 (75.8)	273 (47.6)	<0.0001

Values in parentheses indicate column percentage. The associations between *bla*$_{NDM-1}$, and other ARGs were only mentioned if *p* values were <0.05.

Table 3.12 The associations of prevalent ARGs with *bla*$_{NDM-5}$-positive clinical isolates compared to *bla*$_{NDM-5}$-negative clinical isolates.

ARGs	Presence of ARG, n (%)		*p* value
	bla$_{NDM-5}$-positive isolates (n=97)	*bla*$_{NDM-5}$-negative isolates (n=539)	
aadA2	75 (77.3)	107 (19.9)	<0.0001
APH(6)-Id	44 (45.4)	142 (26.3)	0.0001
armA	26 (26.8)	85 (15.8)	0.008
rmtB	72 (74.2)	19 (3.5)	<0.0001
bla$_{ampC1}$	10 (10.3)	18 (3.3)	0.002
bla$_{ampH}$	11 (11.3)	20 (3.7)	0.001
bla$_{CMY-59}$	28 (28.9)	49 (9.1)	<0.0001
bla$_{CTX-M-15}$	81(83.5)	329 (61)	<0.0001
bla$_{OXA-1}$	57 (58.8)	187 (34.7)	<0.0001
bla$_{SHV-1}$	7 (7.2)	13 (2.4)	0.013
bla$_{SHV-182}$	8 (8.2)	19 (3.5)	0.034
bla$_{TEM-1}$	85 (87.6)	220 (40.8)	<0.0001
bla$_{VEB-5}$	20 (20.6)	11 (2)	<0.0001
catI	26 (26.8)	44 (8.2)	<0.0001
mphE	31 (32)	125 (23.2)	0.056
qnrS1	38 (39.2)	107 (19.9)	<0.0001
sul1	87 (89.7)	233 (43.2)	<0.0001
sul2	48 (49.5)	144 (26.7)	<0.0001
dfrA1	10 (10.3)	21 (3.9)	0.008
dfrA12	77 (79.4)	98 (18.2)	<0.0001

Values in parentheses indicate column percentage. The associations between *bla*$_{NDM-5}$, and other ARGs were only mentioned if *p* values were <0.05.

Table 3.13 The associations of prevalent ARGs with *bla*$_{OXA-181}$-positive clinical isolates compared to *bla*$_{OXA-181}$-negative clinical isolates.

ARGs	**Presence of ARG, n (%)**		***p* value**
	bla$_{OXA-181}$-positive isolates (n=24)	*bla*$_{OXA-181}$-negative isolates (n=612)	
aadA2	12 (50)	170 (27.8)	0.018
rmtF	10 (41.7)	30 (4.9)	<0.0001
bla$_{CMY-59}$	9 (37.5)	68 (11.1)	0.0003
bla$_{CTX-M-15}$	22 (91.7)	388 (63.4)	0.005
bla$_{SHV-182}$	4 (16.7)	23 (3.8)	0.003
arr2	13 (54.2)	59 (9.6)	<0.0001
arr3	3 (12.5)	20 (3.3)	0.019
mphA	11 (45.8)	119 (19.4)	0.003
dfrA1	5 (20.8)	26 (4.2)	0.0005
dfrA12	13 (54.2)	162 (26.5)	0.004

Values in parentheses indicate column percentage. The associations between *bla*$_{OXA-181}$, and other ARGs were only mentioned if *p* values were <0.05.

3.2.5 Baseline data of patients with Enterobacterales infections

Of the 534 patients, 343 (64.2%) were male and 191 (35.8%) were female and the mean (±SD) age was 32.3 (±21.6). The distribution of age and sex are depicted in Figure 3.5. Although the majority (n=308, 58%) of the participants in this study were from Dhaka division, patients from all over the country attended to DMCH for medical care (Figure 3.6). Most of the participants belonged to low socio-economic group [BPL, 45.9% (245/534); poor, 39% (208/534)] (Figure 3.7). Patients with Enterobacterales infections were distributed in different wards of DMCH (Figure 3.8). There were no associations between admission to a certain ward and particular age group except NICU with 0-5 years (Figure 3.8). Ceftriaxone was the commonly prescribed antimicrobial (61.4%) among the participants followed by metronidazole (29.8%), ciprofloxacin (23%) and amikacin (19.3%). Carbapenem usage was 13.1% (meropenem) and 1.5% (imipenem). The usage of colistin was 3.2%. Ceftriaxone is the most frequently prescribed antibiotics in burn (82.5%, 118/143), surgery (69%, 60/87), and urology (31%, 27/87). The frequency of ceftriaxone, and meropenem usage was the same in ICU (58.3%, 35/60). Meropenem usage was the highest in ICU (50%, 35/70) (Figure 3.9). Our data indicates that 94.2% (503/534) of the patients with Enterobacterales infections were treated with empirical antibiotics on admission at DMCH. Table 3.14 demonstrates the data on the frequency patients with at least one effective antimicrobial, however, susceptibility patterns of azithromycin, cefixime, cefuroxime, clindamycin, doxycycline, flucloxacillin, linezolid, metronidazole, moxifloxacin, Penicillin G, streptomycin, and vancomycin were not tested in this study. Based on data available, only 27% (144/534) of the patients were found to be prescribed with at least one antibiotic to which the respective Enterobacterales infections were susceptible (Table 3.14). The mortality among the patients with Enterobacterales infections was 17.6% (n=94) and in-hospital 30-days mortality was 17.2% (n=92). 74.9% (n=400) of the participants were discharged alive and 7.5% (n=40) were DAMA (Figure 3.10). This study recorded name of antibiotics prescribed to the patients; however, data lacked the course of antibiotics therapy (dosage, date of start, and duration) or whether the antibiotics were changed according to clinical reports. Statistically, there was increased probability of overall mortality among the patients with at least one effective antimicrobials, however the result was not significant (p=0.315) (Table 3.15).

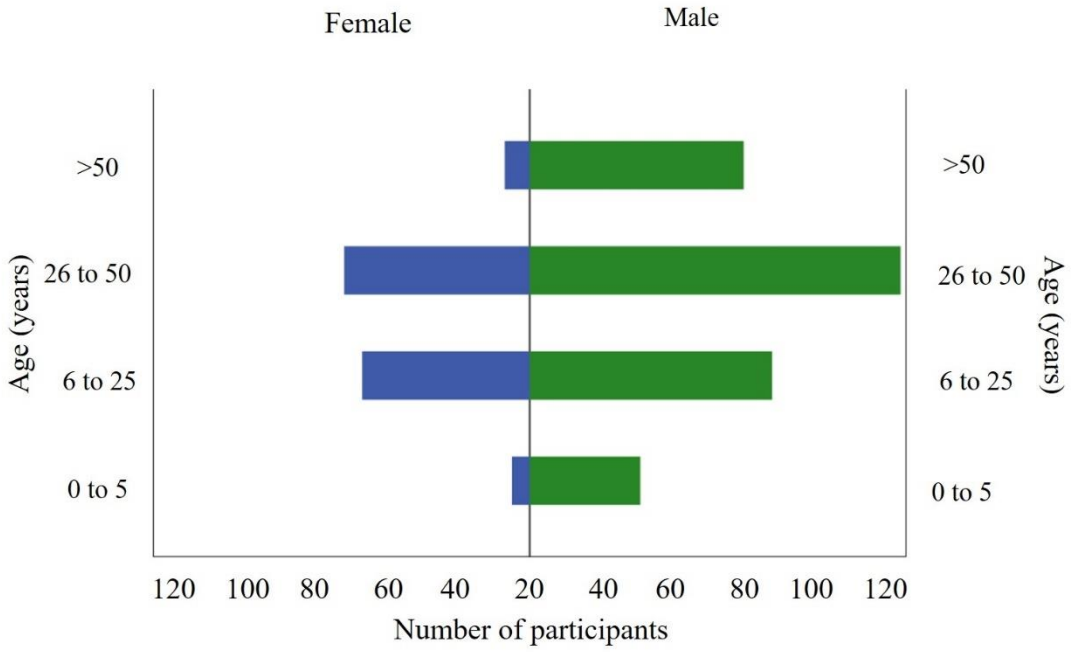


Figure 3.5 Distribution of age and sex among the patients with Enterobacterales infections.

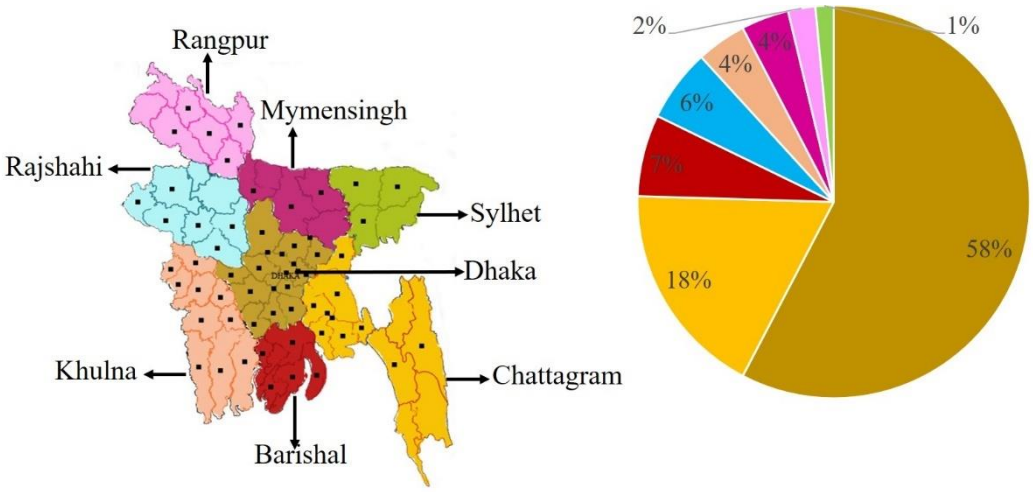


Figure 3.6 Locality of the participants enrolled in this study. Districts of Bangladesh are separated by lines in the map. Black dots in the map denotes distribution of participants' locality. Pie chart represents the proportion of participants from each division of Bangladesh. Each division is represented by identical colour in the map and pie chart.

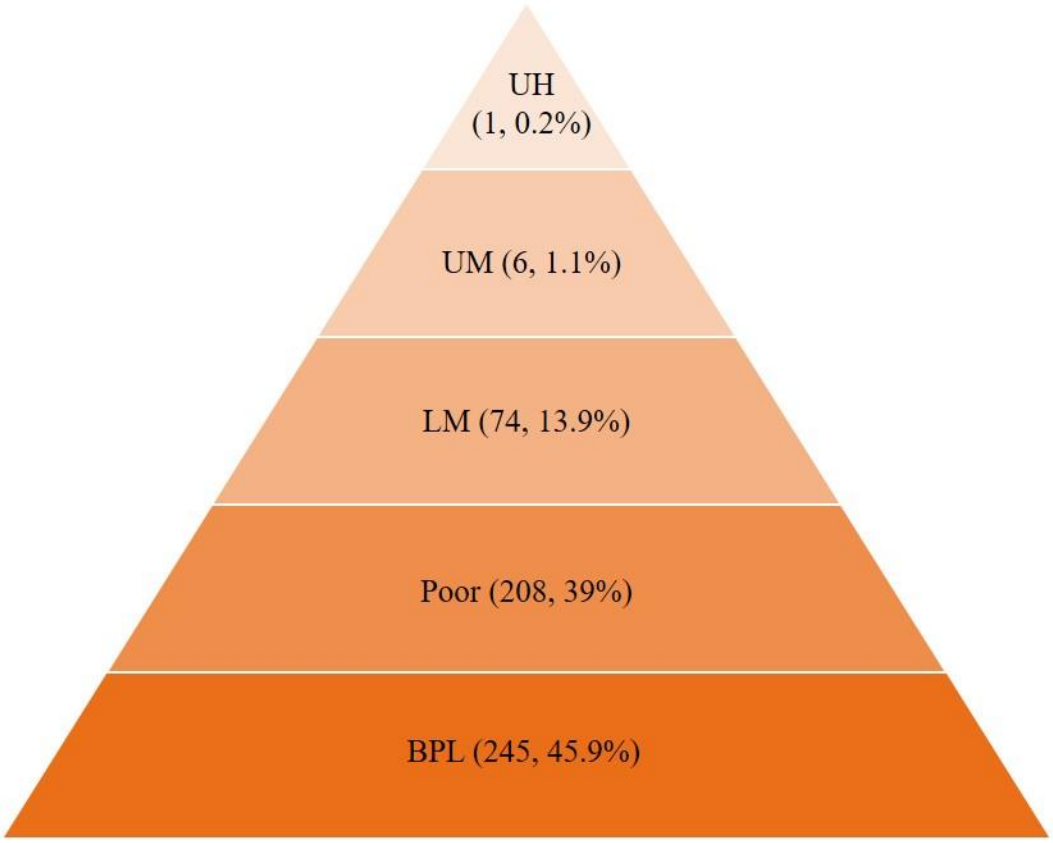


Figure 3.7 Overview of socio-economic status of the patients admitted at DMCH. UH, upper high; UM, upper middle; LM, lower middle; BPL, below the poverty level.

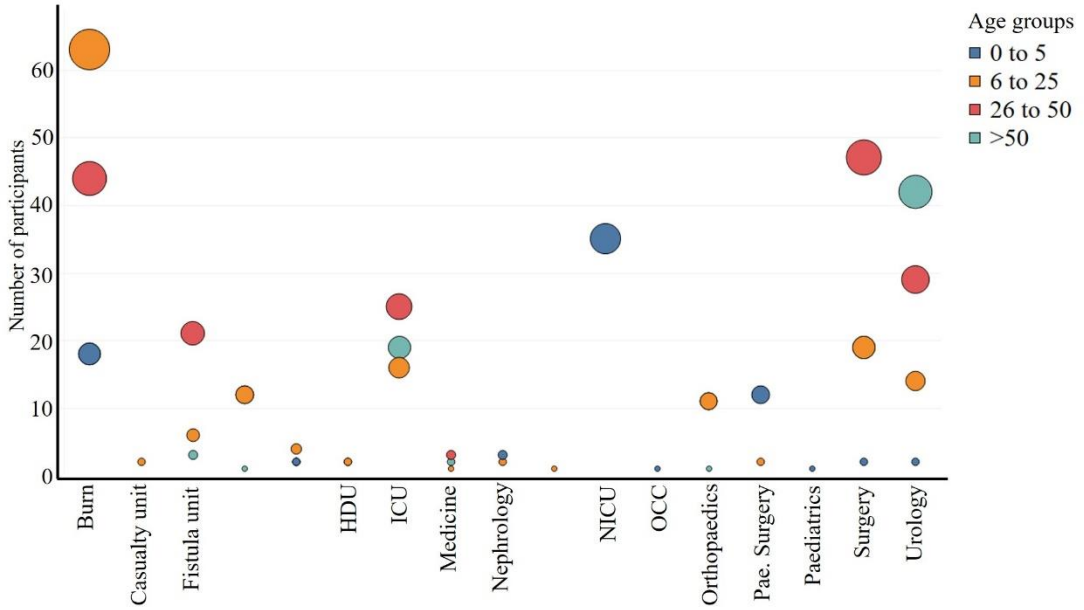


Figure 3.8 The distribution of Enterobacterales isolation from the patients admitted in different wards of DMCH according to age groups. The bubble size is proportional to the number of participants.

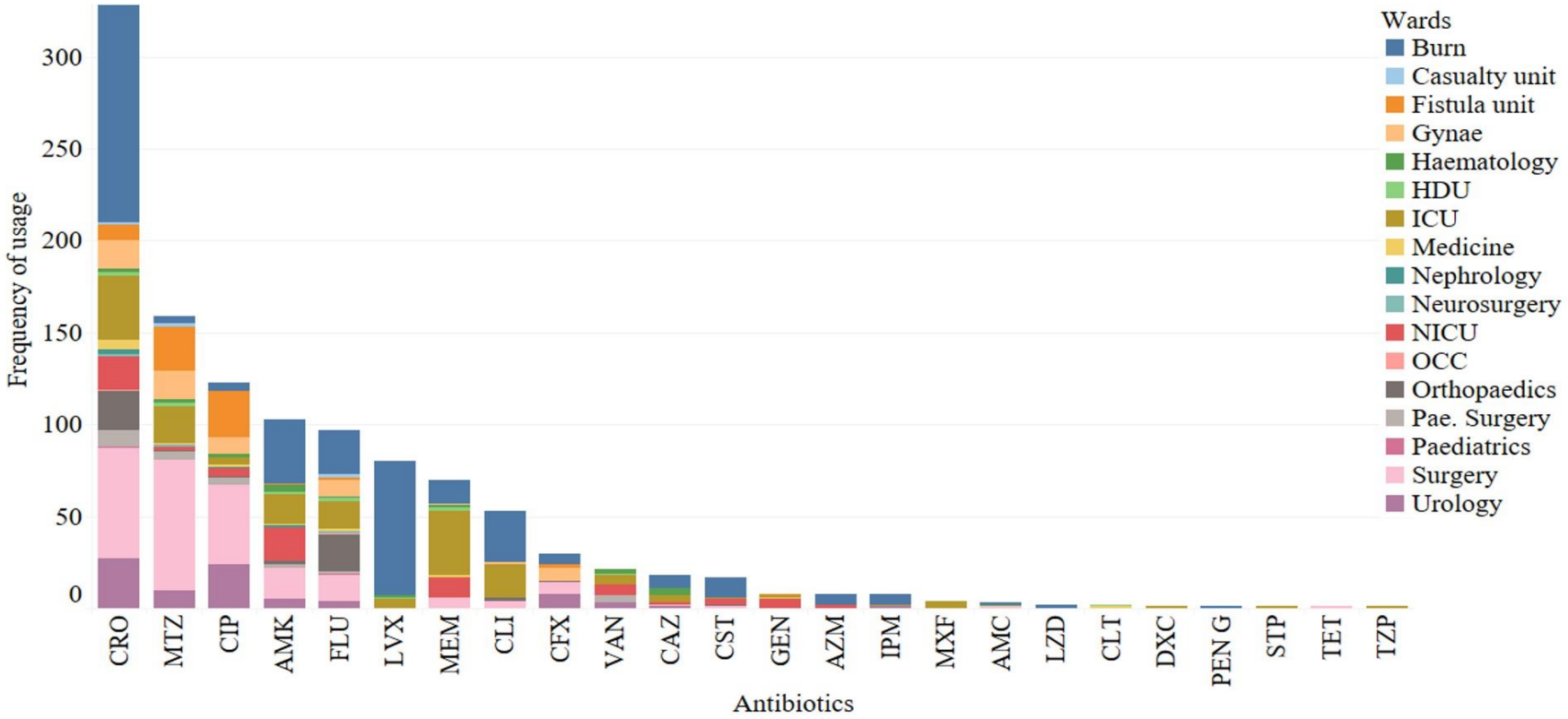


Figure 3.9 Antibiotics usage in different wards of DMCH. AMC, amoxicillin-clavulanic acid; AMK, Amikacin; AZM, azithromycin; CAZ, ceftazidime; CFX, cefixime; CIP, ciprofloxacin; CLI, clindamycin; CLT, clarithromycin; CRO, ceftriaxone; CST, colistin; CTX, cefotaxime; DXC, doxycycline; FLU, flucloxacillin; FOF, fosfomycin; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; LZD, linezolid; MEM, meropenem; MTZ, metronidazole; MXF, moxifloxacin; PEN G, Penicillin G; STP, streptomycin; TET, tetracycline; TZP, piperacillin-tazobactam; VAN, vancomycin.

Table 3.14 The frequency of patients with at least one effective antimicrobial stratified based on patients' outcome.

Clustering of patients based on usage of antimicrobials*	Number of patients with at least one effective antimicrobial tested in this study		
	DAMA	Died	Discharge
AMC (n=1)	-	-	0
AMC+CAZ (n=1)	1	-	-
AMC+MTZ (n=1)	-	-	0
AMK (n=1)	-	-	0
AMK+AZM+MTZ (n=2)	1	1	-
AMK+CAZ (n=6)	1	1	1
AMK+CAZ+CLT+LVX (n=1)	-	-	0
AMK+CAZ+CRO+MTZ (n=1)	-	-	1
AMK+CAZ+FLU+MEM+VAN (n=1)	-	-	1
AMK+CAZ+MEM (n=1)	-	-	0
AMK+CFX+CRO+LVX (n=1)	-	-	0
AMK+CIP (n=2)	-	-	0
AMK+CIP+FLU+LVX+LZD+MTZ (n=1)	-	-	0
AMK+CIP+IPM (n=1)	-	0	-
AMK+CIP+MEM (n=1)	-	-	1
AMK+CIP+MTZ (n=2)	-	-	2
AMK+CLI+LVX+MEM (n=1)	-	-	1
AMK+CLT+LVX (n=1)	-	-	0
AMK+CRO (n=25)	2	1	6
AMK+CRO+CIP+MTZ (n=6)	-	0	0
AMK+CRO+FLU (n=6)	-	1	4
AMK+CRO+FLU+CLI (n=1)	-	-	0
AMK+CRO+LVX (n=15)	-	2	6
AMK+CRO+MEM (n=4)	-	0	0
AMK+CRO+MEM+MTZ (n=2)	0	1	-
AMK+CRO+MTZ (n=9)	1	1	4
AMK+CRO+MXF (n=1)	-	0	-
AMK+CST (n=2)	-	1	1
AMK+CST+CRO (n=2)	-	-	1
AMK+MEM (n=4)	-	1	3
AMK+MEM+MTZ (n=3)	-	1	-
AZM+CAZ+FLU (n=1)	-	-	0
AZM+CLI+PEN G (n=1)	-	0	-
AZM+CRO (n=1)	-	-	0
AZM+CRO+MEM (n=1)	-	0	-
AZM+IPM+LVX (n=1)	1	-	-
AZM+LVX (n=1)	-	-	0
CAZ+CLI (n=2)	-	-	0
CAZ+CRO (n=1)	-	-	0

CAZ+CRO+FLU (n=1)	-	-	0
CAZ+CRO+LVX (n=1)	-	-	0
CAZ+STP (n=1)	-	-	0
CFX (n=6)	-	0	0
CFX+CIP+CRO (n=1)	-	-	0
CFX+CIP+CRO+MTZ (n=2)	-	-	0
CFX+CIP+MTZ (n=3)	0	-	1
CFX+CRO+FLU (n=1)	-	-	0
CFX+CRO+LVX (n=2)	1	-	0
CFX+CRO+MTZ (n=4)	-	-	3
CFX+FLU (n=8)	-	-	1
CFX+IPM (n=1)	-	-	0
CFX+LVX (n=1)	-	-	0
CIP (n=23)	0	0	3
CIP+CLI+MEM (n=1)	-	-	1
CIP+CRO (n=3)	-	-	2
CIP+FLU (n=1)	-	-	0
CIP+MEM (n=2)	-	1	1
CIP+MEM+MTZ (n=1)	-	-	1
CIP+MTZ (n=40)	0	0	5
CLI (n=1)	-	-	0
CLI+CRO (n=20)	1	0	2
CLI+CRO+CST (n=1)	-	-	0
CLI+CRO+LVX (n=6)	-	1	1
CLI+CRO+MEM (n=5)	-	0	2
CLI+CRO+MTZ (n=1)	-	0	-
CLI+CST (n=1)	-	-	1
CLI+CST+MEM+MTZ (n=1)	-	1	-
CLI+DXC+LVX+MEM (n=1)	-	-	0
CLI+FLU (n=1)	-	-	0
CLI+FLU+MEM (n=2)	-	0	1
CLI+IPM (n=1)	1	-	-
CLI+LVX (n=1)	-	-	0
CLI+MEM+MXF (n=1)	-	0	-
CLI+MEM+MXF+TZP (n=1)	-	0	-
CRO (n=25)	0	0	0
CRO+CIP+FLU (n=2)	-	-	0
CRO+CIP+MTZ (n=31)	0	1	4
CRO+CLI+MEM+MTZ (n=1)	-	0	-
CRO+CLI+MEM+VAN (n=1)	-	-	1
CRO+CST+IPM+LVX (n=2)	-	1	1
CRO+CST+LVX (n=2)	-	-	2
CRO+CST+LZD (n=1)	-	0	-
CRO+CST+MEM (n=1)	-	-	1
CRO+FLU (n=43)	2	1	5

CRO+FLU+LVX (n=2)	-	0	0
CRO+FLU+MEM (n=4)	-	2	1
CRO+FLU+MEM+MTZ (n=2)	0	0	-
CRO+FLU+MTZ (n=10)	0	-	1
CRO+GEN (n=7)	1	1	3
CRO+GEN+MTZ (n=1)	1	-	-
CRO+IPM (n=1)	0	-	-
CRO+LVX (n=34)	-	3	3
CRO+LVX+MEM (n=1)	-	0	-
CRO+MEM+MTZ (n=2)	-	1	0
CRO+MEM+MTZ+VAN (n=1)	-	0	-
CRO+MTZ (n=17)	0	-	4
CRO+MTZ+LVX (n=1)	-	0	-
CRO+VAN (n=12)	-	0	2
CST+FLU (n=1)	-	-	1
CST+LVX+MEM (n=1)	-	1	-
CST+MEM (n=1)	-	-	1
CST+MEM+MTZ (n=1)	-	-	1
CXM (n=2)	1	-	1
FLU (n=1)	-	0	-
FLU+IPM (n=1)	-	-	1
FLU+LVX (n=1)	-	-	0
FLU+MEM (n=3)	-	1	2
FLU+MEM+MTZ+VAN (n=1)	-	-	1
FLU+MTZ (n=2)	-	-	0
LVX+MTZ (n=1)	-	-	0
MEM (n=5)	1	1	2
MEM+MTZ (n=5)	-	0	2
MEM+MTZ+LVX (n=1)	-	1	-
MEM+MTZ+VAN (n=1)	-	-	0
MEM+MXF (n=1)	-	0	-
MEM+VAN (n=4)	1	-	2
MTZ (n=3)	0	-	0
TET (n=1)	-	-	0
No antibiotic (n=31)	0	-	0
Total (n=534)	17	28	99

n, number of patients. AMC, amoxicillin-clavulanic acid; AMK, Amikacin; AZM, azithromycin; CAZ, ceftazidime; CFX, cefixime; CXM, cefuroxime; CIP, ciprofloxacin; CLI, clindamycin; CLT, clarithromycin; CRO, ceftriaxone; CST, colistin; CTX, cefotaxime; DXC, doxycycline; FEP, cefepime; FLU, flucloxacillin; FOF, fosfomycin; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; LZD, linezolid; MEM, meropenem; MTZ, metronidazole; MXF, moxifloxacin; PEN G,

Penicillin G; STP, streptomycin; SXT, sulfamethoxazole-trimethoprim; TET, tetracycline; TZP, piperacillin-tazobactam; VAN, vancomycin. *Total number of patients received respective antimicrobials. Antibiotics in the first column are highlighted whether the susceptibility patterns were tested in this study.

Table 3.15 Comparative analysis to assess the associations of patients' overall mortality with at least one effective antimicrobial therapy.

At least one effective antimicrobials	Died (n=94)	Discharge (n=400)	*p* value	Odd ratio	95% CI
Yes (n=127)	28 (29.8)	99 (24.8)	0.315	1.290	0.785-2.120
No (n=367)	66 (70.2)	301 (75.3)			

Values in parentheses indicate column percentage. Patients with DAMA (n=40) were excluded from the outcome analysis.

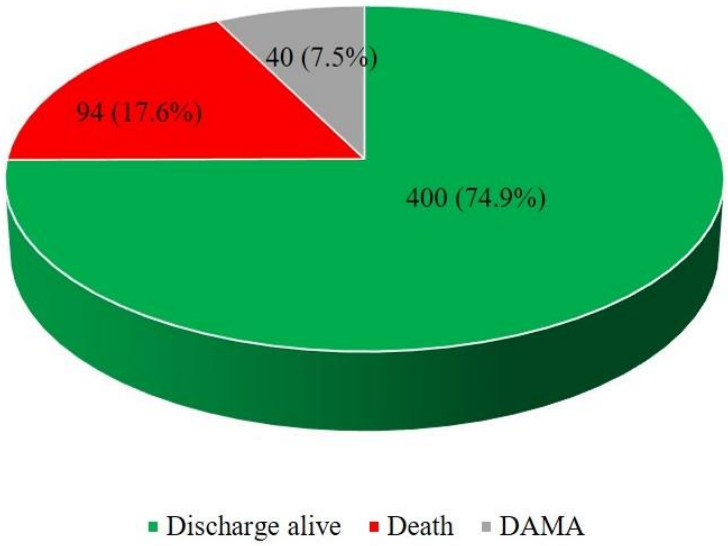


Figure 3.10 The overall outcome of the participants with Enterobacterale infections. DAMA, discharge against medical advice.

3.2.6 Risk assessment of baseline variables

The exposure of interest of the risk measurement was carbapenem resistance. A patient, positive for at least one CRE positive culture was considered as CRE infection and/or colonisation. Whether a patient had positive culture with Enterobacterales, but the respective Enterobacterales was sensitive to carbapenem, was regarded as CSE infection and/or colonisation. Out of 534 cases, 194 (36.3%) were identified as CRE cases, and 340 (63.7%) were as CSE cases (Figure 3.1).

Compared to CSE cases, CRE were significantly associated with age group of 6 to 25 years, in female, patients admitted to burn unit and ICU, and among the patients with history of levofloxacin, amikacin, clindamycin, and meropenem administration during hospital stay ($p<0.05$); however, usage of multiple antibiotics did not significantly alter the acquisition of CRE infections (Table 3.16). Given that burn itself is an immunocompromised condition, and long-term hospital care is usual for burn patients (Gallaher *et al.,* 2018; Stanojcic *et al.,* 2018). Showing a large proportion of clinical specimens in this study from burn patients (26.8%, 143/534), patients' attributes for CRE acquisition were reassessed statistically with the dataset with burn cases only and the dataset excluding burn cases (Table 3.17; Table 3.18). The findings of entire dataset (Table 3.16), and that of the dataset excluding burn cases (Table 3.18) on statistical perspectives were nearly consistent. Dataset excluding burn cases showed significant association of carbapenem resistance with LM socio-economic group ($p<0.05$) and no statistical significance between female and carbapenem resistance. Including burn cases only, no risk was found with carbapenem resistance (Table 3.17).

Table 3.16 Descriptive statistics for risk assessment of CRE clinical cases compared to CSE cases.

Attributes		CRE (n=194)	CSE (n=340)	*p* value
Age (years)	0 to 5	27 (13.9)	49 (14.4)	0.875
	6 to 25	67 (34.5)	88 (25.9)	0.034
	26 to 50	66 (34)	130 (38.2)	0.331
	>50	34 (17.5)	73 (21.5)	0.273
Sex	Female	81 (41.8)	110 (32.4)	0.029
	Male	113 (58.2)	230 (67.6)	
Socioeconomic group	BPL	84 (43.3)	161 (47.4)	0.366
	Poor	74 (38.1)	134 (39.4)	0.773
	LM	34 (17.5)	40 (11.8)	0.064
	UM	2 (1)	4 (1.2)	0.878
	UH	0 (0)	1 (0.3)	-
Admitting wards	Burn	74 (38.1)	69 (20.3)	<0.0001
	Surgery	14 (7.2)	73 (21.5)	<0.0001
	Urology	23 (11.9)	64 (18.8)	0.036
	ICU	33 (17)	27 (7.9)	0.001
	Other wards	50 (25.8)	107 (31.5)	0.165
Comorbidity	DM	15 (7.7)	58 (17.1)	0.003
	Malignancy	7 (3.6)	15 (4.4)	0.653
Antibiotics exposure during hospital stay before sampling*	Ceftriaxone	128 (66)	200 (58.8)	0.102
	Metronidazole	49 (25.3)	110 (32.4)	0.085
	Ciprofloxacin	27 (13.9)	96 (28.2)	0.0001
	Amikacin	49 (25.3)	54 (15.9%)	0.008
	Meropenem	33 (17)	37 (10.9	0.044
	Flucloxacillin	27 (13.9)	70 (20.6)	0.054
	Levofloxacin	45 (23.2)	35 (10.3)	<0.0001
	Clindamycin	28 (14.4)	25 (7.4)	0.008
Number of antibiotics prescribed**	Monotherapy	19 (10.3)	50 (15.7)	0.093
	More than one drug	165 (89.7)	269 (84.3)	
Hospital stay before sampling	≤7 days	68 (35.1)	136 (40)	0.258
	>7 days	126 (64.9)	204 (60)	

Values in parentheses indicate column percentage. BPL, below the poverty level; P, poor; LM, lower middle; UM, upper middle; UH, upper high; ICU, intensive care unit; DM, diabetes mellitus. Cells are highlighted whether any variable was significantly associated with CRE. Bivariate analysis was performed to identify the risks for CRE. *Eight common antibiotics prescribed at DMCH are included in this descriptive analysis. **Patients without any antibiotic (n=31) were excluded from the analysis.

Table 3.17 Descriptive statistics for risk assessment of CRE clinical cases compared to CSE cases with burn infections.

Attributes		CRE (n=74)	CSE (n=69)	*p* value
Age (years)	0 to 5	12 (16.2)	6 (8.7)	0.175
	6 to 25	30 (40.5	33 (47.8)	0.381
	26 to 50	24 (32.4)	20 (29)	0.655
	>50	8 (10.8)	10 (14.5)	0.507
Sex	Female	35 (47.3)	23 (33.3)	0.089
	Male	39 (52.7)	46 (66.7%)	
Socioeconomic group	BPL	30 (40.5)	33 (47.8)	0.381
	Poor	32 (43.2)	27 (39.1)	0.618
	LM	11 (14.9)	9 (13)	0.754
	UM	1 (1.4)	0 (0)	0.333
Comorbidity	DM	3 (4.1)	5 (7.2)	0.407
	Malignancy	0 (0)	2 (2)	0.140
Antibiotics exposure during hospital stay before sampling*	Ceftriaxone	59 (79.7)	59 (85.5)	0.363
	Metronidazole	4 (5.4)	0 (0)	0.050
	Ciprofloxacin	2 (2.7)	3 (4.3)	0.593
	Amikacin	22 (29.7)	13 (18.8)	0.130
	Meropenem	8 (10.8)	5 (7.2)	0.459
	Flucloxacillin	4 (5.4)	20 (29)	<0.0001
	Levofloxacin	42 (56.8)	31 (44.9)	0.157
	Clindamycin	15 (20.3)	13 (18.8)	0.830
Number of antibiotics prescribed	Monotherapy	5 (6.8)	2 (2.9)	0.285
	More than one drug	69 (93.2)	67 (97.1)	
Hospital stay before sampling	≤7 days	21 (28.4)	18 (26.1)	0.759
	>7 days	53 (71.6)	51 (73.9)	

Values in parentheses indicate column percentage. BPL, below the poverty level; P, poor; LM, lower middle; UM, upper middle; UH, upper high; ICU, intensive care unit; DM, diabetes mellitus. Bivariate analysis was performed to identify the risks for CRE. *Eight common antibiotics prescribed at DMCH are included in this descriptive analysis.

Table 3.18 Descriptive statistics for risk assessment of CRE clinical cases compared to CSE cases excluding burn cases.

Attributes		CRE (n=120)	CSE (n=271)	*p* value
Age (years)	0 to 5	15 (12.5)	43 (15.9)	0.388
	6 to 25	37 (30.8)	55 (20.3)	0.023
	26 to 50	42 (35)	110 (40.6)	0.296
	>50	26 (21.7)	63 (23.2)	0.731
Sex	Female	46 (38.3)	87 (32.1)	0.230
	Male	74 (61.7)	184 (67.9)	
Socioeconomic group	BPL	54 (45)	128 (47.2)	0.683
	Poor	42 (35)	107 (39.5)	0.400
	LM	23 (19.2)	31 (11.4)	0.041
	UM	1 (0.8)	4 (1.5)	0.602
	UH	0 (0)	1 (0.3)	-
Admitting wards	Surgery	14 (11.7)	73 (26.9)	0.001
	Urology	23 (19.2)	64 (23.6)	0.329
	ICU	33 (27.5)	27 (10)	<0.0001
Comorbidity	DM	12 (10)	53 (19.6)	0.019
	Malignancy	7 (5.8)	13 (4.8)	0.668
Antibiotics exposure during hospital stay before sampling*	Ceftriaxone	69 (57.5)	141 (52)	0.317
	Metronidazole	45 (37.5)	110 (40.6)	0.564
	Ciprofloxacin	25 (20.8)	93 (34.3)	0.007
	Amikacin	27 (22.5)	41 (15.1)	0.076
	Meropenem	25 (20.8)	32 (11.8)	0.020
	Flucloxacillin	23 (19.2)	50 (18.5)	0.867
	Levofloxacin	3 (2.5)	4 (1.5)	0.481
	Clindamycin	13 (10.8)	12 (4.4)	0.017
Number of antibiotics prescribed**	Monotherapy	14 (12.7)	48 (19.2)	0.134
	More than one drug	96 (87.3)	202 (80.8)	
Hospital stay before sampling	≤7 days	47 (39.2)	118 (43.5)	0.419
	>7 days	73 (60.8)	153 (56.5)	

Values in parentheses indicate column percentage. BPL, below the poverty level; P, poor; LM, lower middle; UM, upper middle; UH, upper high; ICU, intensive care unit; DM, diabetes mellitus. Cells are highlighted whether any variable was significantly associated with CRE. Bivariate analysis was performed to identify the risks for CRE. *Eight common antibiotics prescribed at DMCH are included in this descriptive analysis. **Patients without any antibiotic (n=31) were excluded from the analysis.

3.2.7 Outcome analysis of CRE cases compared to CSE

To investigate the impact of carbapenem resistance on patients' outcome, CRE cases were assessed for in-hospital 30-days mortality and length of hospital stay (LoS) in comparison to CSE group. Two time points were considered for the analysis: 'time from admission' (time zero is admission) and 'time from infection' with Enterobacterales (time zero is infection). Cox proportional hazards model was fitted with 'time from infection' to outcome as 'time-to-event' and 'time from admission' to infection as 'covariate'. Confounders such as age, gender, comorbidity, admission to ICU and burn unit, and association of *E. coli* and *K. pneumoniae* infections were adjusted in the model and compared to the unadjusted model. Patients discharged alive or in-hospital mortality after 30 days was used as competing variable for outcome analysis. Patients with DAMA (n=40) and outlier cases (n=2) (hospital stay >100 days from 'time from infection' to outcome) were excluded from the outcome analysis.

All-cause in-hospital 30-days mortality occurred in 50 (27.8%) of 180 patients with CRE cases and in 42 (13.5%) of 312 patients with CSE cases. CRE cases had significant impact on in-hospital 30-days mortality (p=0.001) (Figure 3.11). Adjusted subdistribution hazard ratio (SHR) inferred that confounders did not affect the statistical significance of in-hospital 30-days mortality due to carbapenem resistance except model 4 (adjusted with ICU admissions), but covariates reduced the strength of associations. The covariates, *E. coli* infections (model 5), wound infections (model 8), and UTIs (model 9) were inversely related to CRE with 30-days mortality. SHR estimated the increase probability of excess LoS in relation to CSE cases than CRE (Table 3.18).

We compared resistance and virulence profile of *E. coli* and *K. pneumoniae* against patients' mortality using patients discharged alive or in-hospital mortality after 30 days as competing outcome which showed the significant association only between VF scores of *E. coli* and patients' mortality (p=0.008) (Table 3.20). The cumulative number of ARGs of *E. coli* and *K. pneumoniae* had positive correlation with the total number of VF of the respective species (r=0.138) (Figure 3.12).

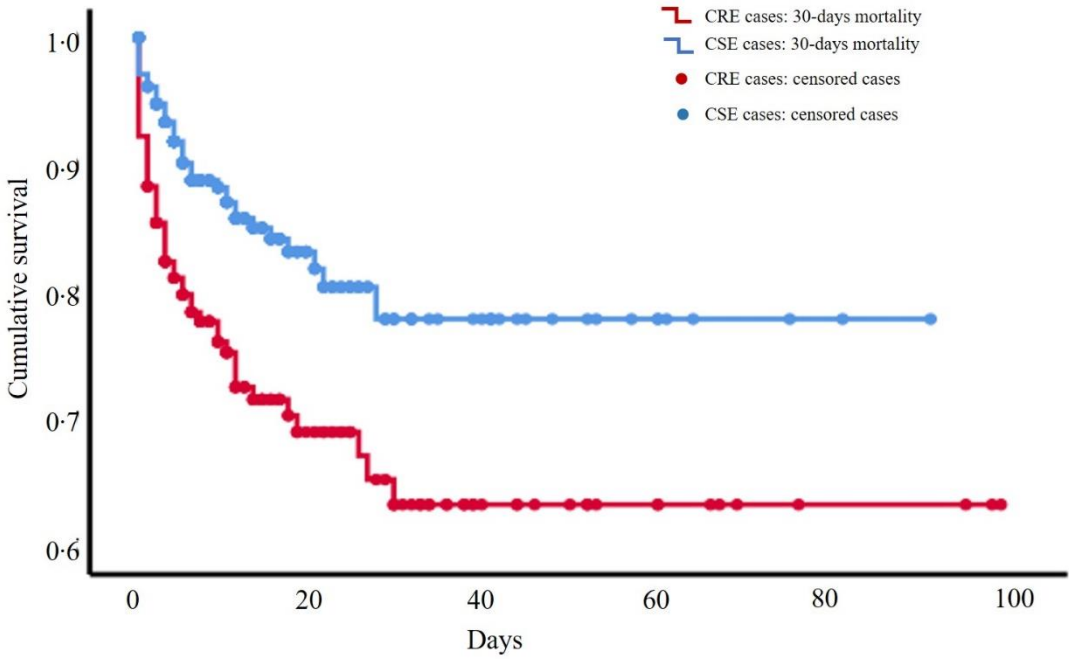


Figure 3.11 Kaplan–Meier plot representing the cumulative proportion of all-cause in-hospital 30-days mortality of CRE patients compared to CSE patients with a timescale 'time from infection' to outcome. Differences in mortality were calculated by log-rank (Mantel-Cox). CRE infections were significantly associated with in-hospital 30-days mortality than CSE patients (p=0·001). Patients with DAMA (n=40) and outlier cases (n=2) (hospital stay >100 days from 'time from infection' to outcome) were excluded from the outcome analysis.

Table 3.19 Cox proportional hazards models to analyse for the impact carbapenem resistance on patients' outcome.

Description		Inference			*p* value	SHR	95% CI
		Increased probability	Associations*	Inverse relation*			
All-cause in-hospital 30-days mortality	Model 1: Adjusted by time from admission to infection	CRE			0.001	0.491	0.325-0.741
	Model 2: Model 1 plus age, gender, comorbidity (DM & malignancy)	CRE	Increased age, female, malignancy	DM	0.002	0.519	0.342-0.789
	Model 3: Model 2 plus admission to burn	CRE	Admission to burn		0.009	0.569	0.372-0.871
	Model 4: Model 2 plus admission to ICU	CRE	Admission to ICU		0.072	0.677	0.442-1.131
	Model 5: Model 2 plus association of *E. coli* infections	CRE		*E. coli* infections	0.004	0.539	0.355-0.819
	Model 6: Model 2 plus association of *K. pneumoniae* infections	CRE	*K. pneumoniae* infections		0.014	0.568	0.362-0.891
	Model 7: Model 2 plus BSIs	CRE	BSIs		0.013	0584	0.382-0.893
	Model 8: Model 2 plus wound infections	CRE		Wound infections	0.002	0.519	0.342-0.788
	Model 9: Model 2 plus UTIs	CRE		UTIs	0.024	0.617	0.406-0.938
LoS	Model 1: Adjusted by time from admission to infection	CSE			0.056	1.199	0.995-1.444
	Model 2: Model 1 plus age, gender, comorbidity (DM & malignancy)	CSE	Increased age, female, DM	Malignancy	0.072	1.191	0.985-1.440
	Model 3: Model 2 plus admission to burn	CSE		Admission to burn	0.235	1.122	0.928-1.358
	Model 4: Model 2 plus admission to ICU	CSE	Admission to ICU		0.051	1.211	0.999-1.467
	Model 5: Model 2 plus association of *E. coli* infections	CSE	*E. coli* infections		0.071	1.191	0.985-1.441
	Model 6: Model 2 plus association of *K. pneumoniae* infections	CSE		*K. pneumoniae* infections	0.365	1.100	0.895-1.351
	Model 7: Model 2 plus BSIs	CSE		BSIs	0.075	1.188	0.983-1.437
	Model 8: Model 2 plus wound infections	CSE		Wound infections	0.071	1.191	0.985-1.440
	Model 9: Model 2 plus UTIs	CSE	UTIs		0.101	1.174	0.969-1.422

*Towards increased probability. Patients with DAMA (n=40) and outlier cases (n=2) (hospital stay >100 days from 'time from infection' to outcome) were excluded from the outcome analysis.

Table 3.20 Comparative analysis to assess the associations of all-cause 30-days mortality with genomic profiles (ARG and VF scores) of Enterobacterales isolated from respective patients.

		30-days mortality	**Competing outcome**	***p* value**
E. coli	ARG score [mean(±SD)]	8 (±5)	5.6 (±3.62)	0.080
	VF scores [mean(±SD)]	14.8 (±19.7)	13.8 (±14.3)	0.008
K. pneumoniae	ARG score [mean(±SD)]	9.4 (±4.4)	8.5 (±4.3)	0.608
	VF scores [mean(±SD)]	55.4 (±20.6)	51.4 (±22.9)	0.846

Patients discharged alive or in-hospital mortality after 30 days was used as competing variable. ARG and VF scores in this study denotes the number of known ARGs, and virulence genes in a strain, respectively. Known ARGs, and VFs were retrieved using 'CARD' and 'VFDB' database, accordingly. Number of efflux pumps are not included in ARG scoring.

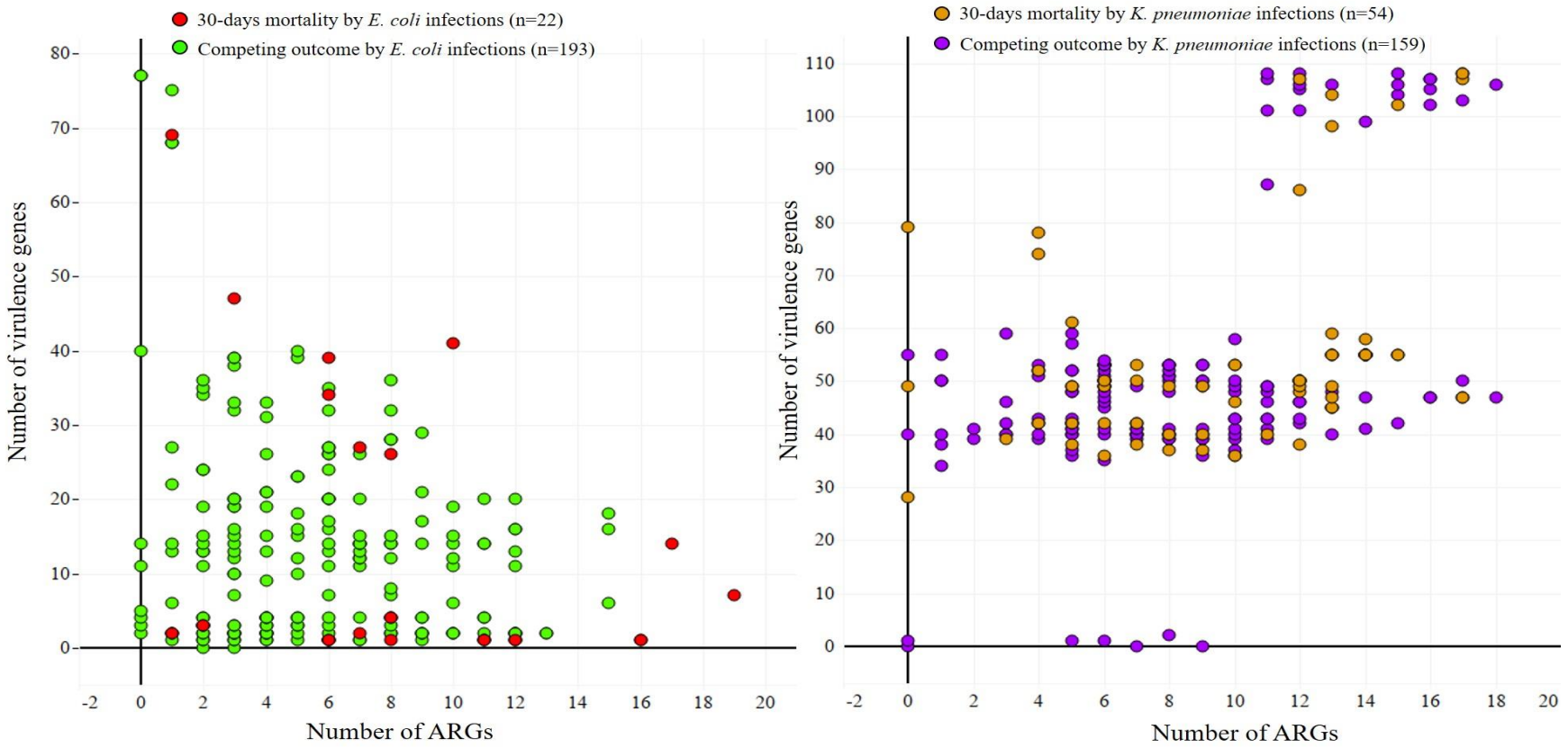


Figure 3.12 Scattered plot representing the distribution of patients' outcome data by merging with genomic profile (the relation between the cumulative number of ARGs and that of VF of respective isolate) of *E. coli* and *K. pneumoniae* isolated from the respective patient. Known ARGs and VFs were retrieved using 'CARD' and 'VFDB' database, accordingly. Number of efflux pumps are not included in the analysis.

3.3 Discussion

Addressing the extent of any health-related issue in a country requires detailed background information which can be considered as real time evidence to develop effective interventions in the future. The enormity of the situation of AMR in Bangladesh is still a notion based on narrow scale sporadic reports (Farzana *et al.,* 2013; Islam *et al.,* 2013; Rakhi *et al.,* 2019; Hoque *et al.,* 2020). To date, this is the only comprehensive study in Bangladesh describing clinical associations and outcomes in relation to a high-end resistance mechanism.

The prevalence Enterobacterales infections in the study was 29.2% (534/1830). Traditionally, majorities of infections are treated empirically at DMCH. The choice of antibiotics mostly depends on the availabilities of the drug in hospital supply. Clinical specimens are usually referred for microbiology if the infections are suspected clinically or when the empirical therapy is failed. In Bangladeshi health settings, physicians are only responsible for taking decision of treatment, laboratory testing, or any sort of medical interventions. Nurses or ward-attendants are only allowed for caregiving according to doctors' instructions (Rahman, personal communication). This study did not involve with any issue related to patients' management, but the project activities were aligned with the DMCH's usual practice of infection management. Provided the facts and our consultations with the local physicians on several occasions, positive cultures from clinical specimens included in this study were more likely the indicative of true infections. In Bangladesh, there is no facility in the public system to capture or record patients' data electronically. Two third of the admitted patients do not have any allocated bed rather stay in ward floor, hospital corridors, or even in the stairwells who are assigned as 'extra' by the hospital. Each ward maintains logbook to record patients' turnover data with some basic information only, and patients' clinical documents are transferred to store when the patient either died or is discharged (Scoping findings by T. R. Walsh explained in general discussion). This study recorded clinical data from the patients with positive cultures only which was realistic, achievable within time limit, and aided to avoid lots of missing data in the dataset.

In general, this study provided some contextual information about DMCH: **1.** The age distribution was wide, and no ward was dominated by a certain age group

except NICU and distribution of sex was almost equivalent (Figure 3.5; Figure 3.8). **2.** DMCH served the patients from all over the country, particularly a low socio-economic group (Figure 3.7). Therefore, the burden of AMR in the hospital would have huge impact in the country. **3.** Antibiotics usage in the hospital was generally high and the choice of antibiotics did not differ much from one unit to other (Figure 3.9) which reflects a lack of disease-specific antimicrobial guidelines in the hospital. **4.** The mortality of patients with Enterobacterales infections was 17.6% (Figure 3.10). The overall mortality among the study population was not recorded. **5.** Forty patients (7.5%) with Enterobacterales infections left the hospital against the doctor's advice (Figure 3.10). DAMA from the hospital is a usual event in SA which happened most often due to financial constrains (Bhoomadevi *et al.,* 2019). There is no health insurance system in Bangladesh which can be afforded by low socio-economic group (Fahim *et al.,* 2019). As public health facilities do not allow always standard care for general freely (only 3% of GDP expenditure to public health by Bangladesh Government) (WHO, 2015), paying for medicine, or other necessities during patients' hospitalisation is a huge burden for a family belonged to low-economic status. The detailed on the topic has been described in '*Chapter 10*' (General discussion).

The prevalence of carbapenem resistance among the species of Enterobacterales was 32.6% (210/643) (Figure 3.1). The rate of carbapenem resistance varies among species based on the site of infections (Nordmann and Poirel, 2019). Report of national surveillance in Bangladesh from 2017 to 2019 revealed that 10% of *E. coli* and 25% of *K. pneumoniae* were carbapenem resistant (WHO, 2020a). In this study, the rate of carbapenem resistance was significantly higher for *K. pneumoniae* than other species ($p<0.05$) (Table 3.5). The genomic profile also indicated that $bla_{\text{NDM-1}}$, $bla_{\text{NDM-5}}$, and $bla_{\text{OXA-181}}$ were significantly more associated with *K. pneumoniae* than other species ($p<0.05$) (Figure 3.3). Provided the previous reports and findings of this study, it can be implied that CRE in nosocomial infections is highly prevalent in the region of SA, whereas nosocomial carbapenem resistance in the USA and Europe is mostly associated with non-fermenters (Nordmann and Poirel, 2019; Walia *et al.,* 2019). It has been also speculated that Indian subcontinent is the source of bla_{NDM} and $bla_{\text{OXA-181,}}$ and data revealed the wide dissemination of the genes in this region. $bla_{\text{OXA-48-like}}$ such $bla_{\text{OXA-232}}$ is also found to be prevalent in *K. pneumoniae* in the region (Yong *et al.,* 2009; Kumarasamy *et al.,* 2010; Farzana *et al.,* 2013, Islam *et*

al., 2013; Mohanty *et al.,* 2017; Garg *et al.,* 2019; Jaggi *et al.,* 2019; Shankar *et al.,* 2019; Suay-García and Pérez-Gracia, 2019). Present findings were not an exception; bla_{NDM} (180/643, 28%) was the most common carbapenemase in clinical Enterobacterales followed by $bla_{OXA-232}$ (26/643, 4%), and $bla_{OXA-181}$ (24/643, 3.7%) (Table 3.5). *K. pneumoniae* and *E. coli* were the most common reservoir of the carbapenemase genes (Figure 3.3). The magnitude of the problem is extremely worrisome due to inevitable gut replacement MDR bacteria which has been discussed in the later chapters (Carlet, 2012; van Schaik, 2015; Gupta *et al.,* 2019).

The risks of CRE infections documented by earlier studies are recent history of hospitalisation or travel in the region of SA, recent antibiotics exposure, introduction of artificial devices, and longer hospital stay (Chiotos *et al.,* 2017; van Loon *et al.,* 2017; Asai e*t al.,* 2018; Nicolas-Chanoine *et al.,* 2019; Richter *et al.,* 2019). A report on risk assessment of AMR in SA estimated that poor IPC, low antimicrobial stewardship, and selective antibiotic pressure are highly related to human-to-human transmission of AMR in the region (Chereau *et al.,* 2017). Widespread empirical antibiotic prescription in the hospital setting of SA is prominent, much of which is likely to be inappropriate, and is a key driver of resistant bacteria in the hospital environment (Hsu *et al.,* 2017). In this study, patients admitted in burn unit, and ICU were considered as high risk for acquiring CRE during their hospital stay ($p<0.05$) (Table 3.16). The finding is not unexpected as several factors such as impaired immunity, comorbidities, prolonged hospital stay and close contact with hospital staffs, receipt of artificial devices, antibiotics exposures are commonplace in burn, and ICU patients which facilitate the colonisation, and/or infections by resistant bacteria (Lachiewicz *et al.,* 2017; Tamma *et al.,* 2019). Our study recorded inpatient empirical antibiotic exposure on admission which was 94.2%. There were significant associations between receipt of amikacin, meropenem, levofloxacin and clindamycin, and CRE cases ($p<0.05$) (Table 3.16). Age group of 6 to 25 years, and female were also significantly more associated with CRE infections (Table 3.16). These factors were independently considered as risk for CRE in bivariate analysis. The subpopulation of cases (excluding burn cases) had the statistically consistent findings except sex 'female' as a risk (Table 3.18). Nevertheless, lower middle economic group were significantly more linked to CRE infections rather than poor or BPL in subpopulation analysis (excluding burn cases) (Table 3.18). It has been anticipated that

the poor or BPL have less access to antibiotics due to lack of affordability which might explain the relation between antibiotics usage and acquisition of resistance (Vanderhaeghen and Dewulf, 2017). Generally, majority of the participants in this study (98.7%) belonged to very low to lower-middle socio-economic status (Figure 3.7). A study by World Bank suggested that there is strong correlation of sanitation facilities and personal hygiene with socio-economic status in Bangladesh (Mahmud and Mbuya, 2016). Poor individual hygiene status along with lack of access to hand washing materials, hospital infrastructure, inadequate sanitation, improper disposal of medical waste, and hospital infrastructure at DMCH have constant impact in the transmission of infectious diseases (Shahida *et al.,* 2016).

Overall global report suggested that CRE are associated with 3-fold greater mortality than the susceptible counterpart. Cassini *et al.* (2019) reported about 13% death due to carbapenem-resistant *K. pneumoniae* and 5% due to carbapenem-resistant *E. coli* in Europe. Studies in SA estimated CRE-attributable mortality of 56.7% to 84.2%. The analysis on the CRE-associated health burden has been mostly performed with specific cohort of BSIs, *K. pneumoniae* infections, and comorbid or critically ill patients (Kaur *et al.,* 2017; Kohler *et al.,* 2017; Mariappan *et al.,* 2017; Righi *et al.,* 2017; Martin *et al.,* 2018; Shankar *et al.,* 2018; Stewardson e*t al.,* 2019 Liu *et al.,* 2020). The all-cause in-hospital 30-days mortality due to CRE infections (27.8%) was lower than the rate reported in SA. The lower mortality in this study may be driven by the inclusion of CRE of any species of Enterobacterales from all types of clinical specimens. Even so, the death rate among CRE cases was substantially higher (27.8%) compared to the CSE cases (13.5%) ($p<0.05$) (Figure 3.11; Table 3.19). Comorbidities can influence the likelihood of poor outcome. Clinical information collected in this study were devoid of data on indicators to evaluate the comorbid status of a patient such as Charlson comorbidity score, Pitt bacteraemia score, and APACHE II score (van Duin *et al.,* 2013; Stewardson *et al.,* 2019). All-cause 30-days mortality was adjusted with time from admission, and other available covariates which showed diminished associations. The regression analysis also demonstrated the negative relation of CRE-attributable 30-days mortality with *E. coli* infections, would infections, and UTIs which supports the relatively lower death rate with CRE in this study than the other studies (Table 3.19). The worse patients' outcome with CRE are invariably associated with the limited therapeutic options (van Duin *et al.,* 2013;

Stewardson *et al.,* 2019). The data on antibiotics usage clearly demonstrated that overall, 73% of the patients did not receive at least one appropriate clinically important antibiotic (Table 3.14). However, no statistical significance was observed between overall mortality and devoid of access to essential antimicrobials rather there was decease probability of mortality with the patients without any effective antimicrobials (Table 3.15). Given the lack of data on course of antibiotic therapy to the patients, this study unable to assess the effect of treatment strategies. Moreover, this study also did not test susceptibilities of some antimicrobials pertinent to the patients' usage, although the usage of those antimicrobials was less frequent except metronidazole (29.8%) (Table 3.14). Infections by MDR bacteria typically lengthen patients' hospital days (Naylor *et al.,* 2019; Stewardson *et al.,* 2019). This study conversely estimated increase probability of LoS with the CSE group than CRE (Table 3.19). Perhaps strong association between 30-days mortality and CRE reduced the probabilities of excess hospital stay in CRE group.

Apart from that, a positive correlation between resistance, and virulence ($r=0.138$) was found among clinical Enterobacterales (Figure 3.12). Acquisition of resistance leads to fitness costs for bacteria (Yang *et al.,* 2017; Wyres *et al.,* 2020). However, convergence of virulence, and resistance might occur due to horizontal acquisition of both resistance, and virulence determinants, or adaptive mutations by the host (Giraud *et al.,* 2017).

Significant resistance to non-β-lactams (ciprofloxacin, levofloxacin, amikacin, gentamicin, and sulfamethoxazole-trimethoprim) was observed in CRE irrespective of species ($p<0.05$) (Table 3.8; Table 3.9; Table 3.10). Genomic data on the associations of multiple ARGs with CRE were consistent the phenotypic resistance (Table 3.11-3.13). The associations between carbapenem resistance and other antimicrobial resistance have been illustrated in ***Chapter 4*** by merging the data of clinical and faecal isolates. The overall most effective antibiotics against clinical CRE in this study were colistin, and fosfomycin (Table 3.4). Taken together, the findings of this study infer a high CRE prevalence in Bangladeshi health setting with poor clinical outcome, and selective antibiotic pressure is a potential driver for the spread of AMR.

Section Four

Molecular Epidemiology and Risk Analysis of Carbapenem-Resistant Enterobacterales in Faecal Carriage

4.1 Introduction

An estimated 10^{14} symbionts, commensals and opportunistic pathogens are the inhabitants of human body. The human gut microbiota comprises diverse range of species which are remarkably stable in healthy adults. Generally, the human gut is exposed to a substantial number of bacteria through hands, pharyngeal and nasal secretions, water, food, and beverages. The gut microbiota acts as barrier for intestinal colonisation of pathogens. While the composition of gut microbiota depends on dietary habit or environmental insult, antibiotic exposure holistically alters taxonomic diversity of microbiota, increasing the vulnerability to infection by pathogens. The major concerns owing to the alteration are the persistence of taxonomic changes for protracted periods and inter-strain horizontal gene transfer. Conjugation and transduction contribute a major role to the spread of AMR genes in the gut microbiota, and human gut provides the optimal conditions for the transfer. Intestinal flora can be reservoir of MDR gene pool in otherwise healthy individual called 'gut resistome' (Carlet, 2012; van Schaik, 2015; Gupta *et al.*, 2019).

The major human gut commensals are the members of two phyla, the Bacteroidetes and Firmicutes. Enterobacteriaceae families are also present as normal flora in the intestinal tract which are of particular interest in terms of resulting nosocomial infections, and ability to take part in horizontal gene transfer. Selective pressure driven disruption of gut flora exacerbates the number of resistant bacteria in the gut ecosystem. The emergence of CRE in health care setting is a major public health challenge, has been associated with limited therapeutic options and increased mortality and morbidity (van Duin *et al.*, 2013; Stewardson *et al.*, 2019). Gut colonisation with CRE can be the source of transmission of CRE in hospital settings, and the community (Huddleston, 2014; Gupta *et al.*, 2019; Jeong *et al.*, 2019; Tamma *et al.*, 2019).

Scrutinizing faecal specimens for AMR periodically can predict the trend of any resistance mechanism in a community. Provided the fact of limited data on AMR, and anticipated endemicity of some resistance mechanisms in the region of SA, wide scale systematic national AMR survey can be the option to understand about the existent scenario (Zaidah *et al.*, 2017; Gupta *et al.*, 2019; Kłudkowska *et al.*, 2019). This chapter investigates the prevalence of faecal carriage of CRE in Bangladesh and

the risks for CRE colonisation in hospital settings, has accomplish '**objectives #5'** of this project.

4.2 Results

4.2.1 Study outline and prevalence of CRE carriage in a clinical setting of Bangladesh

A total of 700 RSs from 13th May 2018 to the17th June 2018 from patients attending DMCH were included. The study outline is depicted in Figure 4.1. The frequency of Enterobacterales isolation from the media containing vancomycin (10 mg/L), and ertapenem (2 mg/l) was 47.7% (334/700). The most common Enterobacterales were *E. coli* (41.8%, 293/700) followed by *K. pneumoniae* (11.6%, 81/700), *Citrobacter* spp. (2.7%, 19/700), *Enterobacter* spp. (2.1%, 15/700), and others (2.4%, 17/700) (Figure 4.2).

The frequency of carbapenem resistance in each species identified was: *K. pneumoniae*, 88.9% (72/81); *E. coli*, 72% (211/293); and others, 68.6% (35/51) (Table 4.1; Table 4.2). *S. marcescens* (n=1) was not assessed for MIC_{50} and MIC_{90}, but resistant to amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftazidime, cefotaxime, sulfamethoxazole-trimethoprim, and colistin and sensitive to ceftriaxone, cefepime, imipenem, meropenem, ciprofloxacin, levofloxacin, amikacin, gentamicin, and fosfomycin.

The prevalence of CRE in faecal carriage was 34.8% (244/700). The prevalence was significantly higher among inpatients (206/383, 53.8%) than the outpatients (38/317, 12%) ($p<0.05$) (Figure 4.3).

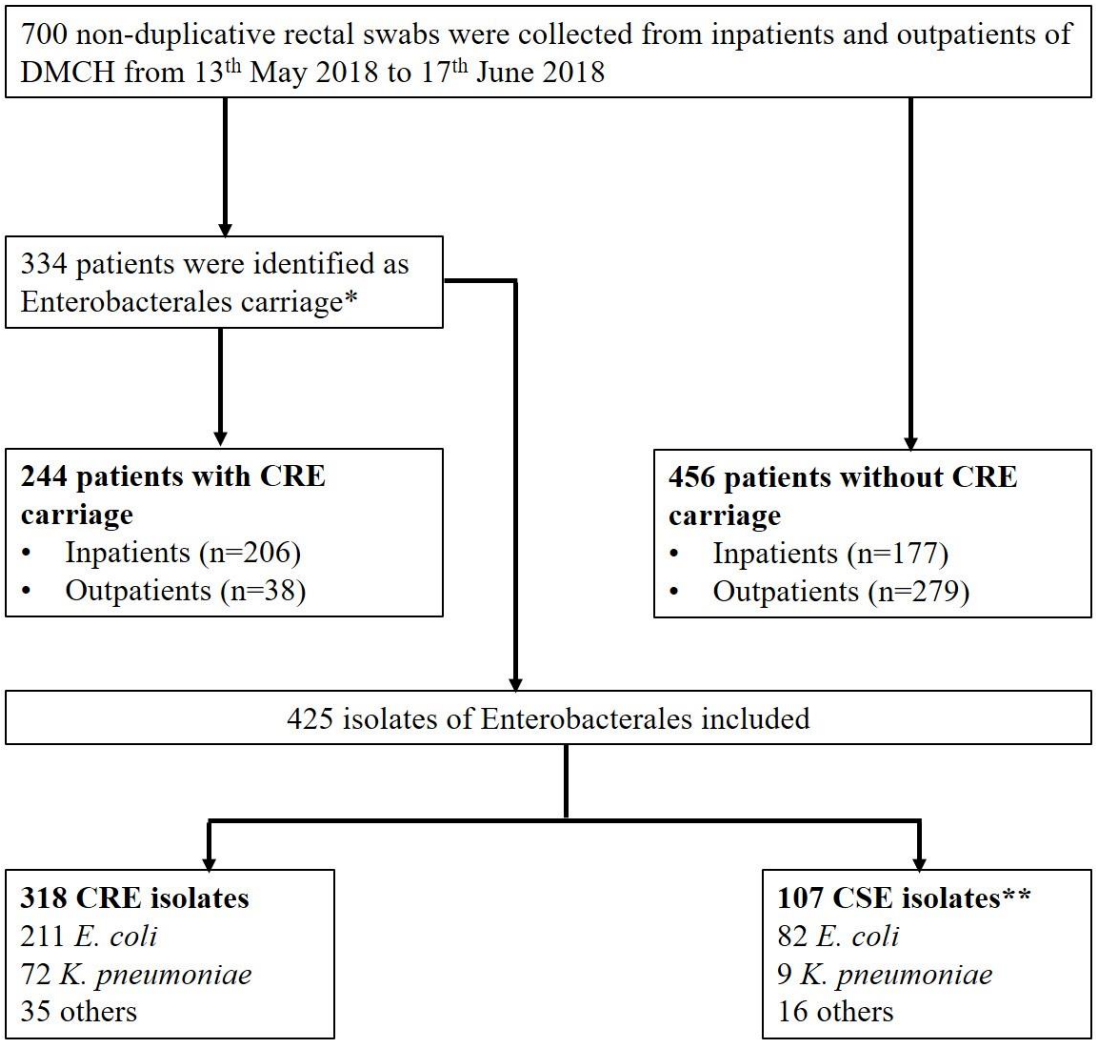


Figure 4.1 Flowchart diagram of colonisation study at DMCH. *The Enterobacterales from RSs were screened in vancomycin (10 mg/l), and ertapenem (2 mg/l) containing media. **CSE identified in this study were used as comparators to compare phenotypic and genomic profile between CRE and CSE.

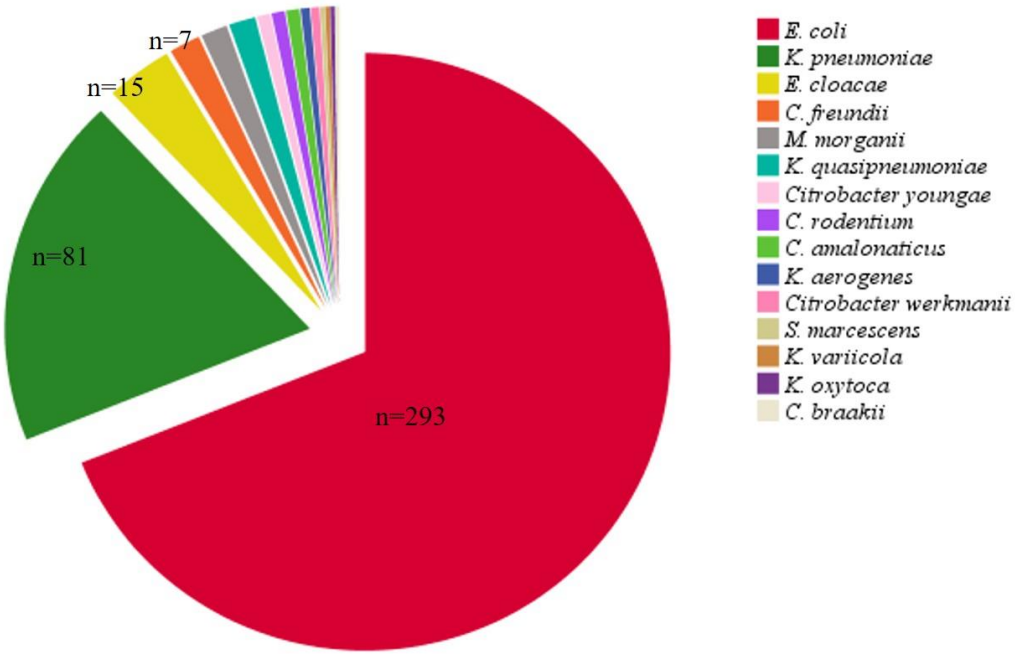


Figure 4.2 The frequency of isolation of different species of Enterobacterales from RSs at DMCH (n=700). The Enterobacterales from RSs were screened in vancomycin (10 mg/l), and ertapenem (2 mg/l) containing media.

Table 4.1 Antimicrobial susceptibility pattern of faecal Enterobacterales.

	Antibiotics	MIC_{50} (mg/l)	MIC_{90} (mg/l)	Range of MIC (mg/l)	n (%) Resistance
E. coli (n=293)	AMC	>256	>256	8 to >256	287 (98)
	TZP	>256	>256	0·125 to >256	252 (86)
	CRO	>256	>256	≤0·06 to >256	283 (96·6)
	CAZ	>256	>256	0·125 to >256	283 (96·6)
	CTX	>256	>256	≤0·06 to >256	281 (95·9)
	FEP	>256	>256	≤0·06 to >256	273 (93·2)
	IPM	8	32	≤0·06 to 128	200 (68·3)
	MEM	16	64	≤0·06 to 128	211 (72)
	CIP	128	>256	≤0·06 to >256	268 (91·5)
	LVX	32	64	≤0·06 to 256	258 (88·1)
	AMK	16	>256	0·5 to >256	147 (50·2)
	GEN	256	>256	0·25 to >256	172 (58·7)
	SXT	256	256	1 to >256	240 (81·9)
	FOF	0·25	1	≤0·06 to >256	5 (1·7)
	CST	0·25	0·25	0·125 to 8	1 (0·3)
K. pneumoniae (n=81)	AMC	>256	>256	256 to >256	81 (100)
	TZP	>256	>256	1 to >256	72 (88·9)
	CRO	>256	>256	1 to >256	78 (96·3)
	CAZ	>256	>256	0·125 to >256	80 (98·8)
	CTX	>256	>256	0·5 to >256	80 (98·8)
	FEP	64	>256	≤0·06 to >256	78 (96·3)
	IPM	4	32	≤0·06 to >256	51 (63)
	MEM	8	64	≤0·06 to >256	69 (85·2)
	CIP	64	>256	0·125 to >256	78 (96·3)
	LVX	8	256	0·125 to >256	73 (90·1)
	AMK	>256	>256	0·125 to >256	72 (88·9)
	GEN	>256	>256	0·125 to >256	70 (86·4)
	SXT	256	>256	0·125 to >256	78 (96·3)
	FOF	16	64	0·125 to 256	16 (19·7)
	CST	0·5	1	0·125 to 128	6 (7·4)
Klebsiella other than *K. pneumoniae* (n=10)	AMC	>256	>256	>256	10 (100)
	TZP	>256	>256	4 to >256	8 (80)
	CRO	256	>256	1 to >256	9 (90)
	CAZ	>256	>256	1 to >256	9 (90)
	CTX	>256	>256	4 to >256	10 (100)
	FEP	32	>256	≤0·06 to >256	8 (80)
	IPM	8	64	0·125 to 64	6 (60)
	MEM	16	32	≤0·06 to 32	8 (80)
	CIP	4	>256	0·125 to >256	8 (80)
	LVX	1	128	0·125 to 128	8 (80)
	AMK	>256	>256	1 to >256	7 (70)
	GEN	>256	>256	0·5 to >256	7 (70)
	SXT	>256	>256	4 to >256	10 (100)
	FOF	16	64	4 to 64	1 (10)
	CST	0·5	1	0·125 to 1	0 (0)
Enterobacter spp. (n=45)	AMC	>256	>256	>256	15 (100)
	TZP	256	>256	4 to >256	12 (80)
	CRO	>256	>256	1 to >256	15 (100)
	CAZ	>256	>256	8 to >256	15 (100)
	CTX	64	256	128 to >256	15 (100)
	FEP	64	64	8 to 128	15 (100)
	IPM	8	64	0·5 to 64	13 (86·7)

	MEM	16	64	≤0·06 to 64	14 (93·3)
	CIP	4	32	0·25 to 64	13 (86·7)
	LVX	1	64	0·125 to 64	12 (80)
	AMK	16	>256	4 to >256	10 (66·7)
	GEN	4	>256	1 to >256	9 (60)
	SXT	>256	>256	2 to >256	14 (93·3)
	FOF	32	64	0·5 to 256	7 (46·7)
	CST	0·25	1	0·25 to 16	1 (6·7)
Citrobacter spp. (n=19)	AMC	>256	>256	16 to >256	19 (100)
	TZP	4	>256	1 to >256	9 (47·4)
	CRO	16	>256	0·25 to >256	13 (68·4)
	CAZ	64	>256	0·125 to >256	12 (63·2)
	CTX	64	>256	0·125 to >256	13 (68·4)
	FEP	16	128	≤0·06 to 256	12 (63·2)
	IPM	0·5	16	0·125 to 16	8 (42·1)
	MEM	≤0·06	16	≤0·06 to 16	7 (36·8)
	CIP	1	64	≤0·06 to 64	11 (57·9)
	LVX	1	16	≤0·06 to 64	10 (52·6)
	AMK	2	>256	2 to >256	6 (31·6)
	GEN	0·5	>256	0·25 to >256	6 (31·6)
	SXT	2	256	1 to 256	8 (42·1)
	FOF	0·5	2	0·5 to 2	0 (0)
	CST	0·25	0·25	≤0·06 to 0·5	0 (0)
M. morganii (n=6)	AMC	128	>256	16 to >256	6 (100)
	TZP	128	256	2 to 256	5 (83·3)
	CRO	>256	>256	16 to >256	6 (100)
	CAZ	256	>256	8 to >256	6 (100)
	CTX	16	>256	4 to >256	6 (100)
	FEP	4	64	4 to 64	6 (100)
	IPM	2	16	1 to 16	6 (100)
	MEM	4	>256	0·25 to >256	5 (83·3)
	CIP	128	>256	128 to >256	6 (100)
	LVX	64	>256	32 to >256	6 (100)
	AMK	>256	>256	4 to >256	5 (83·3)
	GEN	256	>256	2 to >256	5 (83·3)
	SXT	256	256	256	6 (100)
	FOF	256	>256	64 to >256	6 (100)
	CST	>256	>256	>256	6 (100)

n, number of resistant isolates to respective antibiotic; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin. Data on *S. marcescens* (n=1) was not included in the table. The Enterobacterales from RSs were screened in vancomycin (10mg/L), and ertapenem (2 mg/l) containing media.

Table 4.2 Antimicrobial susceptibility patterns of faecal Enterobacterales.

Species	Resistance to respective antibiotics, n (%)														
	AMC	TZP	CRO	CAZ	CTX	FEP	IPM	MEM	CIP	LVX	AMK	GEN	SXT	FOF	CST
E. coli (n=293)	287 (98)	252 (86)	283 (96·6)	283 (96·6)	281 (95·9)	273 (93·2)	200 (68·3)	211 (72)	268 (91·5)	258 (88·1)	147 (50·2)	172 (58·7)	240 (81·9)	5 (1·7)	1 (0·3)
K. pneumoniae (n=81)	81 (100)	72 (88·9)	78 (96·3)	80 (98·8)	80 (98·8)	78 (96·3)	51 (63)	69 (85·2)	78 (96·3)	73 (90·1)	72 (88·9)	70 (86·4)	78 (96·3)	16 (19·7)	6 (7·4)
Other *Klebsiella* spp. (n=10)*	10 (100)	8 (80)	9 (90)	9 (90)	10 (100)	8 (80)	6 (60)	8 (80)	8 (80)	8 (80)	7 (70)	7 (70)	10 (100)	1 (10)	0 (0)
Enterobacter spp. (n=45)	15 (100)	12 (80)	15 (100)	15 (100)	15 (100)	15 (100)	13 (86·7)	14 (93·3)	13 (86·7)	12 (80)	10 (66·7)	9 (60)	14 (93·3)	7 (46·7)	1 (6·7)
Citrobacter spp. (n=19)	19 (100)	9 (47·4)	13 (68·4)	12 (63·2)	13 (68·4)	12 (63·2)	8 (42·1)	7 (36·8)	11 (57·9)	10 (52·6)	6 (31·6)	6 (31·6)	8 (42·1)	0 (0)	0 (0)
M. morganii (n=6)	6 (100)	5 (83·3)	6 (100)	6 (100)	6 (100)	6 (100)	6 (100)	5 (83·3)	6 (100)	6 (100)	5 (83·3)	5 (83·3)	6 (100)	6 (100)	6 (100)

Values in parentheses indicate row percentage. AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin. Data on *Salmonella* spp. (n=5), *P. agglomerans* (n=1) and *E. hermannii* (n=1) are not included in this table. **Klebsiella* species other than *K. pneumoniae*. Cells are highlighted according to the proportion of resistance of the species of Enterobacterales against respective antibiotics: ≥90% are represented by red (●); 60% to 89% by amber (●); 30% to 59% by yellow (●); <30% by green (●); intrinsic resistance by grey (●). The Enterobacterales from RSs were screened in vancomycin (10mg/L), and ertapenem (2 mg/l) containing media.

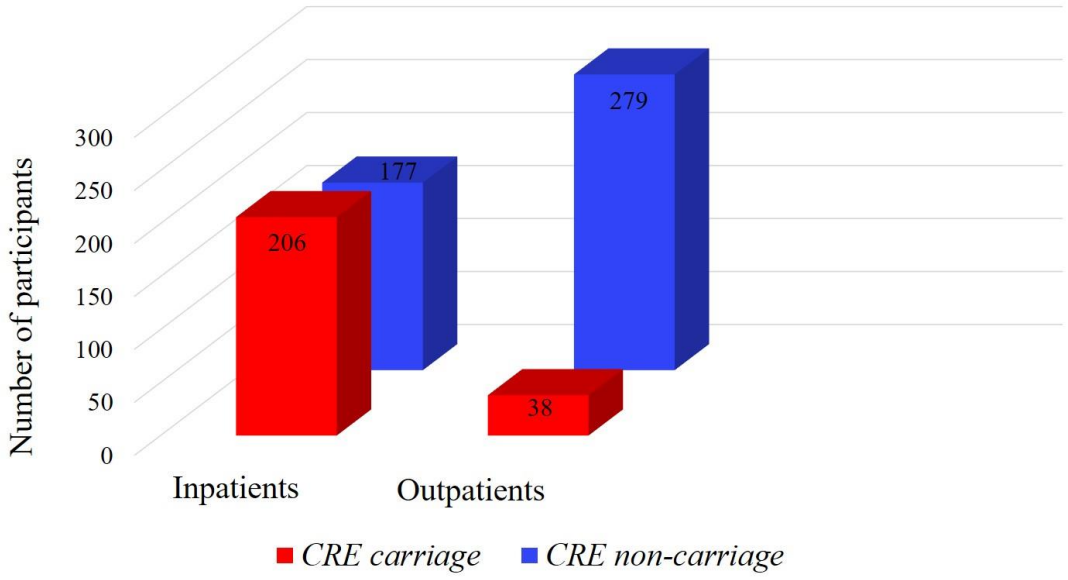


Figure 4.3 The prevalence CRE carriage among inpatients versus the prevalence among outpatients. The prevalence of CRE was significantly higher among inpatients (206/383, 53.8%) than outpatients (38/317, 12%) (p<0.0001).

4.2.2 The prevalence of carbapenemases alleles in faecal Enterobacterales

The distribution of carbapenemase alleles among the Enterobacterales was *bla*$_{NDM-5}$ (228/425, 53·6%) followed by *bla*$_{NDM-1}$ (56/425, 13·2%), *bla*$_{OXA-181}$ (41/425, 9·6%), *bla*$_{NDM-7}$ (14/425, 3·3%), *bla*$_{OXA-232}$ (5/425, 1·2%), *bla*$_{NDM-4}$ (4/425, 0·9%), and novel NDM variants (10/425, 2·3%) (Table 4.3). The combinations of carbapenemase alleles recovered were: *bla*$_{NDM-5}$+*bla*$_{OXA-181}$ (3/425, 0.7%), *bla*$_{NDM-1}$+*bla*$_{OXA-181}$ (1/425, 0.2%), and *bla*$_{NDM-1}$+*bla*$_{OXA-232}$ (1/425, 0.2%). Among the faecal Enterobacterales, *bla*$_{NDM-5}$ was significantly more likely to be found in *E. coli* (171/293, 58·4%) than others (57/132, 43·2%) (p<0.05), and *bla*$_{NDM-7}$ was only identified from *E. coli*. Significant prevalence of carbapenemase alleles in faecal *K. pneumoniae* was: *bla*$_{OXA-232}$ in *K. pneumoniae* (4/81, 4·9%) than others (1/344, 0·3%) (p<0.05). The prevalence of *bla*$_{NDM-1}$ in faecal *E. coli* (n=17) and *K. pneumoniae* (n=17) was the same (Figure 4.4).

Four of faecal *E. coli* phenotypically resistant to both imipenem (MIC range: 4 mg/l to 32 mg/l), and meropenem (MIC range: 16 mg/l to 64 mg/l) but were not positive any known carbapenemase. We found the presence of carbapenemase in phenotypically carbapenem-susceptible faecal isolates (low clinical breakpoint according to EUCAST) (n=8) (Table 4.4).

We considered the alleles as novel NDM variants when anonymous base substitutions were found in 813 bp NDM-encoded ORF, compared to known *bla*$_{NDM}$ variants (data not shown). Elucidating functional and molecular properties of the novel NDM variants was beyond the scope of this study. Further study has been planned to further analyse these novel variants.

Table 4.3 The distributions of carbapenemases alleles among the species of faecal Enterobacterales (n=425).

Carbapenemase alleles	Host species (n)	n (%)
NDM-5	CRo (1), CWe (1), EC (171), EnC (1), EnH (5), KP (46), KQ (1), MM (2)	228 (53·6)
NDM-1	CAm (3), CFr (3), CRo (2), EC (17), EnC (7), KA (1), KP (17), KQ (4), KV (1), MM (1)	56 (13·2)
OXA-181	CFr (2), EC (29), KA (1), KP (9)	41 (9·6)
NDM-7	EC (14)	14 (3·3)
Novel NDM variants	EC (4), EnC (1), KP (3), KQ (1), MM (1)	10 (2·3)
OXA-232	EC (1), KP (4)	5 (1·2)
NDM-4	EC (3), MM (1)	4 (0·9)

n, number of isolates; CAm, *C. amalonaticus;* CFr, *C. freundii;* CRo, *C. rodentium;* CWe, *C. werkmanii;* EC, *E. coli;* EnA, *E. aerogenes;* EnC, *E. cloacae;* KA, *K. aerogenes;* KP, *K. pneumoniae;* KQ, *K. quasipneumoniae;* KV, *K. variicola;* MM, *M. morganii*.

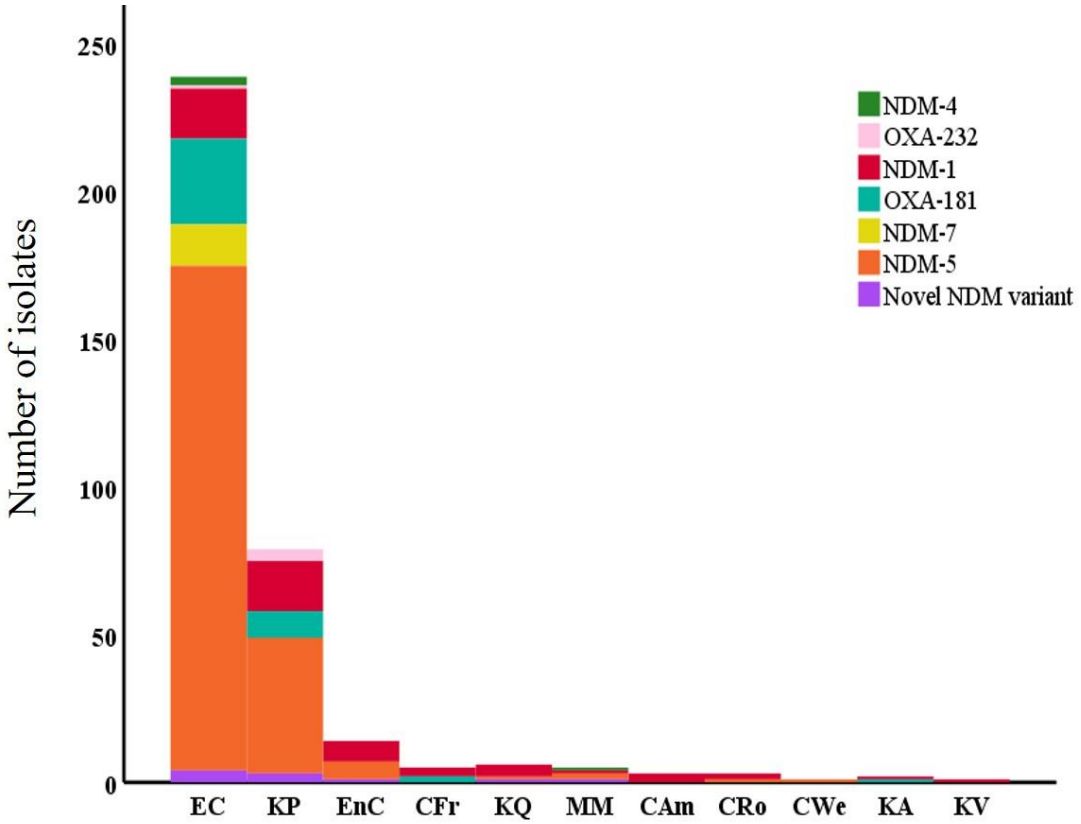


Figure 4.4 Comparative distribution of carbapenemase alleles among different species of faecal Enterobacterales. CAm, *C. amalonaticus;* CFr, *C. freundii;* CRo, *C. rodentium;* CWe, *C. werkmanii;* EC, *E. coli;* EnA, *E. aerogenes;* EnC, *E. cloacae;* KA, *K. aerogenes;* KP, *K. pneumoniae;* KQ, *K. quasipneumoniae;* KV, *K. variicola;* MM, *M. morganii*. Significant associations were observed in *E. coli* for $bla_{\text{NDM-5}}$ (171/293, 58·4%) than others (57/132, 43·2%) (p=0.004), and in *K. pneumoniae* for $bla_{\text{OXA-232}}$ (4/81, 4·9%) than others (1/344, 0·3%) (p=0·0004).

Table 4.4 Range of carbapenems' MIC of carbapenemase producing Enterobacterales having lower clinical breakpoints.

Carbapenemase variants	Carbapenems	Range of MIC (mg/l)
bla$_{\text{OXA-181}}$ (n=3)	IPM	0.25 to 1
	MEM	0.125 to 1
bla$_{\text{NDM-5}}$ (n=3)	IPM	0.125 to 2
	MEM	0.25
bla$_{\text{OXA-232}}$ (n=1)	IPM	0.5
	MEM	0.5
bla$_{\text{NDM-1}}$ (n=1)	IPM	0.125
	MEM	0.06

IPM, imipenem; MEM, meropenem.

4.2.3 Investigating the associations of non-β-lactam resistance in faecal CRE

The overall phenotypic resistance of the faecal Enterobacterales is depicted in Figure 4.5. The most sensitive antimicrobial against CRE were colistin (3.8%) followed by fosfomycin (9.7%). For colistin, this resistance rate reflected inclusion of a small number of isolates for which the species is intrinsically resistant. CRE exhibited significantly greater resistance rates to ciprofloxacin, levofloxacin, amikacin, gentamicin, and sulfamethoxazole-trimethoprim than CSE (p<0.05) (Table 4.5).

The associations of prevalent ARGs with the certain prevalent carbapenemase allele (*bla*$_{\text{NDM-1}}$, *bla*$_{\text{NDM-5}}$, *bla*$_{\text{OXA-181}}$) were examined as to clinical study (*Chapter 3*). Significant associations of carbapenemase alleles in faecal Enterobacterales with other ARGs encoding antibiotic degrading enzymes are mentioned in Table 4.6-Table 4.8. The ARGs were only considered for the analysis if the frequency of any gene was more than 10. Known ARGs were retrieved using CARD with a cut off ≥99.8% coverage, and ≥99.8% identity.

NDM-5 was shown to be the most common carbapenem resistance mechanism in both clinical, and carriage study followed by NDM-1 (Table 3.6; Table 4.3). Heatmaps were generated with the isolates harbouring *bla*$_{\text{NDM-5}}$, and *bla*$_{\text{NDM-1}}$, respectively based on presence ARGs showed significant associations with the respective carbapenemase alleles in both clinical, and carriage studies to identify any putative cluster of isolates having common mode of transmission of carbapenem resistance (Figure 4.6).

CRE harbouring *bla*$_{\text{NDM-5}}$ were significantly associated with *bla*$_{\text{ampC1}}$, *bla*$_{\text{ampH}}$, *bla*$_{\text{CMY-59}}$, *bla*$_{\text{TEM-1}}$, *aadA2*, *rmtB*, *sul1*, *sul2*, and *dfrA12* (Table 3.12; Table 4.7), of which *bla*$_{\text{ampC1}}$, and *bla*$_{\text{ampH}}$ were found only in *E. coli*, *bla*$_{\text{CMY-59}}$ was restricted to *E. coli*, and *K. pneumoniae*. The genes, *bla*$_{\text{TEM-1}}$, *aadA2*, *rmtB*, *sul1*, *sul2*, and *dfrA12* were distributed in different species of Enterobacterales (Figure 4.6). We attempted to screen a cluster with isolates shared *bla*$_{\text{NDM-5}}$, *bla*$_{\text{TEM-1}}$, *aadA2*, *rmtB*, *sul1*, and *dfrA12*. Seeing the comparative low prevalence of *sul2* (31.4%, 102/325) among the NDM-5-positive isolates, we did not consider *sul2* to investigate the putative cluster. A cluster consisted of 167 isolates was identified shared the six resistance genes (Figure 4.6).

CRE harbouring *bla*$_{NDM-1}$ were significantly associated with *APH(3')-VI*, *armA*, *rmtF*, *bla*$_{OXA-1}$, *bla*$_{OXA-9}$, *arr2*, and *mphE* (Table 3.11; Table 4.6). We found the combination of *bla*$_{OXA-1}$, *bla*$_{OXA-9}$, and *APH(3')-VI* among *K. variicola*. A group of isolates (n=6) shared *bla*$_{OXA-1}$, *APH(3')-VI*, *armA*, and *mphE*, of which four were *P. stuartii*. The combination of *bla*$_{NDM-1}$, *bla*$_{OXA-1}$, *armA*, and *mphE* was observed in wide range of species, which was considered as a cluster, comprised of 31 isolates (Figure 4.7).

The putative clusters identified in association with *bla*$_{NDM-5}$ (designated as HT5), and *bla*$_{NDM-1}$ (designated as HT1) were evaluated further whether any specific MDR plasmid facilitate the dissemination of carbapenem resistance which have been illustrated in *Chapter 6*.

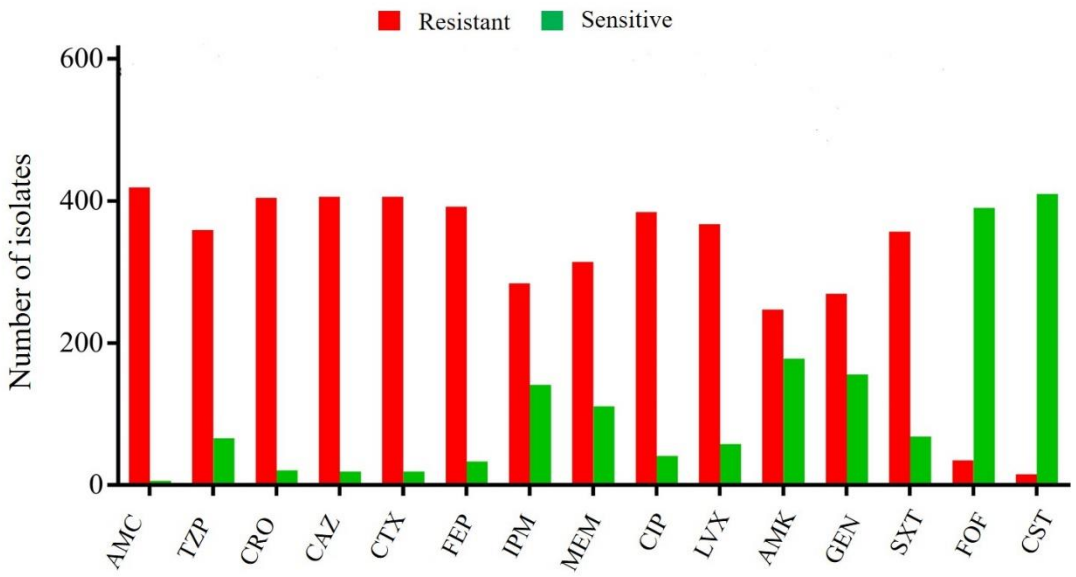


Figure 4.5 Susceptibility patterns of faecal Enterobacterales against the antibiotics tested. *All *M. morganii,* and *S. marcescens* isolated in this study were resistant to colistin. AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin. The Enterobacterales from RSs were screened in vancomycin (10 mg/l), and ertapenem (2 mg/l) containing media.

Table 4.5 Comparative resistance profile to non-β-lactam antibiotics between CRE and CSE.

Antibiotics	CRE (n=318)	CSE (n=107)	*p* value
CIP	311 (97·8)	73 (68·2)	<0·0001
LVX	303 (95·3)	64 (59·8)	<0·0001
AMK	243 (76·4)	4 (3·7)	<0·0001
GEN	251 (78·9)	18 (16·8)	<0·0001
SXT	299 (94·0)	58 (54·2)	<0·0001
FOF*	26/313 (8.3)	3/106 (2.8)	0.05
CST**	7/313 (2.2)	1/105 (1)	0·406

Values in parentheses indicate column percentage. CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin. **M. morganii* (n=6) were excluded from the analysis as the species are intrinsically resistant to fosfomycin. ***M. morganii* (n=6), and *S. marcescens* (n=1) were excluded from the analysis as the species are intrinsically resistant to colistin. Cells are highlighted whether any variable was significantly higher with CRE.

Table 4.6 The associations of prevalent ARGs with *bla*$_{NDM-1}$-positive faecal isolates compared to *bla*$_{NDM-1}$-negative faecal isolates.

ARGs	Presence of ARG, n (%)		*p* value
	bla$_{NDM-1}$-positive isolates (n=56)	*bla*$_{NDM-1}$-negative isolates (n=369)	
AAC(3)-IId	10 (17.9)	22 (6)	0.002
AAC(6')-Ib-cr	25 (44.6)	111 (30.1)	0.030
APH(3')-VI	10 (17.9)	1 (0.3)	<0.0001
APH(6)-Id	16 (28.6)	63 (17.1)	0.039
armA	16 (28.6)	12 (3.3)	<0.0001
rmtF	5 (8.9)	1 (0.3)	<0.0001
bla$_{CTX-M-15}$	52 (92.9)	239 (64.8)	<0.0001
bla$_{OXA-1}$	27 (48.2)	112 (30.4)	0.01
bla$_{OXA-9}$	12 (21.4)	11 (3)	<0.0001
arr2	11 (19.6)	10 (2.7)	<0.0001
mphE	22 (39.3)	34 (9.2)	<0.0001
qnrS1	26 (46.4)	110 (29.8)	0.015

Values in parentheses indicate column percentage. The associations between *bla*$_{NDM-1}$, and other ARGs were only mentioned if *p* values were <0.05.

Table 4.7 The associations of prevalent ARGs with *bla*$_{NDM-5}$-positive faecal isolates compared to *bla*$_{NDM-5}$-negative faecal isolates.

ARGs	Presence of ARG, n (%)		*p* value
	bla$_{NDM-5}$-positive isolates (n=228)	*bla*$_{NDM-5}$-negative isolates (n=197)	
aadA2	184 (80.7)	47 (23.9)	<0.0001
aadA5	38 (16.7)	19 (9.6)	0.037
rmtB	169 (74.1)	13 (6.6)	<0.0001
bla$_{CMY-59}$	42 (18.4)	22 (11.2)	0.037
bla$_{ampC1}$	52 (22.8)	16 (8.1)	<0.0001
bla$_{ampH}$	58 (25.4)	15 (7.6)	<0.0001
bla$_{TEM-1}$	176 (77.2)	68 (34.5)	<0.0001
sul1	199 (87.3)	91 (46.2)	<0.0001
sul2	54 (23.7)	29 (14.7)	0.020
sul3	14 (6.1)	2 (1)	0.007
dfrA12	196 (86)	53 (26.9)	<0.0001
dfrA17	26 (11.4)	8 (4.1)	0.008

Values in parentheses indicate column percentage. The associations between *bla*$_{NDM-5}$, and other ARGs were only mentioned if *p* values were <0.05.

Table 4.8 The associations of prevalent ARGs with *bla*$_{OXA-181}$-positive faecal isolates compared to *bla*$_{NOXA-181}$-negative faecal isolates.

ARGs	Presence of ARG, n (%)		*p* value
	bla$_{OXA-181}$-positive isolates (n=41)	*bla*$_{OXA-181}$-negative isolates (n=384)	
AAC(6')-Ib-cr	19 (46.3)	117 (30.5)	0.046
aadA5	14 (34.1)	43 (11.2)	0.0002
bla$_{CMY-59}$	14 (34.1)	50 (13)	0.001
bla$_{OXA-9}$	5 (12.2)	18 (4.7)	0.047
bla$_{SHV-1}$	5 (12.2)	8 (2.1)	0.001
arr3	3 (7.3)	3 (0.8)	0.001
catB3	4 (9.8)	7 (1.8)	0.004
cat1	8 (19.5)	31 (8.1)	0.021
qnrS1	32 (78)	104 (27.1)	<0.0001
sul1	35 (85.4)	255 (66.4)	0.019
dfrA1	3 (7.3)	8 (2.1)	0.045
dfrA17	9 (22)	25 (6.5)	0.001

Values in parentheses indicate column percentage. The associations between *bla*$_{OXA-181}$, and other ARGs were only mentioned if *p* values were <0.05.

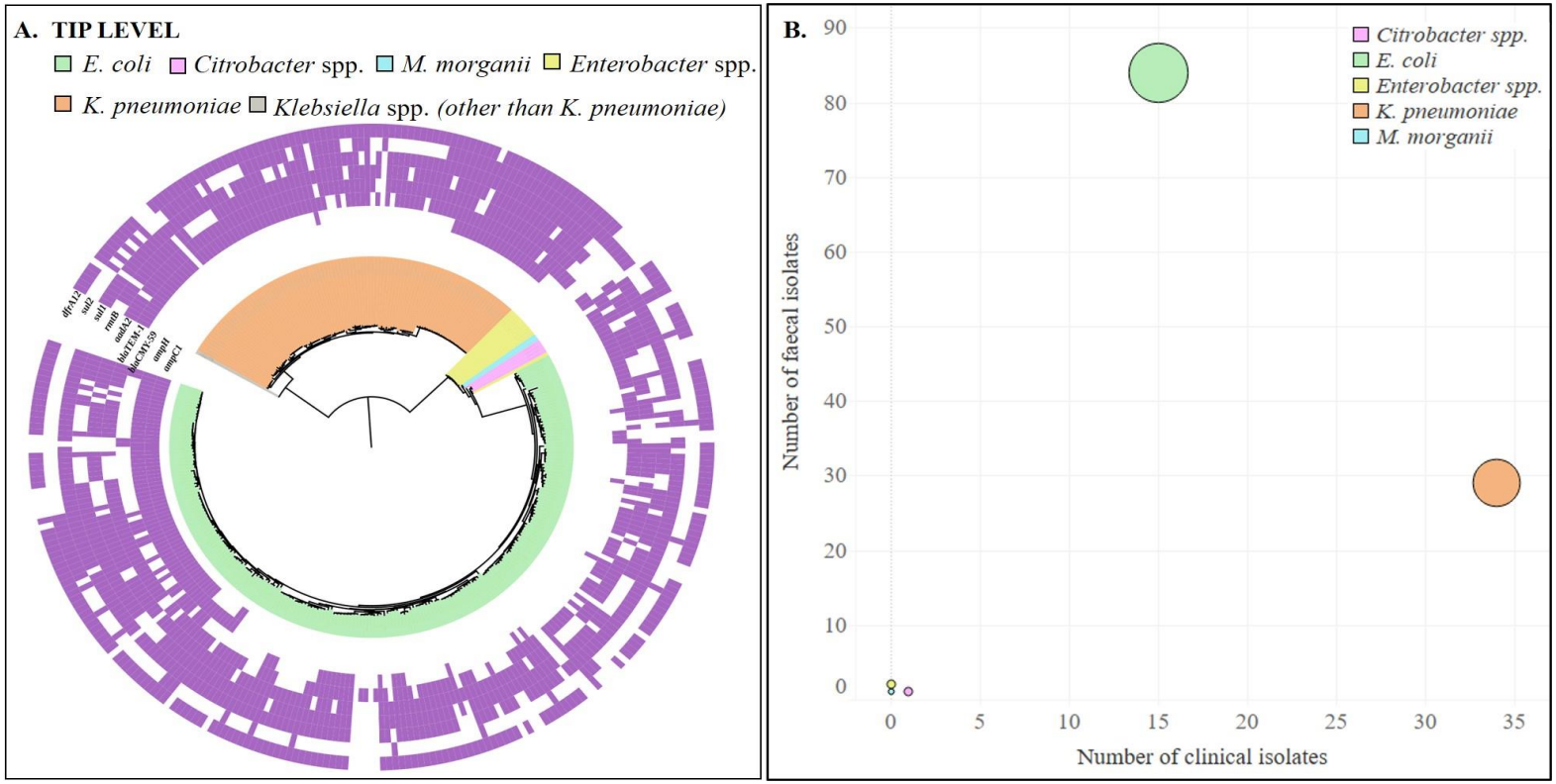


Figure 4.6 A. Phylogenetic tree generated with *bla*$_{\text{NDM-5}}$-positive isolates (n=325) from binary presence and absence of accessory genes using roary (v3.12.0) (core genes, n= 317, soft core genes, n=101, shell genes, n=9270, cloud genes, n=42837). Heatmap showing the presence and absence of respective ARGs. Purple indicates presence of genes, and white indicates absence of genes. **B.** Distribution of isolates (n=167) in the cluster shared *bla*$_{\text{NDM-5}}$, *bla*$_{\text{TEM-1}}$, *aadA2*, *rmtB*, *sul1*, and *dfrA12* among different species isolated from different source. Size of bubble is proportional to the number of isolates.

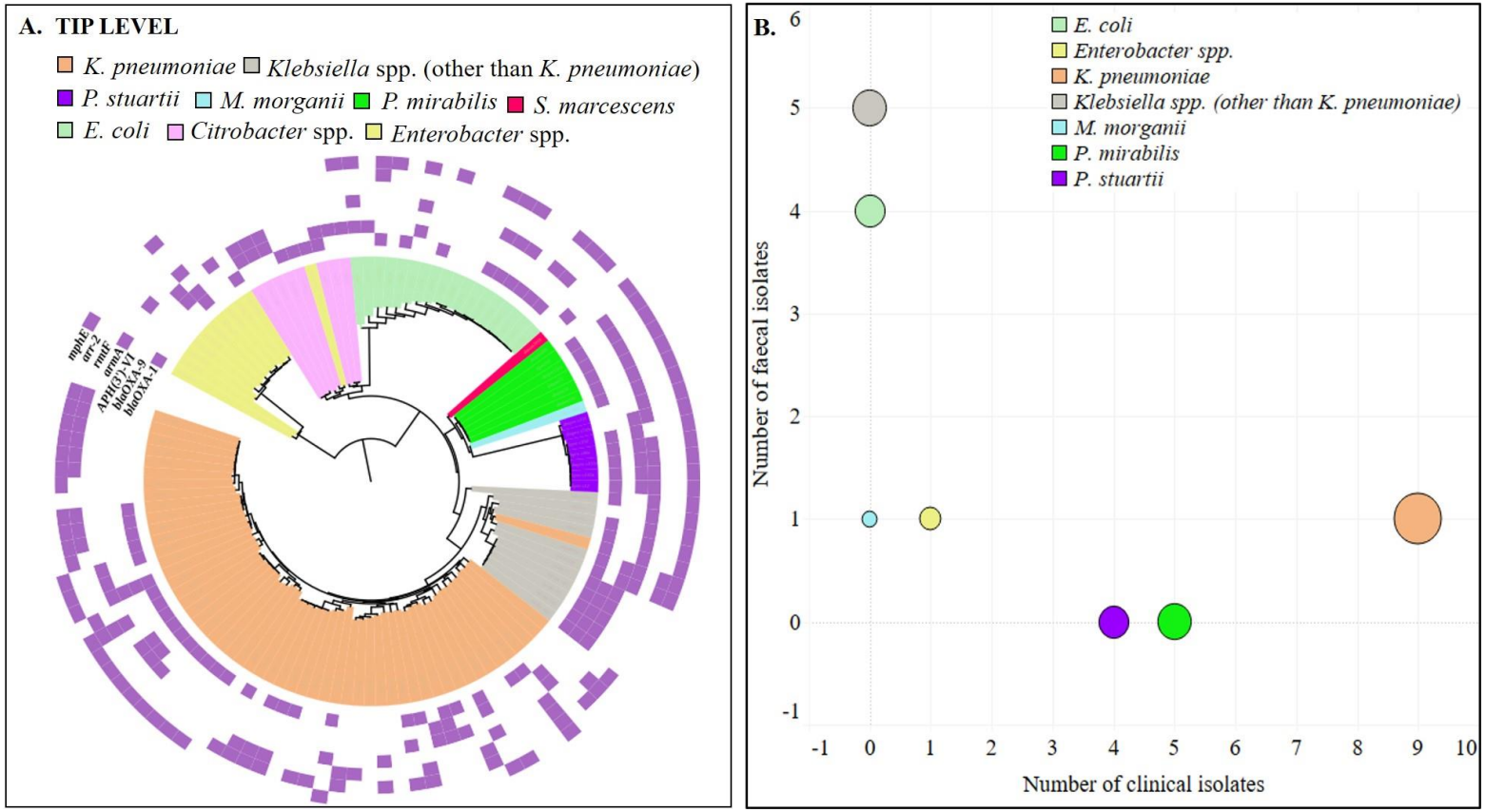


Figure 4.7 A. Phylogenetic tree generated with *bla*$_{\text{NDM-1}}$-positive isolates (n=118) from binary presence and absence of accessory genes using roary (v3.12.0) (core genes, n= 34, soft core genes, n=0, shell genes, n= 8577, cloud genes, n=59496). Heatmap showing the presence and absence of respective ARGs. Purple indicates presence of genes, and white indicates absence of genes. **B.** Distribution of isolates (n=31) in the cluster shared *bla*$_{\text{NDM-1}}$*, bla*$_{\text{OXA-1}}$*, armA, and mph* among different species isolated from different source. Size of bubble is proportional to the number of isolates.

4.2.4 Baseline data of the participants enrolled in carriage study

Of the 383 inpatients, 268 (70%) were male and 115 (30%) were female and the mean (±SD) age was 26.6 (±20.9). Among the outpatients, 204 were female (64.6%), and 112 (35.4%) were male and the mean age was 30.2 (±14.9). The majorities of both inpatients (n=219, 57.2%) and outpatients (n=263, 83.2%) in this study were from Dhaka division followed by other divisions (Figure 4.8). Overall, 67.4% patients belonged to lower-middle group, 20.8% poor, 6.4% BPL, and others (Table 4.9). Several hygiene indicators to determine the hygiene status of the participants were recorded. Participants were stratified based on hygiene indicators and socioeconomic conditions. The highest frequency (45%, 315/700) was observed among the participants of lower-middle group with improved sanitation facilities (access to household water supply, access to septic toilet, access to soap and water in toilet), however, taken together, 43.9% (307/700) of the participants were devoid of household water supply, and 32.1% (225/700) did not have access to water and soap in toilet (Table 4.9). 88.8% (340/383) of the inpatients were with antimicrobial treatment at the time of sampling, and only one outpatient was prescribed with antimicrobial on the day of sampling. The frequency of antimicrobial usage among the participants admitted at different wards of DMCH are summarised in Figure 4.9.

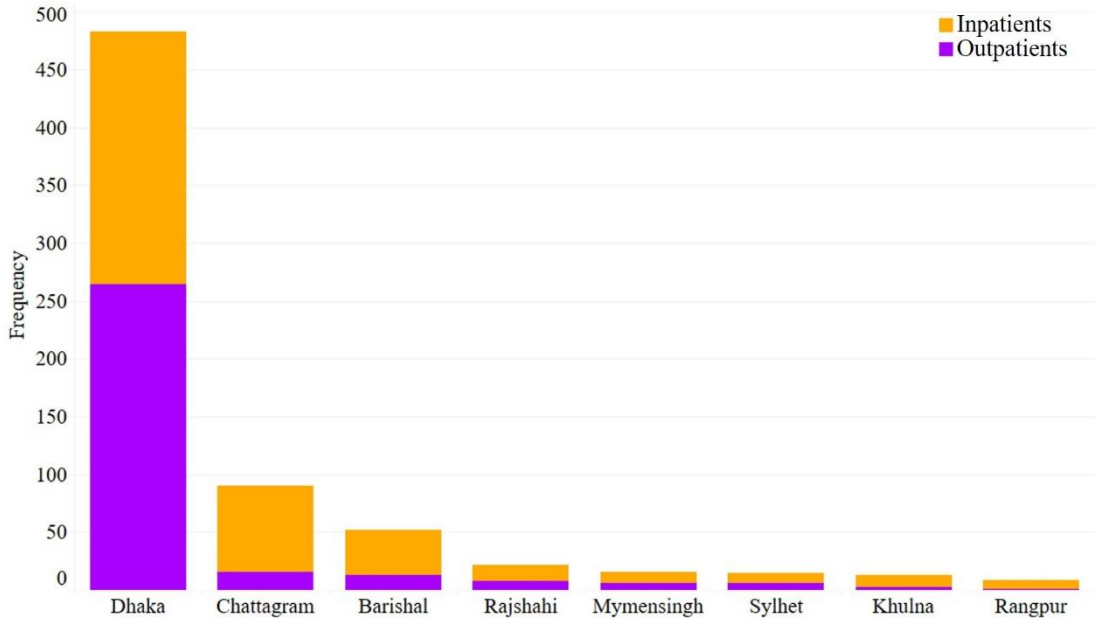


Figure 4.8 Locality of participants enrolled in this study.

Table 4.9 Overall hygiene status of the participants according to socioeconomic condition (n=700).

	Access to household water supply (n=393)			**No access to household water supply (n=307)**					**Total**
	Septic toilet	Pit toilet	Septic toilet	Septic toilet	Pit toilet	Septic toilet	Pit toilet	Open toilet	
BPL	8	0	6	7	1	15	8	0	45
Poor	25	1	3	22	7	57	27	4	146
LM	**315**	2	14	54	9	62	14	2	472
UM	17	0	0	4	1	8	2	2	34
UH	2	0	0	0	0	1	0	0	3

BPL, below the poverty level; LM, lower middle; UM, upper middle; UH, upper high. Green cells denote access to soap and water in toilet, and red cells indicate no access to soap and water in toilet.

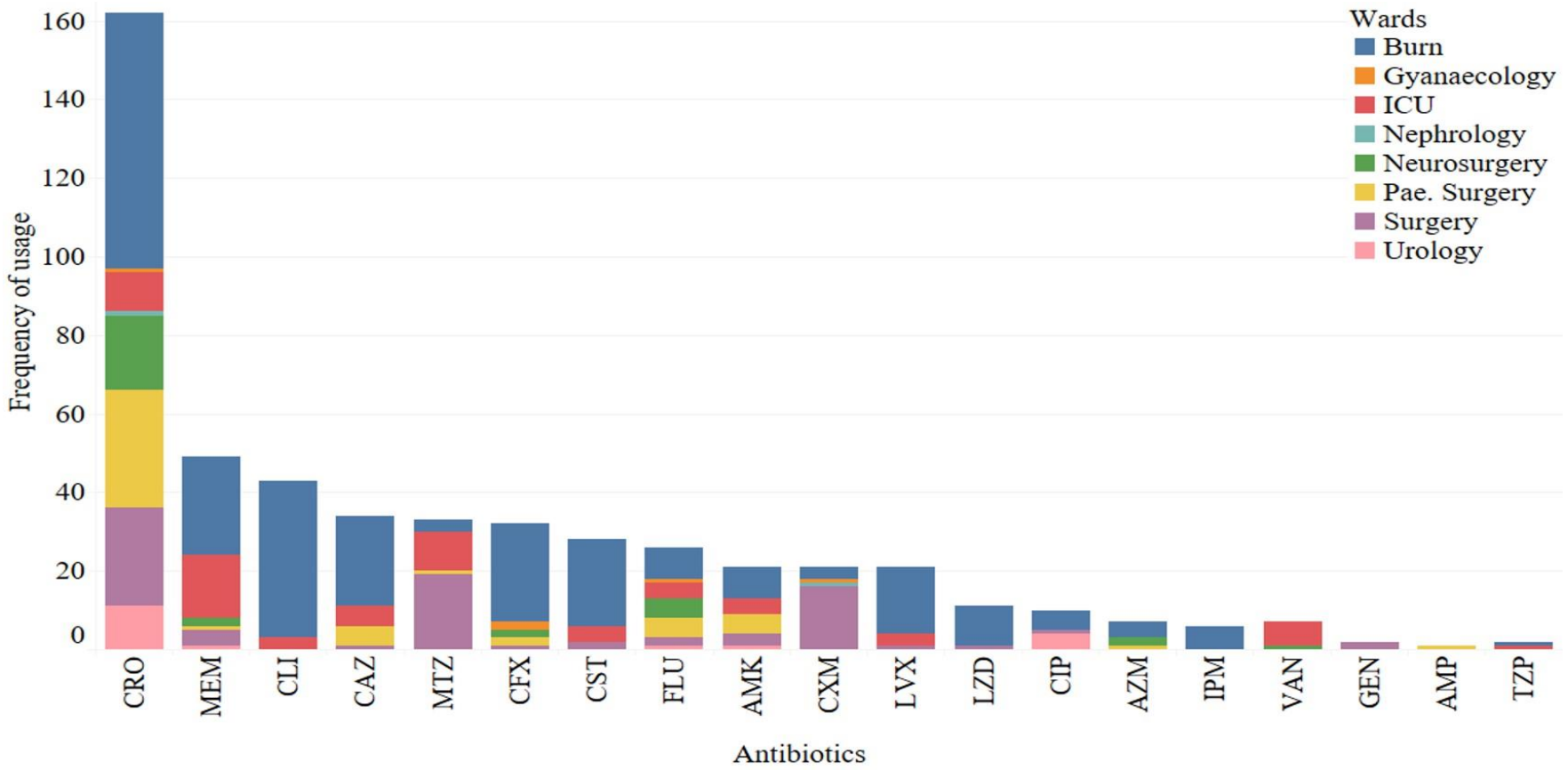


Figure 4.9 Antibiotics usage in different wards of DMCH among the participants enrolled for the colonisation study. AMP, ampicillin AMK, Amikacin; AZM, azithromycin; CAZ, ceftazidime; CFX, cefixime; CXM, cefuroxime; CIP, ciprofloxacin; CLI, clindamycin; CRO, ceftriaxone; CST, colistin; FLU, flucloxacillin; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; LZD, linezolid; MEM, meropenem; MTZ, metronidazole; TZP, piperacillin-tazobactam; VAN, vancomycin.

4.2.5 Risk assessment of baseline variables

The exposure of interest for the risk factor measurement was carbapenem resistance. Participants with at least one CRE positive RSs were considered as CRE carriage. Participants without CRE carriage were used as comparators for the risk assessment. Out of 700 Enterobacterales carriage patients, 244 (34.8%) were identified as CRE carriage and 456 (65.1%) as non-CRE carriage (Figure 4.1). Risk assessments were performed separately for inpatients and outpatients (Table 4.10)

Admission to the burns unit and hospital stay more than 7 days were significantly associated with CRE carriage ($p<0.05$) among the inpatients. Overall antibiotics usage among the inpatients was significantly higher in CRE carriage than the CSE carriage ($p<0.05$). Clindamycin, cefixime, colistin, and levofloxacin exposure had a significant association with CRE carriage ($p<0.05$) (Table 4.10).

Table 4.10 Descriptive statistics for risk assessment of faecal carriage of CRE compared to participants without CRE.

Attributes		**Inpatients (n=383)**			**Outpatients (n=317)**		
		CRE (n=206)	Non-CRE (n=177)	*p* value	CRE (n=38)	Non-CRE (n=279)	*p* value
Age	0 to 5	38 (18.4)	20 (11.3)	0.052	7 (18.4)	17 (6.1)	0.007
	6 to 25	86 (41.7)	63 (35.6)	0.218	14 (36.8)	67 (24)	0.089
	26 to 50	63 (30.6)	59 (33.3)	0.565	16 (42.1)	173 (62)	0.019
	>50	19 (9.2)	35 (19.8)	0.003	1 (2.6)	22 (7.9)	0.241
Sex	Female	67 (32.5)	48 (27.1)	0.250	22 (57.9)	182 (65.2)	0.376
	Male	139 (67.5)	129 (72.9)		16 (42.1)	97 (34.8)	
Socioeconomic group	BPL	21 (10.2)	12 (6.8)	0.235	4 (10.5)	8 (2.9)	0.020
	Poor	47 (22.8)	51 (28.8)	0.180	6 (15.8)	42 (15.1)	0.906
	LM	132 (64.1)	98 (55.4)	0.083	28 (73.7)	214 (76.7)	0.681
	UM	5 (2.4)	15 (8.5)	0.008	0 (0)	14 (5)	0.158
	UH	1 (0.5)	1 (0.5)	0.914	0 (0)	1 (0.4)	0.712
Access to supply water at home	Yes	84 (40.8)	69 (39)	0.721	21 (55.3)	219 (78.5)	0.002
	No	122 (59.2)	108 (61)		17 (44.7)	60 (21.5)	
Type of toilet	Septic toilet	172 (83.5)	148 (83.6)	0.975	34 (89.5)	266 (95.3)	0.132
	Pit toilet	28 (13.6)	27 (15.3)	0.644	4 (10.5)	13 (4.7)	0.132
	Open toilet	6 (2.9)	2 (1.1%)	0.224	-	-	-
Access to water & soap in toilet (at home)	Yes	116 (56.3)	100 (56.5)	0.971	28 (73.7)	231 (82.8)	0.173
	No	90 (43.7)	77 (43.5)		10 (26.3)	48 (17.2)	
Admitting wards (hospitalised patients)[a]	Burn	132 (64.1)	50 (28.2)	<0.0001	-	-	-
	Surgery	20 (9.7)	35 (19.8)	0.005	-	-	-
	PSU	19 (9.2)	28 (15.8)	0.050	-	-	-
	Neurosurgery	8 (3.9)	29 (16.4)	<0.0001	-	-	-
	ICU	19 (9.2)	16 (9)	0.950	-	-	-

Hospital stay before sampling[a]	≤7 days	65 (31.6)	103 (58.2)	<0.0001	-	-	-
	>7 days	141 (68.4)	74 (41.8)		-	-	-
Antibiotics usage during hospital stay (before sampling)[a]	With antibiotics	192 (93.2)	148 (83.6)	0.003	-	-	-
	Without antibiotics	14 (6.8)	29 (16.4)		-	-	-
Antibiotics exposure during hospital stay (before sampling)[a]	Ceftriaxone	78 (37.9)	84 (47.5)	0.058	-	-	-
	Meropenem	32 (15.5)	17 (9.6)	0.083	-	-	-
	Clindamycin	30 (14.6)	13 (7.3)	0.026	-	-	-
	Ceftazidime	19 (9.2)	15 (8.5)	0.797	-	-	-
	Metronidazole	12 (5.8)	21 (11.9)	0.036	-	-	-
	Cefixime	26 (12.6)	6 (3.4)	0.001	-	-	-
	Colistin	22 (10.7)	6 (3.4)	0.006	-	-	-
	Flucloxacillin	18 (8.7)	8 (4.5)	0.102	-	-	-
	Amikacin	12 (5.8)	9 (5.1)	0.751	-	-	-
	Cefuroxime	7 (3.4)	14 (7.9)	0.053	-	-	-
	Levofloxacin	17 (8.3)	4 (2.3)	0.010	-	-	-
Number of antibiotics prescribed (n=340)*[a]	Monotherapy	87 (45.3)	80 (50)	0.110	-	-	-
	More than one drug	105 (54.7)	68 (45.9)		-	-	-

Values in parentheses indicate column percentage. BPL, below the poverty level; LM, lower middle; UM, upper middle; UH, upper high; PSU, paediatric surgery. *Inpatients without any antibiotic among inpatients were excluded from the analysis. [a]Analysis performed for inpatients only. Antibiotics usage among outpatients were not evaluated for CRE colonisation. Cells are highlighted whether any variable was significantly associated with CRE. Bivariate analysis was performed to identify the risks for CRE.

4.3 Discussion

Following the high prevalence [10.6% (194/1831)] of CRE in clinical infections (*Chapter 3*), we aimed to conduct the carriage study to investigate connection between clinical infections and faecal colonisation at DMCH. RSs were screened for CRE on media containing vancomycin (10mg/L), and ertapenem (2 mg/l) to preclude Gram-positive and carbapenem sensitive bacteria. A total of 425 Enterobacterales was screened on media containing vancomycin (10 mg/L), and ertapenem (2 mg/l), however, 107 isolates were considered as CSE due to lower clinical breakpoints of both imipenem (≤2 mg/l) and meropenem imipenem (≤2 mg/l) (Figure 4.1), of which three was positive for *bla*$_{\text{OXA-181}}$, three for *bla*$_{\text{NDM-5,}}$ one for *bla*$_{\text{OXA-232}}$, and one for *bla*$_{\text{NDM-1}}$ (Table 4.4). The remaining 92.5% (99/107) of the isolates were negative for any known carbapenemase. In accordance with the finding, 9 of the clinical isolates carried *bla*$_{\text{OXA-232}}$, and one *bla*$_{\text{OXA-181,}}$ were phenotypically susceptible to both imipenem and meropenem (Table 3.7). Nevertheless, this study considered any species of Enterobacterales as CSE if these were phenotypically carbapenem susceptible (imipenem and meropenem) irrespective of presence of carbapenemase considering very low of such frequency. Carbapenems resistance is mostly mediated by the bacterial production of carbapenemase (Codjoe and Donkor, 2017; Ye *et al.,* 2018). The carbapenemase, *bla*$_{\text{OXA-48-like}}$ can hydrolyse carbapenem at a low level. Hydrolysing capacity of OXA-48-like producers has been often enhanced by the combined effects of other carbapenemases or due to porin loss (Poirel *et al.,* 2012; Fattouh *et al.,* 2015; Lutgring *et al.,* 2018). Although the isolates containing *bla*$_{\text{NDM}}$ unlikely to be susceptible to carbapenem, four faecal NDM-producers showed had low imipenem/meropenem MIC breakpoints (≤2 mg/l) as to previous report by Singh-Moodley and Perovic (2016). Carbapenems' effectivities are also destroyed by the expression of AmpC (chromosomal or acquired) along with the presence of ESBLs or porin mutations. The manifestation of non-carbapenemase mediated resistance mostly reduce the effectivity of ertapenem without compromising the susceptibilities to imipenem or meropenem, elucidating the growth of CSE in ertapenem containing media (Codjoe and Donkor, 2017; Ye *et al.,* 2018).

The overall findings on the patients' demographic data and in-hospital antibiotics usage between clinical and carriage study were consistent (Figure 3.5-Figure 3.7, Figure 3.9; Figure 4.8; Figure 4.9, Table 4.9). This study deployed random

sampling for the carriage study, however, the distribution of participants in different wards was not unform because of patients' refusal to involve. As a considerable number of clinical isolates were recovered from burn unit (Table 3.1), this study approached to include at least one third of inpatients' RSs from burn unit of DMCH. Consequently, a total of 183 RSs from burn patients were collected (Table 4.10). Additionally, participants' hygiene measures were included in the dataset of carriage study which showed that the general hygiene status was poor; 43.9% of the participants did not have access to household supply water, and 32.1% of the participants did not have access to water and soap in toilet (Table 4.9).

The prevalence of CRE in the community setting of Bangladesh is largely unknown (Ahmed *et al.,* 2019; Hasan and Rabbani, 2019). The frequency of faecal CRE carriage among the outpatients in this study reflected the prevalence in the community, was extremely high (12%). Showing that inpatients were more susceptible to acquiring CRE (53.8%) than outpatients (12%) (p<0.05) (Figure 4.3). Literature reviews suggested a varied prevalence of CRE colonisation among hospitalized patients (ranged from 1.6% to 73%) in SA, but it is notable that the higher prevalence is associated with the vulnerable group such as ICU-admitted or cancer patients, and risks for faecal colonisation are similar to that of acquisition of infections such as prolonged hospital stay, introduction of artificial medical devices, and antibiotic exposure (Datta *et al.,* 2015; Saseedharan *et al.,* 2016; Mohan *et al.,* 2017; Bharadwaj *et al.,* 2018; Kumar *et al.,* 2018; Singh *et al.,* 2018). Hospital stay more than 7 days, and antibiotic usage were considered as a significant risk in this study (p<0.05) (Table 4.10). Like clinical study ('*Chapter 3*'), a very high frequency (88.8%) of antibiotic usage at DMCH was observed in the colonisation study from 13th May 2018 to 17th June 2018. Interestingly, significantly associations were found between clindamycin, cefixime, colistin, and levofloxacin usage and CRE colonisation (p<0.05) (Table 4.10). Minimal diagnostic capacity might influence empirical antibiotic prescription in Bangladesh wide verities, leading to colonisation of bacteria with multiple resistance mechanisms (Shahida *et al.,* 2016; Hasan and Rabbani, 2019). Even a very brief successful antibiotic treatment raises the profusion of AMR genes several folds in the gut ecosystem which perhaps persists in asymptomatic host for years or acts as an opportunistic pathogen for severely ill or immunocompromised patients (Jakobsson *et al.,* 2010; Carlet, 2012). A metanalysis documented that the rate of infection

carbapenemase producing bacteria following colonisation varied from 0% to 89% (Tischendorf *et al.,* 2016; Cattaneo *et al.,* 2018). As described before, this PhD project conducted the clinical and carriage study at different time point (Figure 3.1; Figure 4.1). The limitation of this study is inability to estimate the direct impact of colonisation following infections, and *vice versa*. Nevertheless, data demonstrated that admission in a hospital setting like DMCH may act as an amplifier of resistance by a combination of acquisition and increased resistant subpopulations in the gut microbiome due to selection pressure of antibiotics (Carlet, 2012; van Schaik, 2015; Gupta *et al.,* 2019).

Taking account of highest frequency of bla_{NDM} as mode of carbapenem resistance in this study, the possibilities for the transmission of multiple antibiotic resistance through a common means was evaluated which suggested the combination of $bla_{\text{NDM-5}}$, $bla_{\text{TEM-1}}$, *aadA2*, *rmtB*, *sul1*, and *dfrA12* among 167 isolates, and the combination of $bla_{\text{NDM-}}$1, $bla_{\text{OXA-1}}$, *armA*, and *mphE* in 31 isolates (Figure 4.6; Figure 4.7). Supporting the genomic findings, significant more resistance to non-β-lactams against faecal CRE (p<0.05) (Table 4.3) also signifies the associations of multiple ARGs (Nikaido, 2009; Li *et al.,* 2015). The gene, bla_{NDM} has been frequently described in plasmids harbouring the determinants of multi-drug resistance, such as *aadA*, *dfrA*, *mphA*, *APH*, *AAC(6')-Ib-cr*, bla_{OXA}, bla_{TEM} (Poirel *et al.,* 2011; Walsh *et al.,* 2011; Khan *et al.,* 2017; Yoon *et al.,* 2018). The significant linkage of bla_{NDM} with the ARGs encoding aminoglycosides, trimethoprim, and/or other β-lactamase among the different species of Enterobacterales corresponded to the earlier reports, and strongly hypothesized the spread of bla_{NDM} due to some dominant plasmids circulating in Bangladesh.

Faecal carriage of MDR bacteria is a potential reservoir for faeco-oral spread of AMR in the countries with underdeveloped sanitation, poor hygiene practice, inappropriate sewage management, and weak IPC measures in general hospitals. Poor IPC in health settings of the countries of SA is obvious, aggravating the cross transmission of AMR from inter-patients, and/or hospitals environments to patients (Chereau *et al.,* 2017). This chapter demonstrates the prevalence of CRE along with MDR determinants at an alarming level. Health education on hygiene and sanitation, implication of antibiotic stewardship programme, and access of essential antibiotics

can reduce the AMR endemicity in the health setting of the country (O'Neill, 2016; Lee *et al.*, 2013; Cheng *et al.*, 2018). Periodic screening of faecal colonisation of endemic resistance would facilitate to predict the burden of the resistance and to take subsequent action (Ramanathan *et al.*, 2018).

Section Five

Investigating Clonal Dissemination of Carbapenem-Resistant Enterobacterales

5.1 Introduction

A successful MDR bacterial clone is a powerful disseminator of AMR genes. The clone can be propagated by means of strain's survival, expansion and spread. Moreover, the clone possesses an array of genetic elements such as genes, integrons, transposons, and plasmids which can be mobilised horizontally to other clones, species or genera (Moradigaravand *et al.,* 2017; Davin-Regli *et al.,* 2019; Marano *et al.,* 2019). Hospital outbreaks resulting from successful MDR clones are often inevitable when appropriate infection control measures are not in place (Mirande *et al.,* 2018). Additionally, irrational and overuse of antimicrobials facilitate the maintenance of MDR clones in the community as well as the hospital (Barchitta *et al.,* 2019; Tonoyan *et al.,* 2019).

Due to economic globalisation, there has been a considerable increase in global mobility in last two decades including international trade. Travellers may be exposed by a broad range of bacteria including dominant MDR clones which subsequently can be globally disseminated. Colonisation by resistant bacteria of Enterobacterales family in human gut is well documented. Due to genetic plasticity of the species of Enterobacterales, they can readily acquire mobile elements from environmental bacteria and/or transfer to medically important pathogens (Gyles *et al.,* 2014; Hassing *et al.,* 2015; Hawkey, 2015; Davies and Walsh, 2018).

This chapter describes the clonality of Enterobacterales and their role in the dissemination of carbapenem resistance at DMCH. We deployed WGS to investigate clonal relatedness, and spatiotemporal analysis of high-risk clones for carbapenem resistance in the hospital setting. Clones associated with clinical infections were the main focus of interest in this study; however, clinical isolates were matched with faecal isolates to evaluate the persistence of common transmission events. Therefore, this chapter has achieved '**objective #2**', '**objective #3**', and '**objective #5**' of this project.

5.2 Results

5.2.1 Screening of high-risk clones for carbapenem resistance

A comprehensive insight into the clonal relatedness among the isolates was performed by combining the following approaches: **1.** Core-genome alignment following clustering according to the presence and absence of genes, **2.** Genome-wide SNPs analysis, and **3.** MLST profiling, where appropriate.

5.2.1.1 Population structure of *E. coli*

In this study, we found some clusters consisted of isolates with different MLST profiles but differed by ≤100 SNPs. The clusters below (**A** to **G**) were identified which consisted of isolates from diverse STs, differed by ≤100 SNPs: **A.** ST648 (n=40), ST2011 (n=2), ST6870 (n=1), ST8881 (n=1) and ST9666 (n=1); **B.** ST405 (n=14), and ST5954 (n=3); **C.** ST617 (n=3), and ST4981 (n=2); **D1.** ST167 (n=7), ST10 (n=1); **D2.** ST167 (n=53), ST1702 (n=13), and ST9668 (n=1); **E.** ST410 (n=11), and ST2851 (n=3); **F.** ST448 (n=10), and ST2083 (n=7); and **G.** ST224 (n=12), and ST10821 (n=1) (Figure 5.1); however, there was no single pair of isolates from different STs differing by ≤40 SNPs.

The most prevalent STs among clinical *E. coli* was ST131 (10.1%, 23/228) followed by ST405 (9.2%, 21/228), ST648 (9.2%, 21/228), ST410 (9.2%, 21/228), ST167 (7.9%, 18/228), ST101 (5.3%, 12/228), ST38 (3.9%, 9/228), ST448 (2.6%, 6/228), and others (41.4%, 97/228) (Figure 5.1; Figure 5.2). *E. coli* ST167, ST448, ST8346, ST405, and ST648 were significantly associated with carbapenem resistance in the clinical study (p<0.05) (Table 5.1).

Faecal *E. coli* were mostly carbapenem resistant as RSs were screened on media containing vancomycin (10 mg/L), and ertapenem (2 mg/l) (Figure 4.1). Faecal *E. coli* predominantly belonged to ST167 (16.4%, 48/293), ST8346 (8.9%, 26/293), ST405 (8.2%, 24/293), ST648 (6.5%, 19/293), ST448 (6.1%, 18/293), ST410 (5.1%, 15/293), ST38 (4.8%, 14/293), ST1702 (4.4%, 13/293), ST2659 (3.4%, 10/293), and others (36.2%, 110/293) (Figure 5.1; Figure 5.3). Likewise, faecal *E. coli* isolated from outpatients (representing the community prevalence) belonged to wide range of STs. The most frequent were the ST167 (9%), ST405 (9%), and ST648 (9%) followed by ST226 (7.3%), ST8346 (7.3%), and others (Figure 5.3).

There were positive corelations between the prevalence of different *E. coli* clonal types and the frequency of carbapenemase producing *E. coli* to pertinent clones (r=0.662) (Figure 5.4). Faecal *E. coli* were excluded from the correlation analysis because of RSs screening with ertapenem (2 mg/l) selection (Figure 4.1). The carbapenemase alleles were distributed among several STs of *E. coli*; specifically, ST167 with $bla_{\text{NDM-5}}$; ST167 with $bla_{\text{OXA-181}}$; ST405 with $bla_{\text{NDM-5}}$; ST648 with $bla_{\text{NDM-4}}$; ST8346 with $bla_{\text{OXA-181}}$; ST448 with $bla_{\text{NDM-5}}$; ST448 with $bla_{\text{NDM-7}}$; ST448 with $bla_{\text{OXA-181}}$; ST2659 with $bla_{\text{NDM-4}}$; ST101 $bla_{\text{NDM-7}}$; ST1702 with $bla_{\text{NDM-5}}$; and ST617 with $bla_{\text{OXA-181}}$ (p<0·05) (Figure 5.5; Table 5.2).

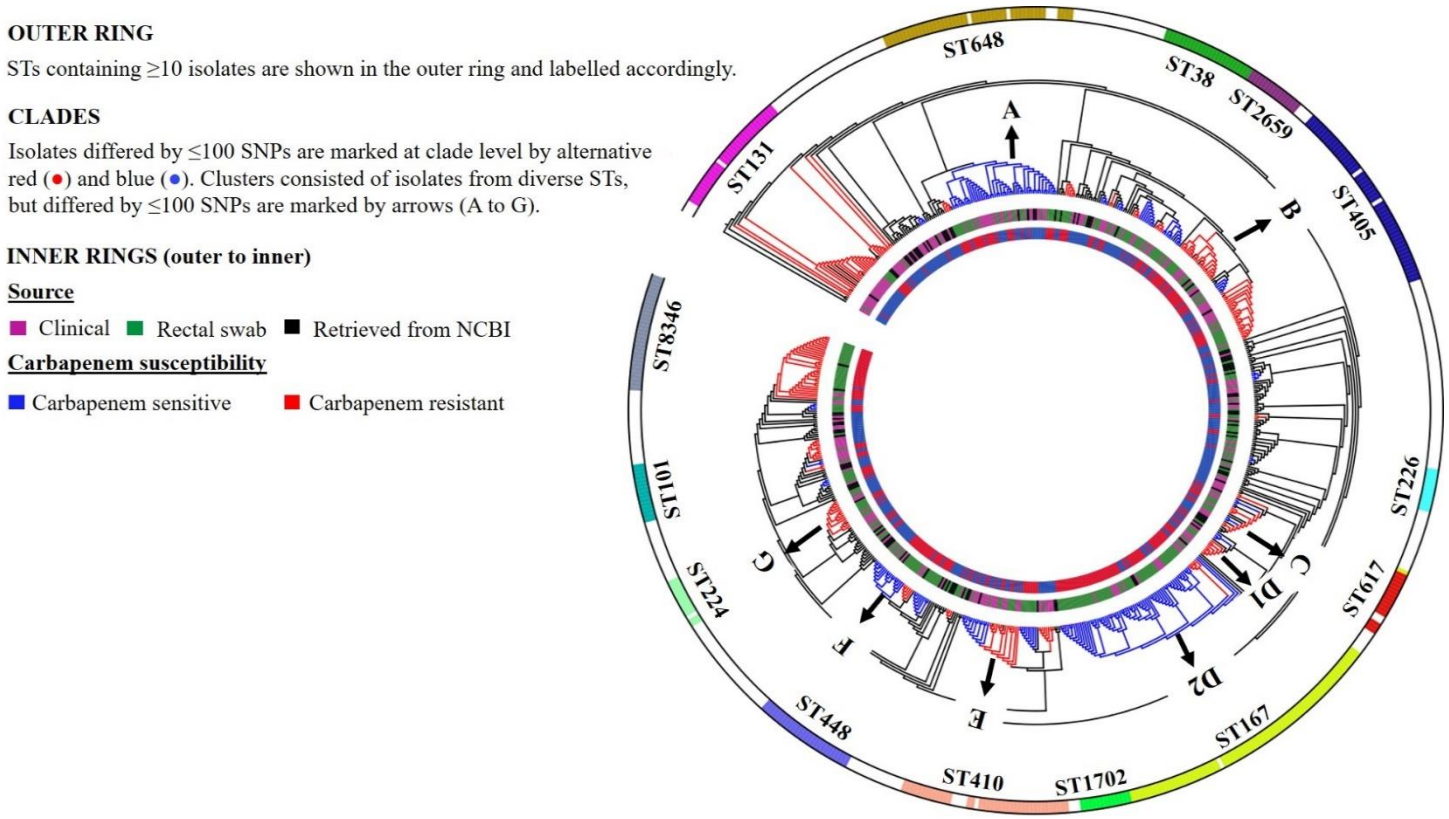


Figure 5.1 ML tree generated from core-genome analysis of *E. coli* isolated in this study. Core-genome alignment was performed using roary (v3.12.0). The ML tree from the core genome was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model and gamma correction with bootstrapping. SNPs calling was performed using Snippy (v4.4.5) followed by recombination removal using Gubbins (v2.3.4) and pair-wise SNPs calculation using pairsnp (v0.0.7). Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

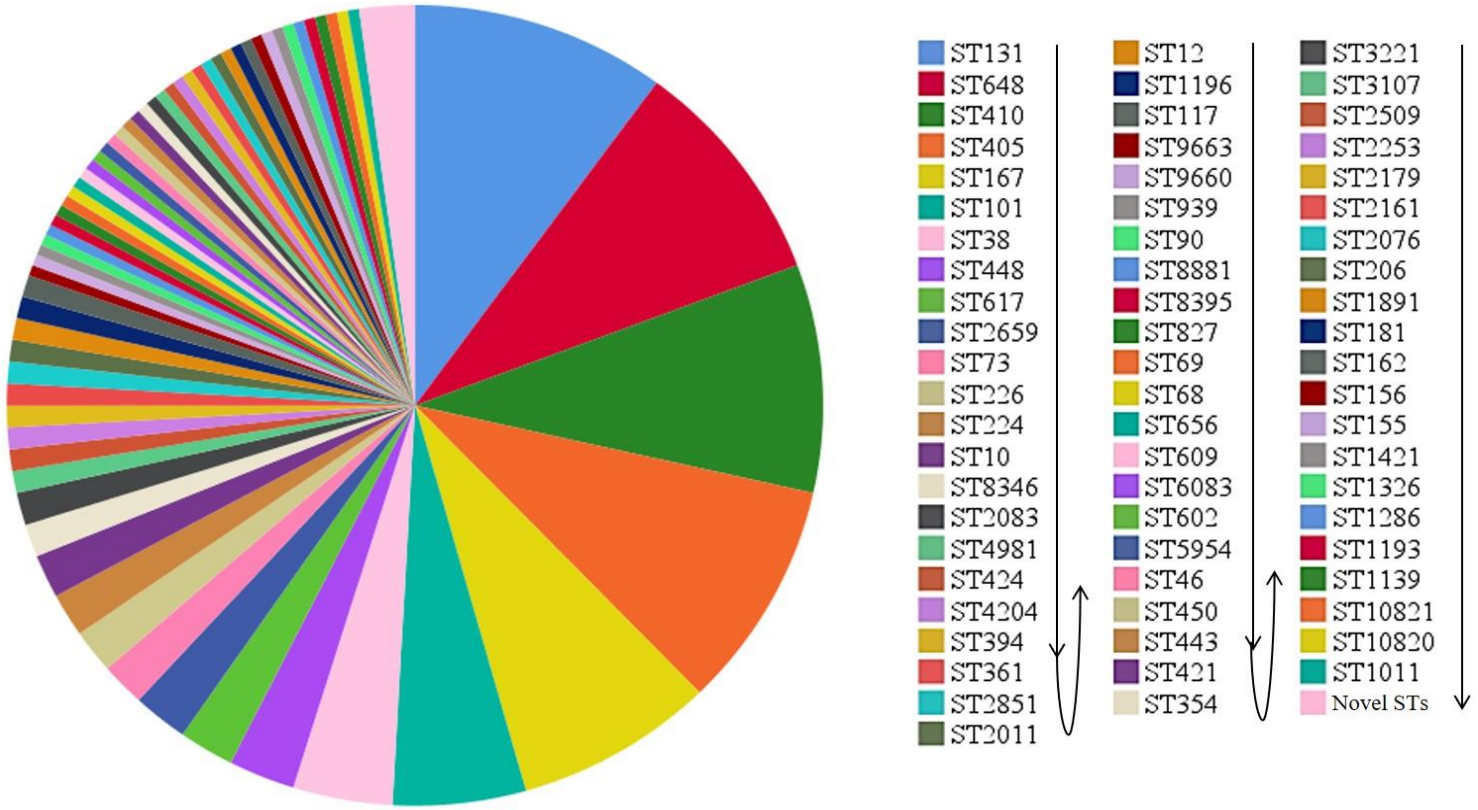


Figure 5.2 The distribution of clinical *E. coli* among different clonal types. Arrow directs from high to low frequency.

Table 5.1 Distribution of clinical carbapenem-resistant *E. coli* among major clonal types compared to carbapenem-sensitive *E. coli*.

	CRE (n=54)	**CSE (n=174)**	***p* value**
ST131	2 (3.7)	21 (12.1)	0.075
ST405	9 (16.7)	12 (6.9)	0.030
ST410	3 (5.6)	18 (10.3)	0.288
ST648	9 (16.7)	12 (6.9)	0.030
ST167	11 (20.4)	7 (4.0)	0.0001
ST101	4 (7.4)	8 (4.6)	0.419
ST38	2 (3.7)	7 (4.0)	0.916
ST448	5 (9.3)	1 (0.6)	0.0004
ST2659	2 (3.7)	3 (1.7)	0.386
ST617	1 (1.9)	4 (2.3)	0.845
ST224	0 (0)	4 (2.3)	0.261
ST226	0 (0)	4 (2.3)	0.261
ST8346	3 (5.6)	0 (0)	0.002

Values in parentheses indicate column percentage. Cells are highlighted whether any ST shows significant association with CRE.

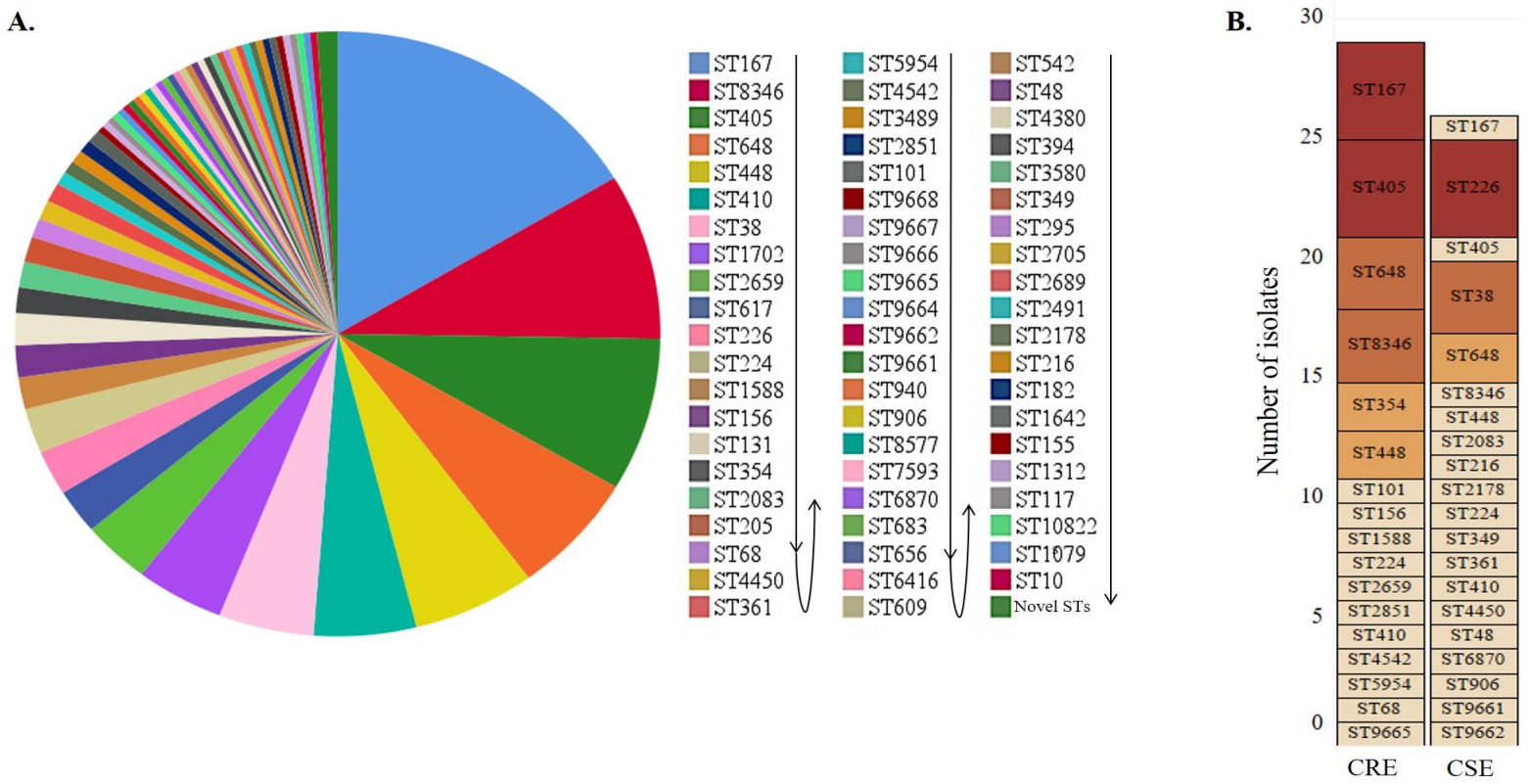


Figure 5.3 A. The distribution of faecal *E. coli* among different clonal types. Arrow directs from high to low frequency. **B.** The distribution of faecal *E. coli* among different clonal types isolated from patients attended to OPD (heatmap is generated based on frequency of isolation). The *E. coli* from RSs were screened in vancomycin (10 mg/l), and ertapenem (2 mg/l) containing media.

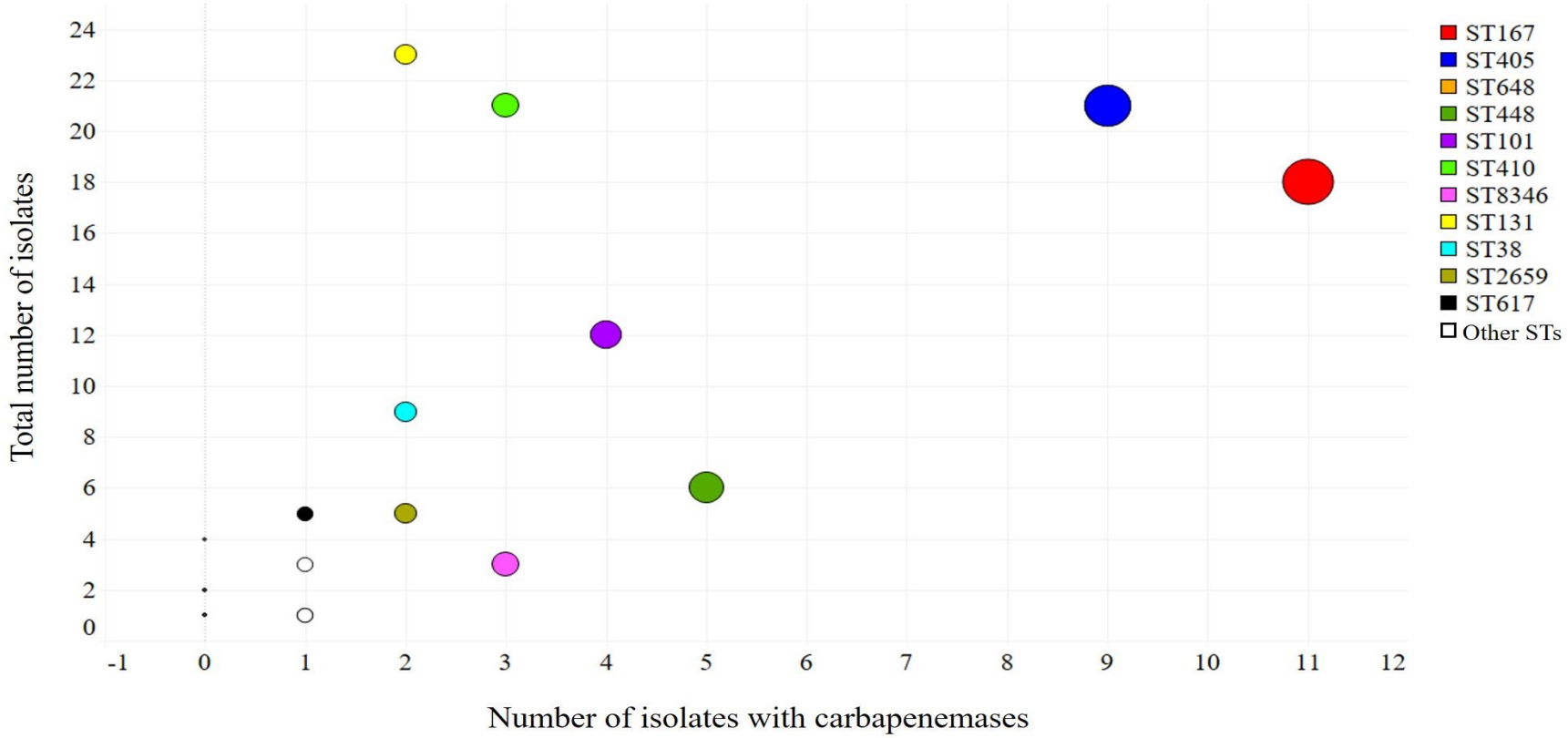


Figure 5.4 Bubble plot representing the relation between the prevalence of different *E. coli* clonal types and the frequency of carbapenemase producers to pertinent clonal types. Pearson correlation coefficient (r) between two variables is 0.662. Only clinical *E. coli* were included in this analysis, but isolates belonged to unknown ST (n=5) were excluded. Size of bubble is proportional to the number of carbapenemase producers.

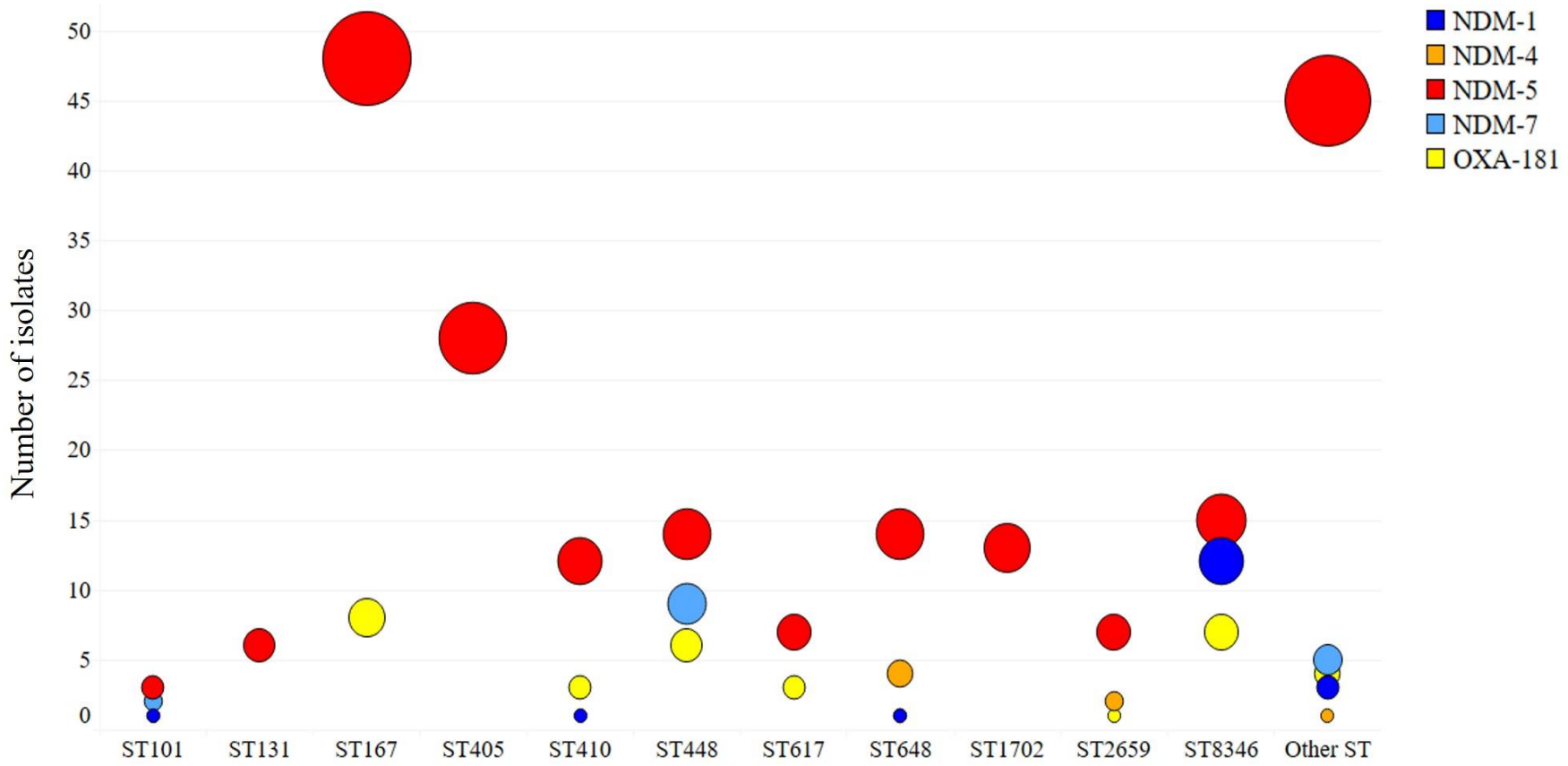


Figure 5.5 The distribution of major carbapenemase alleles among different clonal types of *E. coli*. Isolates were grouped into other STs whether any ST contained ≤5 carbapenemase-positive isolates. Size of bubble is proportional to the number of isolates. Both clinical and faecal *E. coli* were included in this analysis.

Table 5.2 Comparative analysis of distribution of carbapenemase alleles among different STs of *E. coli* (n=521).

	NDM-1 (n=18)	**NDM-4 (n=7)**	**NDM-5 (n=212)**	**NDM-7 (n=15)**	**OXA-181 (n=34)**
ST167 (n=66)	0 (0)	0 (0)	48 (72.7)	0 (0)	8 (12.1)
	p=0.100	*p*=0.310	*p*<0·0001	*P*=0·134	*p*=0.049
ST405 (n=45)	0 (0)	0 (0)	28 (62.2)	0 (0)	0 (0)
	p=0.184	*P*=0·413	*p*=0.002	*p*=0.227	*p*=0.064
ST648 (n=40)	1 (2.5)	4 (10)	14 (35)	1 (2.5)	1 (2.5)
	p=0.731	*p*<0·0001	*P*=0·446	*P*=0·881	*P*=0·283
ST410 (n=36)	1 (2.8)	0 (0)	12 (33.3)	0 (0)	3 (8.3)
	p=0.818	*p*=0.468	*p*=0.352	*p*=0.284	*p*=0.649
ST8346 (n=29)	12 (41.4)	0 (0)	15 (51.7)	0 (0)	7 (24.1)
	p<0·0001	*p*=0.518	*p*=0.213	*p*=0.340	*p*<0·0001
ST131 (n=28)	0 (0)	0 (0)	6 (21.4)	0 (0)	0 (0)
	p=0.303	*p*=0.526	*p*=0.033	*p*=0.349	*p*=0.151
ST448 (n=24)	0 (0)	0 (0)	14 (58.3)	8 (33.3)	6 (25)
	p=0.343	*p*=0.558	*p*=0.072	*p*<0·0001	*p*<0·0001
ST2659 (n=15)	0 (0)	2 (13.3)	7 (46.7)	0 (0)	1 (6.7)
	p=0.457	*p*<0·0001	*p*=0.633	*p*=0.499	*p*=0.982
ST101 (n=14)	1 (7.1)	0 (0)	3 (21.4)	2 (14.3)	1 (7.1)
	p=0.444	*p*=0.658	*p*=0.137	*p*=0.010	*p*=0.925
ST1702 (n=13)	0 (0)	0 (0)	13 (100)	0 (0)	0 (0)
	p=0.490	*p*=0.670	*p*<0·0001	*p*=0.530	*p*=0.335
ST617 (n=12)	0 (0)	0 (0)	7 (58.3)	0 (0)	3 (25)
	p=0.507	*p*=0.683	*p*=0.208	*p*=0.546	*p*=0.009

Values in parentheses indicate row percentage. Cells are highlighted whether any carbapenemase allele shows statistical significance relation to respective ST.

5.2.1.2 Population structure of *K. pneumoniae*

Similar to the *E. coli* population, clusters (**H** to **J**) were recovered which comprised of isolates from different STs of *K. pneumoniae*, differed by ≤100 SNPs. These clonal complexes were: **H.** ST29 (n=7), and ST711 (n=1); **I.** ST16 (n=19), ST17 (n=3), and ST20 (n=2); and **J.** ST147 (n=21), ST1586 (n=1), and ST273 (n=1) (Figure 5.6); however, there was no pair of the isolates from different STs differed by ≤ 40 SNPs.

Clinical *K. pneumoniae* mostly distributed among ST23 (15·4%, 35/228), ST15 (12·7%, 29/228), ST231 (7·9%, 18/228), ST11 (7·5%, 17/228), ST152 (5·3%, 12/228), ST147 (4·8%, 11/228), ST395 (3·9%, 9/228), ST14 (3·9%, 9/228), ST16 (3·5%, 8/228), ST307 (3·1%, 7/228), ST515 (2·6%, 6/228), and others (28·2%, 67/228) (Figure 5.7); however, significant associations of carbapenem resistance were observed with ST16, ST231, ST11, ST515, and ST23 (p<0·05) (Table 5.3).

The clonal distribution of faecal *K. pneumoniae* more likely represented the frequency among the carbapenem resistant isolates as RSs were screened on media containing vancomycin (10 mg/L), and ertapenem (2 mg/l) (Figure 4.1). The majority of *K. pneumoniae* clones from faeces were ST15 (14·8%, 12/81) followed by ST16 (13·6%, 11/81), ST147 (12·3%, 10/81), ST48 (12·3%, 10/81), ST14 (7·4%, 6/81), ST29 (7·4%, 6/81), and others (31·6%, 26/81) (Figure 5.8). The faecal *K. pneumoniae* isolated from outpatients (representing the community prevalence), associated with carbapenem resistance distributed in a wide range of STs (Figure 5.8).

There were positive corelations between the prevalence of different *K. pneumoniae* clonal types and the frequency of carbapenemase producing *K. pneumoniae* to pertinent clones (r=0.963) (Figure 5.9). Faecal *K. pneumoniae* were excluded from the correlation analysis because of RSs screening with ertapenem (2 mg/l) selection (Figure 4.1). Carbapenemase alleles were distributed among the different STs of *K. pneumoniae* identified in this study. Significant associations were observed between ST15 and $bla_{\text{NDM-1}}$; ST15 and $bla_{\text{OXA-232}}$; ST23 and $bla_{\text{NDM-5}}$; ST231 and $bla_{\text{OXA-181}}$; ST231 and $bla_{\text{OXA-232}}$; ST231 and $bla_{\text{KPC-2}}$; ST147 and $bla_{\text{NDM-5}}$; ST16 and $bla_{\text{NDM-1}}$; ST16 and $bla_{\text{OXA-181}}$; ST11 and $bla_{\text{OXA-181}}$; ST14 and $bla_{\text{OXA-181}}$; ST14 and $bla_{\text{OXA-232}}$; ST48 and $bla_{\text{NDM-5}}$; ST395 and $bla_{\text{NDM-1}}$; ST29 and $bla_{\text{NDM-5}}$; ST515 and $bla_{\text{NDM-5}}$ (Figure 5.10, Table 5.4).

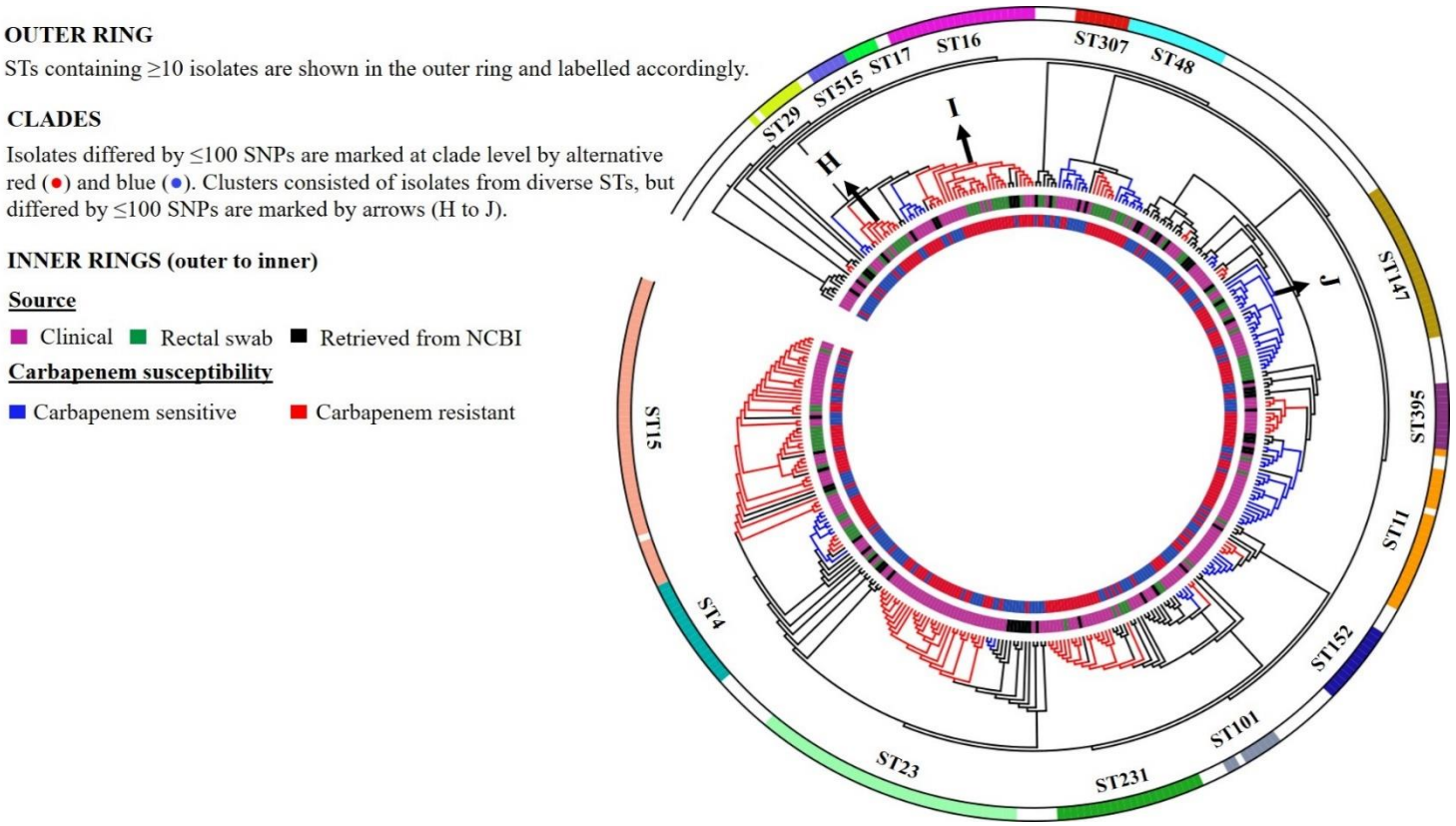


Figure 5.6 ML tree generated from core-genome analysis of *K. pneumoniae* isolated in this study. Core-genome alignment was performed using roary (v3.12.0). The ML tree from the core genome was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model and gamma correction with bootstrapping. SNPs calling was performed using Snippy (v4.4.5) followed by recombination removal using Gubbins (v2.3.4) and pair-wise SNPs calculation using pairsnp (v0.0.7). Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

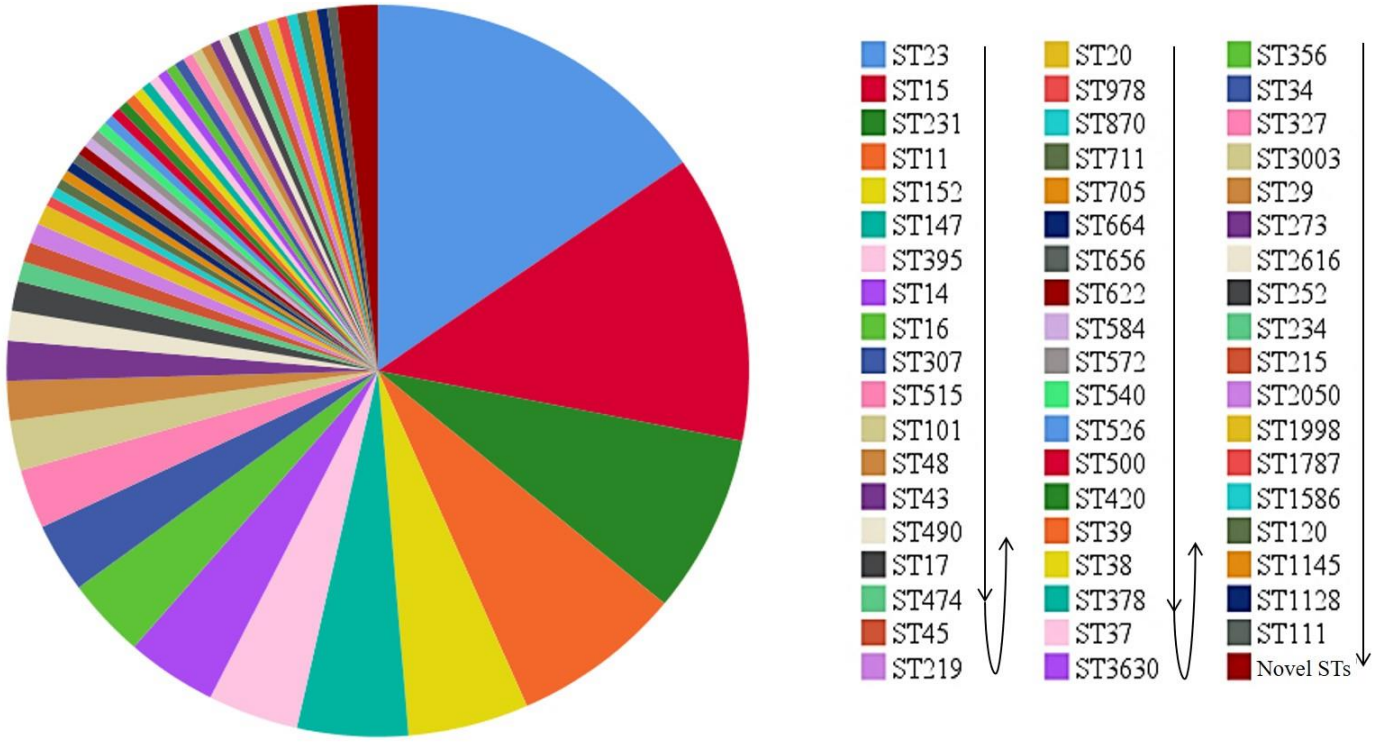


Figure 5.7 The distribution of clinical *K. pneumoniae* among different clonal types. Arrow directs from high to low frequency.

Table 5.3 Distribution of clinical carbapenem resistant *K. pneumoniae* among major clonal types compared to CSE.

	CRE (n=120)	**CSE (n=108)**	***p* value**
ST23	24 (20)	11 (10·2)	0·04
ST15	12 (10)	17 (15·7)	0·194
ST231	15 (12·5)	3 (2·8)	0·007
ST11	14 (11·7)	3 (2·8)	0·011
ST152	6 (5)	6 (5·6)	0·851
ST147	7 (5·8)	4 (3·7)	0·454
ST14	6 (5)	3 (2·8)	0·39
ST395	6 (5)	3 (2·8)	0·39
ST16	8 (6·7)	0 (0)	0·006
ST307	2 (1·7)	5 (4·6)	0·195
ST515	6 (5)	0 (0)	0·019
ST101	0 (0)	6 (5·6)	0·009
ST43	4 (3·3)	0 (0)	0·056
ST48	3 (2·5)	1 (0·9)	0·366

Values in parentheses indicate column percentage. Cells are highlighted whether any ST shows significant association with CRE.

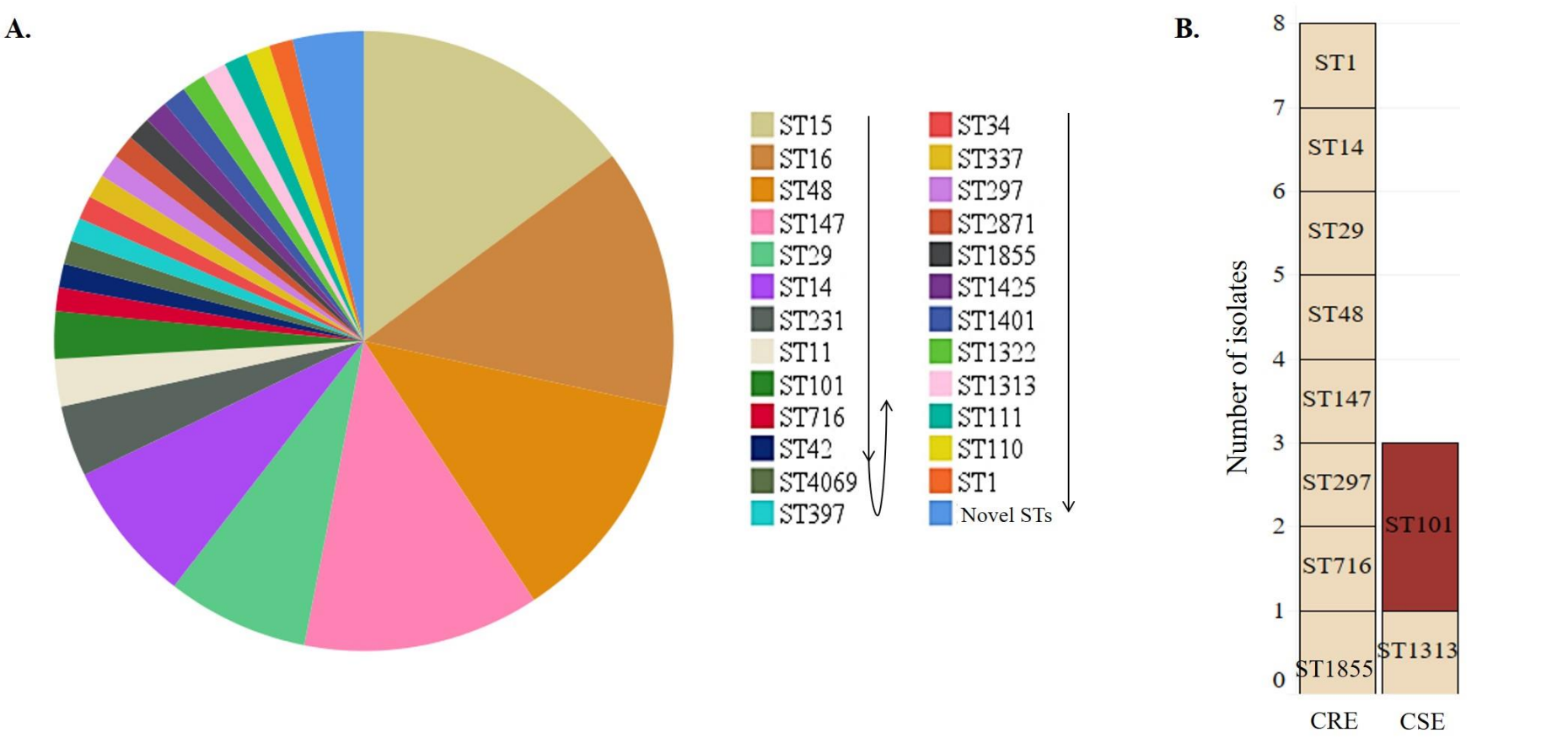


Figure 5.8 A. The distribution of faecal *K. pneumoniae* among different clonal types. Arrow directs from high to low frequency. **B.** The distribution of faecal *K. pneumoniae* among different clonal types isolated from patients attended in OPD (heatmap is generated based on frequency of isolation). The *K. pneumoniae* from RSs were screened in vancomycin (10 mg/l), and ertapenem (2 mg/l) containing media.

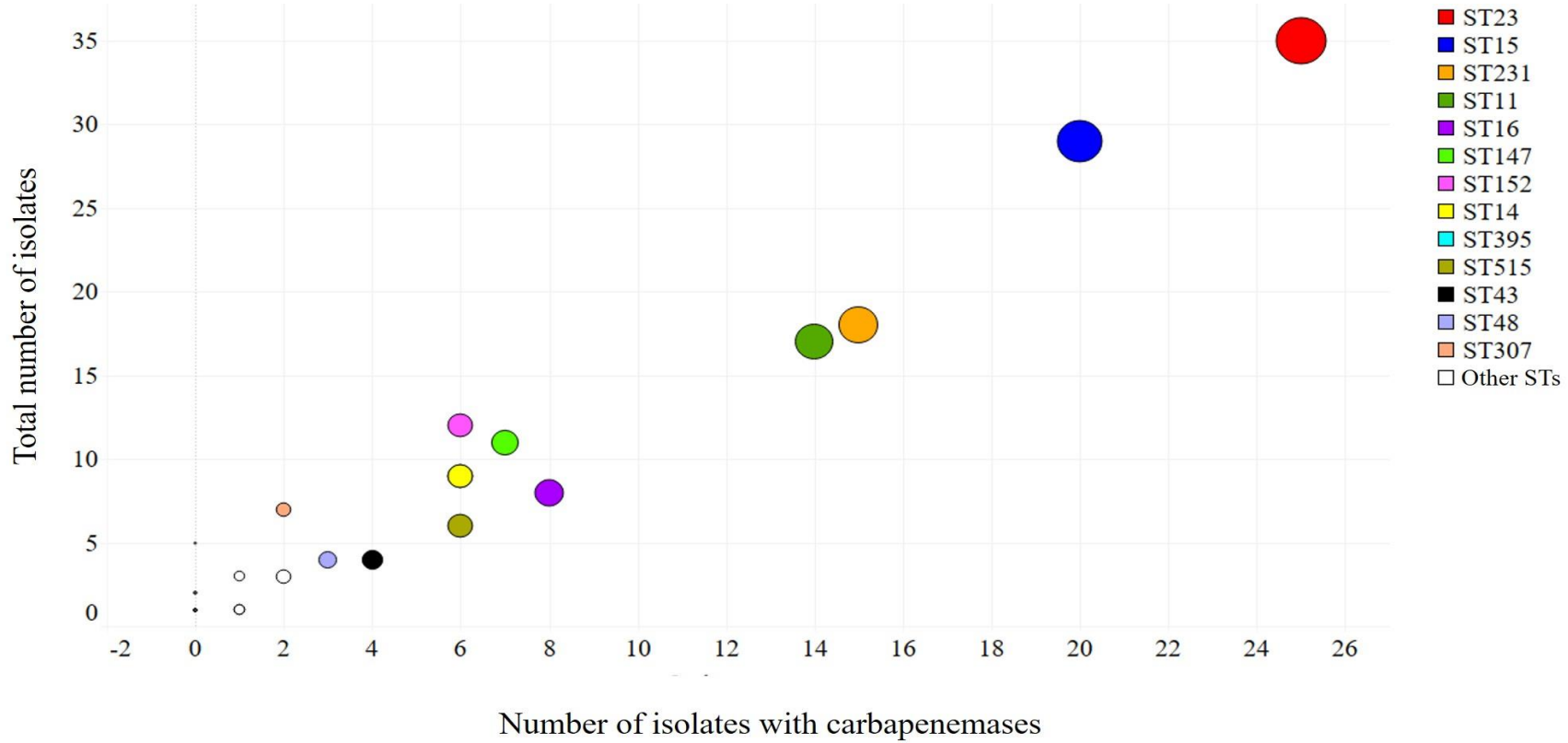


Figure 5.9 Bubble plot representing the relation between the prevalence of different *K. pneumoniae* clonal types of and the frequency of carbapenemase producers to pertinent clonal types. Pearson correlation coefficient (r) between two variables is 0.963. Only clinical *K. pneumoniae* were included in this analysis, but isolates belonged to unknown ST (n=4) were excluded. Size of bubble is proportional to the number of carbapenemase producers.

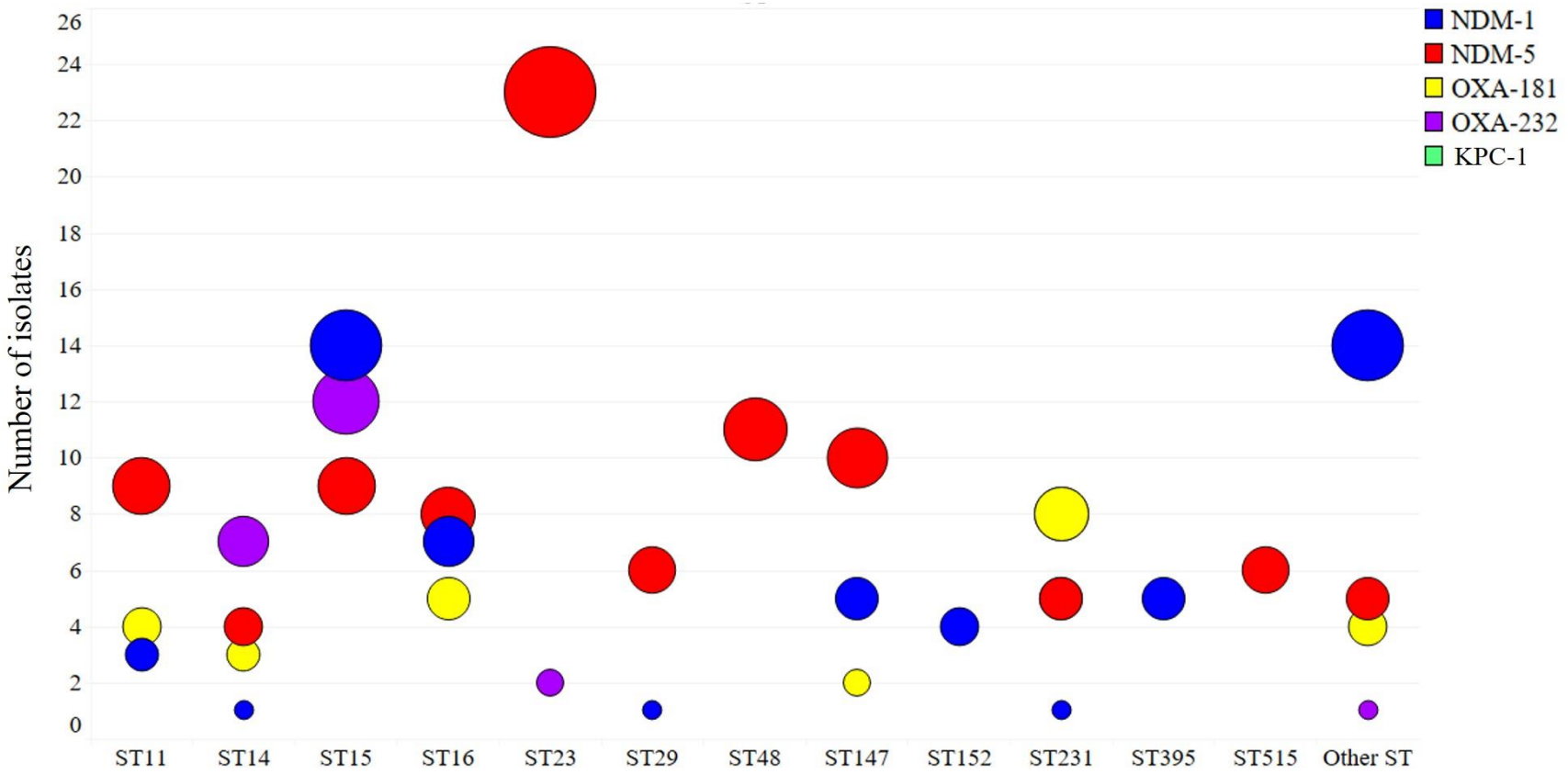


Figure 5.10 The distribution of major carbapenemase alleles among different clonal types of *K. pneumoniae*. Isolates were grouped into other STs whether any ST contained ≤5 carbapenemase-positive isolates. Size of bubble is proportional to the number of isolates. Both clinical and faecal *K. pneumoniae* were included in this analysis.

Table 5.4 Comparative analysis of distribution of carbapenemase alleles among different STs of *K. pneumoniae* (n=309).

	NDM-1 (n=55)	**NDM-5 (n=96)**	**OXA-181 (n=26)**	**OXA-232 (n=30)**	**KPC-2 (n=5)**
ST15	14 (34.1)	9 (9.4)	0 (0)	12 (29.3)	0 (0)
(n=41)	*p*=0.003	*p*=0.176	*p*=0.037	*p*<0·0001	*p*=0.378
ST23	0 (0)	23 (65.7)	0 (0)	2 (5.7)	0 (0)
(n=35)	*p*=0.003	*p*<0·0001	*p*=0.050	*p*=0.397	*p*=0.420
ST231	1 (4.8)	5 (23.8)	8 (38.1)	8 (38.1)	5 (23.8)
(n=21)	*p*=0.106	*p*=0.457	*p*<0·0001	*p*<0·0001	*p*<0·0001
ST147	5 (23.8)	10 (47.6)	2 (9.5)	0 (0)	0 (0)
(n=21)	*p*=0.456	*p*=0.090	*p*=0.850	*p*=0.120	*p*=0.543
ST16	7 (36.8)	8 (42.1)	5 (26.3)	0 (0)	0 (0)
(n=19)	*p*=0.025	*p*=0.283	*p*=0.004	*p*=0.140	*p*=0.564
ST11	3 (15.8)	9 (47.4)	4 (21.1)	0 (0)	0 (0)
(n=19)	*p*=0.813	*p*=0.113	*p*=0.041	*p*=0.140	*p*=0.564
ST14	1 (6.7)	4 (26.7)	3 (20)	7 (46.7)	0 (0)
(n=15)	*p*=0.248	*p*=0.706	*p*=0.097	*p*<0·0001	*p*=0.611
ST48	0 (0)	11 (78.6)	0 (0)	0 (0)	0 (0)
(n=14)	*p*=0.075	*p*<0·0001	*p*=0.246	*p*=0.209	*p*=0.623
ST152	4 (33.3)	0 (0)	0 (0)	0 (0)	0 (0)
(n=12)	*p*=0.151	*p*=0.018	*p*=0.284	*p*=0.247	*p*=0.650
ST395	5 (55.6)	0 (0)	0 (0)	0 (0)	0 (0)
(n=9)	*p*=0.003	*p*=0.041	*p*=0.356	*p*=0.318	*p*=0.696
ST29	1 (14.3)	6 (85.7)	0 (0)	0 (0)	0 (0)
(n=7)	*p*=0.806	*p*=0.002	*p*=0.417	*p*=0.380	*p*=0.731
ST515	0 (0)	6 (100)	0 (0)	0 (0)	0 (0)
(n=6)	*p*=0.250	*p*<0·0001	*p*=0.453	*p*=0.417	*p*=0.751

Values in parentheses indicate row percentage. Cells are highlighted whether any carbapenemase allele shows statistical significance relation to respective ST.

5.2.1.3 Population structure of other species of Enterobacterales

Core-genome analysis of *Proteus* spp. (n=66) divided the isolates into 15 clades, designated as A-J, and 5 more clades consisted of single isolate. One strain of *Proteus* sp. (due to low quality sequence data) was not included in the analysis. Clade D entailed with the isolates of *P. mirabilis* which was significantly associated with carbapenem resistance (71.4%, 5/7) compared to having CSE (3.4%, 2/59) (p<0.05). No *Proteus* spp. were identified from faeces (Figure 5.11)

Enterobacter spp. (n=55) isolated in this study were distributed into 26 known STs and 12 isolates of unknown STs according to *E. cloacae* MLST scheme. The most frequent ST was ST113 (n=11), consisted of both clinical (n=6), and faecal (n=5). There was significant association between ST113 and CRE (40%, 10/25) than CSE (3.3%, 1/30) (p<0.05). We did not find any other STs which comprised of more than three isolates (Figure 5.12).

Core-genome analysis of *Citrobacter* spp. (n=30) split up six clades (A-F), of which clade E consisted of more CRE (66.7%, 8/12) than CSE (27.8%, 5/18) (p<0.05). Seven clinical isolates and six faecal isolates belonged to clade E (Figure 5.13).

Core-genome analysis of *Providencia* spp. (n=23) split up five clades (A-E), of which clade C contained significantly more CRE (71.4%, 5/7) than CSE (6.3%, 1/16) (p<0.05). No *Providencia* spp. was identified from faeces (Figure 5.14).

S. marcescens (n=14), and *M. morganii* (n=12) isolated in this study were also evaluated. Only two of *S. marcescens* were carbapenem resistant and isolates differed by 35,070 SNPs. No clinical *M. morganii* was found to be resistant to carbapenem. An outbreak of *K. variicola* at NICU of DMCH was identified which has been described in Chapter 7.

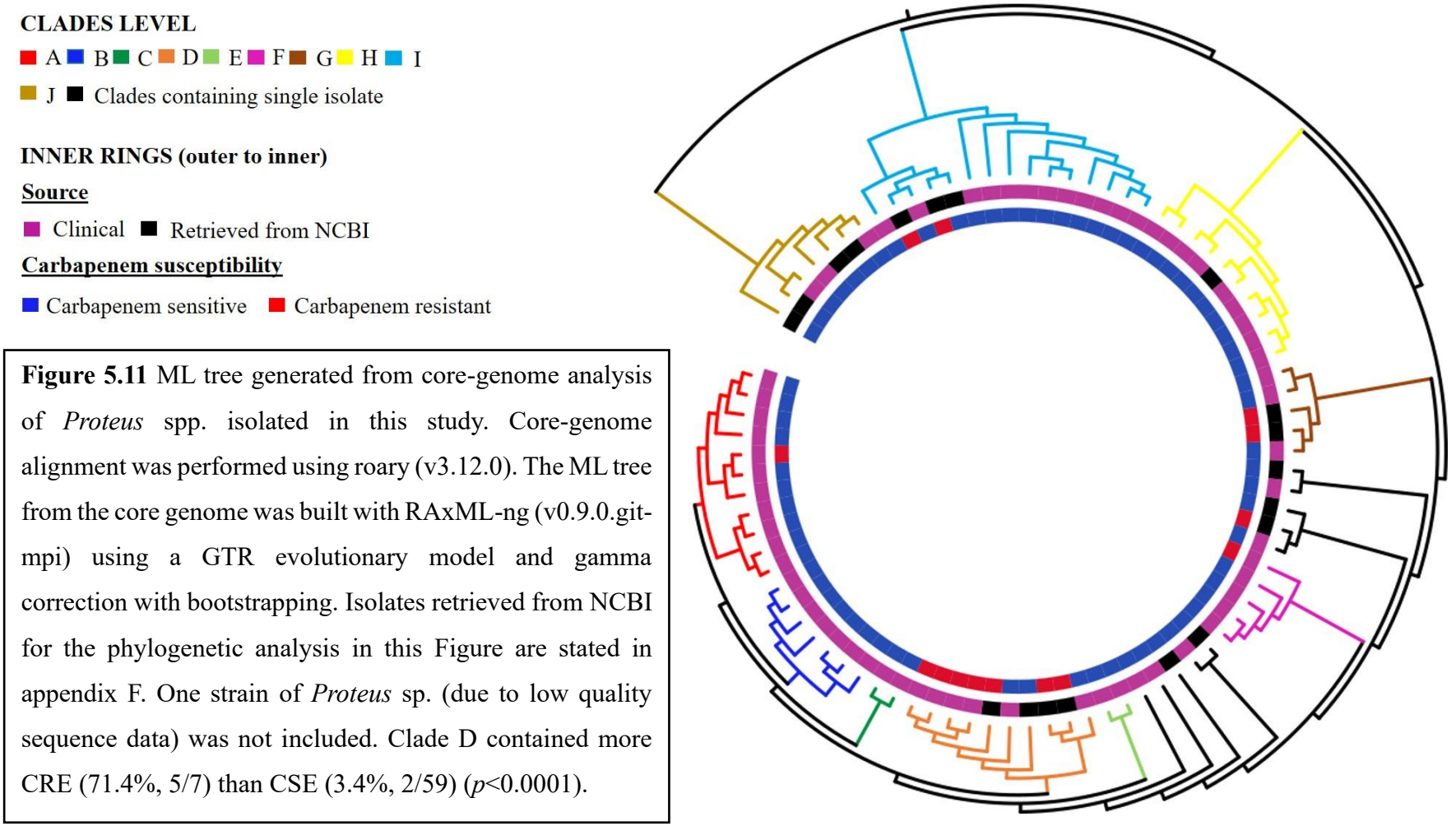


Figure 5.11 ML tree generated from core-genome analysis of *Proteus* spp. isolated in this study. Core-genome alignment was performed using roary (v3.12.0). The ML tree from the core genome was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model and gamma correction with bootstrapping. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F. One strain of *Proteus* sp. (due to low quality sequence data) was not included. Clade D contained more CRE (71.4%, 5/7) than CSE (3.4%, 2/59) (p<0.0001).

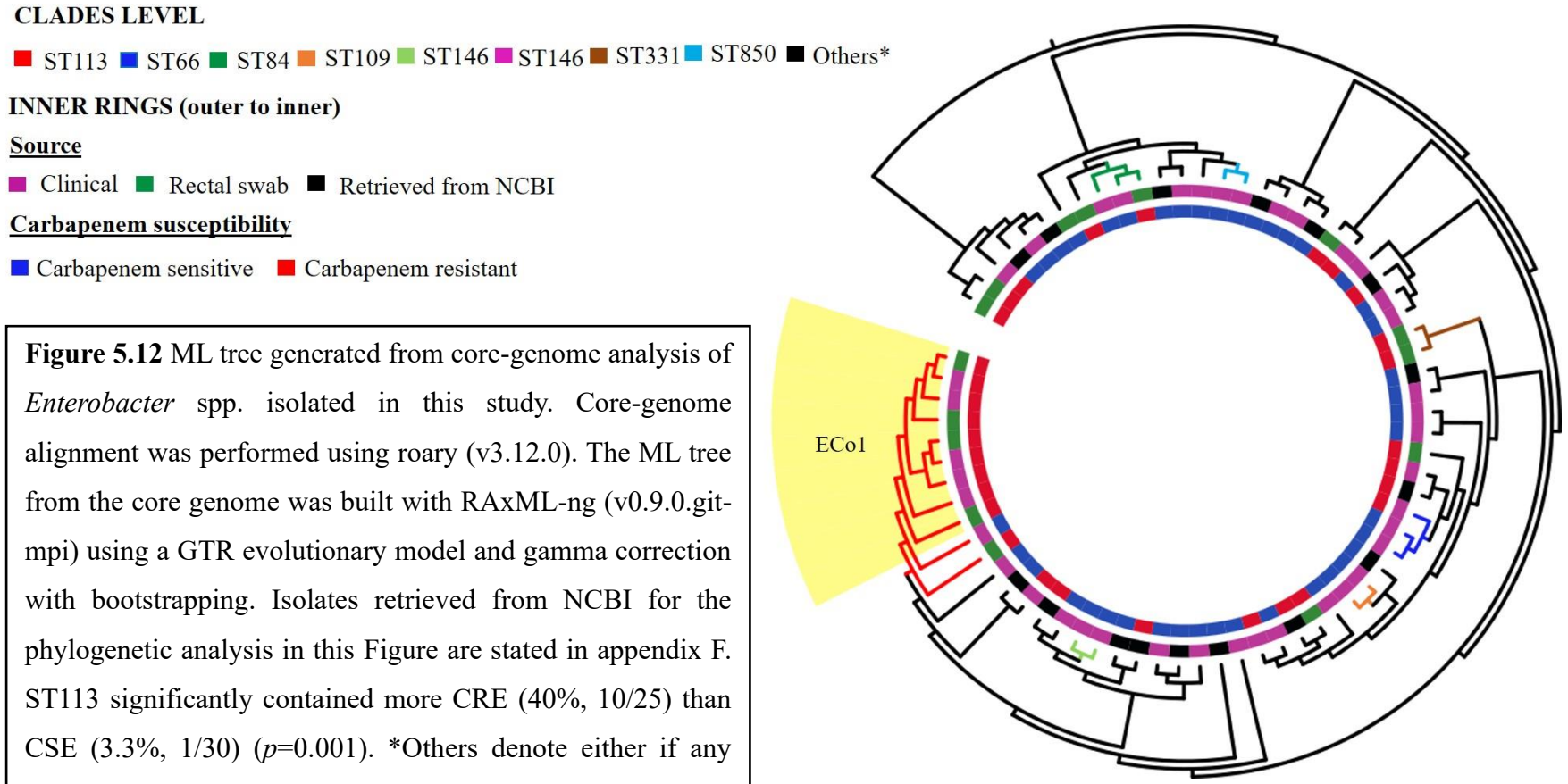


Figure 5.12 ML tree generated from core-genome analysis of *Enterobacter* spp. isolated in this study. Core-genome alignment was performed using roary (v3.12.0). The ML tree from the core genome was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model and gamma correction with bootstrapping. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F. ST113 significantly contained more CRE (40%, 10/25) than CSE (3.3%, 1/30) (p=0.001). *Others denote either if any isolate belonged to novel STs or any ST contained only one isolate. Putative outbreak cluster (Eco1) is highlighted by yellow (●).

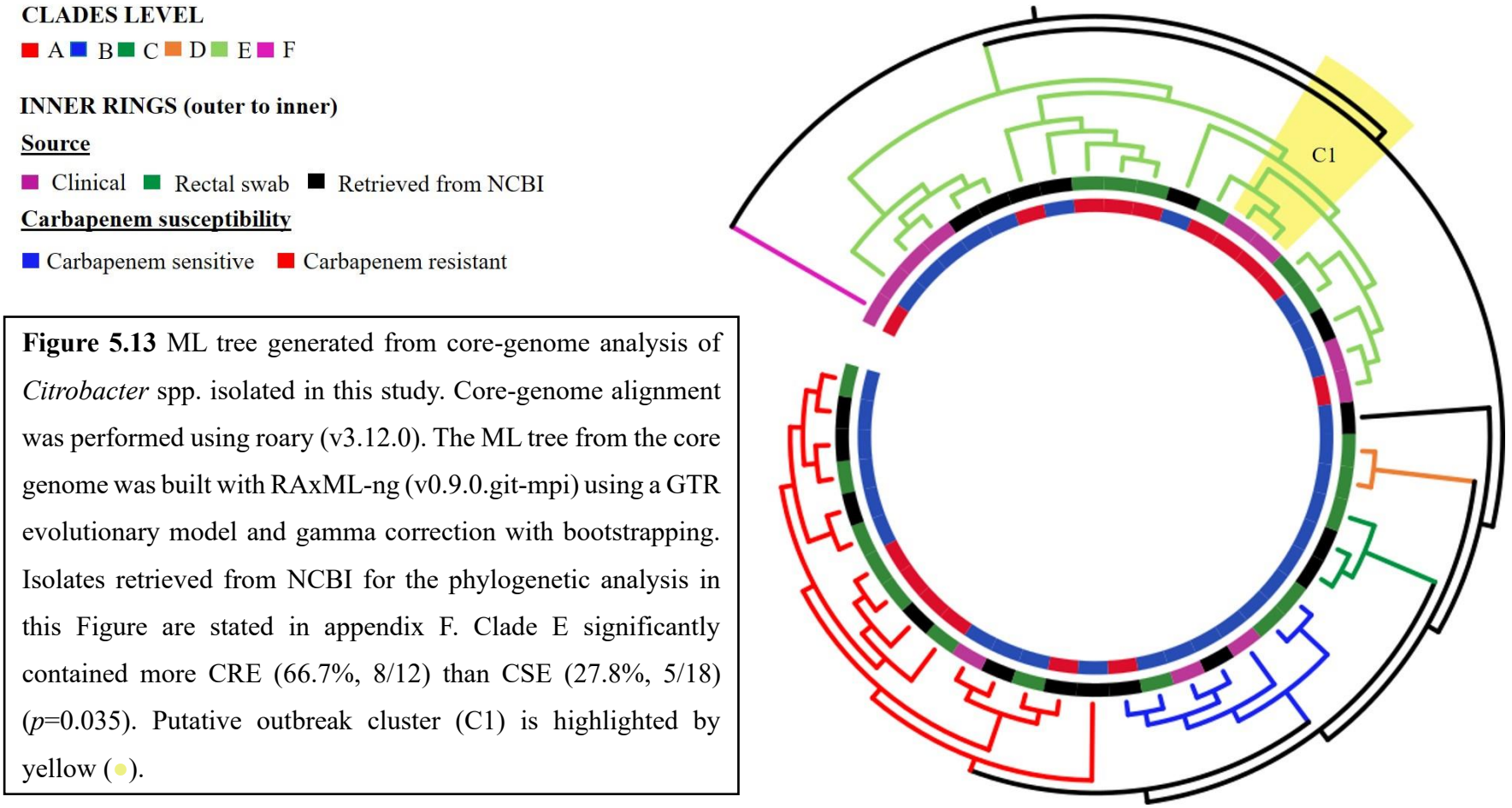


Figure 5.13 ML tree generated from core-genome analysis of *Citrobacter* spp. isolated in this study. Core-genome alignment was performed using roary (v3.12.0). The ML tree from the core genome was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model and gamma correction with bootstrapping. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F. Clade E significantly contained more CRE (66.7%, 8/12) than CSE (27.8%, 5/18) (p=0.035). Putative outbreak cluster (C1) is highlighted by yellow (●).

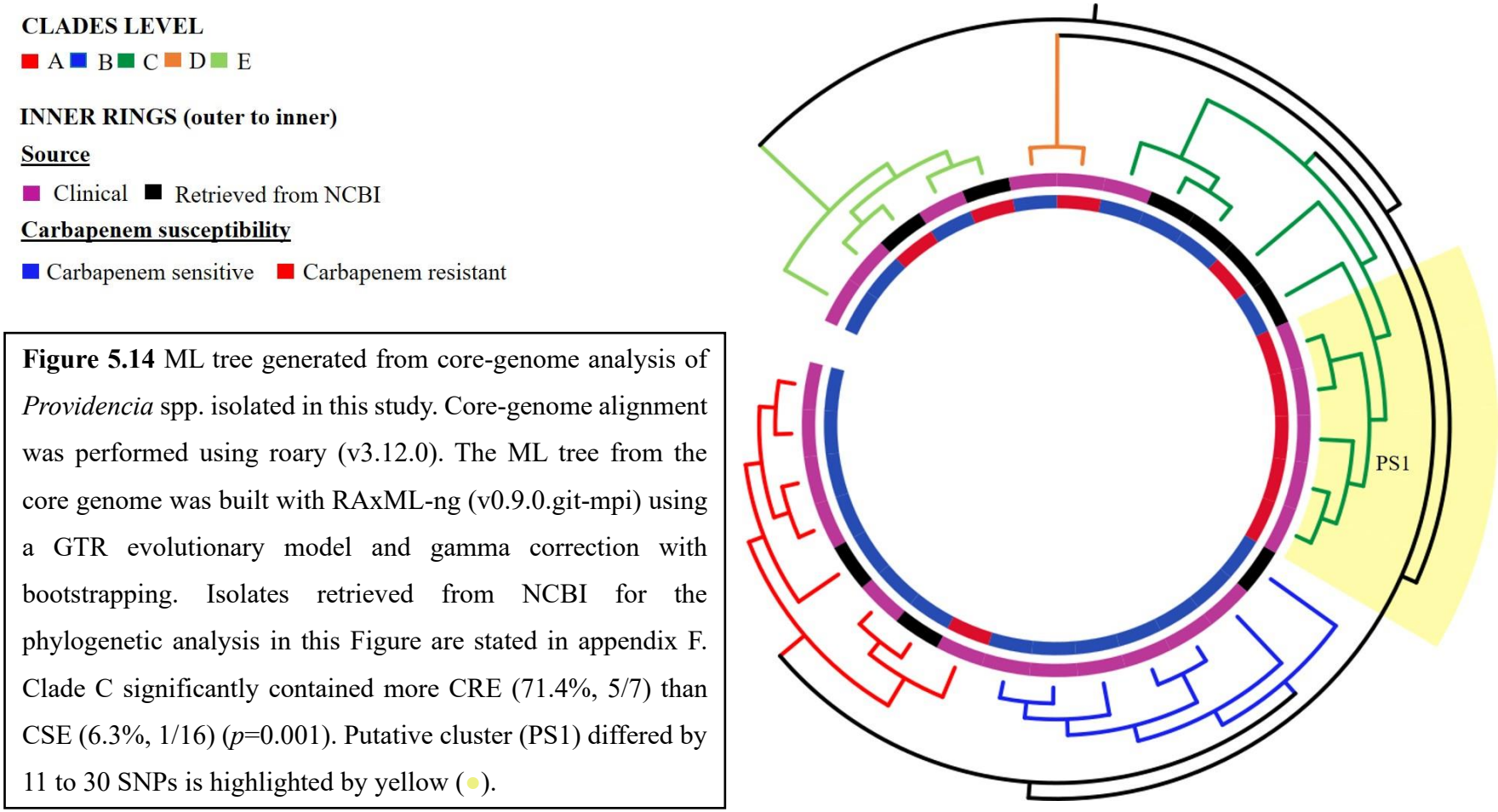


Figure 5.14 ML tree generated from core-genome analysis of *Providencia* spp. isolated in this study. Core-genome alignment was performed using roary (v3.12.0). The ML tree from the core genome was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model and gamma correction with bootstrapping. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F. Clade C significantly contained more CRE (71.4%, 5/7) than CSE (6.3%, 1/16) (p=0.001). Putative cluster (PS1) differed by 11 to 30 SNPs is highlighted by yellow (●).

5.2.2 Investigating possible transmissibility in putative outbreak clusters associated with carbapenem resistance

We used combination of genomic approach and epidemiological data to identify putative transmission clusters. Cut-offs zero to 20 SNPs were set out to screen possible transmission (Snitkin *et al.*, 2012; De Maio *et al.*, 2016; Moradigaravand *et al.*, 2016; Moradigaravand *et al.*, 2017; Marsh *et al.*, 2019). In this study, the clusters of interest were the clusters comprised of at least one clinical isolate, and at least one CRE.

We found 10 putative outbreak clusters of *E. coli*, of which two clusters (EC1: 2 to 10 SNPs differences, and EC2: one SNP difference) belonged to ST167, one cluster to ST448 (EC3: 1 to 2 SNPs differences), one cluster (EC4: 1 to 18 SNPs differences [clinical and faecal isolates by 20 SNPs]) to ST8346, two clusters (EC5: 3 to 9 SNPs differences, and EC6: 4 to 8 SNPs differences [clinical and faecal isolates differed by 8]) to ST405, one in ST5954 (EC7: 3 to 12 SNPs differences [clinical and faecal isolates differed by 12]), and three clusters (EC8: 18 SNPs differences, EC9: 0 to 10 SNPs differences, and EC10: 6 SNPs differences) to ST648. Four of the clusters of *E. coli* contained closely related isolates from both clinical and faecal specimens (Figure 5.15). The largest cluster of *E. coli* was EC9, consisting of seven clinical isolates (between 18th Feb 2017 to 8th May 2017). Epidemiological assessment showed overlapping of patients' hospital stay correlating with clustering of related isolates and that the possible transmission was not confined to a single ward (Figure 5.16). Similarities of carbapenemases were among the isolates in EC1, EC2, EC3, EC4, EC6, EC7, EC9 and EC10. However, EC8 and EC9 consisted of isolates with varied carbapenem resistance patterns. E4, EC5, and EC9 contained some isolates without any carbapenemase gene (Figure 5.17-Figure 5.21).

Fourteen putative outbreak clusters were identified in *K. pneumoniae*, of which three clusters were found in ST15 (KP1: 1 to 7 SNPs differences [faecal and clinical isolates differed by 3 SNPs], KP2: 1 to 2 SNPs differences, and, KP3: 8 SNPs difference), one in ST14 (KP4: 3 SNPs differences), one in ST23 (KP5: 0 to 18 SNPs differences), two in ST231 (KP6: 14 SNPs, and KP7: 16 to 20 SNPs differences), two in ST16 (KP8: 10 to 20 SNPs differences [clinical and faecal isolates differed by 10], and KP9: 1 to 4 SNPs differences), one in ST515 (KP10: 4 to 13 SNPs differences),

one in ST11 (KP11: 9 to 14 SNPs differences), one in ST395 (KP12: 2 to 18 SNPs differences), one in ST147 (KP13: 16 SNPs differences), and one in ST152 (KP14: 2 to 13 SNPs differences) (Figure 5.22). The largest cluster in this study (KP5) was found in ST23 which was composed of 30 clinical isolates (between 31st Oct 2016 to 17th Aug 2017) (Figure 5.22; Figure 5.23). We recovered two more sizable clusters of 17 (KP1) and 8 (KP8) isolates in *K. pneumoniae* ST15 (between 16th Dec 2016 to 2018) and ST16 (between 18th Mar 2017 to 2018), comprising both clinical and faecal isolates (Figure 5.24; Figure 5.25). None of the large cluster in *K. pneumoniae* was ward specific; however, there were linkages between the patients' hospital stay and recovery of the isolates. Communalities of carbapenem resistance variants were found in the clusters of *K. pneumoniae*; however, KP8, KP9, KP12, and KP14 also contained isolates with diverse NDM variants, and KP1, KP5, and KP14 possessed isolates without any carbapenemase genes (Figure 5.26). Interestingly, isolates belonged to KP1 (ST15) harboured carbapenemases three variations (only *bla*$_{\text{NDM-1}}$, only *bla*$_{\text{OXA-181}}$, and combination of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-181}}$) (Figure 5.24).

A cluster consisted of nine isolates from both clinical infections and faeces were retrieved in *E. cloacae*, belonged to ST113 (Eco1: 1 to 7 SNPs differences [clinical and faecal isolates differed by 6]) (between 16th Nov 2016 to 15th July 2017). Interestingly, two of isolates in this cluster were identified from faeces of patients attending the outpatient department (OPD) along with the remaining seven strains from different wards at DMCH (Figure 5.12; Figure 5.25). Eight of the isolates in the clusters harboured *bla*$_{\text{NDM-5}}$, and one carried a novel NDM variant. Two small clusters consisted of two and three clinical isolates of *C. rodentium* and (C1: 5 SNPs differences) and *P. stuartii* (PS1: 11 to 12 SNPs differences), respectively were also found (Figure 5.13; Figure 5.14).

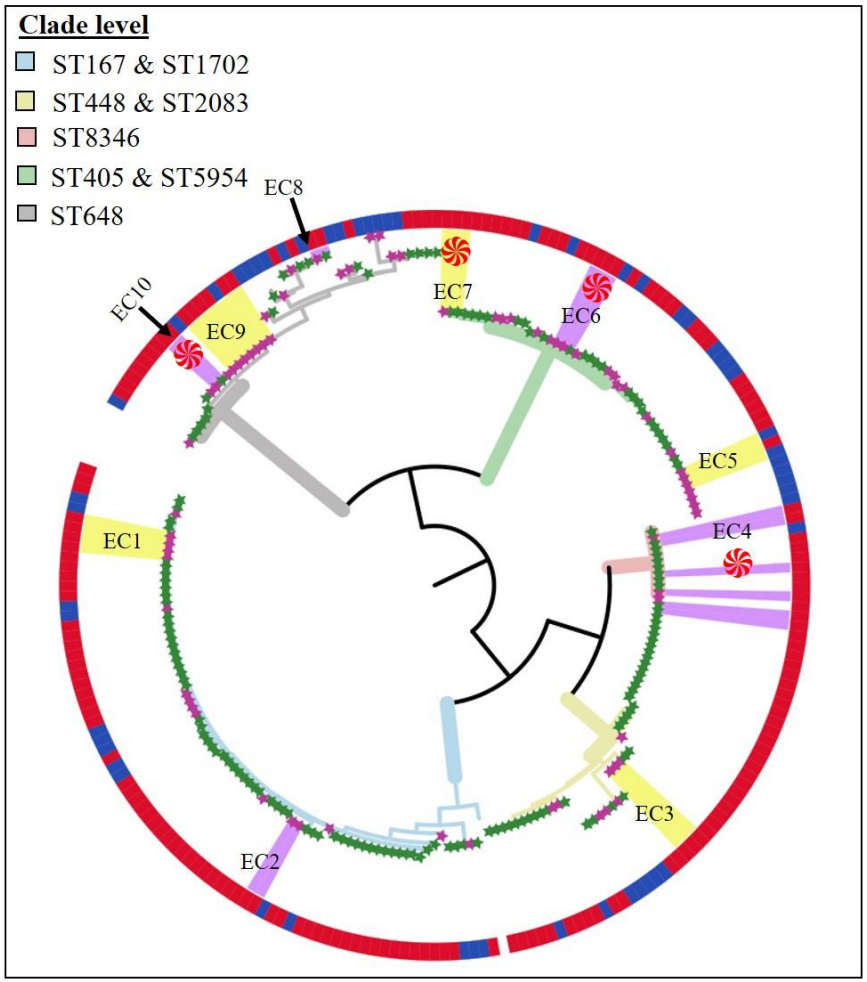


Figure 5.15 Core-genome SNP tree of isolates belonged to major clones of *E. coli* associated with carbapenem resistance and screening of outbreaks in *E. coli*. SNPs calling followed by alignment of core SNPs was performed using Snippy (v4.4.5). The ML tree was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model, and gamma correction with bootstrapping. Recombination removal using Gubbins (v2.3.4), and pair-wise SNPs calculation using pairsnp (v0.0.7). Outbreaks were suspected if the isolates in a cluster differed by ≤20 SNPs. Putative outbreak clusters are highlighted by alternative purple (●) and yellow (○) and designated as EC1 to EC10. STs of respective isolates have been labelled at clade level. Branch symbols, outer ring in the phylogenetic tree denote source of isolation, and carbapenem susceptibility of respective isolates, respectively. Pink (■) indicates clinical isolates, and green (■) indicates faecal isolates. Blue (■) denotes carbapenem sensitive, and red (■) denotes carbapenem resistant. Clusters consisted of both clinical, and faecal isolates are marked by red symbol (🍥).

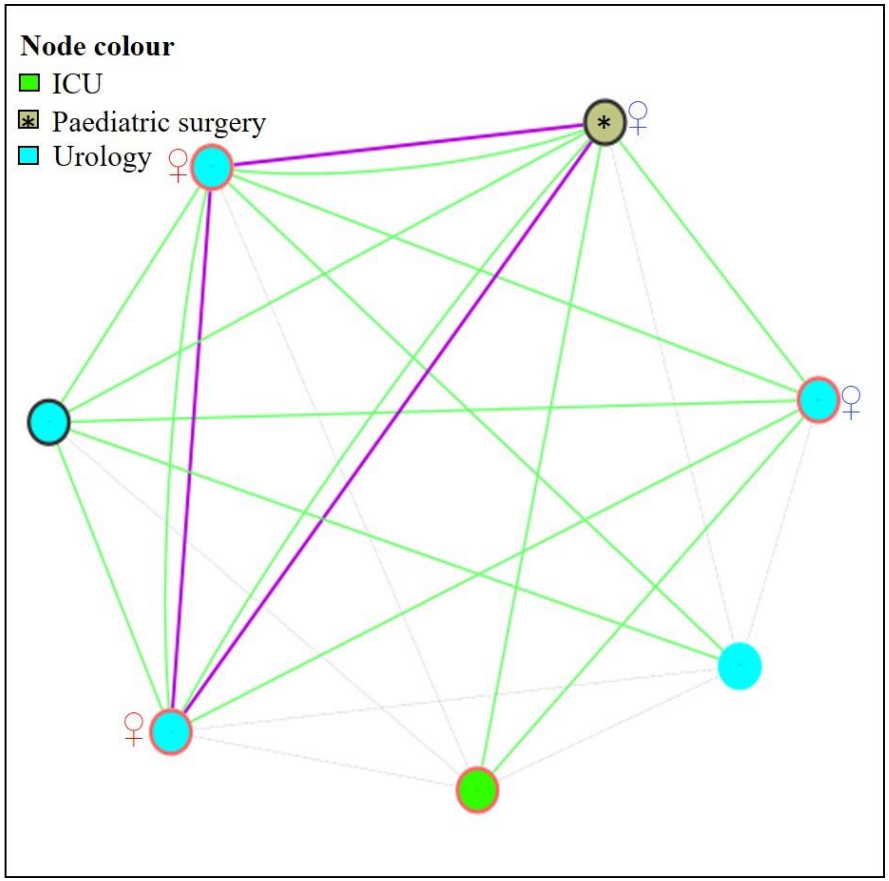


Figure 5.16 Network map generated with the isolates of a putative outbreak cluster of *E. coli* ST648. Isolates are connected where they differed by ≤20 SNPs. Round node shape denotes clinical isolates. Node border denotes presence of carbapenemase alleles, and nodes without border indicate absence of carbapenemase allele. Red border represents presence of $bla_{\text{NDM-4}}$, and black border represents novel bla_{NDM}. Wards of isolation are represented by different node colour. Isolates differed by ≤10 SNPs are connected by green (–) edges and differed by 11 to 20 SNPs by grey (–) edges. Edges are coloured by purple (–) where there was overlapping of patients' hospital stay pertinent to the isolates differed by ≤20 SNPs. Isolates are marked by symbol (♀) where they differed by 0 to 2 SNPs (pairing of isolates by fewer SNPs are represented by individual symbol colour).

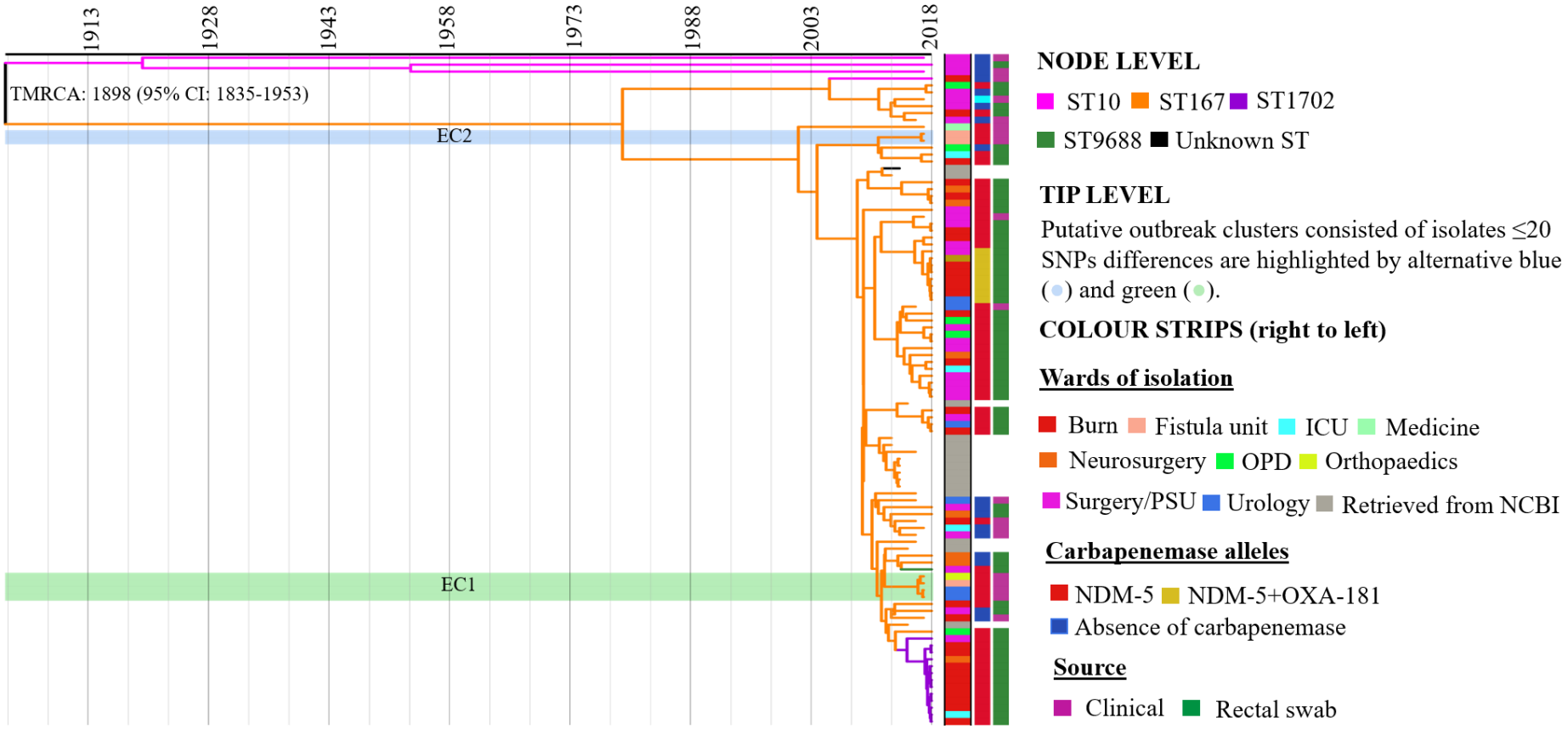


Figure 5.17 Time calibrated phylogenetic tree generated from of *E. coli* genomes belonged to ST167 along with closely related isolates from other STs (ST10, ST1702, and novel allele) (n=97). PSU, paediatric surgery. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

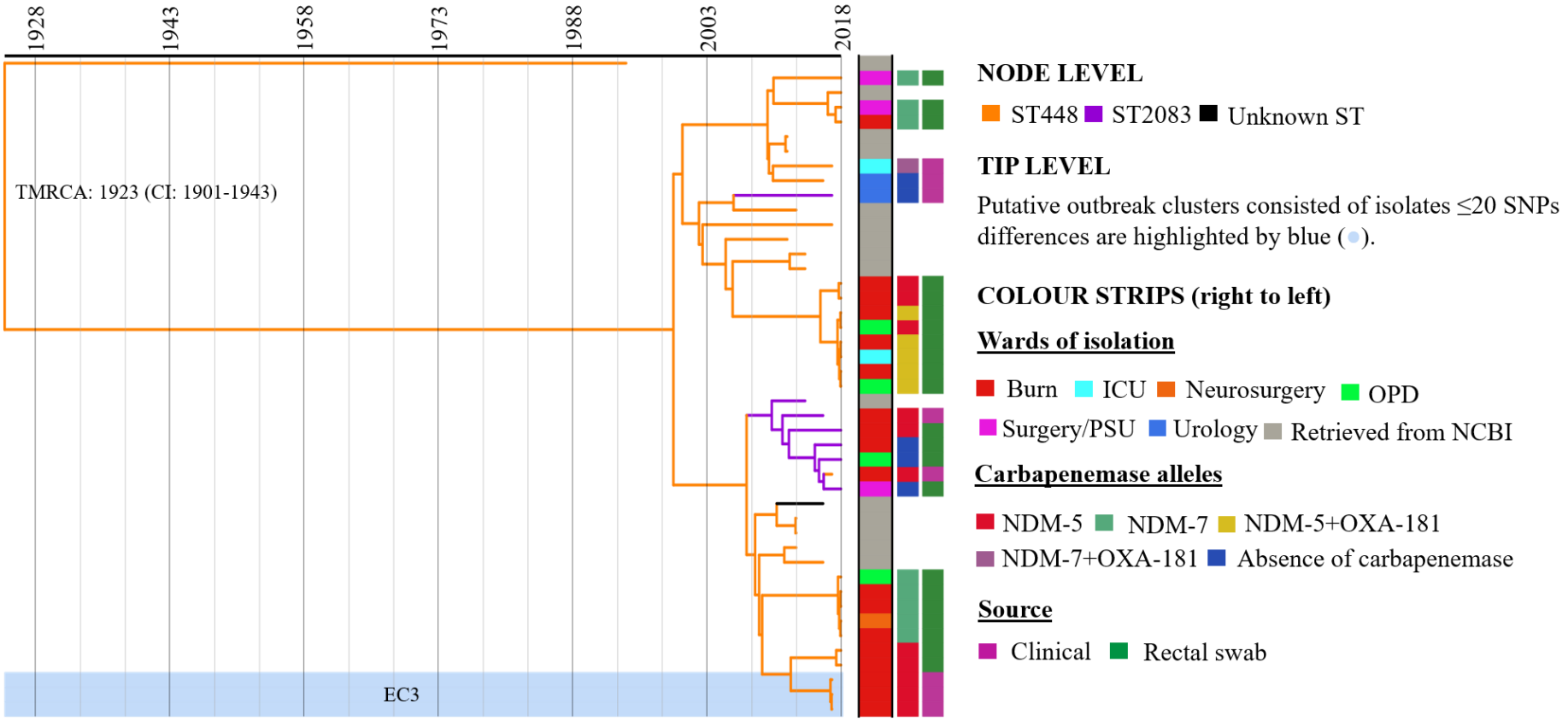


Figure 5.18 Time calibrated phylogenetic tree generated from of *E. coli* genomes belonged to ST448 along with closely related isolates from other STs (ST2083, ST1702, and novel allele) (n=45). PSU, paediatric surgery. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

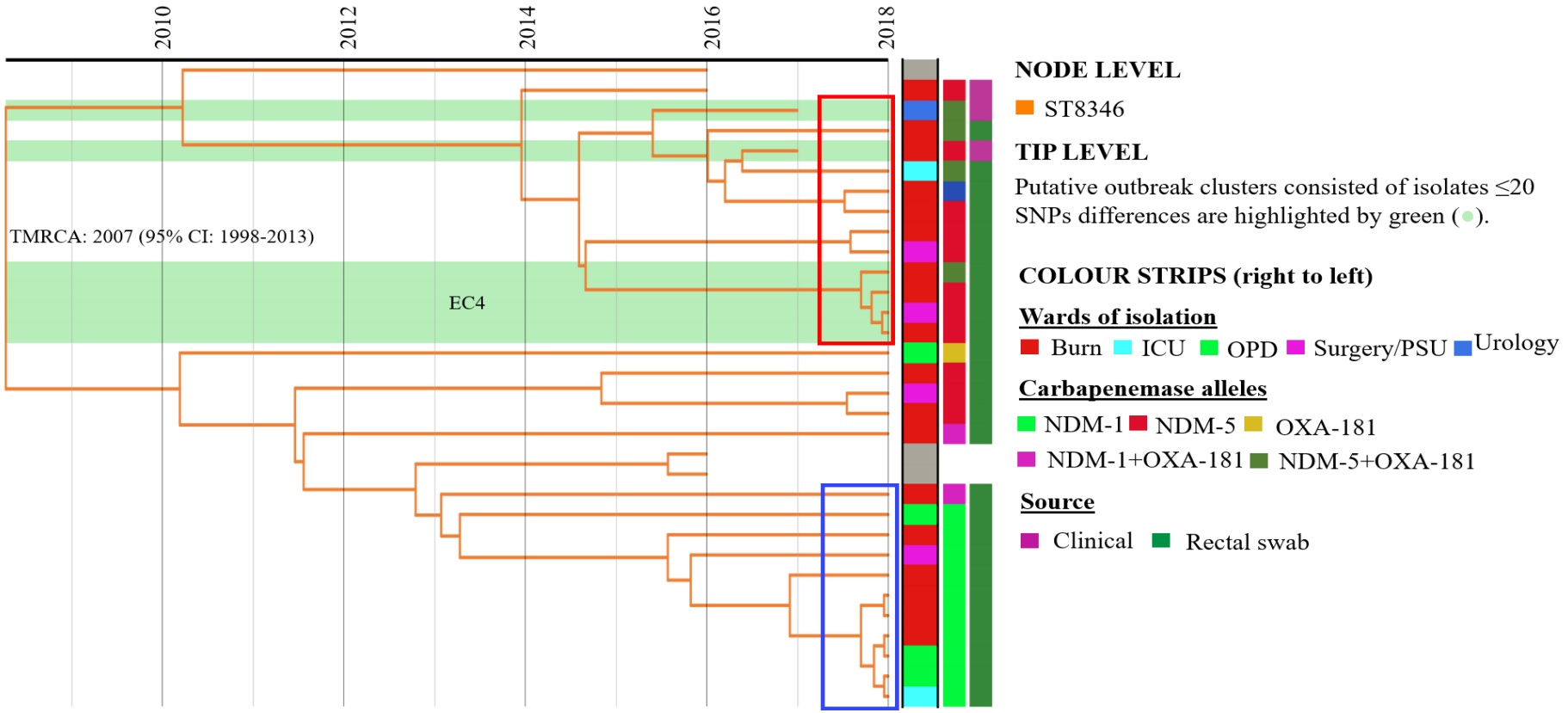


Figure 5.19 Time calibrated phylogenetic tree generated from of *E. coli* genomes belonged to ST8346 (n=32). *E. coli* ST8346 was recognised as an emerging clone in Bangladesh. A putative outbreak cluster (EC4) consisted of clinical, and faecal isolates is marked by red, and a cluster consisted of faecal isolates (differed by ≤10) only is marked by blue. PSU, paediatric surgery. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

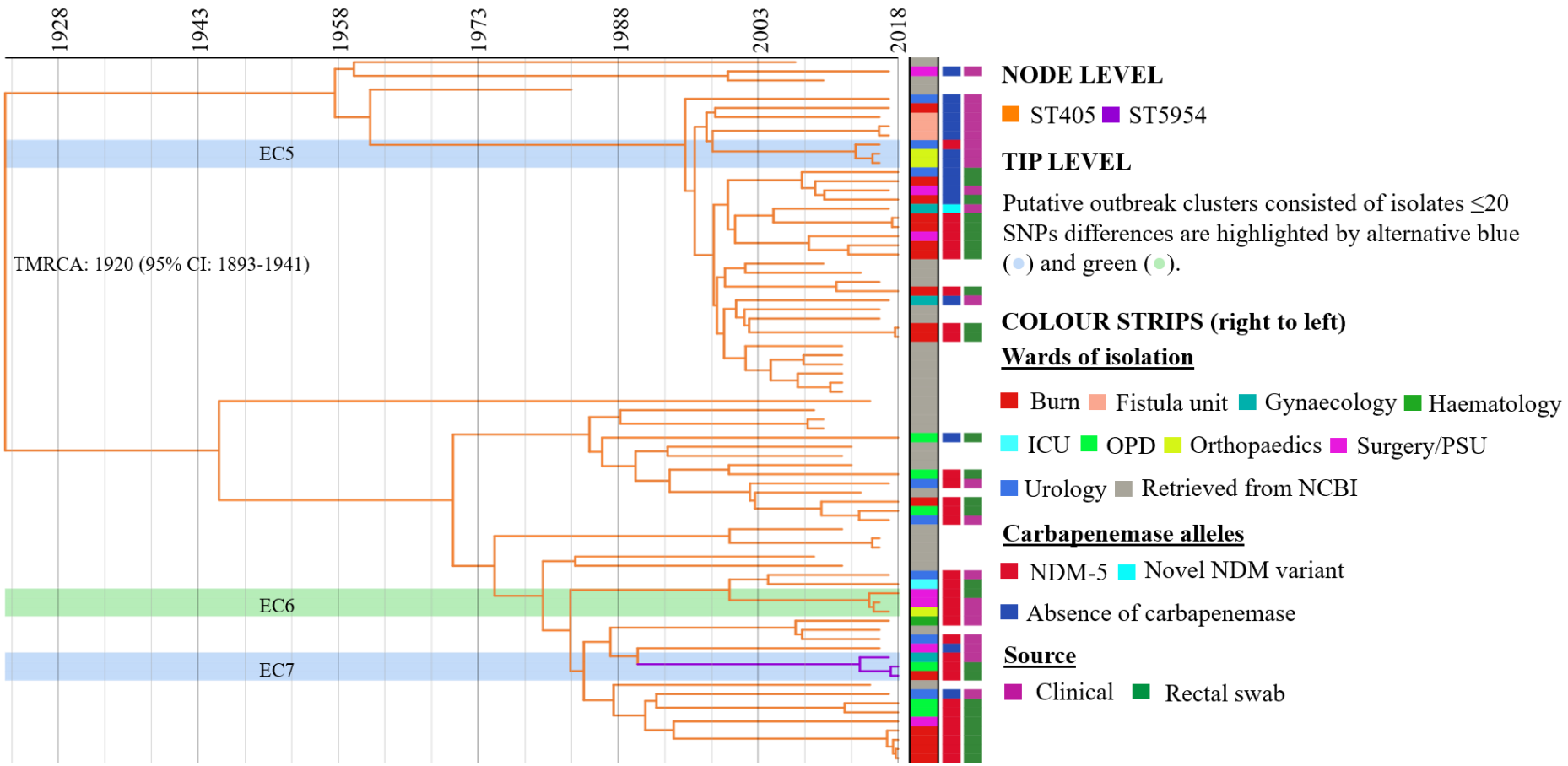


Figure 5.20 Time calibrated phylogenetic tree generated from of *E. coli* genomes belonged to ST405 along with closely related isolates from ST5954 (n=77). PSU, paediatric surgery. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

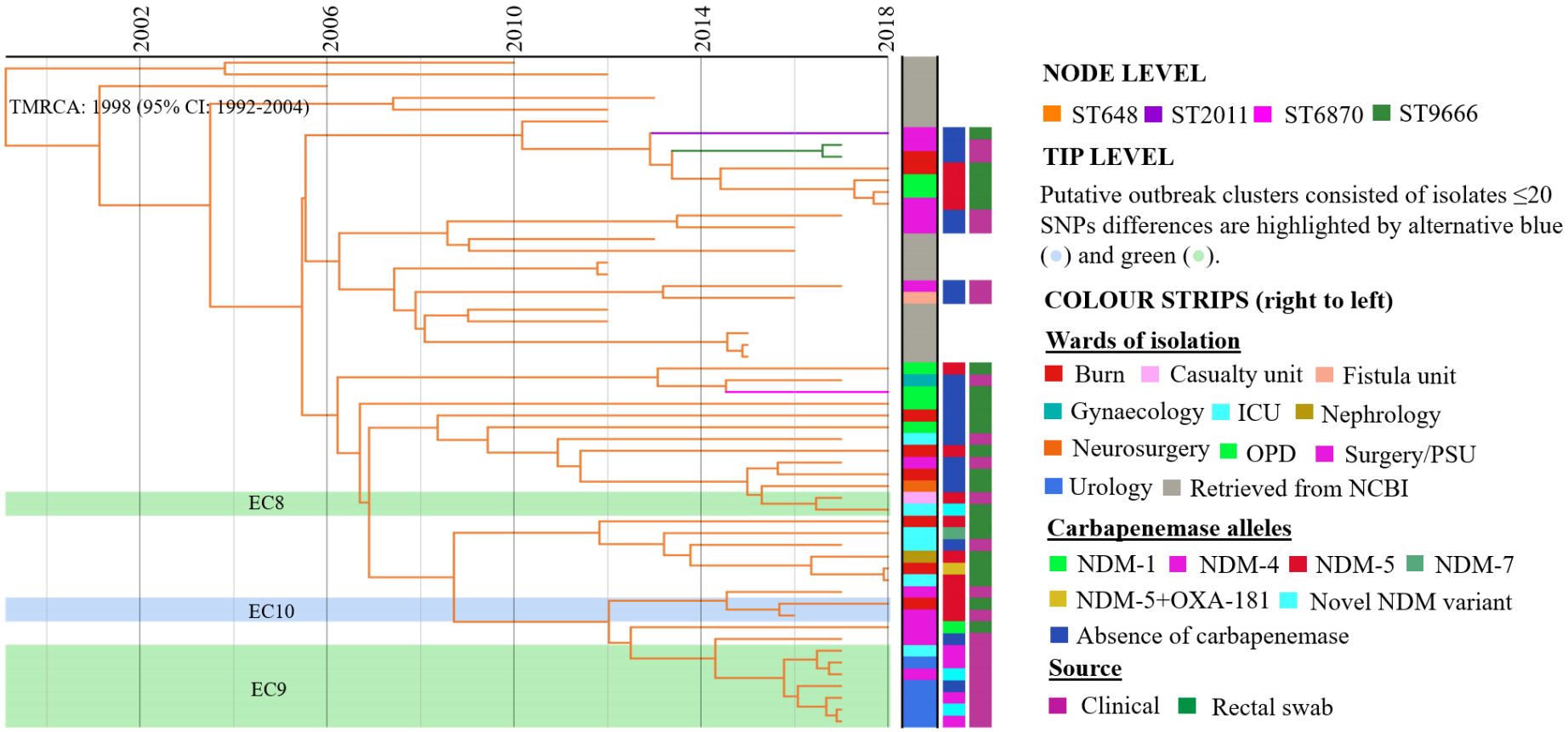


Figure 5.21 Time calibrated phylogenetic tree generated from of *E. coli* genomes belonged to ST648 along with closely related isolates from other STs (ST2011, ST6870, and ST9666) (n=57). PSU, paediatric surgery. Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

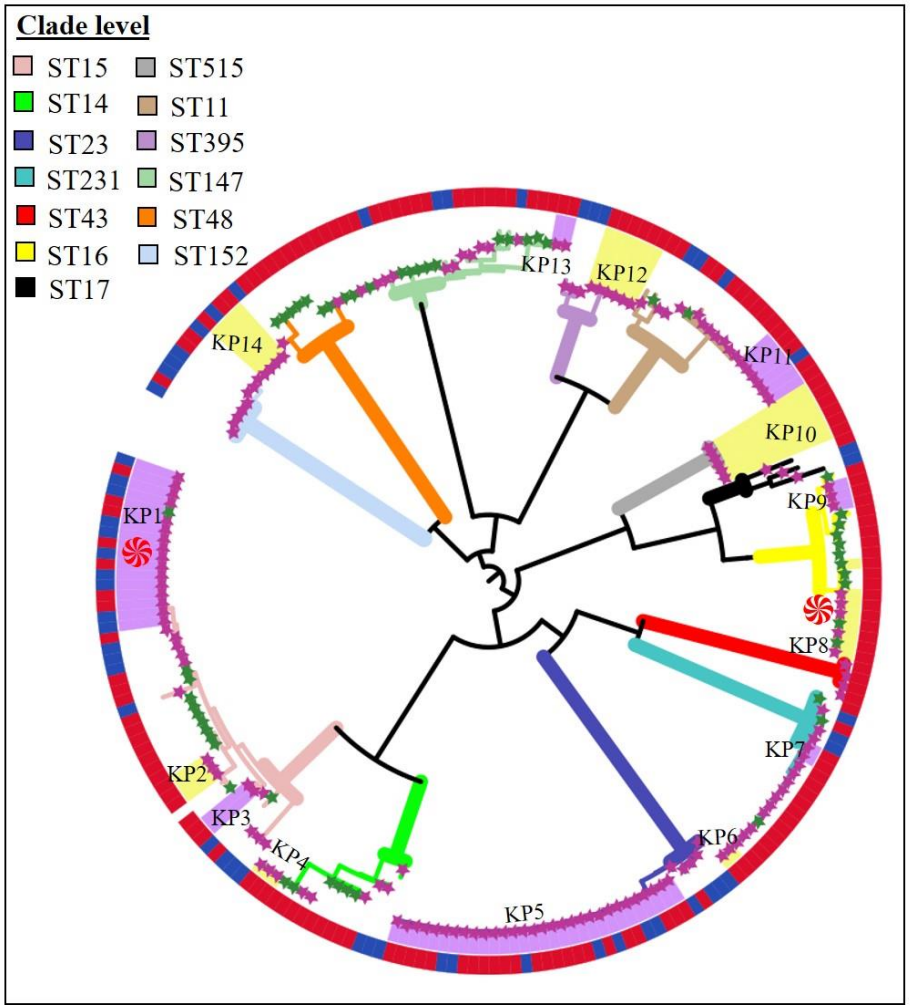


Figure 5.22 Core-genome SNP tree of isolates of major clones of *K. pneumoniae* associated with carbapenem resistance, and screening of outbreaks in *K. pneumoniae*. SNPs calling followed by alignment of core SNPs was performed using Snippy (v4.4.5). The ML tree was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model, and gamma correction with bootstrapping. Recombination removal using Gubbins (v2.3.4), and pair-wise SNPs calculation using pairsnp (v0.0.7). Outbreaks were suspected if the isolates in a cluster differed by ≤20 SNPs. Putative outbreak clusters are highlighted by alternative purple (●) and yellow (●) and designated as KP1 to KP14. STs of respective isolates have been labelled at clade level. Branch symbols, outer ring in the phylogenetic tree denote source of isolation, and carbapenem susceptibility of respective isolates, respectively. Pink (■) indicates clinical isolates, and green (■) indicates faecal isolates. Blue (■) denotes carbapenem sensitive, and red (■) denotes carbapenem resistant. Clusters consisted of both clinical, and faecal isolates are marked by red symbol (❁).

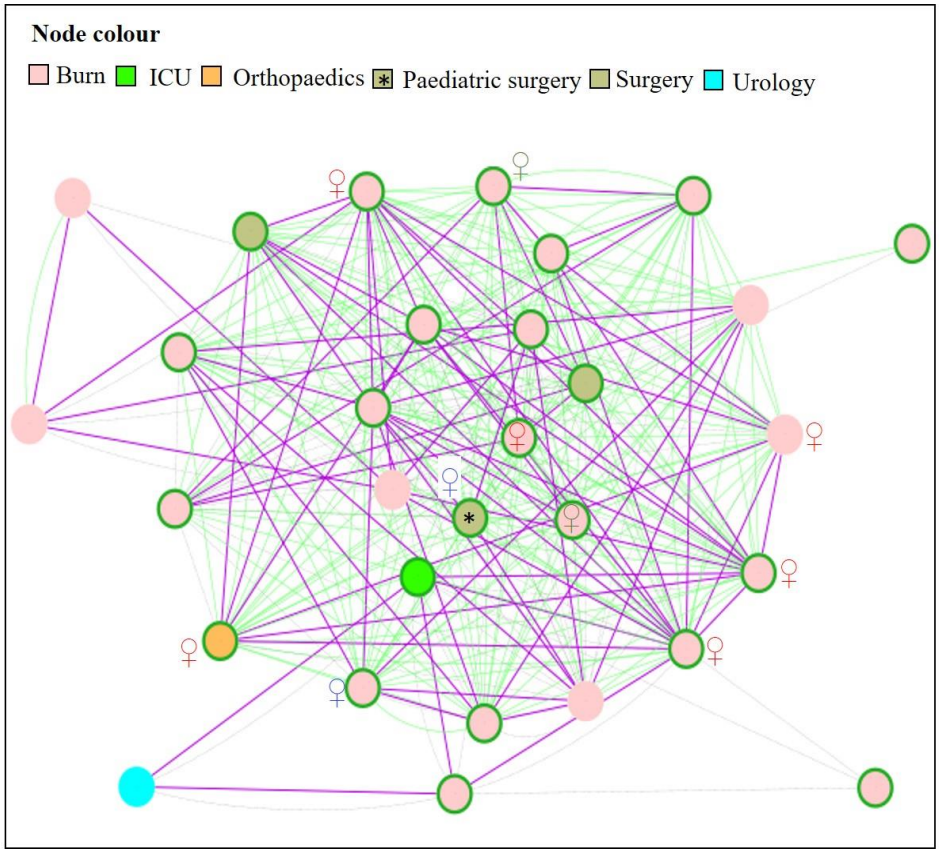


Figure 5.23 Network map generated with the isolates of an outbreak cluster of *K. pneumoniae* ST23. Isolates are connected where they differed by ≤20 SNPs. Round node shape denotes clinical isolates. Node border denotes presence of carbapenemase alleles, and nodes without border indicate absence of carbapenemase allele. Green border represents presence of $bla_{\text{NDM-5}}$. Wards of isolation are represented by different node colour. Isolates differed by ≤10 SNPs are connected by green (–) edges and differed by 11 to 20 SNPs by grey (–) edges. Edges are coloured by purple (–) where there was overlapping of patients' hospital stay pertinent to the isolates differed by ≤20 SNPs. Isolates are marked by symbol (♀) where they differed by 0 to 2 SNPs (pairing of isolates by fewer SNPs are represented by individual symbol colour).

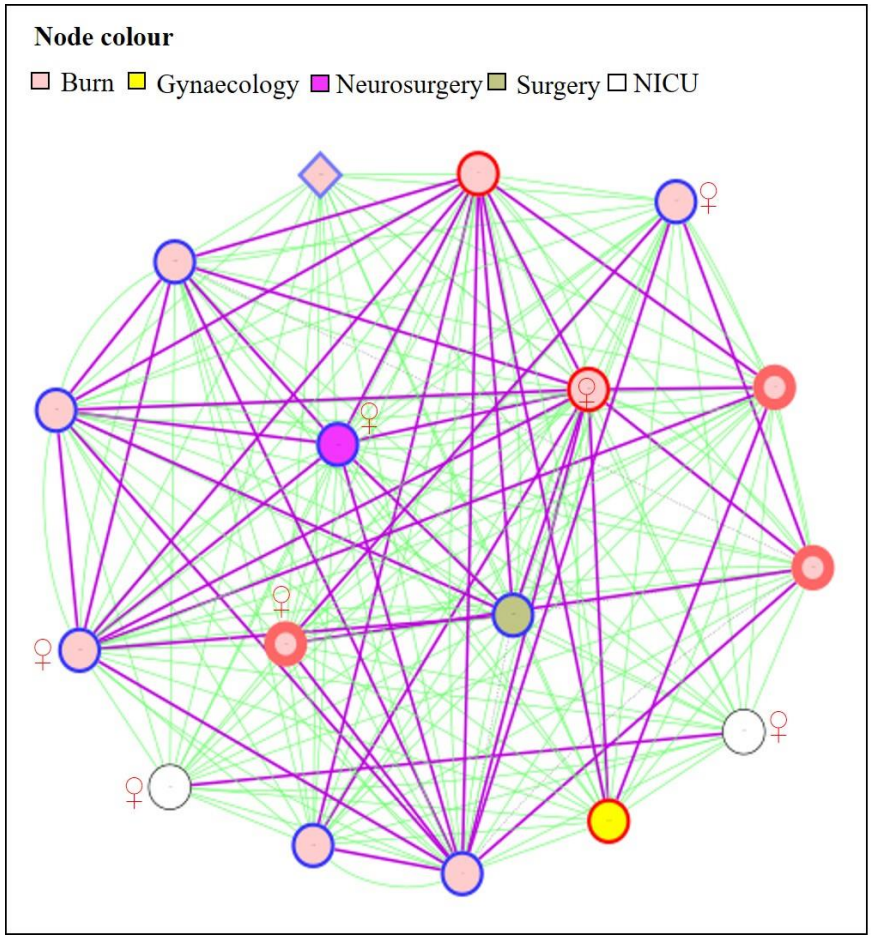


Figure 5.24 Network map generated with the isolates of a putative outbreak cluster of *K. pneumoniae* ST15. Isolates are connected where they differed by ≤20 SNPs. Round node shape denotes clinical isolates, and diamond shape indicates faecal isolates. Node border denotes presence of carbapenemase alleles, and nodes without border indicate absence of carbapenemase allele. Red border represents presence of $bla_{\text{NDM-1}}$, blue border denotes $bla_{\text{OXA-181}}$, and red border with increased width represents the presence of both $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-181}}$. Wards of isolation are represented by different node colour. Isolates differed by ≤10 SNPs are connected by green (–) edges and differed by 11 to 20 SNPs by grey (–) edges. Edges are coloured by purple (–) where there was overlapping of patients' hospital stay pertinent to the isolates differed by ≤20 SNPs. Isolates are marked by symbol (♀) where they differed by 0 to 2 SNPs (pairing of isolates by fewer SNPs are represented by individual symbol colour).

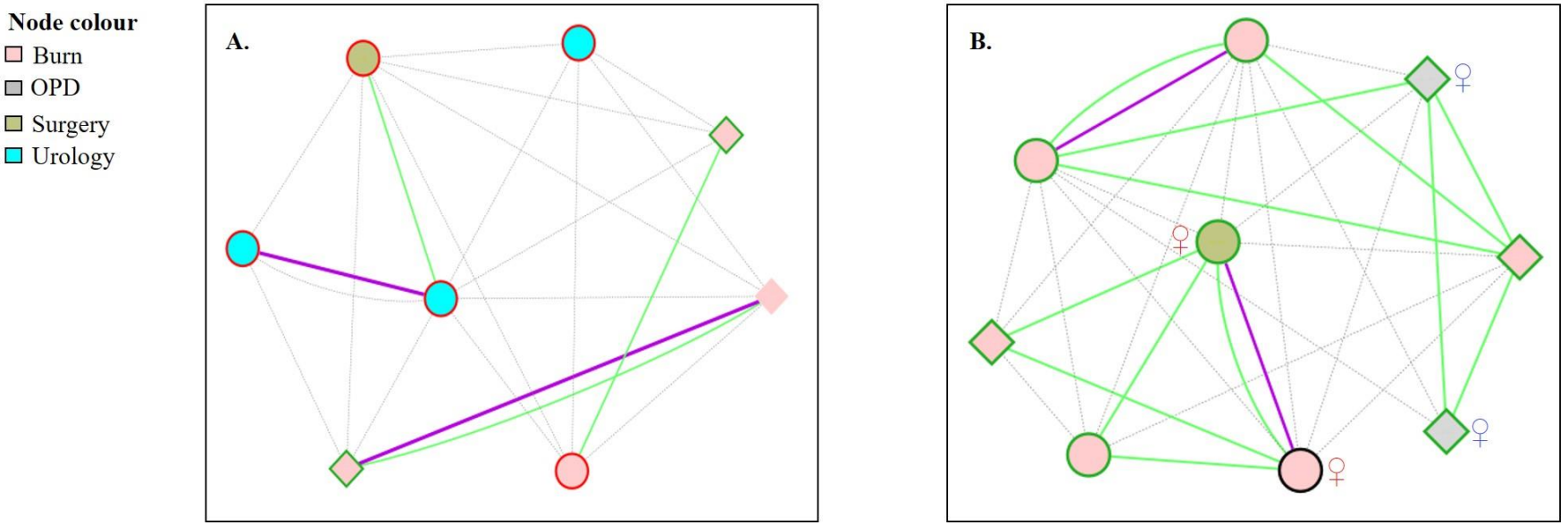


Figure 5.25 Network map generated with the isolates of putative outbreak cluster of *K. pneumoniae* ST16 [**A**], and *E. cloacae* ST113 [**B**]. Isolates are connected where they differed by ≤20 SNPs. Round node shape denotes clinical isolates, and diamond shape indicates faecal isolates. Node border denotes presence of carbapenemase alleles, and nodes without border indicate absence of carbapenemase allele. Red border represents presence of *bla*$_{\text{NDM-1}}$, green border denotes *bla*$_{\text{NDM-5}}$, and black border represents novel *bla*$_{\text{NDM}}$. Wards of isolation are represented by different node colour. Isolates differed by ≤10 SNPs are connected by green (–) edges and differed by 11 to 20 SNPs by grey (–) edges. Edges are coloured by purple (–) where there was overlapping of patients' hospital stay pertinent to the isolates differed by ≤20 SNPs. Isolates are marked by symbol (♀) where they differed by 0 to 2 SNPs (pairing of isolates by fewer SNPs are represented by individual symbol colour).

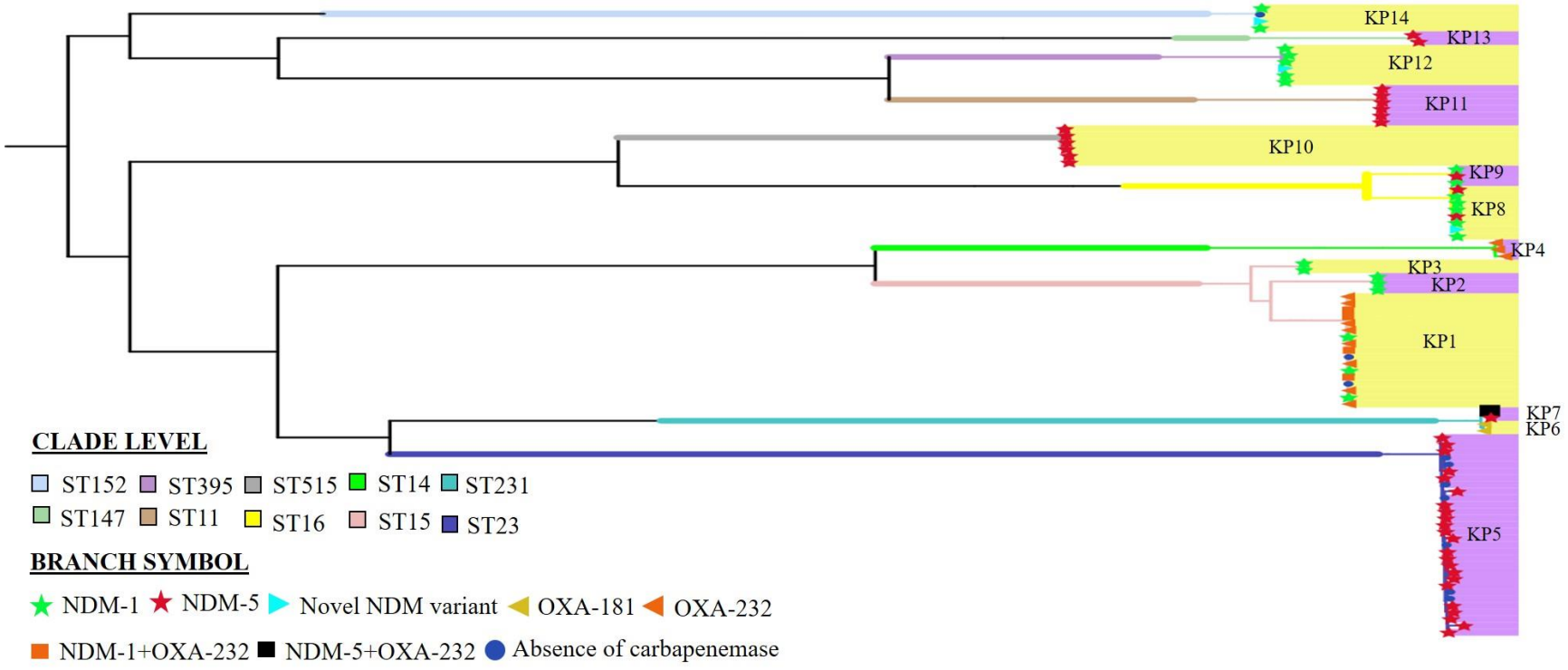


Figure 5.26 ML tree generated from core-genome SNP alignment of *K. pneumoniae* isolates found in putative outbreak clusters. SNPs calling followed by alignment of core SNPs was performed using Snippy (v4.4.5). The ML tree was built with RAxML-ng (v0.9.0.git-mpi) using a GTR evolutionary model, and gamma correction with bootstrapping. Putative outbreak clusters are highlighted by alternative purple (●) and yellow (●).

5.2.3 Time-calibrated evolutionary analysis of high-risk clones emerged with carbapenem resistance

Time-scale phylogenetic analysis was performed using BEAST with the major clones of *E. coli* (ST167, ST448, ST8346, ST405, and ST648), and *K. pneumoniae* (ST15, ST23, and ST16) associated with carbapenem resistance to predict the temporal trajectory of the clone in the hospital and to estimate the date of the most recent common ancestor (TMRCA). The analysis performed with *K. pneumoniae* ST23 was not included due to incoherent BEAST output.

Bayesian phylogenetic analysis suggested that the populations of *E. coli* ST167, ST448, ST8346, ST405, and ST648 had TMRCAs around 1978, 1923, 2007, 1920, and 1998, respectively (including isolates from NCBI). Dates of TMCRAs for DMCH-only subclades ranged from 2004 to 2017 (ST167), 2009 to 2017 (ST448), 2013 to 2014 (ST8346), 1990 to 2017 (ST405), and 2006 to 2013 (ST648). It was predicted that *E. coli* ST167 was evolved from ST10 and diverged into two clades from the TMRCA on 1898 (Figure 5.17). The major putative outbreak clusters associated with carbapenem resistance in *E. coli*, EC9 (ST648), and EC4 (ST8346) were predicted to have emerged in the hospital between 2014 and 2015 (Figure 5.17-Figure 5.21). The mean clock rates per site per year were 6×10^{-7} for ST167 (along with ST10, ST1702, and novel allele), 6.9×10^{-7} for ST448 STs (along with S2083, ST1702, and novel allele), 9.3×10^{-7} for ST8346, 2.5×10^{-7} for ST405 (along with ST5954), and 7.2×10^{-7} for ST648 (along with ST2011, ST6870, and ST9666).

E. coli ST8346 was shown to be an emerging high-risk clone for the dissemination of carbapenem resistance at DMCH. The dated phylogenetic tree revealed that ST8346 population diverged into clades around 2007 (Figure 5.19). One clade evolved with the acquisition of $bla_{\text{NDM-5}}$ and $bla_{\text{OXA-181}}$, and this clade included two clinical isolates differed by 18 SNPs. The other clade progressed with the acquisition of $bla_{\text{NDM-1}}$, $bla_{\text{NDM-5}}$, and $bla_{\text{OXA-181}}$. A faecal cluster of six $bla_{\text{NDM-1}}$-positive isolates possess ≤10 SNPs (Figure 5.19).

TMRCAs for *K. pneumoniae* ST15, and ST16 corresponded to 1980, and 1932, accordingly (including isolates from NCBI). Dates of TMCRAs for DMCH-only subclades ranged from 1998 to 2016 (ST15), and 2009 to 2007 (ST16). ST15, and ST16 included substantial outbreak clusters, KP1, and KP8 were predicted to have

been introduced into the DMCH around 2014 (Figure 5.27; Figure 5.28). The mean clock rates per site per year were 2.8×10^{-7} for ST15, and 6.3×10^{-7} for ST16.

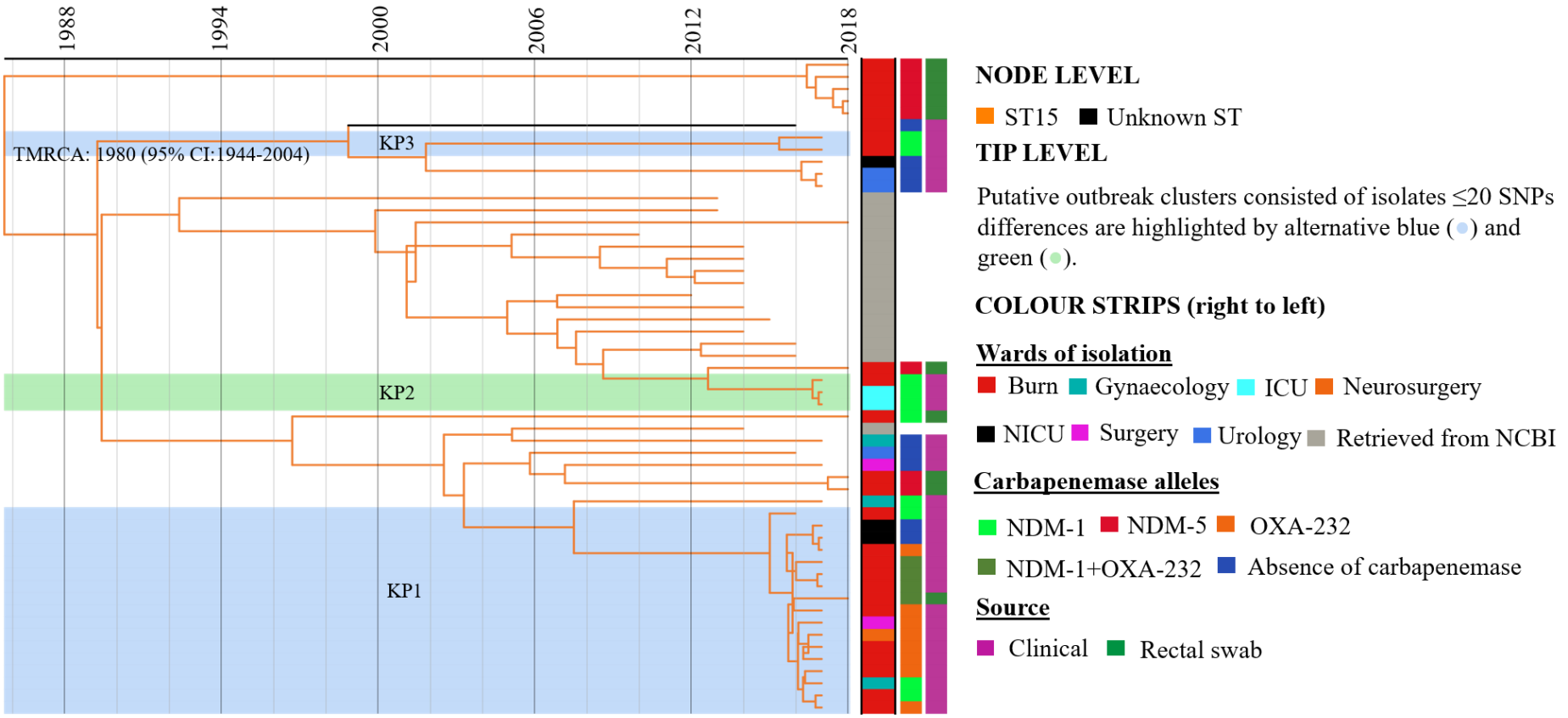


Figure 5.27 Time calibrated phylogenetic tree generated from of *K. pneumoniae* genomes belonged to ST15 along with a closely related isolate of novel allele (n=54). Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

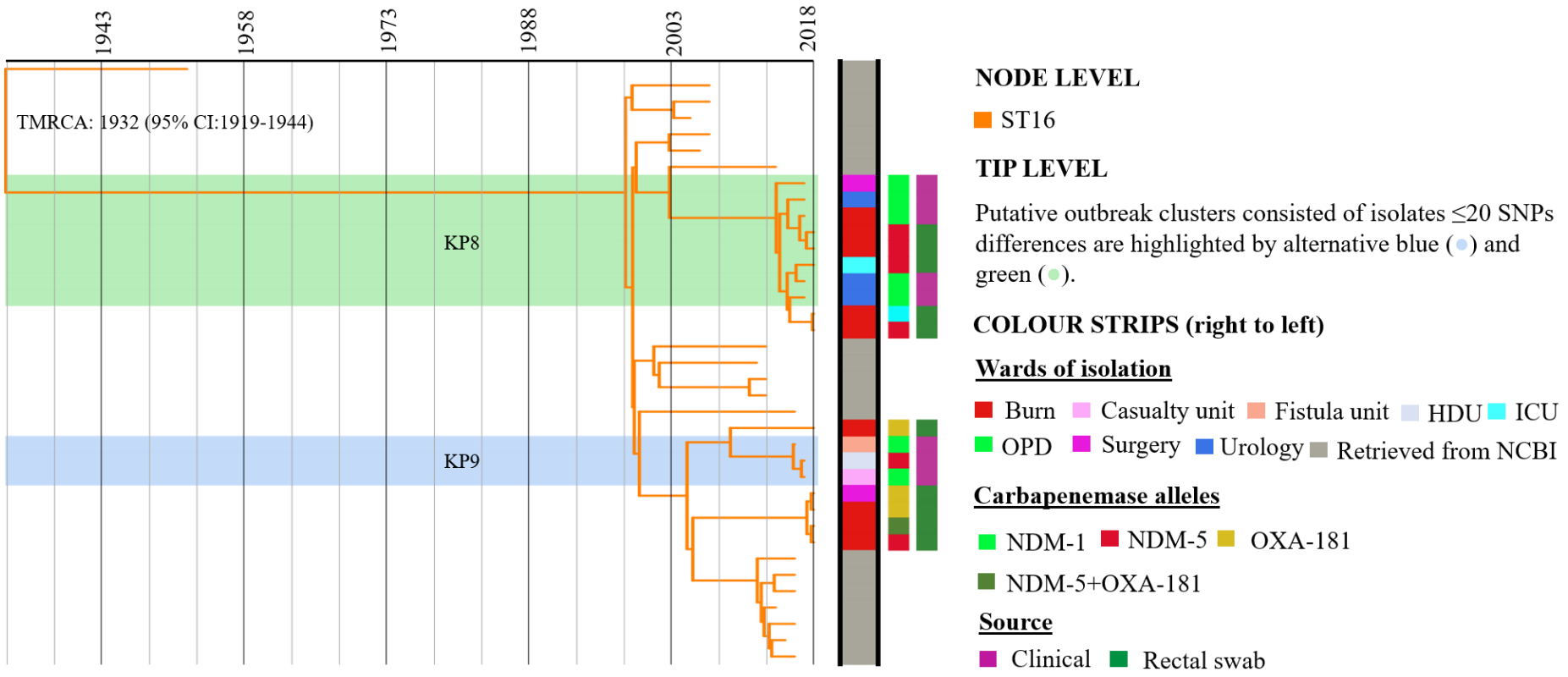


Figure 5.28 Time calibrated phylogenetic tree generated from of *K. pneumoniae* genomes belonged to ST16 (n=37). Isolates retrieved from NCBI for the phylogenetic analysis in this Figure are stated in appendix F.

5.2.4 Impact of high-risk clone on patients' outcome

The impact of high-risk clones was assessed for in-hospital 30-days mortality. Statistical significance was observed for *E. coli* ST8346 ($p<0.05$) (Table 5.5), but the frequency of ST8346 among clinical *E. coli* was very low (n=3). No significant associations were found between other STs of *E. coli* or any ST of *K. pneumoniae* and in-hospital 30-days mortality (Table 5.5; Table 5.6).

Table 5.5 Comparative analysis to assess the associations of in-hospital 30-days mortality among major clonal types of clinical *E. coli*.

	30-days mortality (n=23)	**Competing outcome (n=192)**	***p* value**
ST131	5 (21·7)	18 (9·4)	0·070
ST405	0 (0)	19 (9·9)	0·114
ST410	2 (8·7)	16 (8·3)	0·953
ST648	1 (4·3)	20 (10·4)	0·354
ST167	1 (4·3)	17 (8·9)	0·461
ST101	1 (4·3)	11 (5·7)	0·785
ST38	2 (8·7)	7 (3·6)	0·253
ST448	2 (8·7)	4 (2·1)	0·069
ST2659	1 (4·3)	3 (1·6)	0·350
ST617	0 (0)	5 (2·6)	0·434
ST224	0 (0)	4 (2·1)	0·485
ST226	0 (0)	4 (2·1)	0·485
ST8346	2 (8·7)	1 (0·5)	0·002

Patients discharged alive or in-hospital mortality after 30 days was used as competing outcome variable. Patients with DAMA (n=13) were excluded from the outcome analysis. Values in parentheses indicate column percentage. Cells are highlighted if statistical significance was observed in relation to respective ST.

Table 5.6 Comparative analysis to assess the associations of in-hospital 30-days mortality among major clonal types of clinical *K. pneumoniae*.

	30-days mortality (n=54)	**Competing outcome (n=159)**	***p* value**
ST23	10 (18·5)	20 (12·6)	0·278
ST15	4 (7·4)	24 (15·1)	0·149
ST231	6 (11·1)	11 (6·9)	0·326
ST11	6 (11·1)	10 (6·3)	0·245
ST152	2 (3·7)	9 (5·7)	0·575
ST147	4 (7·4)	7 (4·4)	0·389
ST14	2 (3·7)	7 (4·4)	0·825
ST395	1 (1·9)	7 (4·4)	0·394
ST16	0 (0)	7 (4·4)	0·117
ST307	1 (1·9)	6 (3·8)	0·494
ST101	1 (1·9)	3 (1·9)	0·987
ST48	2 (3·7)	2 (1·3)	0·253
ST43	2 (3·7)	2 (1·3)	0·253

Patients discharged alive or in-hospital mortality after 30 days was used as competing outcome variable. Patients with DAMA (n=15) were excluded from the outcome analysis. Figures in parentheses indicate column percentage. Cells are highlighted if statistical significance was observed in relation to respective ST.

5.3 Discussion

The global dissemination of CRE has been propagated either by transfer of mobile element carrying carbapenemase genes among a wide array of species or clonal spread of bacteria harbouring the resistance determinants (Hassing *et al.,* 2015; Hawkey, 2015; Moradigaravand *et al.,* 2017; Davin-Regli *et al.,* 2019; Marano *et al.,* 2019). In this study, certain clones of *E. coli* (ST167, ST448, ST8346, ST405, and ST648) and *K. pneumoniae* (ST16, ST231, ST11, ST515, and ST23) had significant associations with carbapenem resistance (p<0·05) (Table 5.1; Table 5.2; Table 5.4; Table 5.5). Chapter 3 and 4 have describe the molecular epidemiology of carbapenem resistance in Bangladesh. The most prevalent carbapenem resistance mechanism in this study was the variants of *bla*$_{\text{NDM}}$ followed by *bla*$_{\text{OXA-181}}$ (Table 3.5; Table 4.3). Previous data suggested a strong association of particular resistance patterns with the clonal backgrounds. ST167 of *E. coli*, and ST11, ST14, ST15, ST23, ST147 of *K. pneumoniae* have been reported episodically as the frequent host of *bla*$_{\text{NDM}}$ (Huang *et al.,* 2016; Wu *et al.,* 2019). Our data suggested that the frequency of carbapenem resistance in a certain STs depend on the overall burden of said clone in clinical infections (Figure 5.4; Figure 5.9). Nevertheless, clinical *E. coli* ST131 was inversely related to carbapenem resistance (p<0·05) (Table 5.1; Table 5.2). *E. coli* ST131 is an epidemic high-risk clone for the dissemination of many resistance gene, particularly for *bla*$_{\text{CTX-M-15}}$, however, the incidence of *bla*$_{\text{NDM}}$ in the population of *E. coli* ST131 happened less likely (Peirano *et al.,* 2014; Chen *et al.,* 2019). Only few data from Bangladesh are available to predict about the prevalent *E. coli* clonal types in community acquired infections (CAI) or healthy human gut and in the environment and no data available for the *K. pneumoniae*. The previous studies mostly described the associations of *E. coli* clonal types with several resistance mechanisms such as *bla*$_{\text{CTX-M}}$ or *bla*$_{\text{NDM}}$, and the clonal diversity was found to be very high (Hasan *et al.,* 2012; Haque *et al.,* 2014; Rashid *et al,* 2015; Toleman *et al.,* 2015; Montealegre *et al.,* 2020). This project found the associations of *bla*$_{\text{NDM}}$ with *E. coli* ST167, ST405, ST648, and ST8346 among the outpatients' faecal colonisation which were consistent with the data from the hospitalised patients (Figure 5.2; Figure 5.3; Figure 5.17; Figure 5.19; Figure 5.20; Figure 5.21). Toleman *et al.* (2015) also reported the high level of environmental contamination of *bla*$_{\text{NDM}}$ in Bangladesh which invariably recovered from *E. coli* ST405, ST648, and ST101. A possible link of spread of *bla*$_{\text{NDM}}$ through

clonal lineage between hospital and community is obvious in Bangladesh. Time-phylogenetic analysis of some high-risk clones for carbapenem resistance (including isolates from this study and NCBI isolates) indicated that the clones did not form locally, however, established gradually, and expanded. *E. coli* ST405 and *K. pneumoniae* ST15 were found to be old clones at DMCH, subclades containing local isolates emerged on 1990 and 1998, respectively, and *E. coli*, ST8346 was predicted to be a newly formed high-risk clone for carbapenem resistance (local clades were formed between 2013 and 2014) (Figure 5.17-Figure 5.21; Figure 5.27; Figure 5.28). The mean mutation rates of these clones (2.5×10^{-7} to 9.3×10^{-7} site/year) were consistent with previous reports (Duchêne *et al.*, 2016; Gibson *et al.*, 2018). Plasmid transfer/mutations can result in resistance from antibiotic pressure but may possess a fitness costs; however, secondary compensatory mutation can occur which can alleviate the fitness cost without compromising resistance (Melnyk *et al.,* 2015; Hernando-Amado *et al.,* 2017). Therefore, it is possible, that the aforementioned clones have been established in these clinical and community settings through compensatory mutations.

There is no predefined cut-off of SNP difference for isolates to be considered as the same outbreak. Isolates with fewer SNPs differences can explain putative transmission events of an outbreak by integrating epidemiological information (Snitkin *et al.,* 2012; Moradigaravand *et al.,* 2016; Moradigaravand *et al.,* 2017; Marsh *et al.,* 2019). The most possible transmission events were tracked in this study with SNPs cut-offs of ≤20 followed by merging of epidemiological and genomic data. Clonal transmissions were found to be higher with *K. pneumoniae* (KP1 to KP14) than *E. coli* (EC1 to EC9) (Figure 5.15; Figure 5.22). Moreover, the largest cluster in this study was KP5 followed by KP1, belonged to *K. pneumoniae* ST23, and ST15, respectively (Figure 5.22; Figure 5.23; Figure 5.24). *K. pneumoniae* was reported previously as an extremely transmissible organism and more prone to cause outbreak than other species (Gurieva *et al.,* 2018; Ludden *et al.,* 2020). The remaining considerable larger clusters belonged to *K. pneumoniae* ST16, *E. coli* ST648, ST8346, and *E. cloacae* ST113 (Figure 5.15; Figure 5.22; Figure 5.25). The combined epidemiological and genomic insights inferred the following probable transmission events: **1.** Clusters in common ward, overlapping of patients' hospital stay, and isolates with the SNPs difference of ≤20 was the suggestive of sequential patient-to-patient

transmission **2.** Isolates differed by very few SNPs difference (0 to 2) along with the criteria in **#1** suggested the sequential transmission or concurrent acquisition of identical clone from a common source. The major clusters, KP5 (Figure 5.23), KP1 (Figure 5.24), EC9 (Figure 5.16), and Eco1 (Figure 5.25) might had such kind of transmission event. **3.** Clusters distributed in different wards indicated the probable transmission through health care workers or usage of common device by the patients of different wards. **4.** This study did not investigate the role of carrier state during clinical outbreak. The carrier might be the patients infected with outbreak isolates, other patients, patients' care givers or the health care workers (Hawkey, 2015; Cimmino *et al.*, 2016; Shahida *et al.*, 2016; Sood and Perl, 2016). The clinical and colonisation studies were undertaken about a year interval; however, the analysis revealed the isolates both of clinical and faecal origin with ≤20 SNP gap (EC4, EC6, EC7, KP1, and Eco1 (figure 5.15; Figure 5.22; Figure 5.25), suggested the continuation of putative outbreak for a protracted period of time. Perhaps the involvement of pre-existing exogenous source along with sequential patient-to-patient transmission can be responsible for the occurrences (Roisin *et al.*, 2016). **5.** The limitation of the study is that the linkage of the putative outbreak with hospital environment was not explored. Nevertheless, major clusters presumed to be existed at DMCH for a long time. Time-calibrated phylogeny predicted that TMRCAs for the outbreak cluster, EC9 (ST648), and EC4 (ST8346) corresponded to around 2015 (Figure 5.17-Figure 5.21), and KP1 (ST15), and KP8 (ST16) to between 2014 and 2015 (Figure 5.27; Figure 5.28). The intervals between the first and last case enrolled in this study with the major outbreak clusters were: 17 months for KP1, 14 months for KP8, 11 months for KP5, 8 months for Eco1, and 6 months for EC9. **6.** We found variation of carbapenemase alleles in putative outbreak clusters (Figure 5.17-Figure 5.26) which signifies possible horizontal acquisition of new resistance gene during the event of outbreak. However, certain carbapenemase alleles in this study were clustered significantly in some specific STs (Table 5.3; Table 5.6; Figure 5.5; Figure 5.10) which is suggestive of their likely spread of carbapenem resistance by clonal expansion.

Hospital outbreaks enhance the transmissibility of pathogens among patients, patients to health workers, and/or within the hospital environment. Hospital outbreaks can increase the clinical burden of successful MDR clones which readily can spread between/within hospitals, community via faecal carriage, and between countries

(Koornhof *et al.*, 2001; Zahar *et al.*, 2014; Hassing *et al.*, 2015; Hawkey, 2015; Cimmino *et al.*, 2016; Sood and Perl, 2016). This study estimated significant association of 30-days mortality with *E. coli* ST8346 (Table 5.5). Therefore, *E. coli* ST8346 emerged as high-risk clone for high mortality and carbapenem resistance at DMCH (Table 5.1). Although this study did not find any significant association of mortality with any other ST (Table 5.5; Table 5.6), radical outbreak management and improved IPC measures can significantly reduce the over burden of AMR in clinical settings (Woodford *et al.*, 2011; Zahar *et al.*, 2014; O'Neill, 2016; Sood and Perl, 2016; Babu *et al.*, 2017).

Section Six

Investigating the Spread of New-Delhi metallo-β-lactamase due to Horizontal Gene Transfer

6.1 Introduction

Plasmids are circular extrachromosomal DNA, found in wide range of microorganisms, with the ability to exchange genetic information between bacteria. Some plasmids are 'non-conjugative'; however, many plasmids are transmissible by conjugation, and therefore, influence bacterial evolution (Branger *et al.,* 2019; Shintani *et al.,* 2019). The unique characteristics of plasmids are that plasmids replicate by autonomous self-controlled mechanisms, constituting a substantial amount of the total genetic content of an organism. Plasmid can import and deliver genes as well by means of transposition though IS or transposons, and integrons (Partridge *et al.,* 2018). Acquisition of plasmids can benefit the host bacteria, but plasmid replication and gene expression impose a general metabolic burden in the host, leading to ascendancy of the susceptible bacteria over resistant ones in the absence of selection. However, the fitness costs in the host are alleviated over time through the compensatory mutations in the presence of selection. Therefore, plasmids are generally persisted in the bacterial population regardless of selective pressure. Post-segregational killing of plasmid-free cells during the cell division by the plasmid-encoded addiction system promote the maintenance of plasmid from generation to generation (Melnyk *et al.,* 2015; Hernando-Amado *et al.,* 2017; Tsang, 2017).

Transposons (Tns) or 'jumping gene' are DNA elements which can move from the DNA molecule to other places on the same DNA or other DNA molecules. The transposition events can be manifested by either cut-and-paste, rolling circle, or self-synthesizing mechanism. Transposons can be broadly classified into class I (RTns) and class II (DNA Tns). The role of RTns in the development of AMR is yet to be investigated. DNA Tns have been reported widely in the association of AMR. Four categories of DNA Tns such as 'IS', 'composite Tns', 'non-composite Tns' (Tn*3* family), and 'transposable phage Mu' have been described. ISs are the smallest genetic elements with the length of less than 2500 bp. The components of IS element include an enzyme, transposase, and inverted repeats (IRs). The presence of IS elements in plasmids, composite Tns, and bacteriophage facilitates the spread of AMR. Composite Tns are composed of more than one ISs and an array of genes in between two ISs. Non-composite Tns do not terminate with IS elements rather the gene containing region is flanked by IRs only, capable of mobilising more genes than the composite transposons. Transposable phage Mu differs from other Tns in term of transmission

mechanism, which involve bacterial acquisition of resistance genes by means of lysogenic cycle (Babakhani and Oloomi, 2018; Partridge *et al.,* 2018).

Integrons are bacterial genetic elements, which can promote the acquisition and expression of genes embedded in gene cassettes by site-specific recombination. The components of integrons composed of *IntI* (encode integrase causes integration and excision of genes in gene cassettes), *attI* site (attachment/recombination site where the gene cassettes insert), and promotor region (expression of genes in gene cassettes). Gene cassettes are small, discreate, non-repetitive, mobile genetic element, consist of specific recombination site, called *attC* (also known as 59 base element) and promotorless ORF which might encode resistance or virulence determinants. Gene cassettes can exist as free circular molecule and can mobilise from genetic site to other (Partridge *et al.,* 2018).

The mode of HGT contributes a pivotal role in the spread of AMR in clinical setting (San Millan, 2018; Conlan *et al.,* 2019). Understanding the genetic traits of plasmids has been regarded as powerful tool for epidemiological surveillance of AMR. The well accepted epidemiological categorisation of plasmid includes incompatibility (Inc) typing which was developed based on variation of replication controls. Incompatibility is defined as the inability of two related plasmids to be propagated stably in the same cell line. Therefore, only compatible plasmid existed in transconjugants (Rozwandowicz *et al.,* 2018). Long read sequencing such as PacBio or Oxford Nanopore are the confirmatory approach for full-blown plasmid characterization, providing an insight of epidemic plasmids and dominant transposition spreading high-end resistance. The genomic details would widen future research prospects to combat AMR (Crofts *et al.,* 2017; Berbers *et al.,* 2020).

Given the high prevalence of *bla*$_{\text{NDM}}$ (180/643, 28%) as a mechanism of carbapenem resistance in Enterobacterales infections in Bangladeshi health setting, and followed by the recovery of 44.5% (312/700) NDM-positive Enterobacterales from faecal carriage, a set of NDM-positive Enterobacterales (n=134) was characterized by minION sequencing (Oxford Nanopore technologies, Oxford, UK) in this study. Two isolates of *K. pneumoniae* contained *bla*$_{\text{NDM-5}}$ on the chromosome. Three isolates of *P. mirabilis*, and one of *P. stuartii* carried *bla*$_{\text{NDM-1}}$ on the chromosome. Two copies of *bla*$_{\text{NDM-5}}$ in different genome locations were found in four

isolates, and three copies in one isolate. A total of 133 plasmids harbouring *bla*$_{NDM}$ was closed successfully by hybrid assembly of Illumina short reads and minION long reads. This chapter briefly describes the evaluation of the horizontal mode of transmission of carbapenem resistance in a Bangladeshi setting, based on WGS. Further analysis has been planned beyond the PhD to investigate the comprehensive functional and genomic properties of plasmids harbouring carbapenem resistance.

6.2 Results

6.2.1 Molecular epidemiology of plasmids harbouring *bla*$_{NDM}$ at DMCH

Phylogenetic analysis of plasmids carrying *bla*$_{NDM}$ (n=133) were performed based on presence and absence genes which broadly revealed that plasmids associated with *bla*$_{NDM}$ were highly diverse (Figure 6.1).

Plasmids were divided into 8 groups (A to H) based on phylogenetic ancestry, and each group was assessed individually if any particular plasmid facilitated the dissemination of NDM. Group A belonged to IncFII (n=45), and IncR (n=1); Group B to IncFIA (n=13), IncFIB (n=2), and IncFII (n=1); group C to IncA/C2 (n=10), IncC (n=1), IncHI2 (n=1), IncFIB & IncA/C2 (n=1), IncFII & IncC (n=1), and unknown (n=1); group D to IncH (n=8), IncR (n=2), and IncFIB (n=1); group E to IncX3 (n=17); group F to IncFIB(pQil) (n=10), IncFIB & IncFII (n=2), and IncFIB(pQil) & IncFII (n=1); group G to IncR (n=3), IncFIA (n=2), IncFIB (n=2), and IncFII (n=1), and H to IncFII (n=1), and unknown (n=6)] (Figure 6.1).

Study design to evaluate the horizontal spread of plasmids harbouring *bla*$_{NDM}$ is described in Figure 6.2.

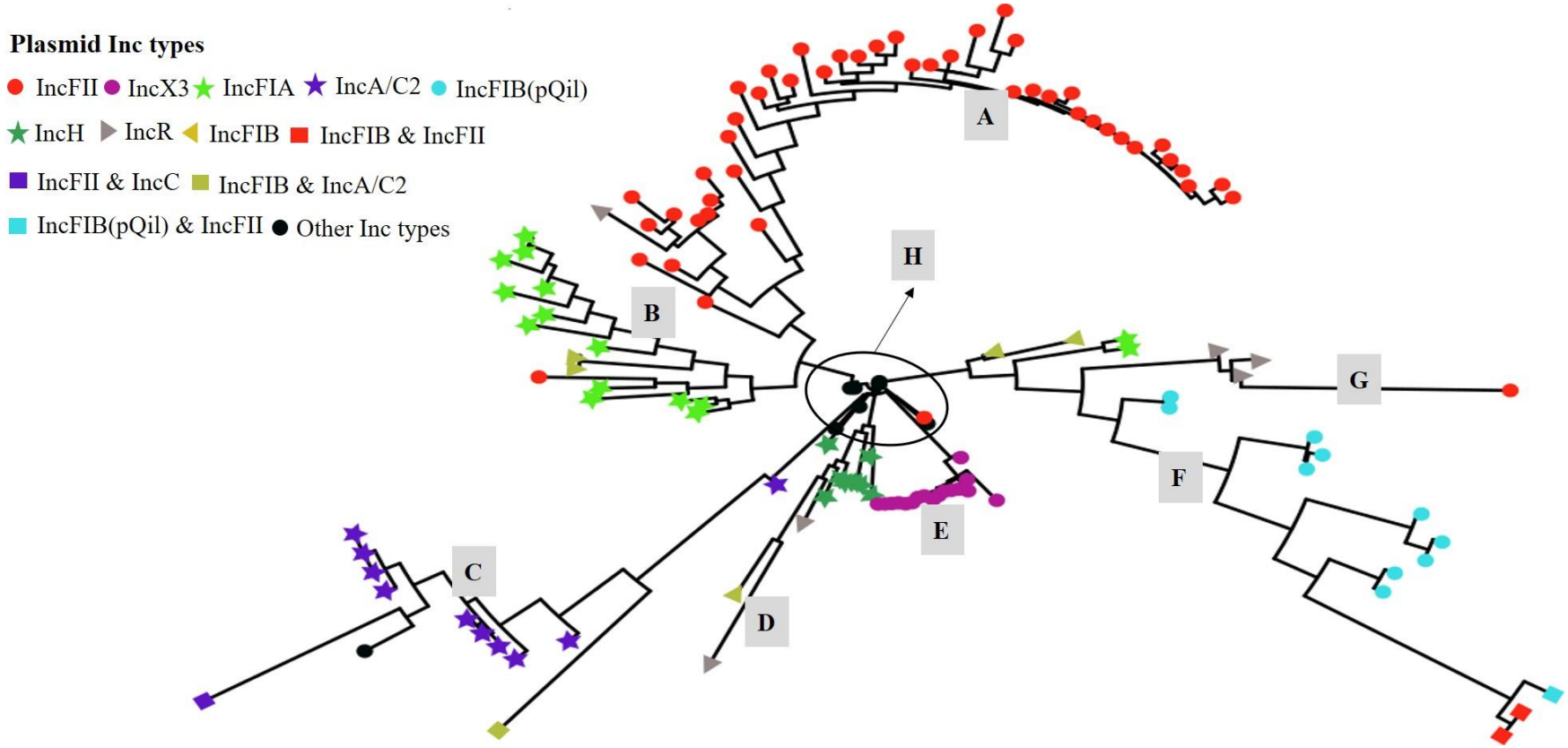


Figure 6.1 Phylogenetic tree was built with the complete, circular sequences of plasmids harbouring *bla*$_{NDM}$ (n=133) based on presence and absence of genes using roary (v3.12.0). To obtain long reads sequence data, minION sequencing was undertaken (Oxford Nanopore technologies, Oxford, UK). Unicycler (0.4.4) was used to yield hybrid assembly using both Illumina short reads and minION long reads, and consequently, plasmid sequences were closed.

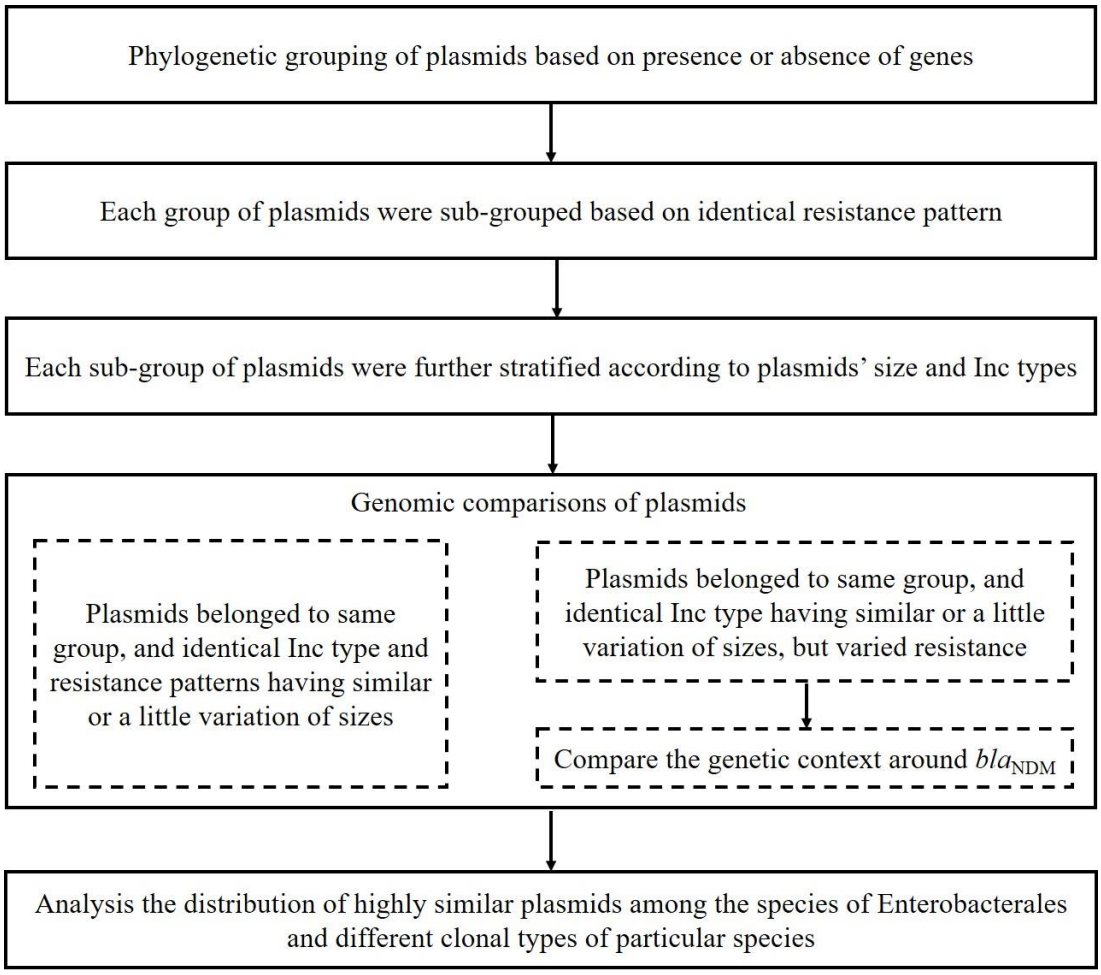


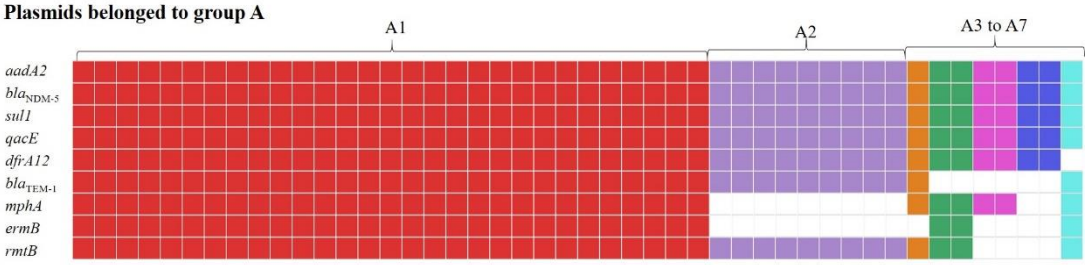
Figure 6.2 Investigating the horizontal transfer plasmids at DMCH.

6.2.2 Analysing the associations of *bla*$_{NDM}$ with other ARGs

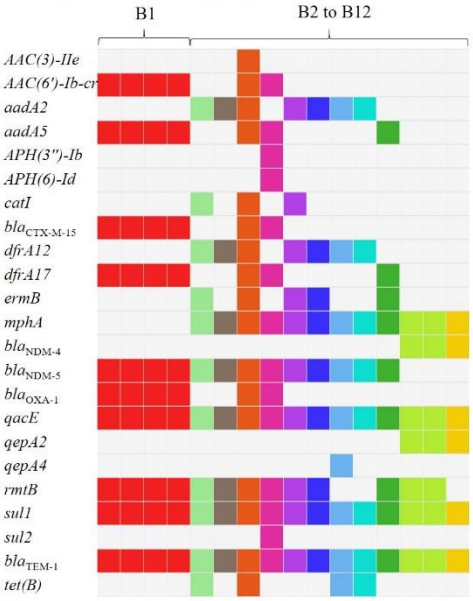
All the plasmids belonged to A to D, and F to H carried the determinants of multidrug resistance (Figure 6.3). Closed, circular sequences belonged to H were retrieved from long read sequencing which were ~8 and ~10 kb in size and did not contain any replication or conjugal protein. All plasmids belonged to E were IncX3; *bla*$_{NDM-5}$ and *bla*$_{NDM-7}$ on IncX3 were not associated with any other ARG, but *bla*$_{NDM-1}$ on IncX3 was associated with *armA*, *msrE*, and *sul*.

Taken together, the most frequent associations of *bla*$_{NDM}$ with the genes against other antimicrobial classes were aminoglycosides (81.2%, 108/133), sulphonamide (75.9%, 101/133), antiseptics (69.9%, 93/133), β-lactams (64.7%, 86/133), and trimethoprim (63.9%, 85/133) followed by macrolides (56.4%, 75/133), efflux pumps (48.1%, 64/133), extended-spectrum β-lactams (15.8%, 21), rifampicin (13.5%, 18), quinolones (9%, 12/133), chloramphenicol (7.5%, 10/133), carbapenems (0.8%, 1/133), and colistin (0.8%, 1/133) (Figure 6.4). The resistance genes found in relation to aforementioned antimicrobials were: *AAC(3), aadA, APH, armA, rmt* for aminoglycosides; *sul* for sulphonamide; *qacE* antiseptics; *bla*$_{TEM-1}$, *bla*$_{CMY-59}$, *bla*$_{OXA-1}$, *bla*$_{OXA-9}$, *bla*$_{SHV}$, and *bla*$_{DHA-1}$ for β-lactams; *dfrA* for trimethoprim; *mph* for macrolides; *tet, erm, cmlA, qepA, msrE* for efflux pumps; *bla*$_{CTX-M-15}$ for extended-spectrum β-lactams; *arr-2* for rifampin; *qnrB, qnrS* for quinolones; *cat* for chloramphenicol; *bla*$_{OXA-232}$ for carbapenems, and *mcr-9* for colistin (Figure 6.3). The colistin resistance determinant, *mcr-9* in association with *bla*$_{NDM}$ harbouring plasmid was recovered from IncH12 plasmid of group C3 from a colistin-susceptible clinical *E. coli* isolate (Figure 6.3). The 5' end of the gene was flanked by IS*1*, and 3' by IS*5* (Figure 6.5).

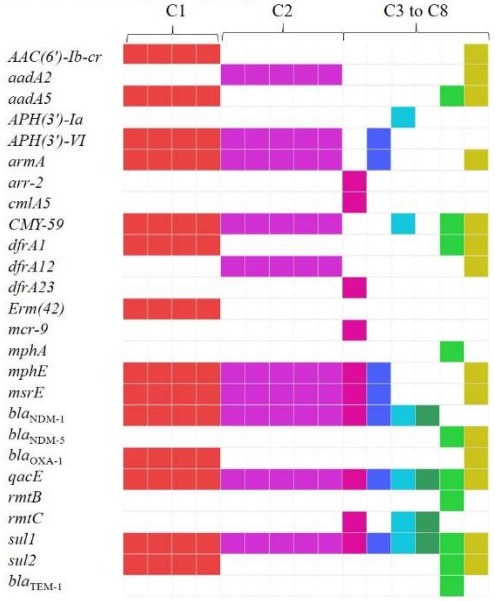
Plasmids belonged to group A



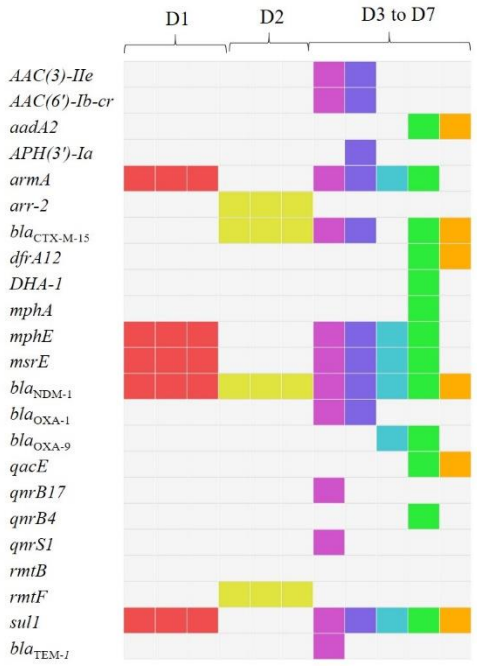
Plasmids belonged to group B



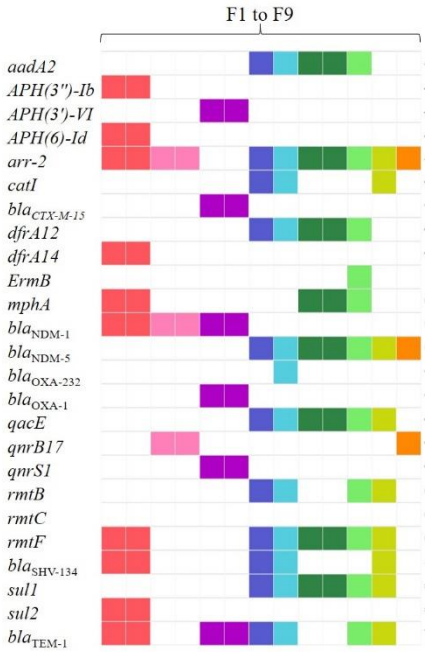
Plasmids belonged to group C



Plasmids belonged to group D



Plasmids belonged to group F



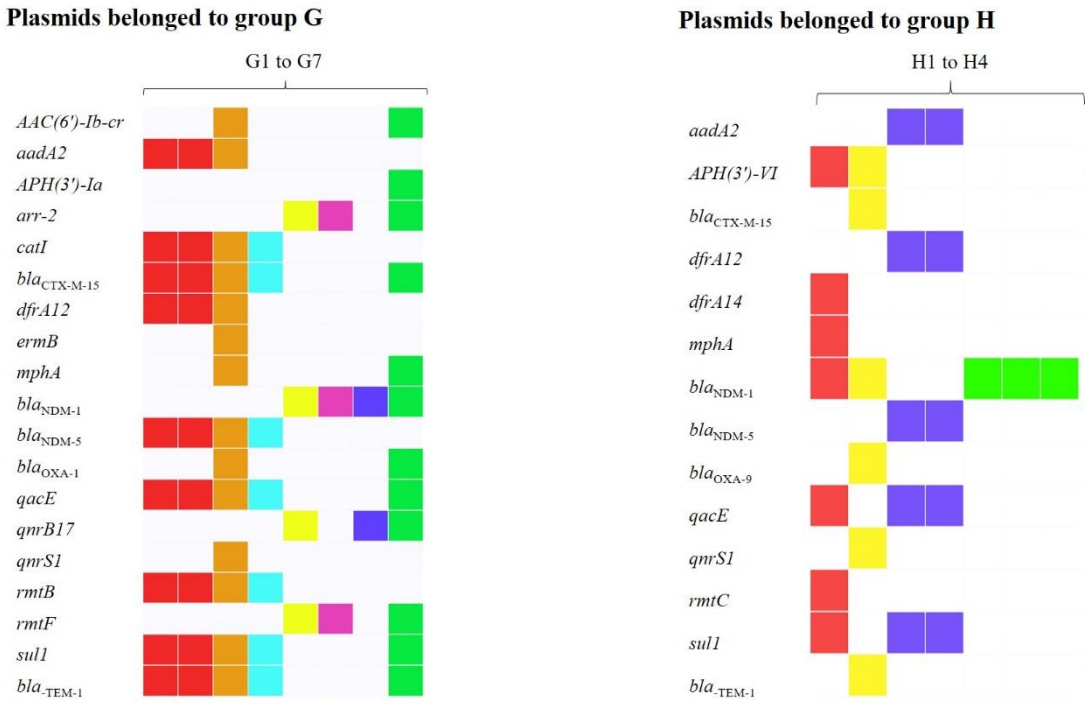


Figure 6.3 Clustering of plasmids in different plasmids' groups based on identical resistance pattern. Plasmids were grouped according to presence or absence of genes, mentioned in Figure 6.1. Complete, circular sequences of plasmids harbouring *bla*$_{NDM}$ (n=133) were screened for the presence of resistance genes. Known ARGs were retrieved using CARD with a cut off ≥99.8% coverage, and ≥99.8% identity. Cells coded by colours indicate the presence of genes, and white cells indicate the absence of respective genes. Plasmids in each group are represented as clusters by individual colour based on identical resistance patterns.

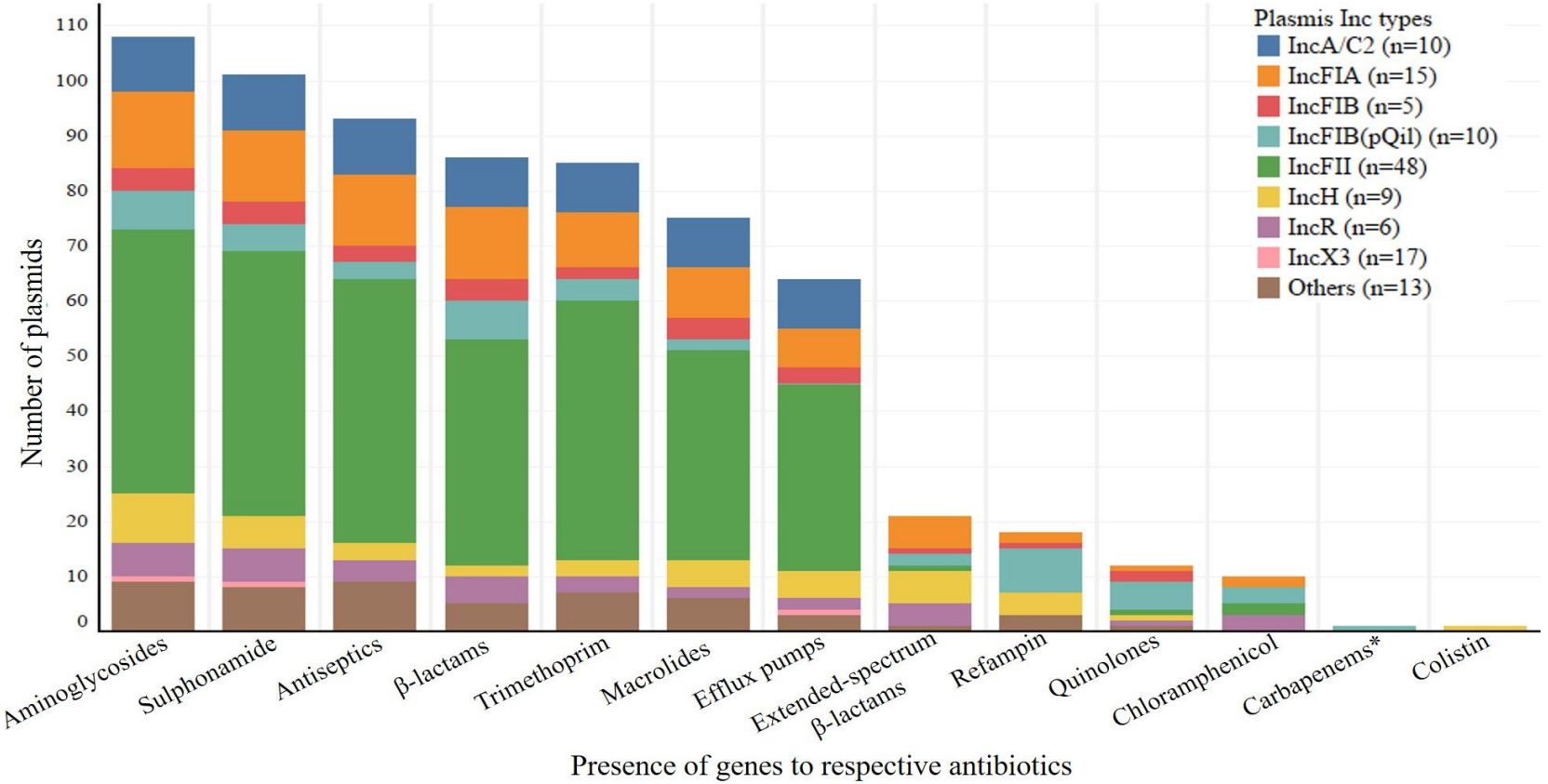


Figure 6.4 The prevalence of resistance to different antimicrobials among the plasmids harbouring *bla*$_{NDM}$. *Resistance to carbapenem indicates the presence of gene other than *bla*$_{NDM}$. Plasmids within others were IncFIB & IncFII, (n=2), IncFIB(pQil) & IncFII (n=1), IncFIB & IncA/C2 (n=1), IncFII & IncC (n=1), IncC (n=1), and unknown (n=7).

Figure 6.5 Genetic context of *mcr-9* in plasmid belonged to C3 (IncHI2).

6.2.3 Investigating transmission of *bla*$_{NDM}$ due to horizontal transfer of plasmid

Sixteen sub-groups of plasmids were screened based on common Inc type, identical resistance patterns and similar molecular weight which contained more than one plasmid. The sub-groups were: A1-b (n=25), A2-b (n=3), A2-c (n=4), A4 (n=2), B1 (n=4), B11 (n=2), C1 (n=4), C2 (n=5), D1-a (n=2), D2 (n=3), E1 (n=10), E2 (n=6), F1 (n=2), F2 (n=2), F3 (n=2), and G1 (n=2) (Table 6.1).

Horizontal transfer of *bla*$_{NDM}$ harbouring plasmids were predicted in A1-b [IncFII (n=25)], A2-b [IncFII (n=3)], E1 [IncX3 (n=10)], E2 [IncX3 (n=6)], and F3 [IncFIB(pQil) (n=2)] considering the distribution of plasmids among different species of Enterobacterale or different clones of species pertinent to each sub-group. Plasmids belonged to A2-c, A4, B1, B11, C1, C2, D1-a, D2, F1, F2, F3, and G1 were specific for a particular clone of Enterobacterales. However, plasmids in group A1-b distributed in *E. coli* [ST10820 (n=1), ST167 (n=1), ST2659 (n=1), ST405 (n=1), ST448 (n=1), ST9665 (n=1)], *K. pneumoniae* [ST23 (n=10), ST515 (n=3), ST147 (n=1), ST16 (n=1), ST490 (n=1)], *E. cloacae* (n=1), a single clone of *C. rodentium* (n=2), A2-b in *E. coli* [ST101 (n=2), ST617 (n=1)], E1 in *E. coli* [ST448 (n=3), ST167 (n=3)], *K. pneumoniae* ST16 (n=1), *E. cloacae* ST113 (n=3), E2 in *E. coli* [ST101 (n=1), ST448 (n=1) ST101 (n=1)], *E. cloacae* ST45 (n=1), *E. cloacae* ST696 (n=1), *C. farmeri* (n=1), and F3 in *K. pneumoniae* ST14 (n=1), *K. variicola* (n=1) (Table 6.1). Genomic comparisons of plasmid sequences were performed which showed >99% identity, and >99% coverage at the nucleotide level among the plasmids in A1-b (Figure 6.6), A2-b (Figure 6.7), E1 (Figure 6.8), E2 (Figure 6.9), and F3 (Figure 6.10).

Plasmids belonged to G4, and G5 were carried by *K. pneumoniae* ST152, and *K. pneumoniae* ST16, respectively which were identical in size, but differed by one resistance gene, *qnrB17* according to 'CARD' database with cut off ≥99.8% coverage, and ≥99.8% identity (Figure 6.3; Table 6.1), however, *qnrB17* with 57% coverage and 100% identity was found in G5 plasmid. Genomic comparison complete, circular sequences of whole plasmids of G4 and G5 plasmids exhibited >99.74% identity, and 97% coverage at the nucleotide level.

Table 6.1 Stratification of plasmids based on resistance patterns, Inc types, and plasmid size (n=133).

Group	Plasmid Inc type	Size of plasmid	Subgroup designation (n)	Distribution of plasmids
A	IncFII	~80 kb	A1-a (1)	*E. coli* ST2083
		~92 kb to ~99 kb	A1-b (25)	*E. coli* [ST10820 (1), ST167 (1), ST2659 (1), ST405 (1), ST448 (1), ST9665 (1)], *K. pneumoniae* [ST23 (10), ST515 (3), ST147 (1), ST16 (1), ST490 (1)], *E. cloacae* (1), *C. rodentium* [(2); corresponding isolates differed by 5 SNPs]
		~101 kb	A1-c (2)	*K. pneumoniae* ST48
		~101 kb	A1-d (1)	*E. coli* ST167
		~70 kb	A2-a (1)	*E. coli* ST410
		~80 to ~82 kb	A2-b (3)	*E. coli* [ST101 (2), ST617 (1)]
		~91 kb to ~93 kb	A2-c (4)	*E. coli* ST8346
		~86 kb	A3 (1)	*E. coli* ST4542
		~95 kb to ~97 kb	A4 (2)	*E. coli* ST5954, *K. pneumoniae* ST23
		~87 kb	A5-a (1)	*E. coli* ST405
		~92 kb	A5-b (1)	*K. pneumoniae* ST11
		~80 kb	A6 (2)	*E. coli* ST405, *K. pneumoniae* ST11
		~93 kb	A7 (1)	*K. pneumoniae* ST147
	IncR	~112 kb	A2-d (1)	*E. coli* ST410
B	IncFIA	~129 kb to ~130kb	B1 (4)	*E. coli* ST167
		~107 kb	B2 (1)	*E. coli* ST648
		~124 kb	B3 (1)	*E. coli* ST1588

		~161 kb	B4 (1)	*E. coli* ST167
		~155 kb	B5 (1)	*E. coli* ST405
		~120 kb	B7 (1)	*E. coli* ST167
		~133 kb	B10 (1)	*E. coli* ST131
		~79 kb	B11 (2)	*E. coli* ST648
		~79 kb	B12 (1)	*E. coli* ST648
	IncFII	~127 kb	B6 (1)	*E. coli* ST648
	IncFIB	~128 kb	B8 (1)	*E. coli* ST405
		~123 kb	B9 (1)	*E. coli* ST405
C	IncA/C2	~287 kb to ~296 kb	C1 (4)	*P. stuartii* [isolates harbouring plasmids were differed by 11 to 28 SNPs]
		~154 kb to ~173 kb	C2 (5)	*K. pneumoniae* ST395
		~72 kb	C6 (1)	*K. pneumoniae* ST11
	IncHI2	~276 kb	C3 (1)	*E. coli* ST38
	Unknown	~111 kb	C4 (1)	*P. stuartii*
	IncFIB & IncA/C2	~304 kb	C5 (1)	*K. pneumoniae* ST15
	IncC	~196 kb	C7 (1)	*K. pneumoniae* ST515
	IncFII & IncC	~275 kb	C8 (1)	*K. pneumoniae* ST515
D	IncHI1B	~251 kb to ~279 kb	D1-a (2)	*K. pneumoniae* ST15
		~345 kb	D2 (3)	*K. pneumoniae* ST15
		~301 kb	D4 (1)	*K. pneumoniae* ST1998
		~242 kb	D6 (1)	*E. cloacae*
	IncR	~70 kb	D1-b (1)	*K. pneumoniae* ST572
		~152 kb	D3 (1)	*K. pneumoniae* ST17
	IncFIB	~215 kb	D5 (1)	*K. pneumoniae* ST147

	IncHI1A	~182 kb	D7 (1)	*E. cloacae*
E	IncX3	~46 kb to ~49 kb	E1 (10)	*E. coli* [ST448 (3), ST167 (3)], *K. pneumoniae* ST16 (1), *E. cloacae* ST113 (3)
		~45 kb to ~46 kb	E2 (6)	*E. coli* [ST101 (1), ST448 (1) ST101 (1)], *E. cloacae* ST45 (1), *E. cloacae* ST696 (1), *C. farmeri* (1)
		~58 kb	E3 (1)	*E. cloacae*
F	IncFIB(pQil)	~135 to ~137 kb	F1 (2)	*K. pneumoniae* ST15
		~168 kb	F2 (2)	*K. pneumoniae* ST16
		~119 kb	F3 (2)	*K. pneumoniae* ST14, *K. variicola*
		~133 kb	F4 (1)	*K. pneumoniae* ST231
		~134 kb	F5 (1)	*K. pneumoniae* ST231
		~133 kb	F8 (1)	*K. pneumoniae* ST231
		~163 kb	F9 (1)	*K. pneumoniae* ST16
	IncFIB & IncFII	~190 kb	F6 (2)	*K. pneumoniae* ST11
	IncFIB(pQil) & IncFII	~203 kb	F7 (1)	*K. pneumoniae* ST11
G	IncR	~143 kb	G1 (2)	*K. pneumoniae* ST23
		~143 kb	G3 (1)	*K. pneumoniae* ST23
	IncFII	~238 kb	G2 (1)	*K. pneumoniae* ST23
	IncFIA	~141 kb	G4 (1)	*K. pneumoniae* ST152
		~141 kb	G5 (1)	*K. pneumoniae* ST16
	IncFIB	~150 kb	G6 (1)	*K. pneumoniae* ST16
		~206 kb	G7 (1)	*K. pneumoniae* ST16
H	IncFII	~158 kb	H1 (1)	*E. cloacae*
	Unknown	~100 kb	H2 (1)	*S. marcescens*
	No plasmid background	~10 kb	H3 (3)	*E. coli* ST167, *E. coli* ST38

	No plasmid background	~8 kb	H4 (3)	*K. pneumoniae* ST15, *K. pneumoniae* ST152, *K. pneumoniae* ST16

Values in parenthesis represents the numbers of plasmids in the respective sub-groups. Sub-groups with possible horizontal transfer of plasmid are highlighted.

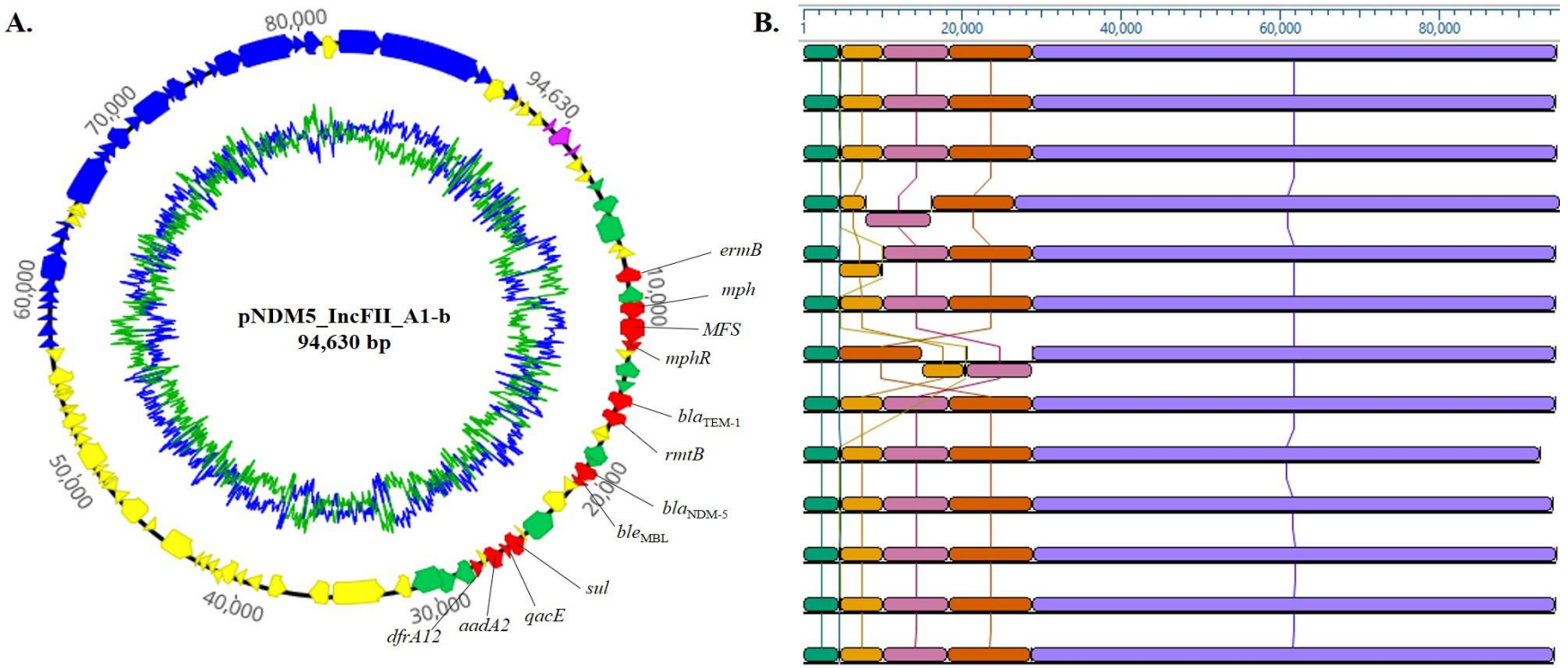


Figure 6.6 A. Schematic layout of IncFII plasmid of subgroup A1-b carrying *bla*$_{NDM-5}$ identified in this study. Arrows represent the position and transcriptional direction of the open reading frames. Resistance genes are represented by red, genes for mobile elements by green, genes associated with conjugation in blue, replication-associated genes in pink, regulatory/accessory/hypothetical proteins in yellow. **B.** Colinear alignment of IncFII plasmids harbouring *bla*$_{NDM-5}$ of sub-group A1-b (n=13). One plasmid from each clonal type was selected for the alignment. Alignment was performed by Mauve using DNASTAR (v17.1).

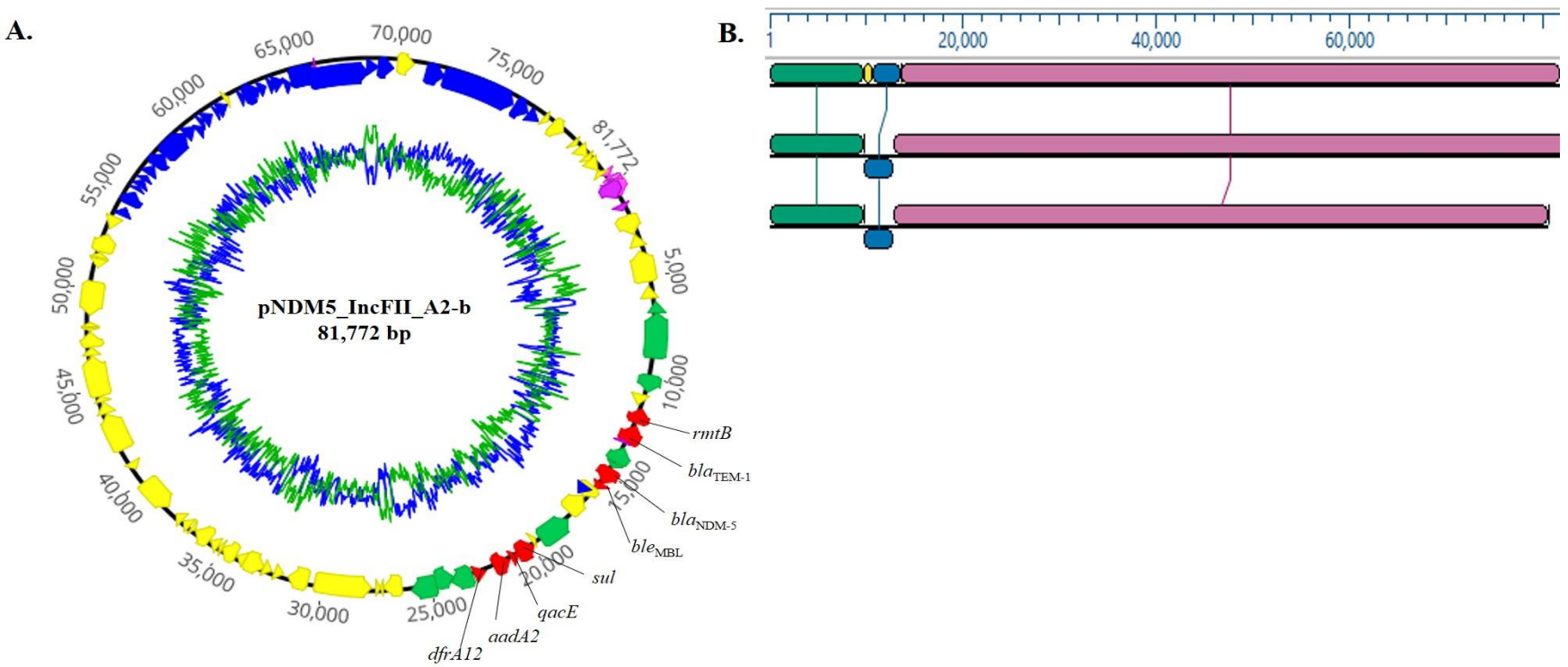
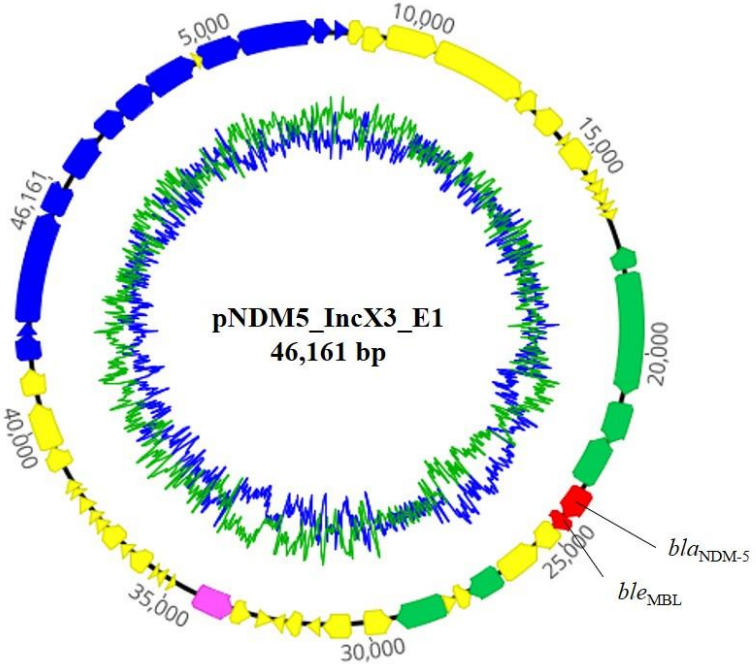


Figure 6.7 A. Schematic layout of IncFII plasmid of subgroup A2-b carrying *bla*$_{\text{NDM-5}}$ identified in this study. Arrows represent the position and transcriptional direction of the open reading frames. Resistance genes are represented by red, genes for mobile elements by green, genes associated with conjugation in blue, replication-associated genes in pink, regulatory/accessory/hypothetical proteins in yellow. **B.** Colinear alignment of IncFII plasmids harbouring *bla*$_{\text{NDM}}$ of sub-group A2-b (n=3). One plasmid from each clonal type was selected for the alignment. Alignment was performed by Mauve using DNASTAR (v17.1).

A.



B.

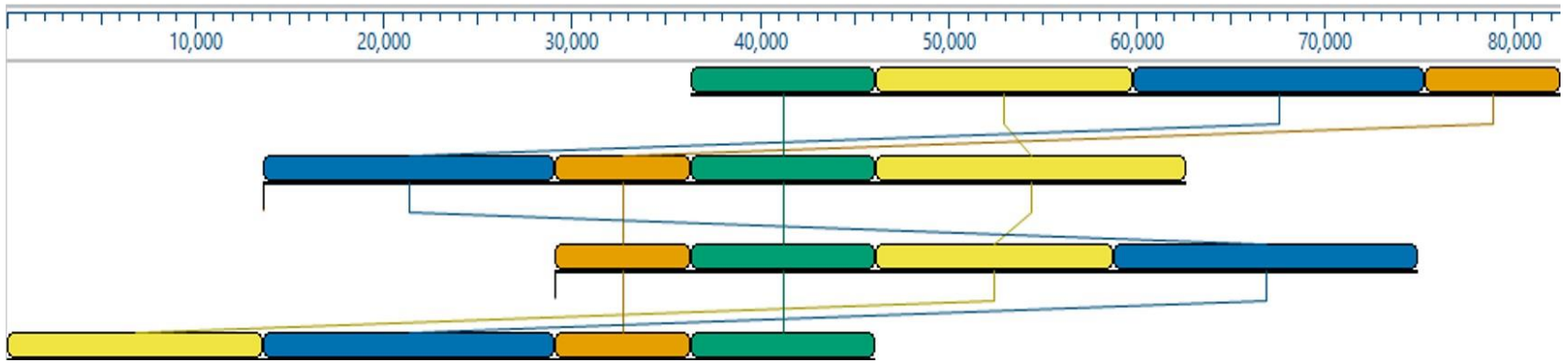


Figure 6.8 A. Schematic layout of IncX3 plasmid of subgroup E1 carrying bla_{NDM} identified in this study. Arrows represent the position and transcriptional direction of the open reading frames. Resistance genes are represented by red, genes for mobile elements by green, genes associated with conjugation in blue, replication-associated genes in pink, regulatory/accessory/hypothetical proteins in yellow. **B.** Colinear alignment of IncFII plasmids harbouring bla_{NDM} of sub-group E1 (n=4). One plasmid from each clonal type was selected for the alignment. Alignment was performed by Mauve using DNASTAR (v17.1).

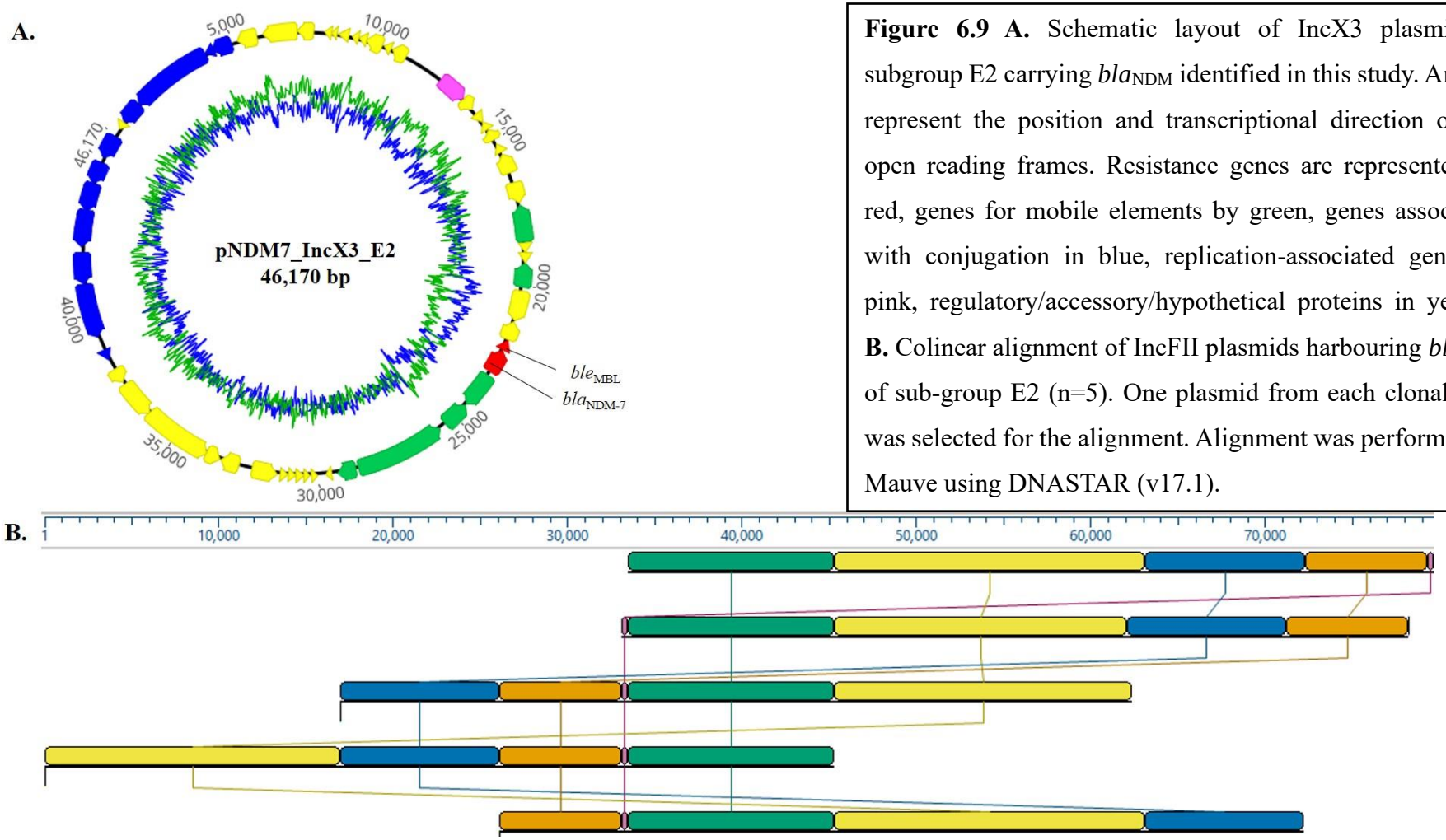


Figure 6.9 A. Schematic layout of IncX3 plasmid of subgroup E2 carrying *bla*$_{NDM}$ identified in this study. Arrows represent the position and transcriptional direction of the open reading frames. Resistance genes are represented by red, genes for mobile elements by green, genes associated with conjugation in blue, replication-associated genes in pink, regulatory/accessory/hypothetical proteins in yellow. **B.** Colinear alignment of IncFII plasmids harbouring *bla*$_{NDM}$ of sub-group E2 (n=5). One plasmid from each clonal type was selected for the alignment. Alignment was performed by Mauve using DNASTAR (v17.1).

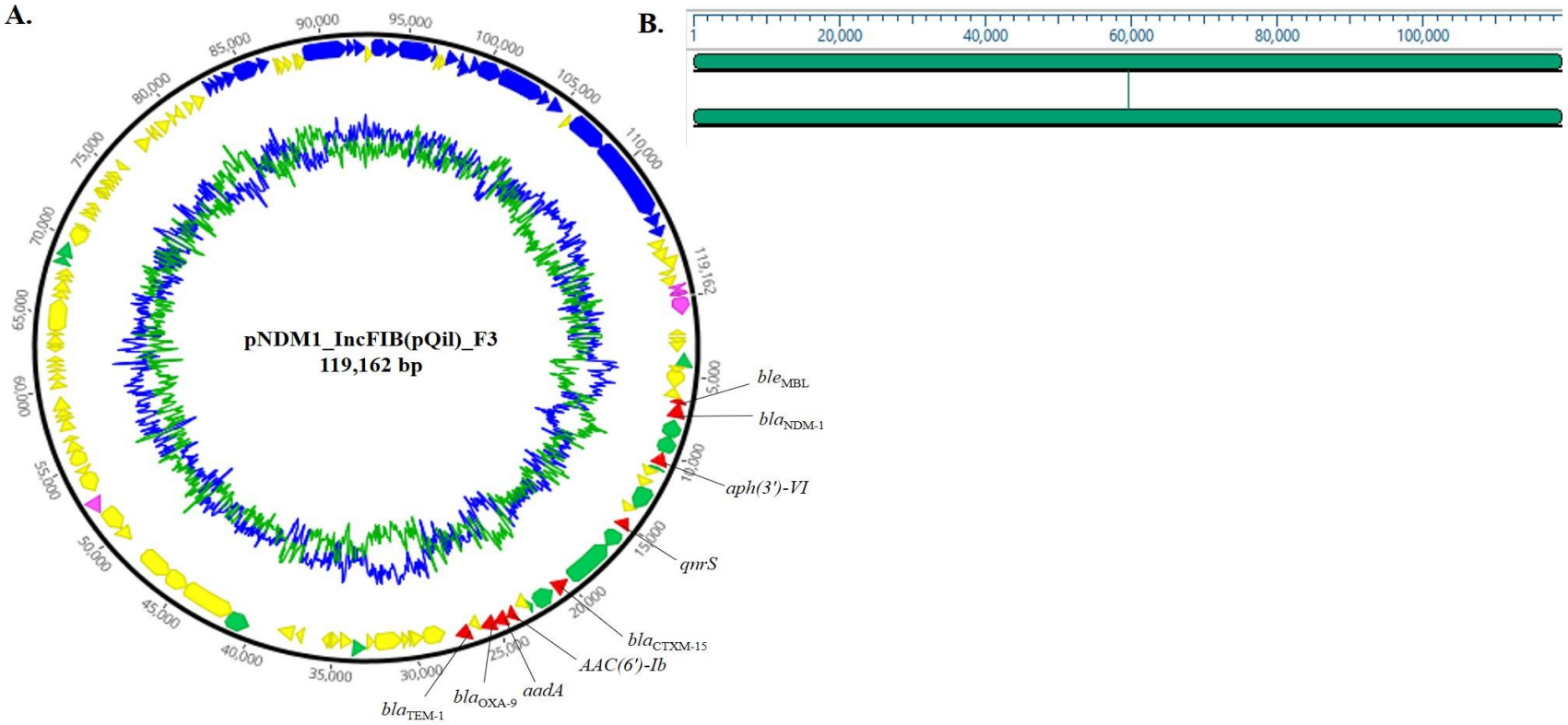


Figure 6.10 A. Schematic layout of IncFIB(pQil) plasmid of subgroup F3 carrying *bla*$_{NDM}$ identified in this study. Arrows represent the position and transcriptional direction of the open reading frames. Resistance genes are represented by red, genes for mobile elements by green, genes associated with conjugation in blue, replication-associated genes in pink, regulatory/accessory/hypothetical proteins in yellow. **B.** Colinear alignment of IncFIB(pQil) plasmids harbouring *bla*$_{NDM}$ of sub-group F3 (n=2). One plasmid from each clonal type was selected for the alignment. Alignment was performed by Mauve using DNASTAR (v17.1).

6.2.4 Investigating possible role of mobile genetic elements in the spread of *bla*$_{NDM}$

The genetic context of each variant of *bla*$_{NDM}$ [*bla*$_{NDM-5}$ (n=84), *bla*$_{NDM-1}$ (n=40), *bla*$_{NDM-7}$ (n=6) and *bla*$_{NDM-4}$ (n=3)] was evaluated separately.

6.2.4.1 Evaluating the genetic context of *bla*$_{NDM-5}$

A conserved region consisted of incomplete IS*Aba125*, *bla*$_{NDM-5}$, *ble*$_{MBL}$, *trpF*, *dsbD* was common across all plasmids harbouring NDM-5 (Figure 6.11).

The conserved region of *bla*$_{NDM-5}$ in association with complex class 1 integron (IS*91*-hypothetical protein (HP)-*sul1-qacE-aadA*-HP-*dfrA*- *intI*), flanked by intact IS*26* at both 3' and 5' end in same orientation was found in group A1 to A6 [IncFII (n=44), IncR (n=1)], B1 [IncFIA (n=4)], B3, B7, B10 [IncFIA (n=3)], F6 [IncFIB & IncFII (n=2)], G1 [IncR (n=2)], G2 [IncFII (n=1)] (Figure 6.11A). Group B5 [IncFIA (n=1)] and B9 [IncFIB (n=1)] plasmids had similar genetic structure around *bla*$_{NDM-5}$, however terminal flanking regions of IS*26* were in opposite direction (Figure 6.11B).

IS*26*-flanked (same direction at each end) conserved region with a little variation of complex class 1 integron (IS*91*-HP-*sul1-qacE-trA-aadA-dfrA-intI*) were found in plasmids belonged to F4 [IncFIB(pQil) (n=1)], F5 [IncFIB(pQil) (n=1)], and F7 [IncFIB(pQil) & IncFII (n=1)] (Figure 6.11C).

IS*26*-flanked (same direction at each end) conserved segment with truncated complex class 1 integron were found in plasmid of A7 [IncFII (n=1)] (Figure 6.11D). Plasmid of group G3 [IncR (n=1)] also had IS*26*-flanked truncated complex class 1 integron, where the IS*26* at both ends were in opposite directions (Figure 6.11E).

An upstream integration of Tn*3*-derived *qnrB* was present with the conserved segment composed of IS*Aba125-bla*$_{NDM-5}$*-ble*$_{MBL}$*-trpF-dsbD-cutA-gorES* in F9 plasmid [IncFIB(pQil) (n=1)]. The intervening region was flanked by IS*26* in opposite direction (Figure 6.11F).

IS*26*-flanked conserved region of *bla*$_{NDM-5}$ with variable downstream was present in B8 [IncFIB (n=1)] (Figure 6.11G), F8 [IncFIB(pQil) (n=1)] (Figure 6.11H), B2 [IncFIA (n=1)], B4 [IncFIA (n=1)], B6 [IncFII (n=1)] (Figure 6.11I), C7 [IncC (n=1)] and C8 [IncFII & IncC (n=1)] (Figure 6.11J).

The conserved region of group E1 [IncX3 (n=10)] also was flanked by IS*5* at the upstream and an intact IS26 at the downstream. An intact IS*Aba125* was flanked upstream of IS*5* (Figure 6.11K).

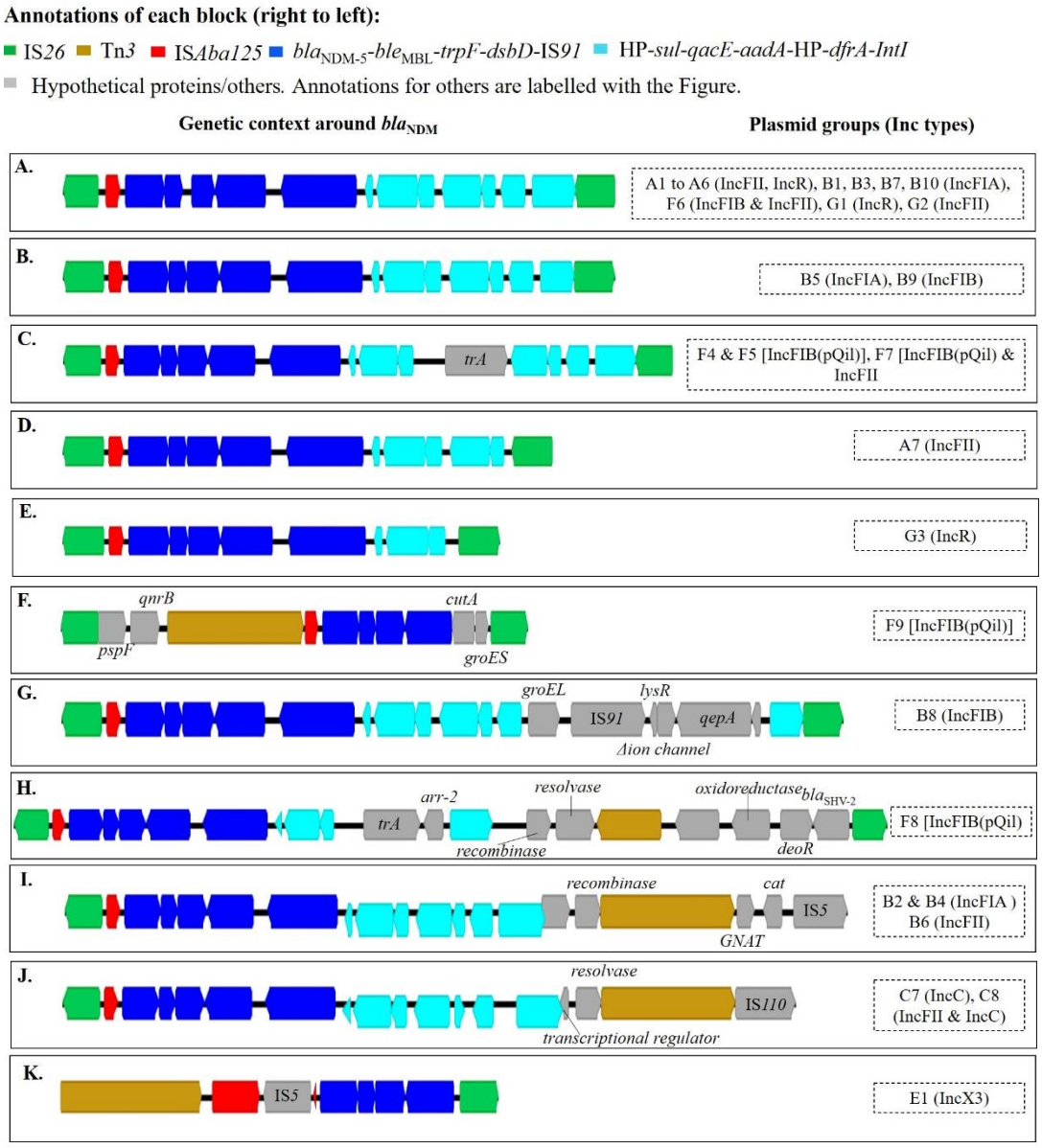


Figure 6.11 Genetic context around *bla*$_{NDM-5}$ in different plasmid backgrounds.

6.2.4.2 Evaluating the genetic context of *bla*$_{NDM-1}$

The genetic structure, Tn*125* [*bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF-dsbD-cutA-groES-groEL*-IS*91*, bordered by intact IS*Aba125* at upstream and downstream] with IS*3*-flanked *aph(3')-VI* at the upstream of Tn*125* was observed in plasmid of C1 (IncA2/C) (n=4) (Figure 6.12A). Similar genetic composition except the flanking region of IS*Aba125* at the downstream was existed among the plasmids of varied Inc types C2 [IncA/C2 (n=5)] (Figure 6.12B), C4 [unknown (n=1)] (Figure 6.12C), C5 [IncFIB & IncA/C2 (n=1)] (Figure 6.12D), and H1 [IncFII (n=1)] (Figure 6.12E), while the region was truncated in F3 [IncFIB(pQil) (n=2)] and H2 [unknown (n=1)] (Figure 6.12F). The intervening *bla*$_{NDM-1}$-containing region including IS*3*-flanked *aph(3')-VI* were shown to be flanked by IS*26* at each end in same direction in group C1 (IncA/C2) (Figure 6.12A) and C4 (unknown background) (Figure 6.12C).

IS*Aba125* at the upstream of conserved segment (*bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF-dsbD-cutA-groES-groEL*-IS*91*) was incomplete in C5 (IncFIB & IncA/C2) (Figure 6.12D), D6 [IncH (n=1)] and D7 [IncH (n=1)] (Figure 6.12G) and original structure of *bla*$_{NDM}$ conserved region was lost in D2 (IncH) by Tn*3*, IS*630*, and IS*3*, and in F3 [IncFIB(pQil)], and H2 (unknown background) by IS*630* and IS*26* (Figure 6.12H).

The conserved region of *bla*$_{NDM-1}$ (incomplete IS*Aba125bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF-dsbD-cutA-groES-groEL*) was located in between two Tn*3* of same direction in IncFIB(pQil), IncFIA, and IncFIB [plasmids belonged to FI (n=2), F2 (n=2), G4 (n=1), G5 (n=1), G6 (n=1), and G7 (n=1), of which upstream was flanked by complete Tn*3* of 3235 bp, while the Tn*3* at downstream was truncated (Figure 6.12I). Two ends of entire region were flanked by IRs of 49 bp.

The genetic structure composed of incomplete IS*Aba125*, *bla*$_{NDM-1}$, *ble*$_{MBL}$, *trpF*, lysR, *qacE*, *sul*, hypothetical protein, and IS*91* was shared by the plasmids belonged to C3 [IncHI2 (n=1), C6 [IncA/C2 (n=1), D1-a [IncH1B (n=2)], D1-b [IncR (n=1)], D3 [IncR (n=1), D4 [IncHI1B (n=1), D5 [IncFIB (n=1), and E3 [IncX3 (n=1)] (Figure 6.12J). Upstream and downstream of this conserved segment were variable among the plasmids.

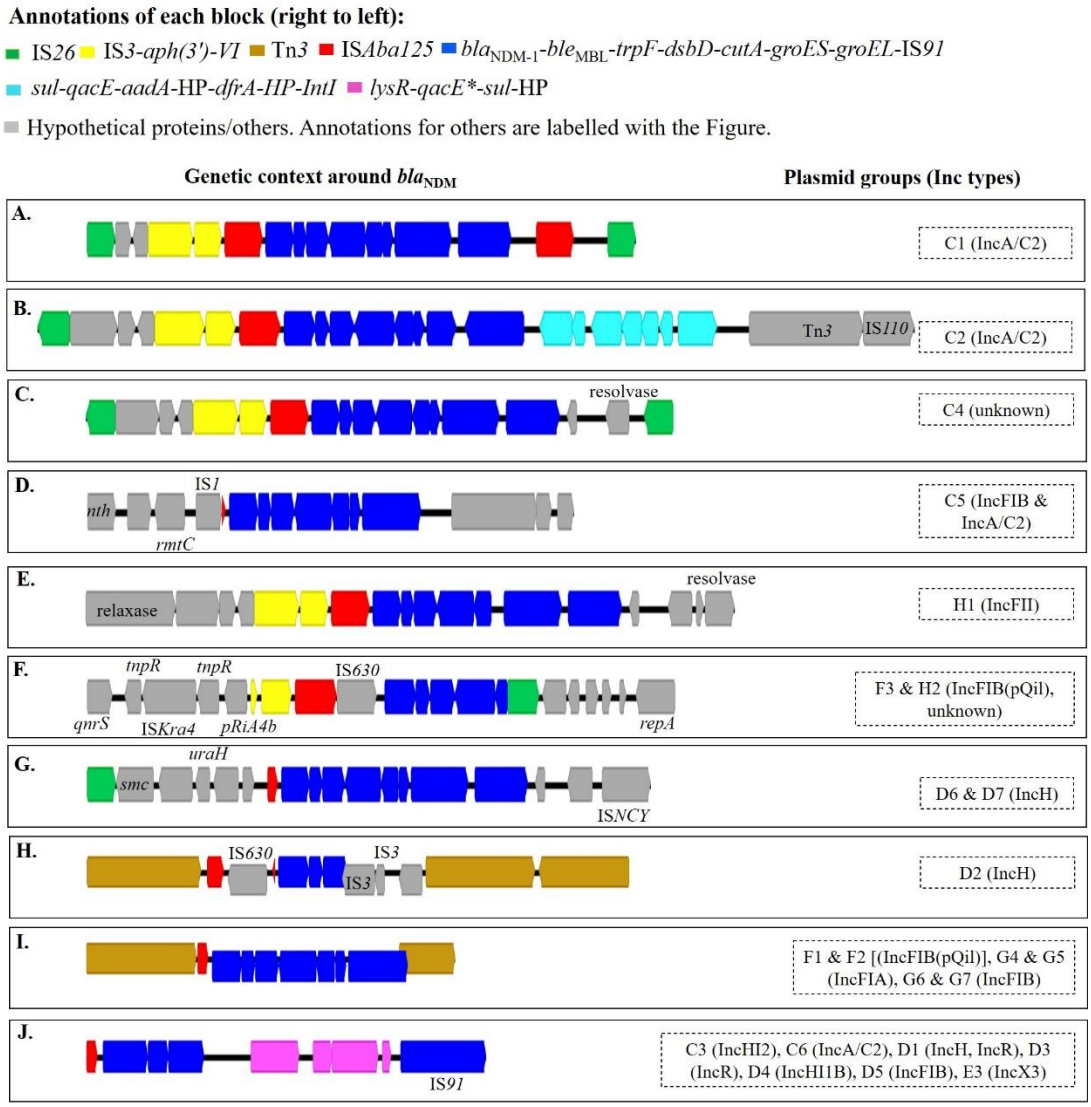


Figure 6.12 Genetic context around *bla*NDM-1 in different plasmid backgrounds. **qacE* was present in the plasmid at 70% coverage.

6.2.4.3 Evaluating the genetic context of *bla*$_{NDM-4}$ and *bla*$_{NDM-7}$

All plasmid harbouring *bla*$_{NDM-7}$ (n=6) belonged to group E2 (IncX3) (Table 6.1). The region consisted of *bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF*-*dsbD* was flanked by IS*26* at the upstream. An intact IS*Aba125* was flanked at the upstream of IS*5* (Figure 6.13A).

Plasmid harbouring *bla*$_{NDM-4}$ belonged to IncFIA (n=3) under the group of B11 and B12 (Table 6.1). The partner components of *bla*NDM (IS*Aba125bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF*-*dsbD*-IS*91*) were flanked by IS*26* at the upstream and downstream in same direction (Figure 6.13B).

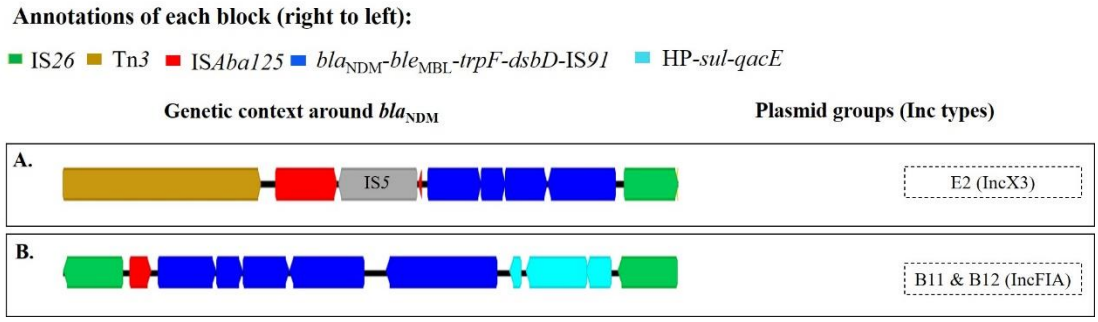


Figure 6.13 Genetic context around *bla*$_{\text{NDM-7}}$ and *bla*$_{\text{NDM-4}}$ in different plasmid backgrounds.

6.3 Discussion

The gene encoding *bla*$_{NDM}$ is mostly located on conjugative plasmids with wide replicon types which are often associated with the determinants of multiple antibiotic resistance. The most frequent plasmid Inc types among the species of Enterobacteriaceae in relation to *bla*$_{NDM}$ were IncX3, IncFII and IncC. The distributions of *bla*$_{NDM}$ among the different Inc types reported are likely to be biased as substantial proportion of *bla*$_{NDM}$ harbouring plasmids were characterized from the Chinese isolates followed by isolates from North America. The plasmids' background related to *bla*$_{NDM}$ from Indian subcontinent and Middle East are largely unknown where the particular resistance was found to be endemic (Khan *et al.,* 2017; Dadashi *et al.,* 2019; Pecora *et al.,* 2019; Wu *et al.,* 2019). In this study, *bla*$_{NDM}$ was most frequently found in IncFII (n=48), IncX3 (n=17), IncFIA (n=15), IncA/C2 (n=10), IncFIB(pQil) (n=10), IncH (n=9) among the plasmids characterized (Figure 6.1; Table 6.1).

Regardless of Inc types, all the NDM-positive plasmids carried the determinants of multidrug resistance except IncX3 (Figure 6.2; Figure 6.3). Referring to '*Chapter 4*', a cluster of 167 isolates (designated as 'HT5') was identified which shared the six resistance genes (*bla*$_{NDM-5}$, *bla*$_{TEM-1}$, *aadA2*, *rmtB*, *sul1*, and *dfrA12*) (Figure 4.6). This chapter documented plasmid-mediated spread of *bla*$_{NDM-5}$ in association with IncFII [A1-b (n=25); A2-b (n=3)] by long read sequencing and further scrutinization (Figure 6.2; Figure 6.3; Figure 6.7; Figure 6.8; Table 6.1). IncFII plasmids belonged to A1 and A2 coharboured *bla*$_{NDM-5}$, *bla*$_{TEM-1}$, *aadA2*, *rmtB*, *sul1*, and *dfrA12* (Figure 6.3), strongly suggesting IncFII as dominant vector of disseminating carbapenem resistance in Bangladeshi hospital. The gene, *bla*$_{NDM-5}$ was present in IncFII plasmids (belonged to A1 and A2) as incomplete IS*Aba125*-*bla*$_{NDM-5}$-*ble*$_{MBL}$-*trpF*-*dsbD*-S*91*-HP-*sul1*-*qacE*-*aadA*-HP-*dfrA*-*intI* which was flanked by IS*26* at both end (Figure 6.11A). IS*26*-flanked regions at both end in same orientation could be circularised and excised the intervening region from donor plasmid by IS*26* and therefore, inserted into plasmids of varied replicon types through IS*91*-mediated homologous recombination (Pecora *et al.,* 2019).

In this study, horizontal transfers of IncX3 (group E1 and E2) were also shown to be responsible for the spread of *bla*$_{NDM-5}$ and *bla*$_{NDM-7}$, respectively in wide host

range (Table 6.2; Figure 6.8; Figure 6.9). IncX3 is highly conjugative plasmid, has been considered as epidemic plasmid for the worldwide dissemination of *bla*$_{NDM}$ (Paul *et al.,* 2017; Li *et al.,* 2018a; Wang *et al.,* 2018c; Liu *et al.,* 2019d). In this study, the plasmids belonged to E1 and E2 were low molecular weight (ranged from ~46 kb to ~49 kb) and carried only *bla*$_{NDM}$ as resistance gene. The conserved region of *bla*$_{NDM}$ (*bla*$_{NDM-1}$-*ble*$_{MBL}$-*trpF-dsbD*) in IncX3 belonged to E1 and E2 was flanked by IS*5* at the upstream. An intact IS*Aba125* was found upstream of IS*5* (Figure 6.11K; Figure 6.13A). Plasmids of group E1 and E2 were highly similar to the plasmids in NCBI archives (accession no: MK317995.1, CP036179.1 MN064714.1, CP040446.1, CP032889.1, CP021692.1). However, IncX3 plasmid harbouring *bla*$_{NDM-1}$ belonged to E3 was exceptional which was associated with resistance determinants, *armA*, *sul*, *msrE* and qacE at a low coverage (Figure 6.12J), however, horizontal transfer of IncX3 carrying *bla*$_{NDM-1}$ was not found. Plasmid-mediated dissemination of *bla*$_{NDM-1}$ occurred at DMCH with IncFIB(pQil) (group F3) and IncFIA (group G3 and G4) (Table 6.1; Figure 6.10).

The most worrying aspect in the clinical setting was that horizontal transfer of MDR plasmids or transposition of MDR determinants among the plasmids limited therapeutic options drastically (Figure 6.3; Figure 6.4). This study also found the presence of both *bla*$_{NDM-1}$ and *mcr-9* in a plasmid of IncH12 (designated as group C3 in this study) recovered from a clinical *E. coli* isolate (Figure 6.3), however, the isolate was shown to be colistin susceptible. MCR-9 is a newly emerging mechanism in Enterobacteriaceae, has been reported previously in colistin susceptible *Salmonella* spp. and *Enterobacter* spp. (Carroll *et al.,* 2019; Chavda *et al.,* 2019). MCR-9 expression is mediated by the TCS (*qseC* and *qseB*), downstream of the gene in colistin-induced cells (Kieffer *et al.,* 2019). In this study, *mcr-9* is located in between IS*1* and IS*5*, and lacked downstream regulatory genes (Figure 6.5). Regardless of colistin susceptibility, the finding warned the impending dissemination of colistin resistance among the carbapenem resistant isolates (Yuan *et al.,* 2019).

It has been postulated that *bla*$_{NDM}$ is the chimera of aminoglycoside resistance gene, *aphA6* and pre-existing *bla*$_{MBL}$, originated in *A. baumannii* and was disseminated to the species of Enterobacterales in the form of composite transposon, Tn*125*. Tn*125* was formed by the fusion of IS*Aba125* at the upstream of *bla*$_{NDM,}$ and a series of genes at the downstream consists of *ble*$_{MBL}$ (responsible for bleomycin

resistance), *trpF* (encoding a phosphoribosylanthranilate isomerase), *dsbD* (encoding a twin-arginine translocation pathway signal sequence domain protein), *cutA1* (encoding a periplasmic divalent cation tolerance protein), *groES-groEL* (encoding chaperonin), IS*CR27* and finally a second IS*Aba125*. An intact or remnants of IS*Aba125* has been found invariably at the upstream of *bla*$_{NDM}$, which acts as promotor for the expression of the gene (Wu *et al.*, 2019). Consistent to the previous reports, similar genetic context immediate to *bla*$_{NDM}$ was observed in this study (Figure 6.11; Figure 6.12; Figure 6.13). The classical partner components of *bla*$_{NDM-1}$, Tn*125* was found in group C1 plasmid (IncA2/C), indicating IS*Aba125*-mediated insertion of the gene into the plasmid background (Figure A). It can be speculated that IS*Aba125* was lost at the downstream in other plasmids due to subsequent recombination. Simultaneously, incomplete IS*Aba125* at the upstream was more frequent among the plasmids characterized in this study (Figure 6.11; Figure 6.12; Figure 6.13), predicting their primitive integration of *bla*$_{NDM-1}$ into plasmid as Tn*125* followed by decaying of adjacent elements and subsequent involvement of other IS in the mobilisation of *bla*$_{NDM}$ (Figure 6.11; Figure 6.12) (Khan *et al.*, 2017; Weber *et al.*, 2019; Wu *et al.*, 2019). Group D2 plasmid (IncH) characterised in this study can be the ideal example of enormous destruction of original *bla*$_{NDM}$ conserved region due to integration of several transposons such as Tn*3*, IS*630*, and IS*3* (Figure 6.12H). The conserved segment consisted of incomplete IS*Aba12*, *bla*$_{NDM-1}$, *ble*$_{MBL}$, *trpF*, *lysR*, *qacE*, *sul*, hypothetical protein and IS*91* was differed from typical *bla*$_{NDM}$ adjacent region, but in this study the region was common in wide range plasmid Inc types (IncHI2, IncA/C2, IncH, IncR, IncHI1B, IncFIB and IncX3) (Figure 6.12J), indicating common mode of transposition of *bla*$_{NDM}$ among the plasmids of varied replicon types. This conserved region was identical to previously described plasmids isolated from *K. pneumoniae* in USA (accession no: CP021709.1) and *E. coli* in Australia (accession no: KC999035.4).

Apart from IncFII, plasmids of wide replicon types [IncR, IncA/C2, IncFIB(pQil), IncC, IncFIA, IncFIB, IncFIB & IncFII, IncFIB(pQil) & IncFII, IncFII & IncC] had conserved segment of *bla*$_{NDM}$ flanked by IS*91* followed by class 1 integron or other partner components and the entire region was flanked by IS*26* at the upstream and downstream in same orientation (Figure 6.11; Figure 6.12; Figure 6.13). It was possible to transposition of IS*26*-flanked intervening segment by two recombination steps by both IS*26* and IS*91* where IS*26* aided to release segment from

donor plasmid and IS*91* facilitated to insert it inro recipient plasmid by rolling circle replication (Pecora *et al.,* 2019). The transposons, 'IS*91*' family differs from typical IS in structure and function, causes transposition though 'rolling circle replication'. IS*91* does not have IRs, the 'transposase' (*tnpA*) contains motif which resembles replication initiator protein *repA,* and IS*91* termini, called *ori91* (where transposition stars), and *ter91* (where transposition stops). However, IS*91* are capable to mobilise genetic elements by one end transposition involving *ori91* only (Garcillán-Barcia *et al.,* 2002; Wawrzyniak *et al.,* 2017). Moreover, IS*26* is the most frequently described transposons, found in association of resistance plasmid in Enterobacterales family which can transposes resistance gene by replicative mechanism in the form of composite transposon (He *et al.,* 2015; Harmer *et al.,* 2016; Takeuchi *et al.,* 2018).

IncFIB(pQil), IncFIA, and IncFIB plasmids belonged to group FI, F2, G4, G5, G6, and G7 contained *bla*$_{NDM}$ partner components in association with an intact Tn*3* at 5' and incomplete Tn*3* at 3' end (Figure 6.12I), and the region was bracketed by Tn*3* IRs of 49 bp. Transposons of Tn*3* family is widely distributed in all bacterial phyla, contributes a major role in spread of resistance gene by replicative transposition. It was possible to mobilise *bla*$_{NDM}$ conserved segment by two terminal unidirectional copies of Tn*3* from donor plasmid and therefore, replicative integration into the recipients (Nicolas *et al.,* 2015).

The findings of this study provided the evidence of multiple mechanisms of HGT for the spread of *bla*$_{NDM}$ at DMCH consistently. However, the possible major trajectories involved in HGT were plasmids of IncFII and IncX3, IS*26*, IS*91* and Tn*3*.

Section Seven

Outbreak of Hypervirulent Multi-Drug Resistant Klebsiella variicola Causing High Mortality in Neonates in Bangladesh

7.1 Introduction

Globally, 2.4 million neonatal death were recorded in 2019 which was approx. 6,700 deaths per day. Neonatal mortality caused by sepsis varies between countries, and according to UNICEF, sepsis accounts for neonatal mortality ranges from 0.4 to 27.4% (UNICEF, 2020a). The global burden of neonatal sepsis is the highest in SA and sub-Saharan Africa. The incidence of confirmed sepsis (culture-positive) in SA is 15.8 per 1,000 live birth although this is thought to be a gross underestimation due to supporting microbiology facilities (Ombelet *et al.,* 2018; Chaurasia *et al.,* 2019). The relentless increase in AMR is complicating the management of neonatal sepsis, particularly in developing societies, which have been compounded by additional factors such as: **1.** Poverty and lack of education **2.** Insufficient antenatal care (ANC) **3.** Poor washing practices and sanitation **4.** Underdeveloped health care systems and limited resources (%GDP spent on public health); **5.** Substandard IPC strategies **6.** Poor antimicrobial stewardship programs (Shane *et al.,* 2017; Bandyopadhyay *et al.,* 2018; Chaurasia *et al.,* 2019). Neonatal sepsis can be categorized broadly into 'early-onset neonatal sepsis' (EOS) whether the sepsis happens in the first 3 days of life by a vertically transmitted pathogens and 'late-onset neonatal sepsis' (LOS) whether the sepsis develops after 3 days or later by vertically or horizontally acquired pathogens. EOS usually develops either from transplacental microbial transmission or ascending entry of bacteria to uterus as a consequence of chorioamnionitis following prolonged rupture of the chorioamniotic membrane (PROM); however, on overcrowded LMIC NICUs or via caesarean sections, EOS can also occur nosocomially. LOS can result from microbial exposure from the surrounding environments such as family members, hospital personnel, nutritional sources, contaminated surfaces, or medical devices. Preterm low birthweight (LBW) neonates have a 3–10 times higher risk of developing sepsis than full-term normal birthweight infants (Shane *et al.,* 2017). Maternal risk factors causing neonatal sepsis includes preterm pregnancy, PROM, chorioamnionitis, and UTI (Simonsen *et al.,* 2014; Shane *et al.,* 2017). Relevant meta-data on Bangladesh are stated in Table 7.1. A report by 'DhakaTribune' in 2019 stated that pre-term birth is the main cause of neonatal mortality in Bangladesh. Additionally, poor nutritional status of pregnant mothers, and early pregnancies result the birth of LBW infants (DhakaTribune, 2019; UNICEF-WHO, 2019; UNICEF, 2020a; UNICEF, 2020b).

Table 7.1 Relevant meta-data for analysing the situation of neonatal sepsis in Bangladesh.

Indicators	Estimates
Birth rate (crude)	18 per 1,000 people (nominal, 2018)
Neonatal mortality rate	17% (nominal, 2019)
Neonatal mortality due to sepsis	19.8% (nominal, 2017)
Percentage of receiving ANC1 at least once	75.2% (nominal, 2019)
Percentage of receiving ANC4 four times	36.9% (nominal, 2019)
Percentage of women who give birth before age 18	24.2% (nominal, 2019)
Percentage of deliveries in a health facility	53.4% (nominal, 2019)
Percentage of deliveries by Caesarean section	36% (nominal, 2019)
Percentage of newborns who have a postnatal contact with a health provider within 2 days of delivery	66.7% (nominal, 2019)
Pre-term birth	20% (nominal, 2019)
Neonates born with LBW	27.8% (nominal, 2015)
Stillbirth	230 per day

Members of the "*K. pneumoniae* complex" can be classified into three phylogenetic groups, termed as KpI, KpII, and KpIII. KpI includes *K. pneumoniae, K. rhinoscleromatis* and *K. ozaenae*. KpII comprises *K. quasipneumoniae* and KpIII comprises *K. variicola* (Brisse *et al.*, 2014). Because of sharing common biochemical and phenotypic properties, *K. variicola* was misdiagnosed as *K pneumoniae* several years ago. *K. variicola* was first described as a novel species in 2004, considered as an endophyte in plant and as pathogen in human. The species, *K. variicola* is distributed in wide range of STs, isolated from a diverse host types such as plants, animals, insects, environments, and human. A total of 166 STs of *K. variicola* has been identified so far from deposited global genomes. *K. variicola* has been now considered as an emerging hypervirulent pathogen for humans, reported worldwide from a variety of infections e.g. BSIs, respiratory tract infections, and UTIs. The added concern is that plasmid borne AMR genes (bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-15}}$, $bla_{\text{KPC-2}}$, $bla_{\text{NDM-1}}$, $bla_{\text{OXA-181}}$) have been found in *K. variicola*-associated infections (Barrios-Camacho *et al.*, 2019; Rodríguez-Medina *et al.*, 2019).

This chapter describes a MDR *K. variicola* outbreak on a NICU illustrated by an ideogram detailing the course of infection, phenotypic and genomic characterization, and clinical outcome, and fulfil '**objectives #2, # 3, and #4'** of the project.

7.2 Results

7.2.1 Tracking the outbreak by *K. variicola*

Blood cultures were taken from neonates (<30 days old) from Oct 2016 to March 2017 presenting with clinical sepsis were included in this cohort. Study outline is represented in Figure 7.1. Additional clinical history related to prenatal risk such as LBW, pre-term, and PROM were collected from culture-positive confirmed cases of neonatal sepsis. The prevalence of confirmed cases of sepsis in this cohort was 24.32% (36/148) (Figure 7.1). The species isolated from the sepsis cases are shown in Figure 7.2. During this period, a sepsis outbreak of *K. variicola* in the NICU was suspected during October 2016 to January 2017 retrospectively. A total of 14 outbreak cases (case 1 to case 14) of *K. variicola* was identified. All *K. variicola* isolates were clonal by PFGE (Figure 7.3). According to 7 loci MLST scheme of *K. pneumoniae*, the isolates belonged to ST771, and belonged to ST60 based on MLST scheme for *K. variicola*, described by Barrios-Camacho *et al.* (2019). Polymorphism variants (SNPs from WGS data on *K. variicola* isolated from case 2 to 14) ranged from 3 to 27 compared to *K. variicola* from case 1 (Table 7.2).

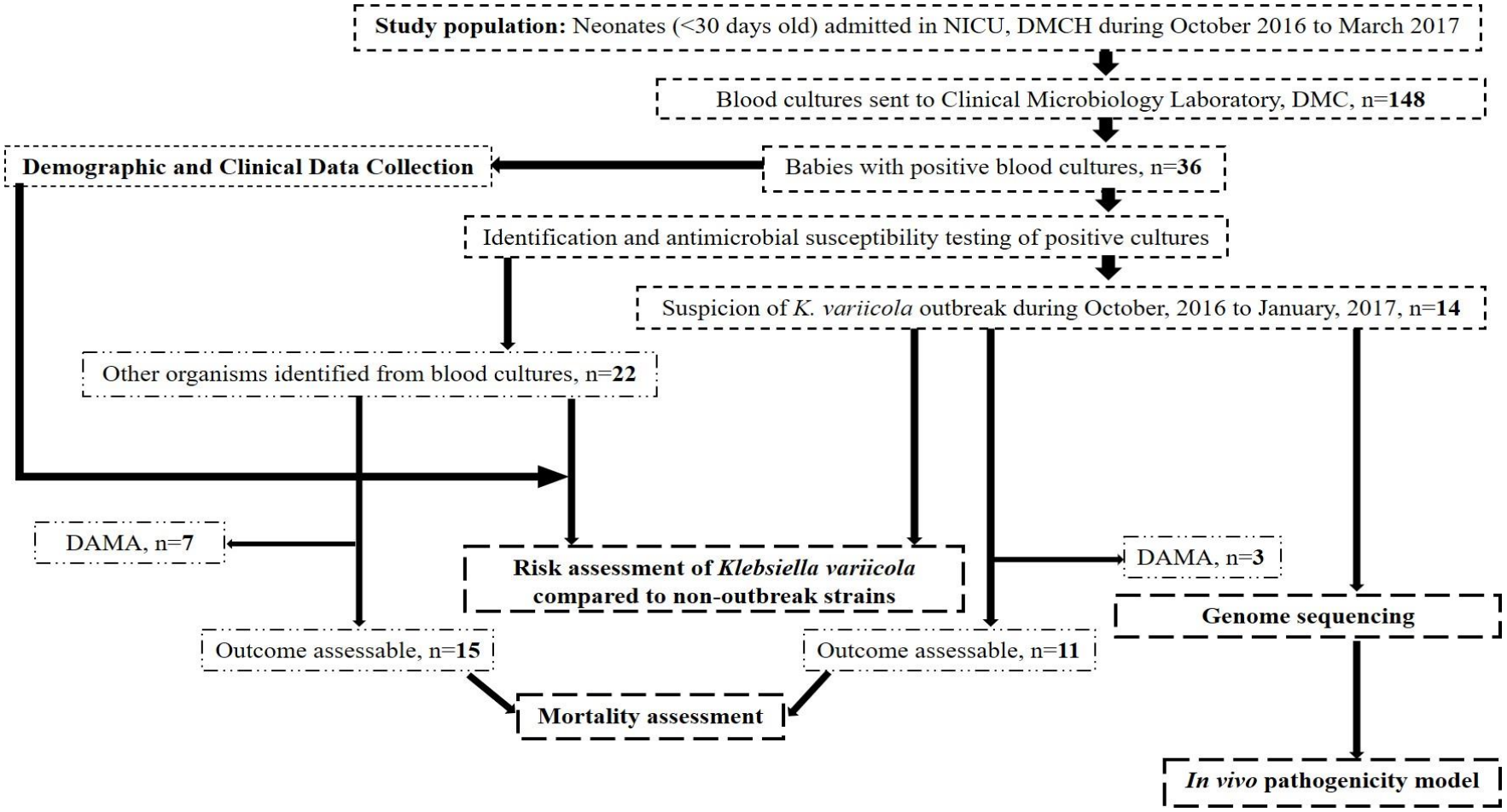


Figure 7.1 Study flowchart.

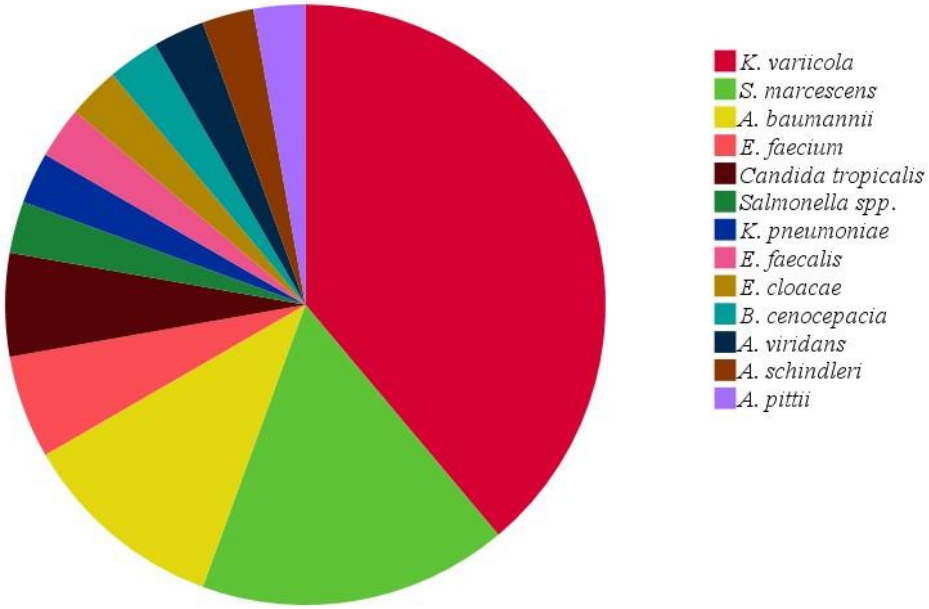


Figure 7.2 The species isolated from blood specimens from neonates during October 2016 to March 2017.

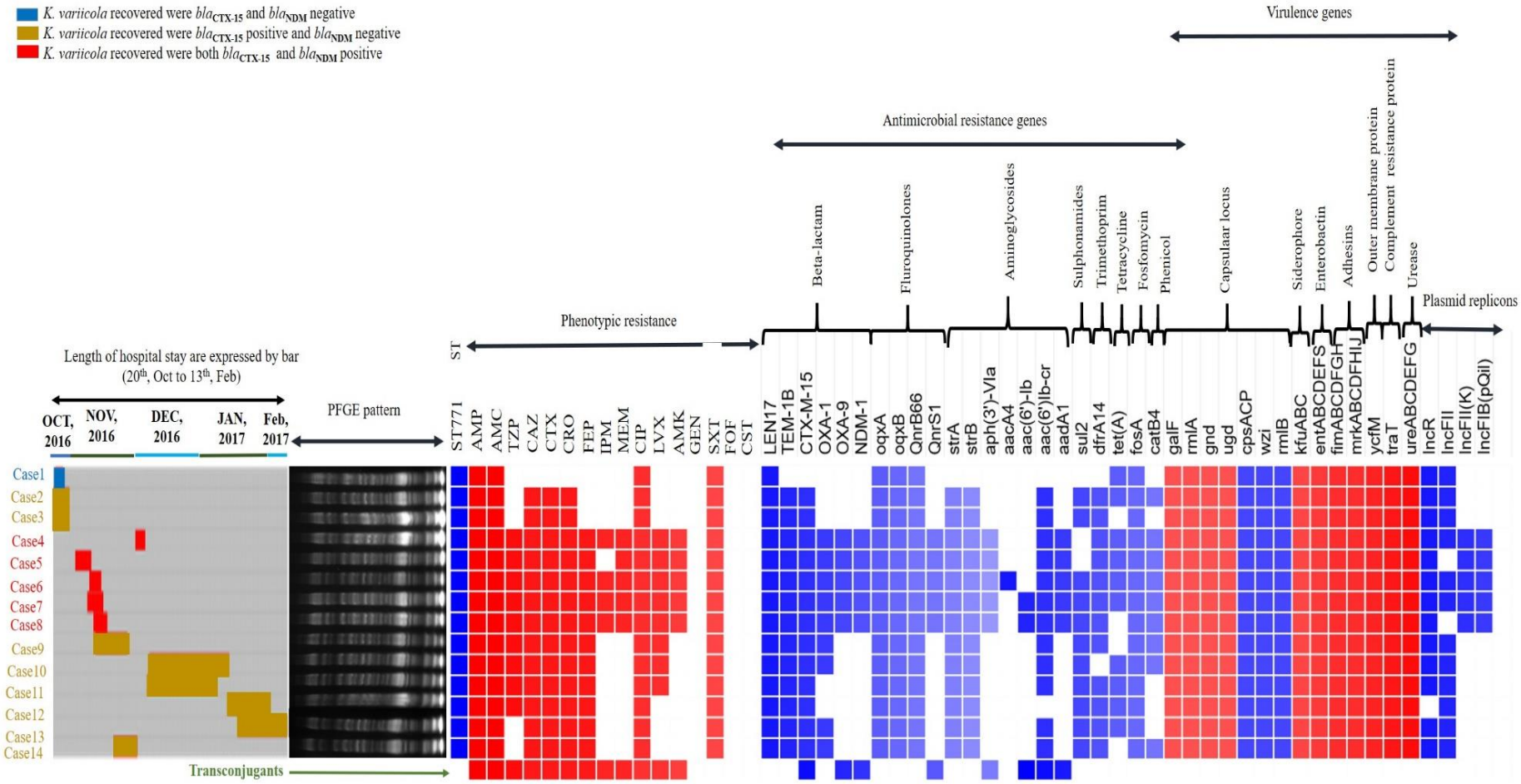


Figure 7.3 Antimicrobial resistance phenotypes and genetic profile of outbreak strains. AMP, ampicillin; AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP,

ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin. Antimicrobial sensitivity and absence of genes are indicated by white cells. Blue and red shades indicate phenotypic resistance and presence of genes by the isolates. Seven capsular genes were identified (*galF*, *rmlA*, *gnd*, *ugd* were identical to KL34 and *cpsACP* matches to KL153, *rmlB* to KL36 and *wzi* to KL114); 12 expected genes did not match to known capsular (KL) type. Capsular operon indicates a novel *K* locus in this clone. Entire capsular loci are depicted in Figure 7.4. Siderophores (yersiniabactin, aerobactin, salmochelin and colibactin) were absent in this clone.

Table 7.2 SNPs of WGS reads to 13 clonal *K. variicola* ST771 compared to first isolated *K. variicola* ST771 in this study.

Case	Strain ID	DOSC	VT	SNP	MNP	INS	DEL	Complex
2	dm-32	27.10.16	20	16	-	-	-	4
3	dm-33	27.10.16	3	3	-	-	-	
4	dm-97	04.12.16	8	7	-	-	-	1
5	dm-115	07.11.16	27	20	2	-	-	5
6	dm-165	12.11.16	13	9	1	-	-	3
7	dm-166	13.11.16	22	18	1	-	1	2
8	dm-167	13.11.16	12	12	-	-	-	
9	dm-169	14.11.16	18	15	1	-	1	1
10	dm-322	19.12.16	7	5	1	-	-	1
11	dm-323	19.12.16	11	11	-	-	-	
12	dm-395	19.01.17	12	10	1	-	-	1
13	dm-405	24.01.17	26	22	-	-	1	3
14	dm-414	24.11.16	20	16	-	-	-	4

DOSC, date of sample collection; VT, variants total; SNP, single nucleotide polymorphism; MNP, multiple nucleotide polymorphism; INS, insertion; DEL, deletion; Complex, combination of SNP/MNP.

7.2.2 Overview of the outbreak by *K. variicola*

K. variicola outbreak strain ST771 was first recovered from a premature (31 weeks), LBW baby admitted to DMCH on 21st of October 2016 and was DAMA (Table 7.3; Figure 7.3). This first isolate was negative for *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{NDM-1}}$. Subsequently two *K. variicola* from twins were identified on 27th October and had acquired a *bla*$_{\text{CTX-M-15}}$ plasmid. One twin died and the other was DAMA (case 2 & 3; Table 7.3). Five *K. variicola* (from cases four to eight; Table 7.3) isolated from 7th November to 4th December had an increased antibiotic resistance profiles, with the acquisition of an additional plasmid carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{NDM-1}}$. Interestingly, this plasmid was also shown to be positive for the resistance genes, *aph(3')-VIa, aadA1, qnrS1, bla*$_{\text{OXA-9}}$ *, aac(6')-Ib* and *aac(6')-Ib-cr* when transferred to *Escherichia coli* J53, and WGS confirmed the presence of these genes in transconjugants (Figure 7.3). One death was recorded among these five cases; three recovered and one DAMA (mortality is 25%, excluding DAMA). During this period (14th to 24th of November), the same clone but with a different resistance genotype (*bla*$_{\text{CTX-M-15}}$ positive, *bla*$_{\text{NDM-1}}$ negative) was isolated for cases 9 to 14. The outbreak cases admitted after 7th December (cases 10 to13; Table 7.3) were infected with *bla*$_{\text{NDM-1}}$-negative *K. variicola*. The mortality for neonates infected with *bla*$_{\text{CTX-M}}$ positive, *bla*$_{\text{NDM-1}}$ negative *K. variicola* was 71.42% (5/7) (not including patients who were DAMA.

All neonates received multiple antibiotics during NICU admission, either in combination or consecutively, with overall antibiotic usage being: 62% ceftriaxone, 53% amikacin, 31% vancomycin, 16% gentamicin, 15% carbapenem, 9% azithromycin, 6% colistin, 6% metronidazole and 3% ciprofloxacin.

Table 7.3 Characteristics of outbreak cases.

Case	Age (days)	Sex	SEC	POS	DOA	DOSC	Outcome	TS	Perinatal risk factors			Ab[c]	AMR genes pertinent to antibiotics used for sepsis			
									PT	PROM	LBW		*bla*$_{CTX-M-15}$	*bla*$_{NDM-1}$	*aph(3')-VIa*	*aadA1*
1	3	M	LM	EOS	21.10.16	23.10.16	DAMA	4	PT[b]	-	LBW	AMK, CAZ	-	-	-	-
2[a]	6	M	LM	LOS	20.10.16	27.10.16	DAMA	8	PT[b]	PROM	LBW	AZT[d], AMK, MT[d]	+	-	-	-
3[a]	6	M	LM	LOS	20.10.16	27.10.16	Died	8	PT[b]	PROM	LBW	AZ[d], AMK, MT[d]	+	-	-	-
4	5	M	Poor	LOS	30.11.16	04.12.16	D/S	5	-	-	-	GEN, CRO	+	+	+	+
5	3	F	BPL	EOS	31.10.16	07.11.16	Died	8	-	-	-	AMK, CRO	+	+	+	+
6	5	F	BPL	LOS	07.11.16	12.11.16	DAMA	6	-	-	LBW	GEN, CRO	+	+	+	+
7	5	F	LM	LOS	06.11.16	13.11.16	D/S	10	PT	-	LBW	AMK, CL	+	+	+	+
8	3	M	BPL	EOS	09.11.16	13.11.16	D/S	7	-	-	LBW	AMK, CRO	+	+	+	+
9	3	M	Poor	EOS	10.11.16	14.11.16	Died	16	PT	-	LBW	AMK, CRO	+	-	-	-
10	7	M	Poor	LOS	07.12.16	19.12.16	Died	40	-	-	LBW	AMK, CRO	+	-	-	-
11	10	M	LM	LOS	06.12.16	19.12.16	D/S	35	-	-	LBW	AMK, CL	+	-	-	-

12	2	M	Poor	EOS	15.01.17	19.01.17	D/S	21	-	-	-	GEN, CRO	+	-	-	-
13	1	M	BPL	EOS	20.01.17	24.01.17	Died	25	PT	-	LBW	MEM, VAN[d]	+	-	-	-
14	7	M	Poor	LOS	19.11.16	24.11.16	Died	12	-	-	LBW	MEM, VAN[d]	+	-	-	-

Abbreviations: M, male, F, female; SEC, socio-economic condition (per capital family income was used to assess socio-economic level); LM, lower middle; BPL, below poverty level; POS, pattern of sepsis; EOS, early onset neonatal sepsis; LOS, late onset neonatal sepsis; DOA, date of admission; DOSC, date of sample collection; DAMA, discharge against medical advice; TS, total stay in hospital; PT, pre-term; PORM, prolonged rupture of membrane; LBW, low birth weight; Ab, antibiotics; AMK, amikacin; CAZ, ceftazidime; AZ, azithromycin; MT, metronidazole; GEN, gentamycin; CRO, ceftriaxone; CL, colistin; MEM, meropenem; VAN, vancomycin. [a]Case 2 and 3 were twins; [b]31 weeks; [c]antibiotics usage during hospital stay (green colour indicates sensitive to respective antibiotics by the isolates recovered and red indicates the resistant); [d]susceptibility were not tested.

7.2.3 Resistance and virulence profiling of *K. variicola*

A total of 21 ARGs was identified, demonstrating concurrence between antimicrobial MICs and presence of known resistance genes (*fosA* did not result in MICs above the resistance break point but this is expected in *Klebsiella* species) (Figure 7.3) (Ito *et al.*, 2017). WGS identified identical putative virulence factors (adhesins, siderophores, invasins etc.) (Li *et al.*, 2014) and a unique capsule locus in *K. variicola* ST771 (Figure 7.3, Figure 7.4).

S1 PFGE analysis revealed that $bla_{\text{CTX-M-15}}$ was carried on ~134 kb and ~158 kb plasmids (Figure 7.5), and $bla_{\text{NDM-1}}$ on an ~134 kb plasmid (Figure 7.6). Conjugation experiments were undertaken with a NDM-positive *K. variicola* donor and *E. coli* J53 recipient. The transconjugants were found to be positive for $bla_{\text{NDM-1}}$ located on a plasmid of ~134 kb (Figure 7.6). The resistance profiles of transconjugants are shown in Figure 7.3.

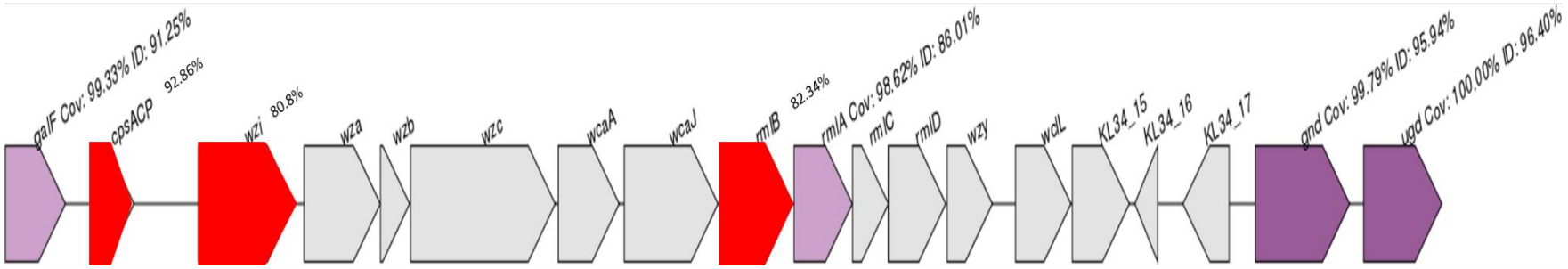


Figure 7.4 Capsular loci (*K*) to *Klebsiella variicola* ST771. Violet shading indicates identity matches with KL34 (*galF*, 99.33%; *rmlA*, 98.62%; *gnd*, 99.79%; *ugd*, 100%); red shading indicates identity matches with other K loci [*cpsACP* matches to KL153 (92.86%), *wzi* to KL114 (80.80%) and *rmlB* to KL36 (82.34%)]. Genes expressed by white arrows did not match to known capsular type. Capsular operon indicates a novel *K* locus in this clone.

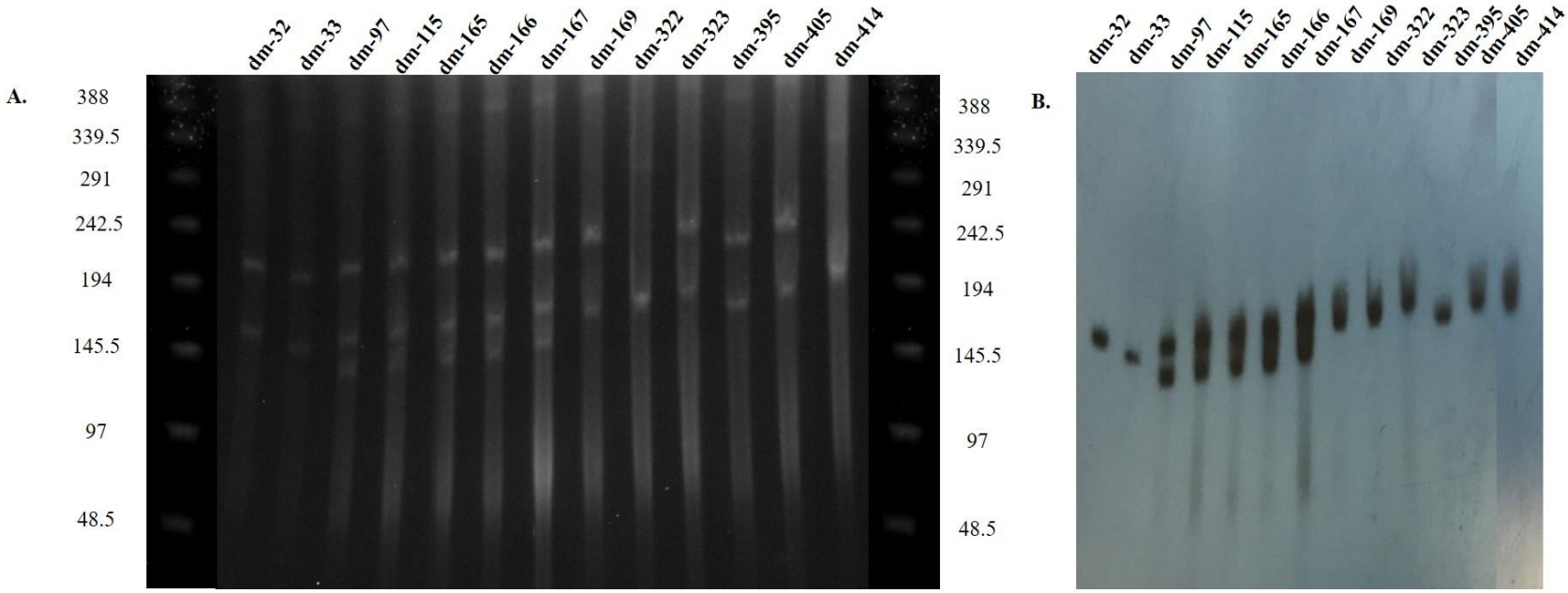


Figure 7.5 Pulsed-field gel of S1 nuclease digested gDNA carrying *bla*$_{\text{CTX-M}}$ and in-gel hybridization with *bla*$_{\text{CTX-M}}$ probe**.** Each lane is represented by specific strain ID. Isolation sources to the strains were: dm.32 from case 2; dm.33 from case 3; dm.97 from case 4; dm.115 from case 5; dm.165 from case 6; dm.166 from case 7; dm.167 from case 8; dm.169 from case 9; dm.322 from case 10; dm.323 from case 11; dm.395 from case 12; dm.405 from case 13; dm.414 from case 14; **A.** S1 PFGE of *K. variicola* isolated from outbreak cases. **B.** In-gel hybridization of outbreak strains with *bla*$_{\text{CTX-M}}$ probe.

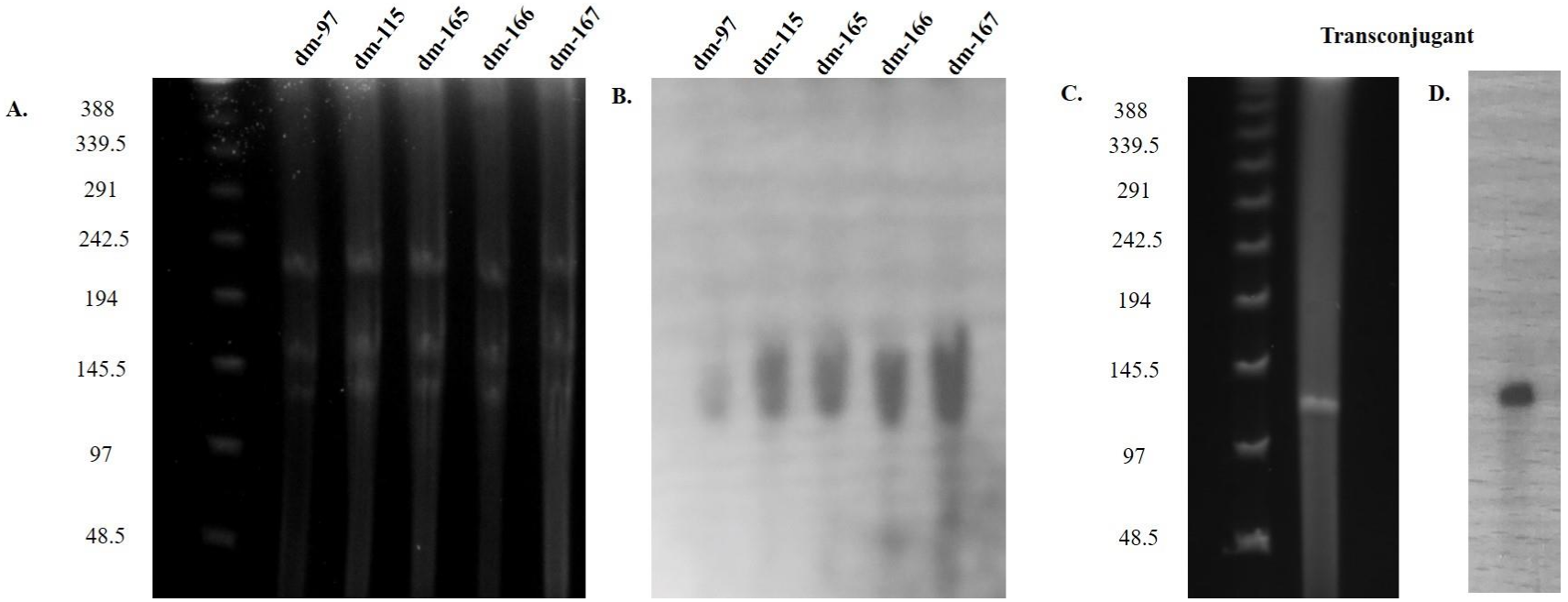


Figure 7.6 Pulsed-field gel of S1 nuclease digested gDNA carrying *bla*$_{NDM}$ and in-gel hybridization with *bla*$_{NDM}$ probe**.** Each lane is represented by specific strain ID. Isolation sources to the strains were: dm.97 from case 4; dm.115 from case 5; dm.165 from case 6; dm.166 from case 7; dm.167 from case 8; **A.** S1 PFGE of *K. variicola* isolated from outbreak cases. **B.** In-gel hybridization of outbreak strains with *bla*$_{NDM}$ probe **C.** S1 PFGE of *bla*$_{NDM}$ transconjugant **D.** In-gel hybridization of *bla*$_{NDM}$ transconjugant**.**

7.2.4 Risk assessment of the outbreak by *K. variicola*

Of the 36 neonates [outbreak (n=14) and non-outbreak (n=22)], 83.3% were male; the mean (SD) age was 5.4 days (±3.6) and 10 (27.8%) were lost to follow up. Babies with LBW was significantly associated with the outbreak ($p<0.05$) (Table 7.4). The overall mortality for *K. variicola* sepsis was 54.5% compared to 33.3% for non-outbreak sepsis cases excluding DAMA [$p>0.01$ (non-significant); OR=2.40; CI= 0.48-11.89] (Table 7.4).

Table 7.4 Risk association of outbreak strains compared to non-outbreak strains (during the outbreak time).

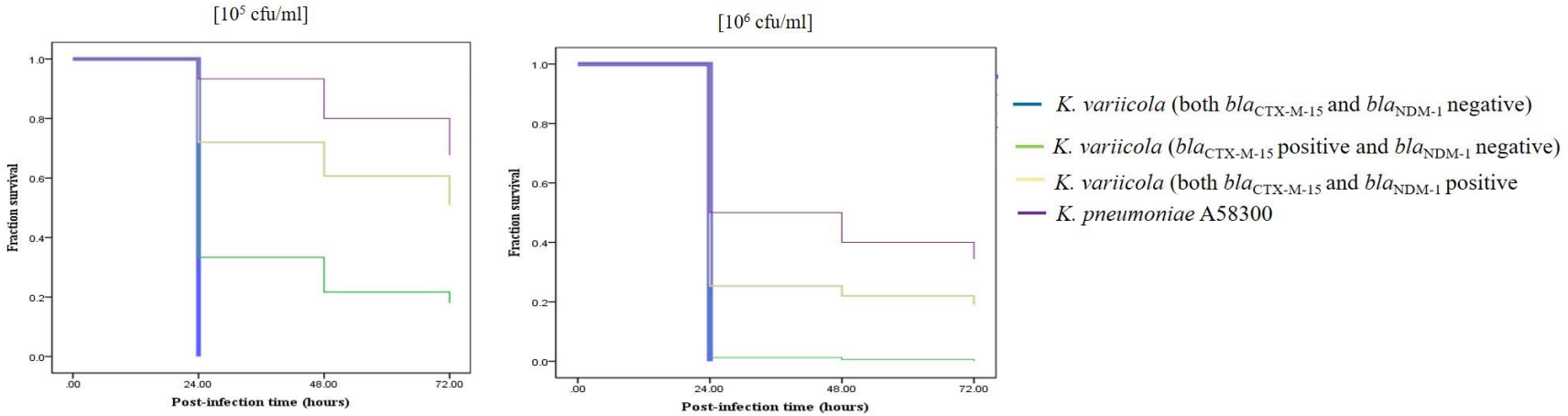
Variables		**Outbreak strains (n=14) n (%)**	**Non-outbreak strains (n=22) n (%)**	***p* value**	**OR**	95% CI
Sex	Male (n=30)	11 (78.57)	19 (86.36)	0.54	1.72	0.30-10.08
	Female (n=6)	3 (21.43)	3 (13.64)			
POS	EOS (n=14)	6 (42.86)	8 (36.36)	0.70	1.31	0.33-5.16
	LOS (n=22)	8 (57.14)	14 (63.64)			
HS [days (mean±SD)]		9.29±8	6.59±3.11	0.16	-	-
Pre-term	Yes (n=13)	6 (42.86)	7 (31.82)	0.50	0.62	0.15-2.50
	No (n=23)	8 (57.14)	15 (68.18)			
PROM	Yes (n=6)	2 (14.29)	4 (18.18)	0.76	1.33	0.21-8.46
	No (n=30)	12 (85.71)	18 (81.82)			
LBW	Yes (n=20)	11 (78.57)	9 (40.91)	0.03	5.30	1.14-24.55
	No (n=16)	3 (21.43)	13 (59.10)			
Mortality[a]	Died (n=11)	6 (54.54)	5 (33.33)	0.28	2.40	0.48-11.89
	Discharge (n=15)	5 (45.46)	10 (66.67)			

POS, pattern of sepsis; HS, hospital stay; chi-square test was used to calculate *p* value of categorical variables and independent t test to quantitative variables. [a]*p* value for mortality was determined by excluding the neonates with DAMA; 3 cases from outbreak group and 7 cases from non-outbreak group lost to follow up. Follow up after discharge was not undertaken in this study.

7.2.5 *In vivo* pathogenicity testing of *K. variicola* in *G. mellonella*

Data from the *G. mellonella* model shows that *K. variicola* ST771 was significantly more virulent than the *K. pneumoniae* comparator at 10^5 and 10^6 cfu/ml ($p<0.0001$).

Similar to the clinical outcome, NDM-1-positive were significantly less pathogenic than NDM-1-negative *K. variicola* isolates ($p<0.000001$) (Figure 7.7).



***p* value to compare pathogenicity in vivo model**

Bacterial inoculum concentration	*K. Pneumoniae* A58300 vs *K. variicola* (irrespective resistance genotype)	*K. Pneumoniae* A58300 vs *K. variicola* (CTX-M & NDM negative)	*K. Pneumoniae* A58300 vs *K. variicola* (CTX-M positive & NDM negative)	*K. Pneumoniae* A58300 vs *K. variicola* (CTX-M & NDM positive)	NDM positive *K. variicola* vs NDM negative *K. variicola*
10^5	0.000016	<0.000001	<0.000001	0.070	<0.000001
10^6	0.000005	0.000009	<0.000001	0.048	<0.000001

Figure 7.7 *In vivo* pathogenicity testing in *Galleria mellonella* model to compare the virulence of *K. Variicola* Vs *K. Pneumoniae*. Kaplan–Meier plots representing the cumulative proportion of *G. mellonella* larvae survival over 72 hours post infection with *K. variicola* and *K. pneumoniae* A58300 [previously described hypervirulent pathogen (Coutinho *et al.*, 2014)]. Differences in survival were calculated by log-rank (Mantel-Cox).

7.3 Discussion

This is the first reported outbreak of neonatal sepsis by MDR *K. variicola* at DMCH, Bangladesh, or for that matter in SA. The findings of this chapter were submitted for publication when the outbreak was identified at CU retrospectively and was accepted by Clinical Infectious Diseases (CID) (Farzana *et al.,* 2019a). The laboratory work, and analysis related to the work have been completely performed by me as a part of this PhD. Only the sequencing services (Illumina MiSeq) were provided by Prof. Walsh's genomic laboratory at CU.

Genomic profile considered all *K. variicola* isolated in this study as clonal, belonged to ST771 based on *K. pneumoniae* MLST scheme, and ST60 by the recently described *K. variicola* scheme (Barrios-Camacho *et al.,* 2019). The outbreak was associated with higher mortality than non-outbreak sepsis cases. Despite the small sample, the confidence interval for the OR was 0.48-11.89, suggesting a likely difference (Table 7.4). *K. variicola* in human was first described in an episode of paediatric outbreak in Mexico. Other reports on *K. variicola* bacteraemia have been consistently associated with higher mortality than *K. pneumoniae*. However, no additional known virulence properties were retrieved from analysis of global genomes of *K. variicola* (Barrios-Camacho *et al.,* 2019; Rodríguez-Medina *et al.,* 2019). The outbreak clone in this study was associated with iron-acquisition (*ent*, *kfu*), adhesins (*fim*, *mrk*) and complement resistance (*traT*), described previously as bacterial virulence determinants (Coutinho *et al.,* 2014; Li *et al.,* 2014; Araújo *et al.,* 2018), and the ST771 *K. variicola* capsular operon suggests a novel *K* locus (Figure 7.3; Figure 7.4), but lacked hypermucoid phenotype and *rmpA* and *magA*, indicators for the hypervirulent K1 capsular phenotypes of *K. pneumoniae* (Coutinho *et al.,* 2014). It is likely that there are others, as yet unidentified, determinants of virulence responsible for the ST771 *K. variicola*. However, the high mortality attributable to the *K. variicola* outbreak strain was supported by *G. mellonella* models, demonstrating high larval death rates compared to the hypervirulent ST23 K1 *K. pneumoniae* strain, A58300 (Figure 7.7).

The isolates of *K. variicola* outbreak exhibited variable resistance patterns. *K. variicola* ST771 acquired antimicrobial resistance genes including $bla_{\text{NDM-1}}$ horizontally during the course of outbreak (Figure 7.3). Exposure to ceftriaxone and/or

ceftazidime and amikacin in the NICU may have favoured the *bla*$_{\text{CTX-M-15}}$ and *aph(3')-VIa*/*bla*$_{\text{NDM-1}}$ acquisition (Figure 7.3, Table 7.3). Acquiring a resistance plasmid is often associated with bacterial fitness costs (Yang *et al.,* 2017). This could explain the lower mortality observed clinically and in the in-vivo virulence model for *bla*$_{\text{NDM-1}}$ positive vs *bla*$_{\text{NDM-1}}$ negative *K. variicola* (Figure 7.7).

Healthcare resources in Bangladesh allow only limited surveillance of healthcare associated infections (WHO, 2020d). The neonatal NICU in DMCH consists of 36 beds, having 3,500 neonatal admissions annually. To overcome the load, the beds are often shared by two or three neonates. There is only two washing stations and the unit is not properly structured according to recommended standards (White, 2013). Blood cultures are not routinely taken at DMCH NICU and neonates are only followed up when empirical antibiotics fail with minimal antibiotic stewardship management or infection control as to other units in the hospital (Rahman, personal communication). The hospital environment including patients and healthcare providers could be the potential reservoirs of MDR infections, and outbreaks are inevitable in these settings (Dramowski *et al.,* 2017). The outbreak by MDR virulent strains, in under-resourced hospitals, where antibiotics usage is empirical and IPC is suboptimal, would pose significant challenges, and as witnessed by this study, result in numerous deaths.

Section Eight

Emergence of Mobile Colistin Resistance in a Clinical Setting in Bangladesh

8.1 Introduction

Colistin is the member of cationic polypeptide, is the last line option for the treatment of MDR infections. The spectrum of colistin is narrow, only active against Gram-negative bacteria. Furthermore, *Proteus* spp., *Neisseria* spp., *Serratia* spp., *Providencia* spp., *Burkholderia pseudomallei*, *M. morganii*, and *E. tarda*, and certain anaerobic bacteria are constitutionally resistant to colistin (Falagas and Kasiakou, 2005; Bialvaei and Samadi, 2015). Widespread colistin usage in agriculture; particularly, for prophylaxis and as a growth feed additive in animal production could have exacerbated the growing prevalence of plasmid-mediated colistin resistance, '*mcr*' throughout China and globally (Shen *et al.,* 2016; Wang *et al.,* 2018; Luo *et al.,* 2020). Although recent reports suggest the emergence of *mcr* in the food-chain, environment, and human in Pakistan, India, and Bangladesh (Azam *et al.,* 2017; Gogry *et al.,* 2019; Palani *et al.,* 2020; Parvin *et al.,* 2020), the epidemiology of *mcr*-like mechanisms in SA is still largely unknown. This chapter summarizes the epidemiology of *mcr*-positive Enterobacterales associated with human infections/colonisation in a Bangladeshi health setting in Dhaka.

8.2 Results

8.2.1 Prevalence of human-associated *mcr* in a Bangladeshi health setting

The prevalence of *mcr* among the patients with Enterobacterales infections was 1.1% (6/534). Three patients with *K. pneumoniae* infections were positive for *mcr-8.1*, two patients with *E. coli* infections were positive for *mcr-9.1*, and one patient with *E. cloacae* was positive for *mcr-9.1*. *K. pneumoniae* and *E. cloacae* positive for *mcr-8.1* and *mcr-9.1*, respectively were phenotypically resistant to colistin, however *E. coli* carrying *mcr-9.1* was phenotypically sensitive to colistin.

As it has been stated previously, the faecal carriage study was undertaken within a year of the clinical study with an aim to investigate the prevalence and transmission dynamics of CRE faecal colonisation in Bangladesh. Therefore, faecal specimens were cultured onto media containing vancomycin (10mg/L), and ertapenem (2mg/L). As this carriage study was not designed to estimate the prevalence of *mcr*, subsequent analysis is likely to be underestimated. Out of 700 non-duplicate faecal specimens, one *E. coli* isolated was positive for *mcr-1.1* which also exhibited colistin resistance phenotypically.

This PhD describes the entire genomic details of *mcr-1.1*-positive *E. coli* (MCRPEC), and *mcr-8.1*-positive *K. pneumoniae* (MCRPKP) by merging clinical profile of pertinent patient. However, the genomic, and functional properties of *mcr-9.1* were not elucidated in this PhD.

8.2.2 Description of the case colonised by MCRPEC

The MCRPEC (RS571; accession no: CP034389- CP034392) was isolated from the rectal swab culture of a 17-year boy who was admitted to the burn's ICU, DMCH with 53% flame burn involving much of the trunk and face. He suffered smoke inhalation injury and became severely hypoxic. The patient worked as a labourer in a garment factory in Munshiganj district and the injury was sustained due to a gas pipe leak.

RS571 was identified 2 days after admission so the patient could have acquired the MCRPEC in the community or the hospital. Clindamycin and ceftriaxone treatment were commenced early in the hospital stay. The patient died on day 5 of admission, with the cause of death given as septicaemia. No clinical specimens were referred for culture during his hospital stay so it is not known whether the patient was colonising flora or whether the organism played a pathogenic role, although RS571 was resistant to all antibiotics the patient received during admission. MCRPEC (RS571) isolated in this study belonged to *E. coli* ST648.

8.2.3 Resistance profile of MCRPEC

E. coli RS571 was resistant to amoxicillin-clavulanate, piperacillin-tazobactam, ceftazidime, cefotaxime, cefepime, ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, and colistin. The isolate was susceptible to imipenem, meropenem, amikacin, fosfomycin, and tigecycline (Table 8.1). WGS revealed a 1626 bp ORF encoding putative phosphoethanolamine transferase, consisting of 541 amino acid which exhibited 100% nucleotide sequence identity to *mcr-1.1*. In addition to *mcr-1*, other resistance genes such as $bla_{\text{TEM-1B}}$, *aac(3)-IId*, *aph(3'')-Ib*, *aph(6)-Id*, *aph(3')-Ia*, *aadA1*, *aadA2*, *sul3*, *dfrA14*, *mph(A)*, *tet(A)*, *cat*B3, *cml* and *floR* (plasmid mediated) and *aac(6')-Ib-cr*, *aac(3)-IIa*, $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{CTXM-15}}$ (chromosomal) were present in the isolate.

The *mcr-1.1* in *E. coli* can be transferred to the recipient *K. variicola* and with a transfer frequency of 8.3×10^{-5}. Transconjugants were more resistant to colistin than donor (MIC 32 µg/ml) and conferred resistance to gentamicin and trimethoprim-sulfamethoxazole as well (Table 8.1).

Table 8.1 MIC of antimicrobials against transconjugants obtained in this study along with donor (RS571, accession no: CP034389- CP034392) and recipient (BD_DM_07, accession no: PJQN00000000).

Antibiotics	**Donor (RS571)[1]**	**Recipient (BD_DM_07)[1]**	**Transconjugant[1]**
Piperacillin-tazobactam	8	4	8
Ceftazidime	32	0.125	0.25
Cefotaxime	>256	0.125	0.25
Cefepime	32	0.125	0.125
Imipenem	0.25	0.125	0.25
Meropenem	0.25	≤0.06	≤0.06
Ciprofloxacin	256	4	4
Levofloxacin	32	0.5	1
Amikacin	8	4	4
Gentamicin	256	4	128
Trimethoprim-sulfamethoxazole	256	64	256
Fosfomycin	1	8	8
Minocycline	1	16	16
Tigecycline	2	2	4
Colistin	16	0.5	32

[1]MIC values for the antibiotics are in µg/ml.

8.2.4 Genetic context of plasmids carrying *mcr-1.1* in this study

The draft genome sequence of *E. coli* strain RS571 was assembled into 4 contigs with an N_{50} length of 5,085,958 bp. A 257,243 bp long plasmid which carried *mcr-1.1* was named pRS571-MCR-1.1 (accession no: CP034390) and belongs to the IncHI2-ST3 replicon type. One copy of *mcr-1* (nucleotide 214,483 to 216,108) was found in pRS571-MCR-1.1, flanked by IS*ApaI1* (nucleotide 213,314 to 214,287) at the 5′ end (Figure 8.1). Other resistance genes such as *tet(A), floR, aph(6)-Id*, *aph(3''), Ib- aph(3'), Ia- aadA1, sul3, aad2*, *bla*$_{\text{TEM-1B,}}$ *mph(A), dfrA14* were in the nucleotide position 123,150 to 159,667 which were associated with several transposable elements, IS*26*, IS*CR2*, IS*1133*, IS*903B*, IS*406* and class 1 integron, In*191*. From nucleotide position 93,220 to 96,205, *aph(3')-Ia, aph(6), Id aph(3'')-Ib* were found which were flanked by IS*26* (Figure 8.1). The variable region of pRS571-MCR-1.1 shared the most identity with pSA186_MCR1 (CP022735.1) and pSA26-MCR-1 (KU743384.1) (88% coverage and 99% nucleotide identity for both), isolated from the Arabian Peninsula (Figure 8.2).

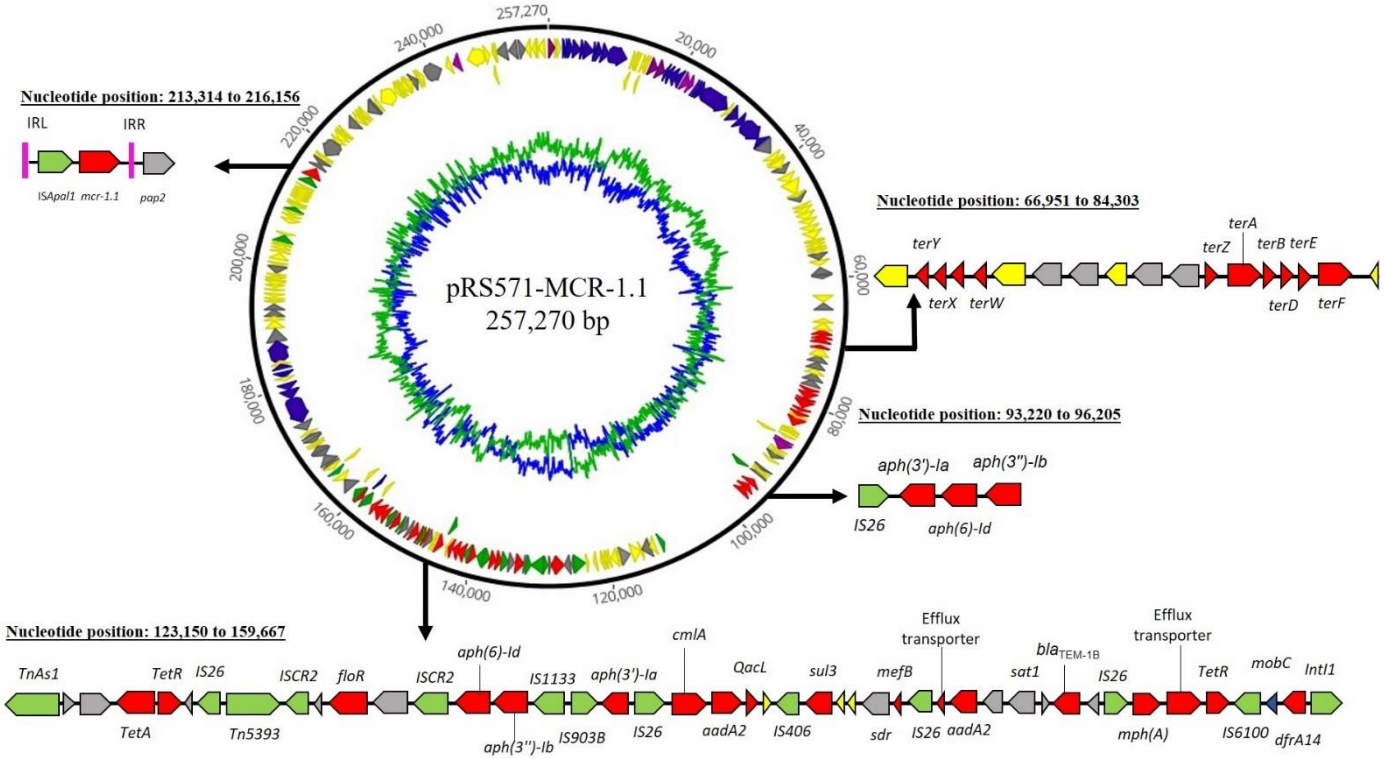


Figure 8.1 Structural organization of plasmid, pRS571-MCR-1.1 (accession no: CP034390). Schematic representation of genetic context of resistance loci are represented in further detail. Arrows represent the position and transcriptional direction of the open reading frames. Resistance genes are represented by red, transposase elements in green, genes associated with conjugation in blue, replication-associated genes in purple, regulatory/accessory genes in grey, hypothetical proteins in yellow and IRs in pink.

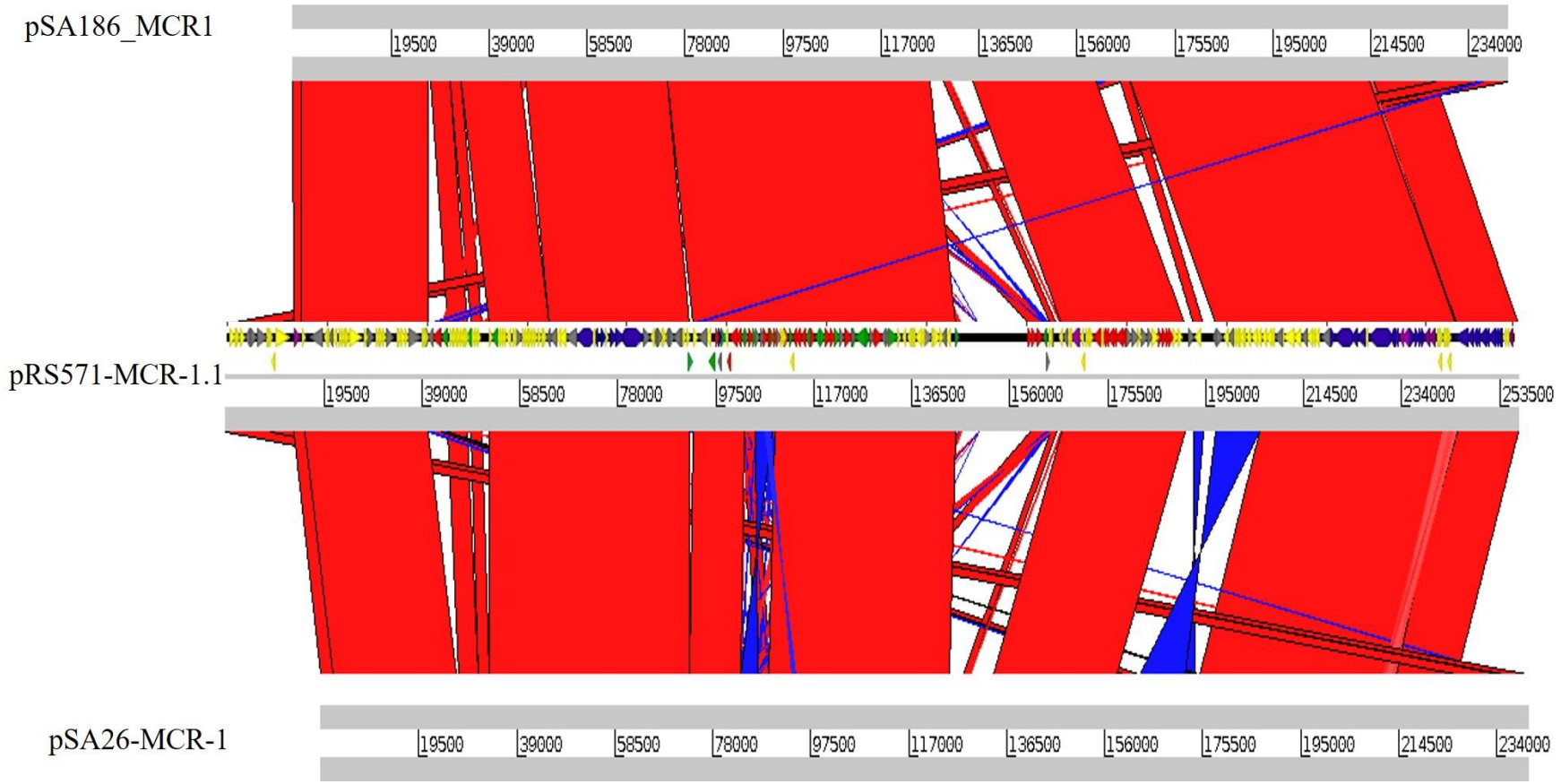


Figure 8.2 Linear comparison of plasmid sequence pRS571-MCR-1.1 (accession no: CP034390), pSA186_MCR1 (accession no: CP022735.1) and pSA26-MCR-1 (accession no: KU743384.1). Forward matches are indicated by red and reverse matches by blue. Region of sequence with

homology to the other genomes are separated by black lines. Genes associated with pRS571-MCR-1.1 are represented by colour. Resistance genes are represented by red, transposase elements in green, genes associated with conjugation in blue, replication-associated genes in purple, regulatory/accessory genes in grey and hypothetical proteins in yellow. Genomic comparison was performed by artemis comparison tool (ACT) (v.18.0.1).

8.2.5 Description of cases with infections by MCRPKP

MCRPKP were recovered from the urine of two patients admitted under urology, and the blood of a third patient in the DMCH NICU. Case 1 (BD_DM_697) was a 55-year-old male with benign enlargement of the prostate with diabetes mellitus, and history of catheterisation for 13 days. Case 2 (BD_DM_782) was a 63-year-old male patient with a left renal tumour, a history of catheterisation for 15 days and haematuria. These patients were discharged on day 20 and 35 of hospitalization, respectively. Case 3 (BD_DM_914) was a 5-day pre-term LBW neonate with late onset of sepsis who died within 18 days after hospital admission. We did not observe any overlapping of hospital stay among the MCRPKP cases (Table 8.2).

Table 8.2 Characteristics of the patients with MCRPKP.

Sample ID	Age	Sex	SEC	Ward	DOA	Diagnosis	Probable risk	Sample	DOSC	Antibiotics prescribed	Outcome	DOO
BD_DM_697	55 years	M	BPL	Urology	3.4.17	BEP	DM, catheterized	Urine	16.4.17	CIP	Discharge	22.4.17
BD_DM_782	63 years	M	BPL	Urology	23.4.17	Left renal tumour	Haematuria, catheterized	Urine	8.5.17	CFX	Discharge	27.5.17
BD_DM_914	5 days	M	Poor	NICU	26.6.17	LONS	PT, LBW	Blood	2.7.17	CRO, VAN	Died	13.7.17

SEC, socio-economic condition; DOA, date of admission; DOSC, date of sample collection; DOO, date of outcome; M, male; DM, diabetes mellitus; CIP, ciprofloxacin; CFX, cefixime; NICU, neonatal intensive care unit; LONS, late onset neonatal sepsis; PT, pre-term; LBW, low birth weight CRO, ceftriaxone; VAN, vancomycin.

8.2.6 Investigation of clonal relatedness of MCRPKP

All MCRPKP isolated in this study belonged to *K. pneumoniae* ST15. The isolates differed by 0 to 1 SNP after recombination removal. Phylogenetic analysis of all *K. pneumoniae* ST15 isolated in this study revealed that all MCRPKP clustered into one clade (Figure 8.3). All MCRPKP were associated with the $bla_{\text{CTX-M-15}}$. It is noted that 96% (18/29) of *K. pneumoniae* ST15 identified from clinical and faecal specimens in this study carried $bla_{\text{CTX-M-15}}$ (Figure 8.3).

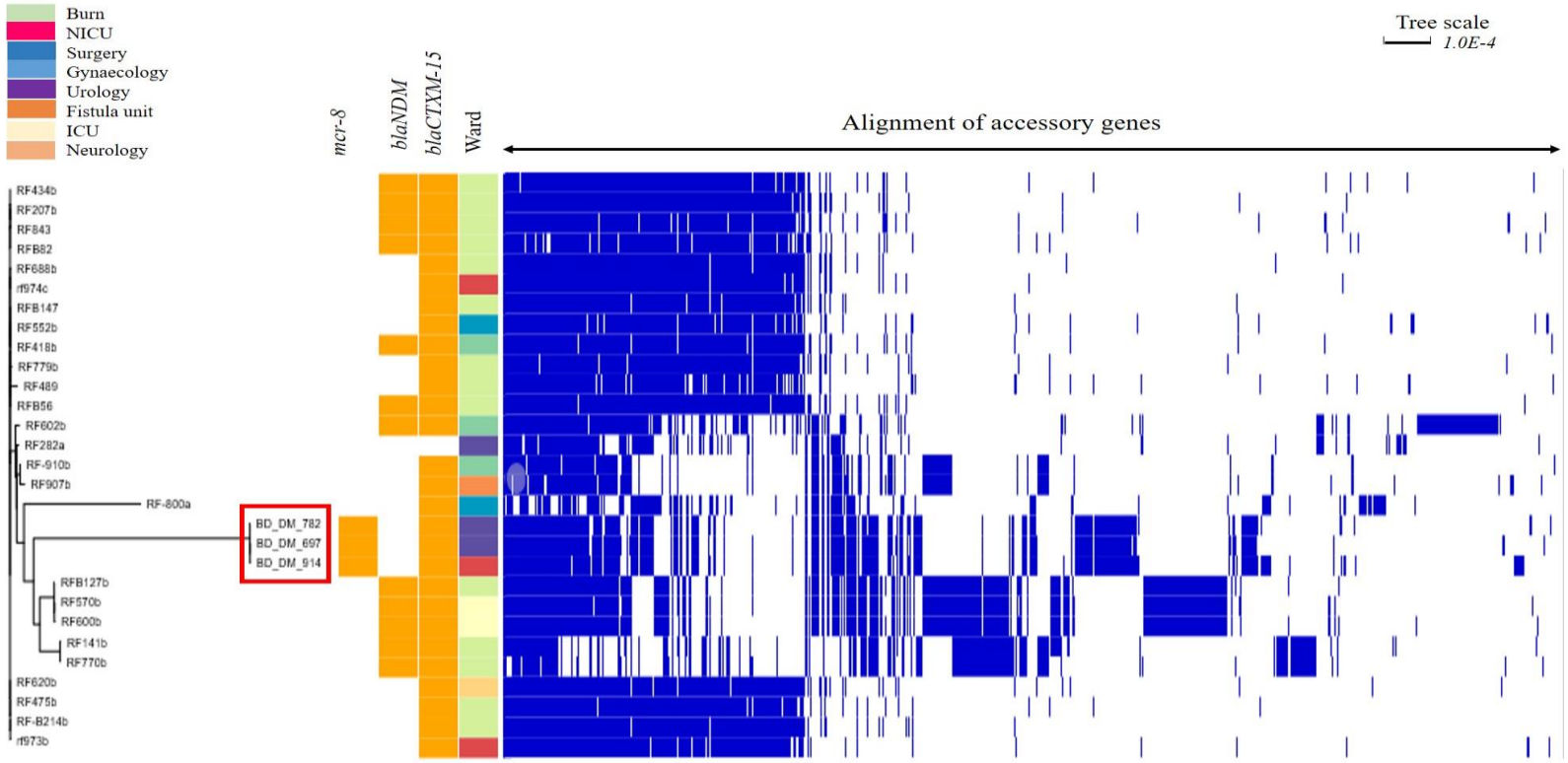


Figure 8.3 Phylogenetic tree of *K. pneumoniae* ST15 identified in this study (n=29). ML phylogenetic tree constructed using core-genome alignment Roary (v3.12.0). Visualisation of phylogenetic trees and incorporation of metadata with the trees were performed using phandango (Hadfield *et al.*, 2018). Epidemiologically important resistance genes are indicated by orange cells, accessory genes by blue cells and absence of genes by white cells.

8.2.7 Resistance profile of MCRPKP

MCRPKP were resistant to amoxicillin-clavulanate, piperacillin-tazobactam, cephalosporins (ceftazidime, cefotaxime), ciprofloxacin, levofloxacin, gentamicin, trimethoprim-sulfamethoxazole and colistin and susceptible to carbapenems, amikacin, fosfomycin and tigecycline (Table 8.3). All the isolates shared common resistance genes. *In silico* genome-wide analysis of MCRPKP detected 1698 bp open reading frame (ORF), encoding a phosphoethanolamine transferase, showing 100% nucleotide identity to *mcr-8.1*. Other ARGs found in the isolates were: $bla_{\text{SHV-106}}$, $bla_{\text{TEM-1B}}$, $bla_{\text{OXA-1}}$, $bla_{\text{CTX-M-15}}$, *aac(6')-Ib-cr aac(3)-IIa, aph(6)-Id, aph(3')-Ia, aph(3'')-Ib, aadA2, qnrB1, sul2, sul3, dfrA14, fosA6, cmlA1, mdf(A), mef(B), oqxA, oqxB,* and *tet(A)*.

The conjugation assay confirmed the transferability of the plasmid containing *mcr-8.1* to *E. coli* J53 with a frequency range of 3.1×10^{-2} to 8×10^{-2}. Phenotypically, the transconjugants were resistant to ampicillin, amoxicillin-clavulanate, 3rd generation cephalosporins, trimethoprim-sulfamethoxazole and colistin (Table 8.3).

Table 8.3 Range of MIC values of transconjugants obtained in this study along with donors and recipient.

Antibiotics	**Donors[1]**	**Recipient (*E. coli* J53)[1]**	**Transconjugants[1]**
Ampicillin	128-≥256	2	64-256
Amoxicillin-clavulanate	256	2	64-256
Piperacillin-tazobactam	16	2	2
Ceftazidime	32	≤0.06	32
Cefotaxime	128	≤0.06	128
Cefepime	16-32	≤0.06	0.25
Imipenem	0.25	≤0.06	≤0.06
Meropenem	≤0.06	≤0.06	≤0.06
Ciprofloxacin	256	≤0.06	≤0.06
Levofloxacin	64	≤0.06	≤0.06
Amikacin	2-4	2	2
Gentamicin	32	0.5	0.5
Trimethoprim-sulfamethoxazole	128	2	32-128
Colistin	8	≤0.06	8

[1]MIC values for the antibiotics are in µg/ml.

8.2.8 Genetic context of plasmids carrying *mcr-8.1* in this study

Complete plasmid sequences were determined for pKP782 (accession no: CP046384) and pKP914 (accession no: CP046952) by hybrid assembly, while pKP697 (accession no: CP046942) was not successfully closed. Genome-wide analysis demonstrated that the plasmids recovered from the MCRPKP were almost identical to each other (Figure 8.4). The draft genome sequences and S1 PFGE indicate that *mcr-8.1* in *K. pneumoniae* were located on identical IncFIB(pQil) plasmids of ~113 kb (accession no: CP046384, CP046952 & CP046942) (Figure 8.5, Figure 8.6). Genetic environment analysis of plasmids with *mcr-8.1* showed that three genes, upstream of *mcr-8.1* (*cobD*-Δ*NAT*-Δ*gtf-mcr-8.1)* was flanked by IS*903B* and the downstream (*mcr-8.1-copR-baeS-dgkA*-Δ*gtf*-ΔHP-ΔHP-bla_{ampC}-*MipA-sbmC*-ΔHP-bla_{ampC}), flanked by IS*Kpn21*. Additional resistance profiles were found: *sul2, APH(6)-1d*, and *APH(3'')-Ib*, flanked by IS*91* at the downstream and $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1b}}$ with composite transposons, flanked by insertion sequences. All resistance components in IncFIB(pQil) plasmids in this study were bracketed by IS*903B* from nucleotide position 20,590 bp to 64,656 bp (Figure 8.7).

MCRPKP and shared 99.72% nucleotide identity at 70% coverage with previously described plasmids (accession no: CP023922.1 and CP024040.1) which were absent for *mcr*-like genes (Figure 8.7). The genetic environment around *mcr-8.1* in IncFIB(pQil)-MCR-8.1 (pKP697, pKP782 & pKP914) shared identity with a previously described *mcr-8.1* containing plasmid isolated from pigs in China (accession no: MG736312.1), although the plasmids harbouring *mcr-8.1* in this study were truncated at the 5' end and IS*903B* at the 3' end was replaced by IS*Kpn21* (Figure 8.8).

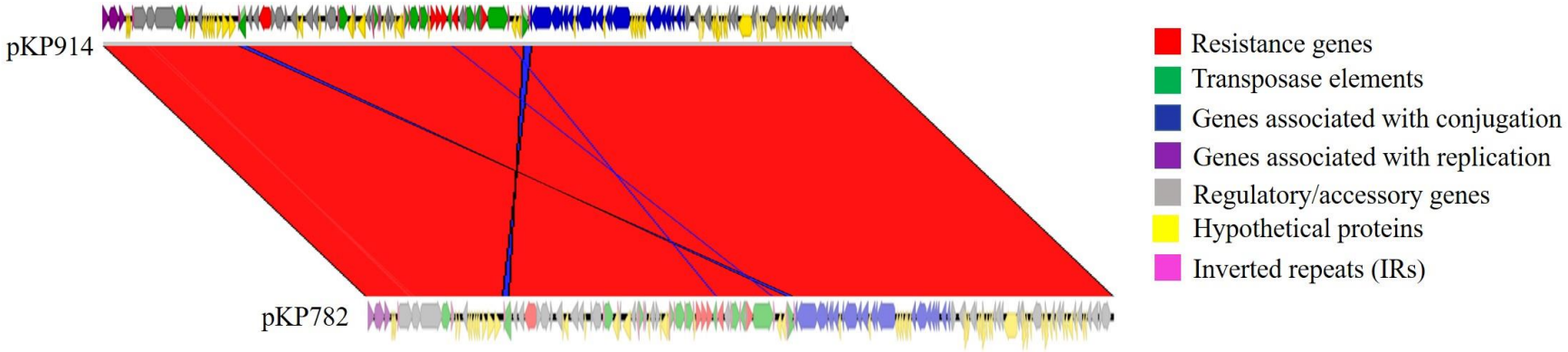


Figure 8.4 Linear comparison of plasmid sequence pKP914 (accession no: CP046952) and pKP782 (accession no: CP046384), recovered in this study. Forward matches are indicated by red and reverse matches by blue. Regions of sequence with homology to the other genomes are separated by black lines. Genomic comparison was performed by ACT (v.18.0.1).

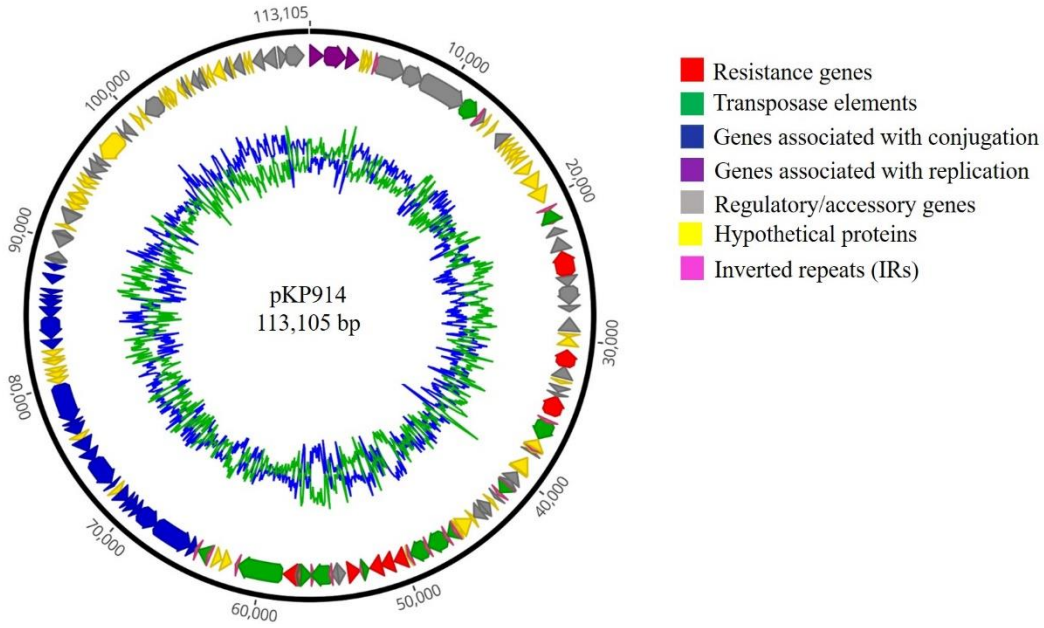


Figure 8.5 Genetic organization of plasmid harbouring of *mcr-8.1*. Circular view of pKP914 (accession no: CP046952).

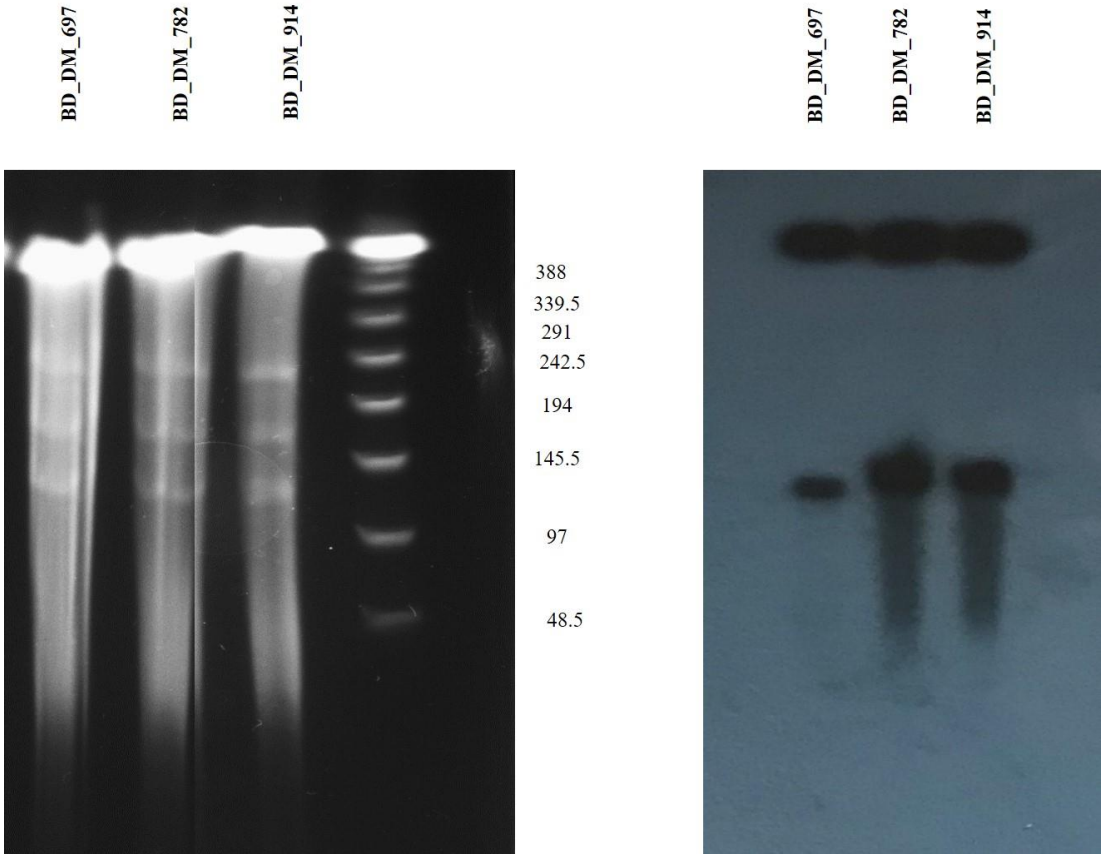


Figure 8.6 Pulsed-field gel of S1 nuclease digested gDNA carrying *mcr-8.1* and in-gel hybridization with *mcr-8.1* probe.

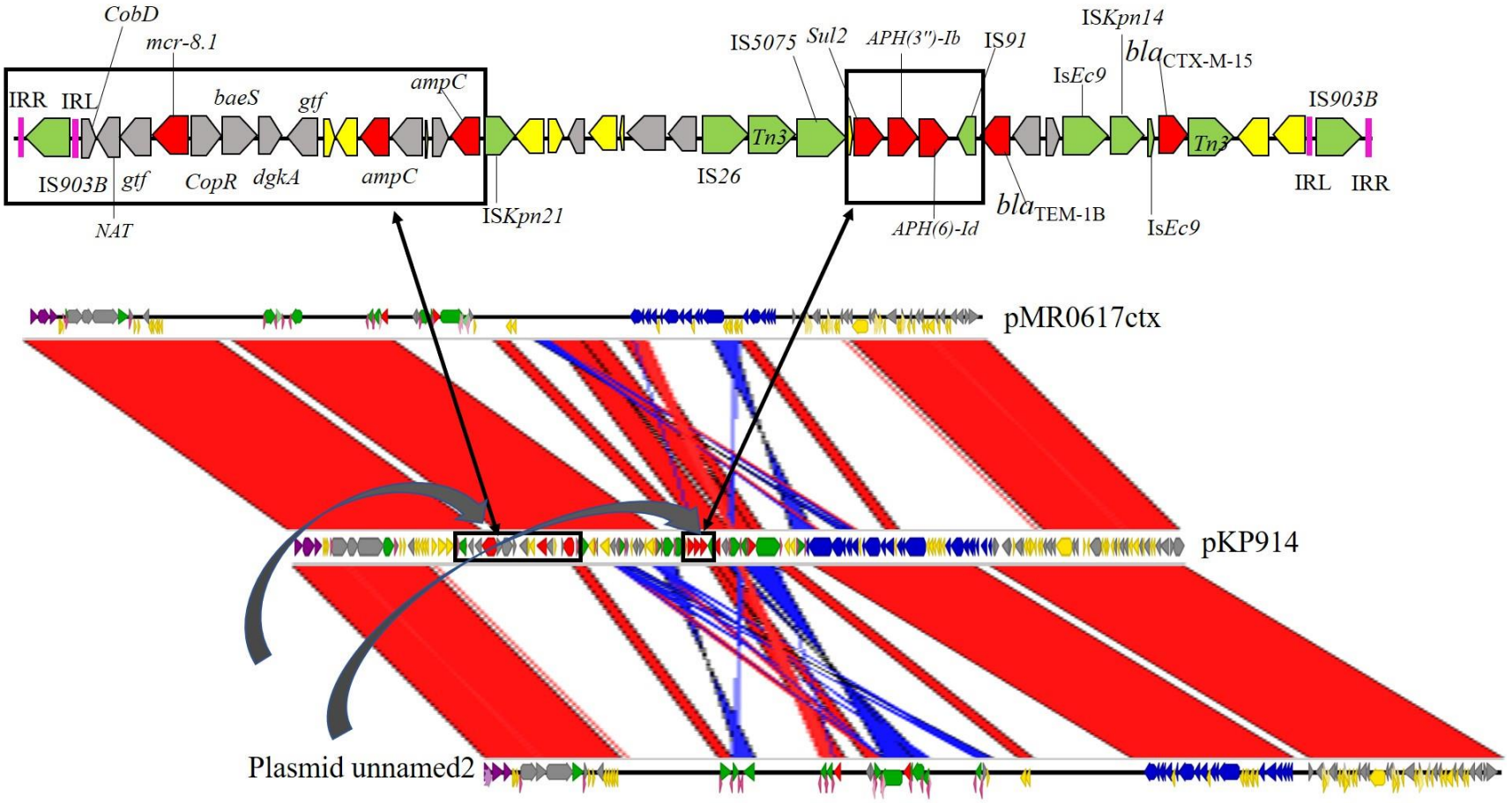
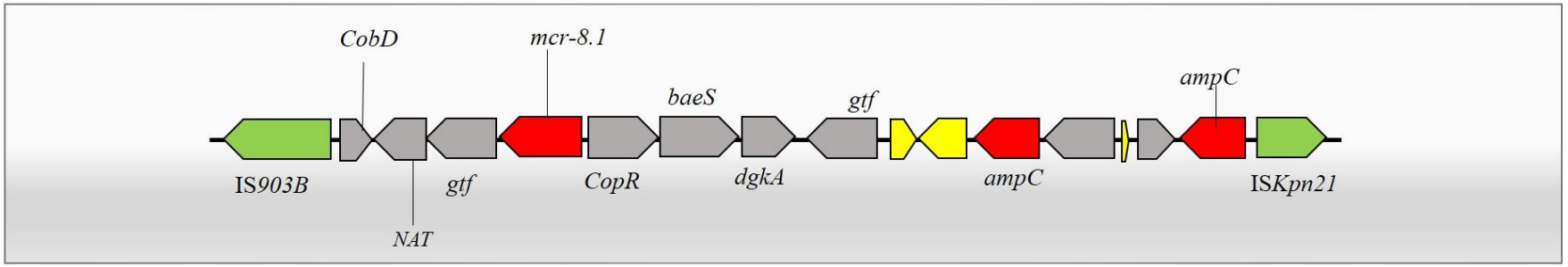


Figure 8.7 Schematic layout of sequence comparison of pKP914 (accession no: CP046952) against FDAARGOS_440 plasmid unnamed2 (accession no: CP023922.1) and pMR0617ctx (accession no: CP024040.1). Arrows represent the position and transcriptional direction of the open

reading frames. Genomic comparison was performed by ACT (v.18.0.1). *APH*, aminoglycoside phosphotransferase; *baeS*, histidine-protein kinase; *bla*, beta-lactamase; *Cob*, cobalamin biosynthesis; *Cop*, copper homeostasis transcription factor; *dgkA*, diacylglycerol kinase; *DJ-1/PfpI*, cysteine peptidase; *gtf*, glucosyltransferase; Hha, Hemolysin expression-modulating protein; IRL, inverted repeat left; IRR, inverted repeat right; IS, insertion sequence; *mcr-8.1*, mobilized colistin resistance; *NAT*, N-Acetyltransferase; *sul2*, dihydropteroate synthase.

Genetic context of *mcr-8.1* [pKP679, Pkp782, pKP914 (this study; accession no: CP046942, CP046384, CP046952)]



Genetic context of *mcr-8.1* [pKP91 (accession no: MG736312.1)]

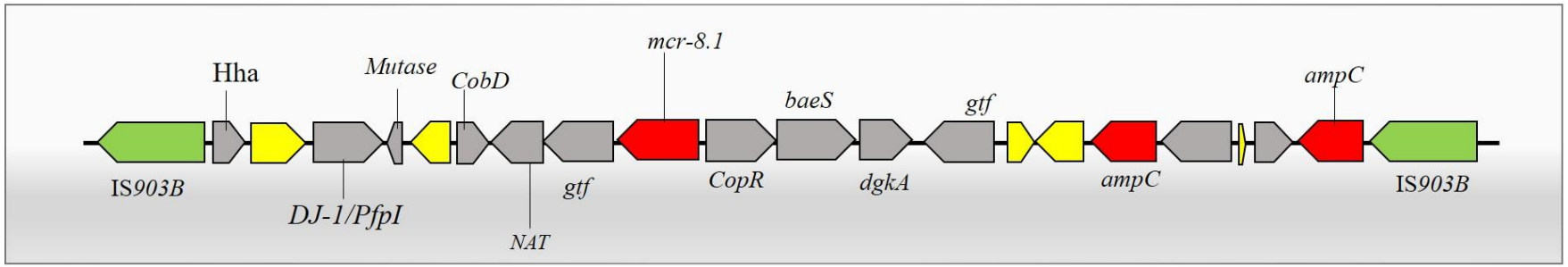


Figure 8.8 Comparison of genetic environments of *mcr-8.1*. *APH*, aminoglycoside phosphotransferase; *baeS*, histidine-protein kinase; *bla*, beta-lactamase; *Cob*, cobalamin biosynthesis; *Cop*, copper homeostasis transcription factor; *dgkA*, diacylglycerol kinase; *DJ-1/PfpI*, cysteine peptidase; *gtf*, glucosyltransferase; Hha, Hemolysin expression-modulating protein; IRL, inverted repeat left; IRR, inverted repeat right; IS, insertion sequence; *mcr-8.1*, mobilized colistin resistance; *NAT*, N-Acetyltransferase; *sul2*, dihydropteroate synthase.

8.2.9 Determining the stability of plasmid carrying *mcr-8.1*

The plasmids harbouring, *mcr-8.1* was stable on serial dilution without any antibiotic challenge. Compared to day 0, the abundance of *mcr-8.1* versus HKG was static up to day 12. Interestingly increased abundance of *mcr-8.1* was observed on day 3 for all three strains (Figure 8.9). We did not find any mutations in AA sequences of PmrA, PmrB, PhoP, PhoQ or *mgrB* of MCRPKP compared to colistin sensitive *K. pneumoniae* isolates from this study.

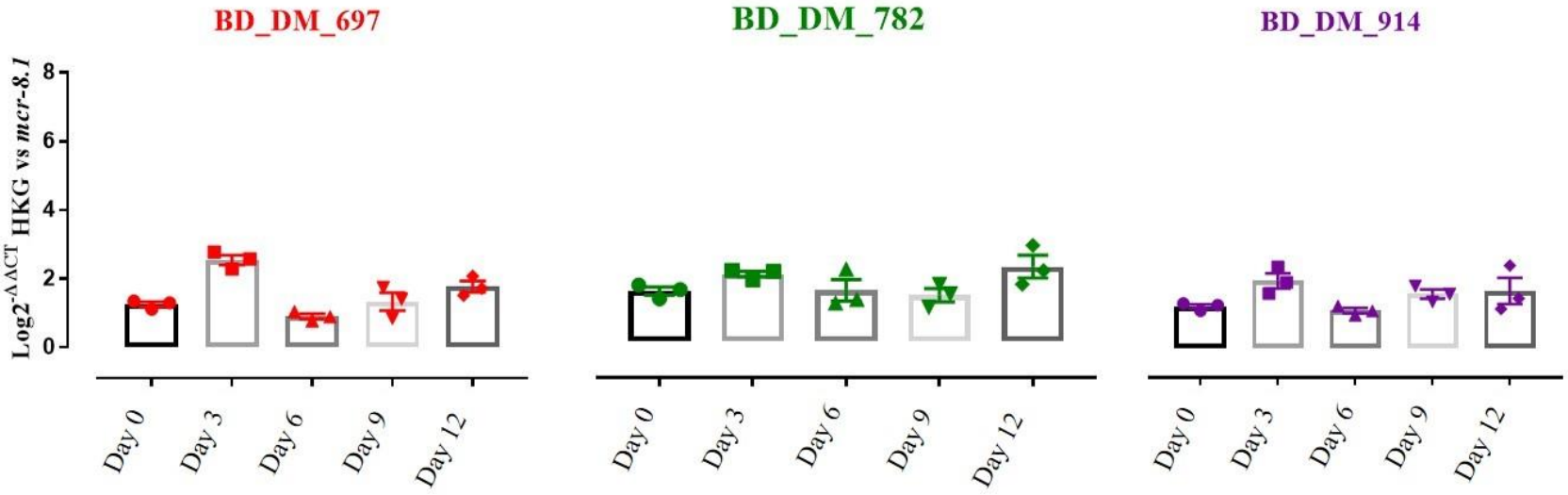


Figure 8.9 Stability of plasmid mediated colistin resistance in *K. pneumoniae*. Colistin resistance mechanism, *mcr-8.1* was highly stable after 12 days of passaging in colistin free medium.

8.2.10 Determining fitness cost of MCRPKP

This study observed significant lower growth over time in transconjugants (TDM697b and TDM914b) than *E. coli* J53 (p<0.0001) (Figure 8.10). However, considerable variabilities on growth were also found among the transconjugants formed due to acquisition of plasmids pKP697, pKP782 & pKP914. Compared to *E. coli* J53 lower growth rate was also observed in TDM782b; however, the fitness cost was not statistically significant (Figure 8.10).

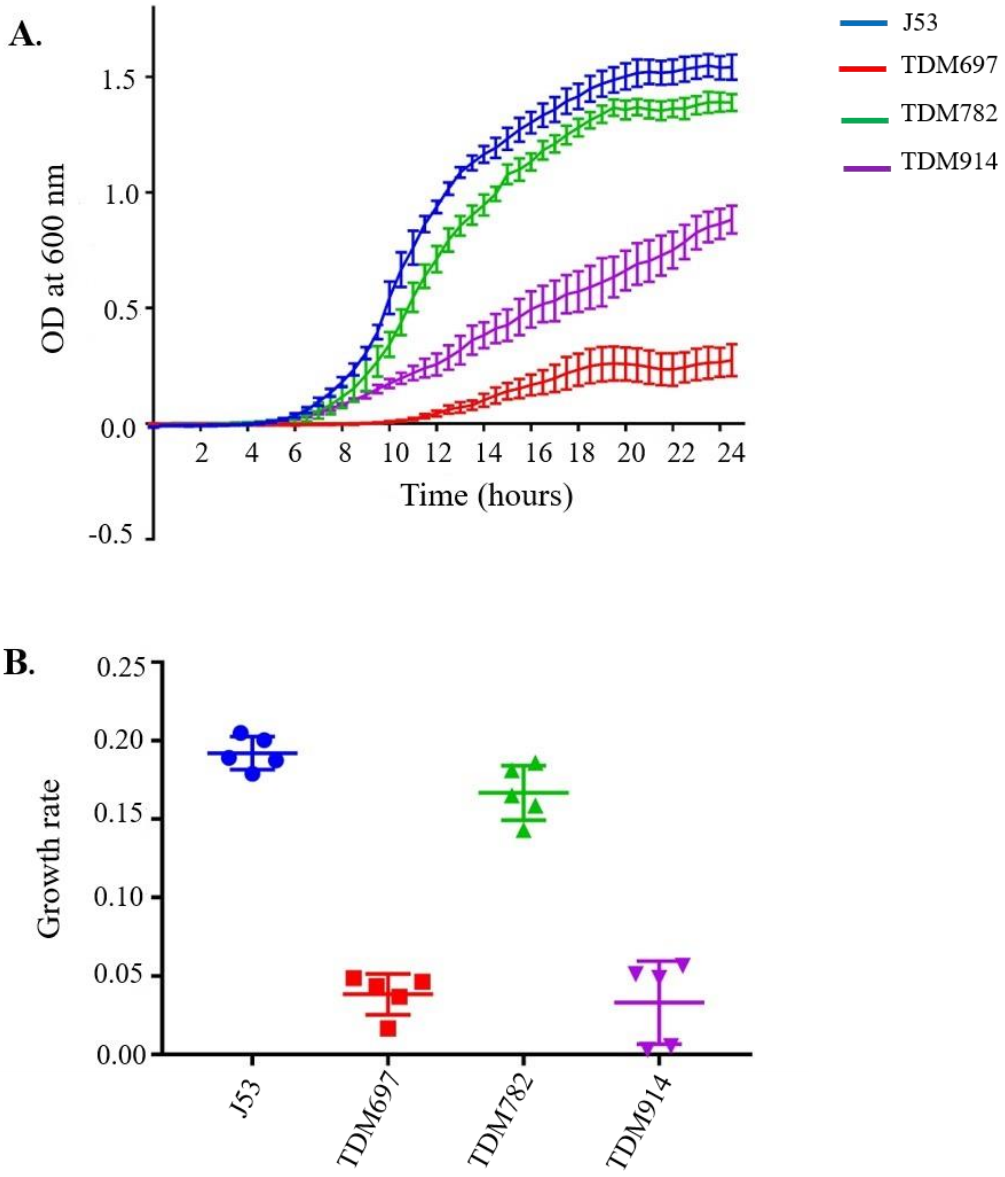


Figure 8.10 Analysis of bacterial fitness cost. **A.** Growth curve of trnasconjugants compared to *E. coli* J53**. B.** Growth rate of trnasconjugants compared to *E. coli* J53, fitted with the Gompertz model.

8.3 Discussion

There is gap in our understanding on the usage of colistin in SA (Lundborg and Tamhankar, 2017). A report by Davies and Walsh (2018) substantiates the export of thousand tonnes of colistin from China to India, Vietnam, and South Korea in 2016. The usage of colistin is apparent as growth promoter in food chain in SA countries (Davies and Walsh, 2018). The main source of active pharmaceutical ingredient (API) for antibiotics manufacture in Bangladesh are India followed by China (OEC, 2020). Before the announcement for banning of colistin in Indian poultry sector by the Indian Government in 2019 (Kannan, 2019), the import of colistin API to Bangladesh was frequent. As the Bangladesh Governmental legislation (2007) directs not to usage of antibiotics in animal feed (Hoque *et al.,* 2020), local farmers often use colistin in drinking water for food animals (Saha, personal communication). The reports of *mcr* in the food chain, environment, and human flora in Bangladesh also signify its usage (Islam *et al.,* 2017; Sobur *et al.,* 2019a; Sobur *et al.,* 2019b; Akter *et al.,* 2020; Amin *et al.,* 2020; Johura *et al.,* 2020). This study demonstrated human-associated *mcr* variants for the first time in SA and the mechanism has been characterized comprehensively. The findings of this chapter have been published upon identification of this high-end resistance mechanism from Bangladeshi specimens (Farzana *et al.,* 2019b; Farzana *et al.,* 2020a). The laboratory work, and analysis related to the work have been completely performed by me as a part of this PhD. Only the sequencing services (Illumina MiSeq) were provided by Prof. Walsh's genomic laboratory at CU.

The population of *mcr-1* carrying *E. coli* is highly diverse (Wise *et al.,* 2018; Long *et al.,* 2019; Hoa *et al.,* 2020; Luo *et al.,* 2020). *mcr-1* described in our study was linked to *E. coli* ST648 on a conjugative IncHI2 plasmid. The clone of *E. coli*, ST648 with IncHI2 has been documented as inter-human *mcr-1* transmission (Hadjadj *et al.,* 2017). In this study, 9.2% of *E. coli* (21/228) recovered from clinical infections 6.5% (19/293) from faecal samples, belonged to ST648. The clone, *E. coli* ST648 was considered as high risk for $bla_{\text{NDM-4}}$ (p<0·0001) (<u>*Chapter 5*</u>). The acquisition of *mcr-1.1* in the clone can be an added concern. The global dissemination of *mcr-1.1* has been described in associations with the plasmid background of IncX4, IncI2 and IncHI2 (Wang *et al.,* 2018a; Luo *et al.,* 2020). It is speculated that 70% of IncHI2 carrying *mcr-1* derived from Europe (Matamoros *et al.,* 2017). The variable region of

pRS571-MCR-1.1 (isolated in this study) shared most identity with pSA186_MCR1 (CP022735.1) and pSA26-MCR-1 (KU743384.1) (88% coverage and 99% nucleotide identity for both), isolated from the Arabian Peninsula (Sonnevend *et al.,* 2016). It is likely that *mcr-1* was initially captured and mobilized by the composite transposon Tn*6330* (IS*Apl1*-*mcr-1*-*pap2*-IS*Apl1*), followed by the loss of IS*Apl1* over time, leading to the stabilisation of *mcr-1* in diverse plasmid backgrounds and therefore dissemination via horizontal transfer (Wang *et al.,* 2018a). In this study, *mcr-1.1* from nucleotide 213,314 to 214,287, flanked by IS*ApaI1* at the 5′ end and other resistance determinants were located separately on the plasmid (Figure 8.1), suggesting independent mobilisation of *mcr-1* into a MDR IncHI2-ST3 plasmid by IS*ApaI1* and inferring further possible dissemination of *mcr-1.1* via insertional transposition or transfer of a highly resistant plasmid in the hospital setting.

The emergence of *mcr-8.1* in both animal, and human from China has been described by several reports (Wang *et al.* 2018b, Wang *et al.,* 2019). The variants of *mcr-8* were further reported from China, Laos, and Algeria (Hadjadj *et al.,* 2019; Nabti *et al.,* 2020; Yang *et al.,* 2020). None of the previous reports found the association of *mcr-8* with a particular clone. Genomic analysis revealed that *mcr-8.1* was circulated at DMCH through the clone of *K. pneumoniae* ST15 (Figure 8.3). The isolates were shown to be differed by 0 to 1 SNP after recombination removal and were not confined to a single ward (Table 8.2). ST15 has been regarded as successful clone in disseminating $bla_{\text{CTX-M-15}}$ globally (Baraniak *et al.,* 2013; Ewers *et al.,* 2014; Harada *et al.,* 2016). It was worrying that *K. pneumoniae* ST15, associated with the highly prevalent resistance gene in the region of SA, is $bla_{\text{CTX-15}}$ positive and has acquired colistin resistance (Bevan *et al.,* 2017). The colistin resistance, *mcr-8.1* in this study was carried by an identical IncFIB(pQil) conjugative plasmid (Table 8.3; Figure 8.6). Earlier studies described the presence of *mcr-8* with IncFII, and IncP (Wang *et al.* 2018b, Hadjadj *et al.,* 2019; Wang *et al.,* 2019; Nabti *et al.,* 2020; Yang *et al.,* 2020). The plasmid background of IncFIB(pQil)-MCR-8.1 was very similar to the previously described plasmids without any *mcr*-like mechanism (Figure 8.7); IncFII plasmids isolated from pigs in China (accession no: MG736312.1). However, IS*903B* at the 3' end have been replaced by IS*Kpn21* (Figure 8.8). It is possible that *mcr-8.1* was originally transposed to the IncFIB(pQil) plasmid by IS*903B* composite transposon (Figure 8.7; Figure 8.8). Incidentally, all resistance components in IncFIB(pQil)

plasmids in this study were bracketed by IS*903B* from nucleotide position 20,590 bp to 64,656 bp, suggesting the potential for transposition of the entire intervening DNA segment (Figure 8.7; Figure 8.8). Our findings demonstrate that the IncFIB(pQil) plasmid harbouring *mcr-8.1* was remarkably stable (Figure 8.9), suggesting adaptive plasmid-host evolution (Stalder *et al.,* 2017). Yang *et al.,* (2017) reported that colistin susceptibility could be attenuated after serial passaging of *mcr-1* positive strains in antibiotic-free medium. *K. pneumoniae* ST15 can be a vector capable of spreading *mcr*-mediated colistin resistance, particularly in a setting with sub-optimal IPC practices (Hawkey, 2015; Cimmino *et al.,* 2016; Sood and Perl, 2016; Cheng *et al.,* 2018). Acquisition of a resistance plasmid may impose a fitness cost, depending on the host and plasmid backbones (Humphrey *et al.,* 2012; Yang *et al.,* 2017). Growth rates over time of transconjugants (TDM697b and TDM914b) were significantly lower than that of *E. coli* J53 (p<0.0001) (Figure 8.10), implying significant fitness cost owing to the acquisition of plasmid harbouring *mcr-8.1*.

This is the first comprehensive report of human-associated *mcr* in humans from SA. These data indicate the need for a "one health" surveillance system in Bangladesh to prevent the spread of *mcr* in human medicine (Walsh, 2018).

Section Nine

Molecular and Epidemiological Analysis of a Burkholderia cepacia sepsis outbreak from a Tertiary Care Hospital in Bangladesh

9.1 Introduction

9.1.1 Bloodstream infections and *Burkholderia cepacia c*omplex

BSI is the presence of viable microorganisms (bacteria or fungi) in blood, eliciting the alteration of clinical, laboratory and hemodynamic parameters as a result of inflammatory response and essentially established by a positive blood culture. Generally, BSI can be categorized based on target groups: i. an immunologically normal host, ii. physiologically immunocompromised patients, e.g. extreme age (newborns, elderly), iii. patients predisposing to infections due to pathological conditions or medications. However, BSI in third category group can vary according to hosts' immune status, underlying disease conditions and environmental factors (Viscoli, 2016).

Burkholderia cepacia complex (Bcc) is considered as opportunistic human pathogen, pose a significant health risk particularly to cystic fibrosis (CF) patients, resulting life threatening lung infection, bacteraemia and death (Dentini *et al.,* 2017; Sfeir, 2018). *B. cepacia* bacteraemia also has been reported in association with nosocomial outbreaks and patients admitted in ICU; however, Bcc is also associated with host and/or environmental risk factors such as immunosuppression, co-morbidities, exposure to medical devices or surgical interventions, use of central venous catheter and prolonged hospital (Baul *et al.,* 2018; Becker *et al.,* 2018; Gupta *et al.,* 2018; Rastogi *et al.,* 2019).

9.1.2 Risk factors for burn infections briefly

Burn sepsis is one of the major complications of burn injury, accounts for 50-60% of death followed by burn, leads to multiple organ dysfunction syndrome (MODS) which is a key indicator for sepsis (Hogan *et al.,* 2012; Manning, 2018).

A variety of factors increase the risk of developing invasive burn wound followed by sepsis includes individual with burn of >20% of total body surface area (TBSA), inhalation injury, delayed in burn wound excision, extreme age (very old, very young) and impaired immunity (Hidalgo *et al.,* 2016; Rech *et al.,* 2019). Increase length of hospital stay, use of artificial medical and ICU admission predispose to sepsis (Dolp *et al.,* 2018; O'Brien *et al.,* 2019).

9.1.3 The genus *Burkholderia*

The genus *Burkholderia* is a member of Proteobacteria, includes species associated with plant, animal and human pathogenesis and symbioses which was identified in 1940 by Walter H. Burkholder as a cause of sour skin of onion (Kenna *et al.,* 2017). *Burkholderia* are Gram negative, catalase-producing, lactose-nonfermenting, obligately aerobic bacilli. *Burkholderia* encompasses species of Bcc, *Burkholderia mallei*, *B. pseudomallei* and *B. gladioli*. All the members of *Burkholderia* are motile except *B. mallei* (Kenna *et al.,* 2017; Wikipedia, 2020f). Three monophyletic clusters have been demonstrated with the genera, consists of Bcc, *B. pseudomallei* and closely related species, and of most of the phytogenic species within the genus, including *Burkholderia glumae* and *Burkholderia gladioli* (Figure 9.1) (Sawana *et al.,* 2014). Bcc and *B. pseudomallei* are human pathogens, causing lung infection, particularly in CF patients and melioidosis, respectively, whereas *B. mallei* is responsible for disease in horses or related animal, causing glanders (Kenna *et al.,* 2017; Wikipedia, 2020f).

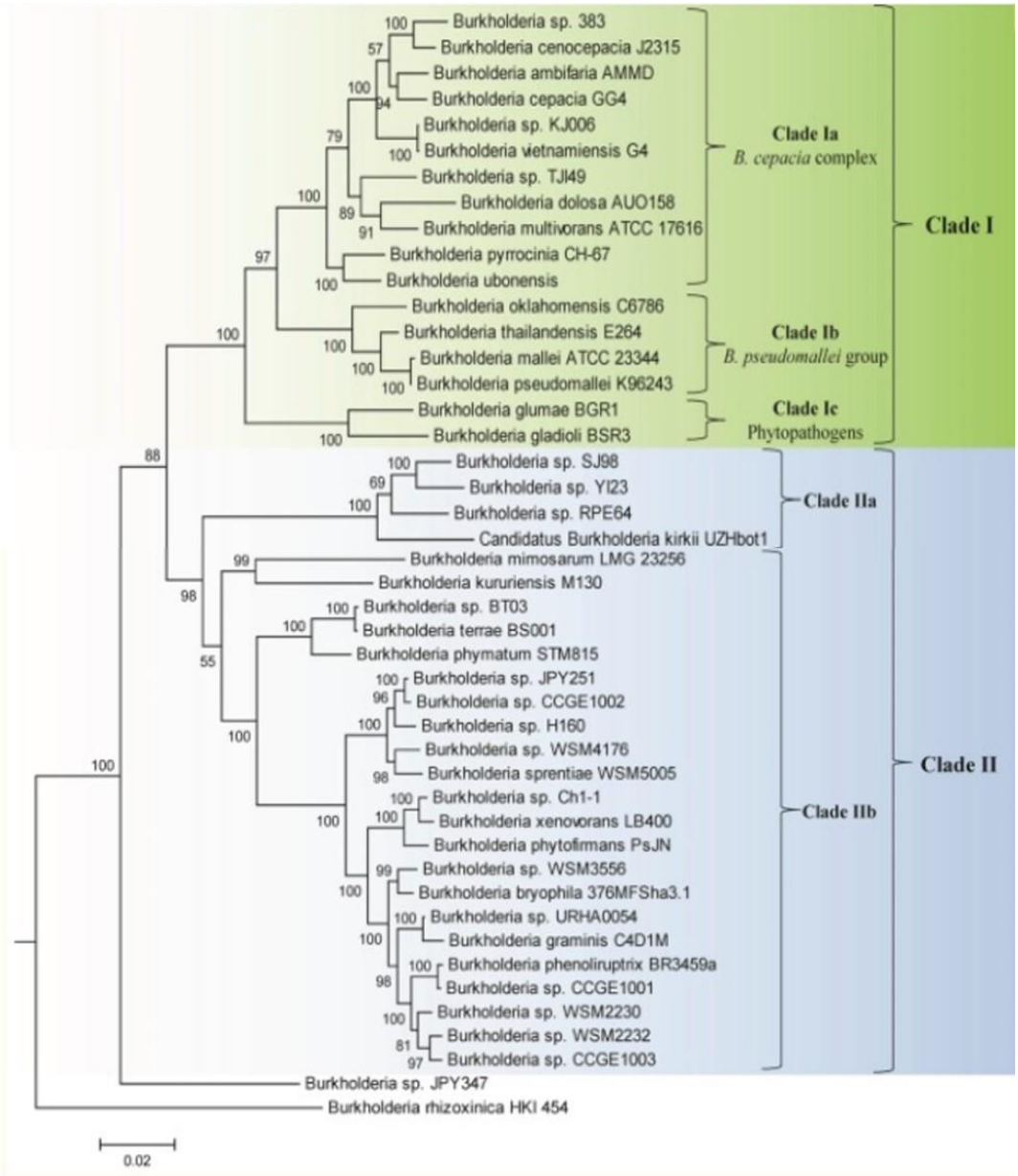


Figure 9.1 A maximum likelihood phylogenetic tree of members of the genus *Burkholderia* based conserved proteins. Reproduced from (Sawana *et al.*, 2014).

9.1.4 *Burkholderia cepacia* complex

Bcc is composed of at least 20 different species (Figure 9.1), including *B. cepacia, B. multivorans, B. cenocepacia, B. vietnamiensis, B. stabilis, B. ambifaria, B. dolosa, B. anthina, B. pyrrocinia,* and *B. ubonensis*. (Kenna *et al.,* 2017; Wikipedia, 2020f). In the mid-1980s, Bcc was described in CF patients as a cause of sepsis called "Cepacia Syndrome", characterized by lung function deterioration, bacteraemia and death (Isles *et al.,* 1984). The members of Bcc are typically found in soil, water, plant, rhizosphere, and in animals and can survive for prolonged periods in moist environments, even in presence of disinfectants, therefore serves as a potential reservoir for nosocomial infections (Donlan and Costerton, 2002; Tsang, 2004). The Bcc large genome (7-9 Mb) consisting of several chromosomes and plasmids support their metabolic versatility and facilitate to adapt in adversarial environment (Holden *et al.,* 2009; Agnoli *et al.,* 2012). Many members of Bcc are inherently virulent and resistant to many antimicrobials (Rhodes and Schweizer, 2016; Bochkareva *et al.,* 2018).

9.1.4.1 Pathogenesis of Bcc

Although the virulence factors associated with Bcc are not well documented, the lung pathogenesis by Bcc has been elicited due to the following factors. Bcc associated with high mortality among the CF patients with lung transplantation (Dentini *et al.,* 2017). The clinical impact of lung infections by different species of *Burkholderia* in CF patients after lung transplantation is variable; *B. cenocepacia* and *B. multivorans* have been shown to be more virulent than other species (Olland *et al.,* 2011; Loutet *et al.,* 2020).

9.1.4.1.1 AMR in Bcc

The genomes of Bcc species contain various β-lactamase genes, includes *ampC*, having hydrolytic activity for expanded-spectrum cephalosporins, and *ampD*, encode cell wall-recycling enzyme (N-acetyl-anhydromuramyl-L-alanine amidase) (Holden *et al.,* 2009). Mutation of *ampD* leads to accumulation of 1,6-anhydro-MurNAc-peptides in the cytoplasm resulting in *penR* (*AmpR*) mediated transcription of *penB* and *ampC* target; therefore, mediates resistance to β-lactams (Figure 9.2) (Harris, 2015; Rhodes and Schweizer, 2016). Efflux pumps in relation to AMR have been studied intricately in *B. cenocepacia*. Several efflux pumps of the resistance nodulation cell division (RND) family have been described in *B.* cenocepacia which have been associated with efflux of antimicrobials (Table 9.1) (Podnecky *et al.,* 2015; Rhodes and Schweizer, 2016). The number of porins in Bcc is like *P. aeruginosa* exhibiting similar permeability of antimicrobials, but 10-fold less than *E. coli*. Hence Bcc is comparatively more resistant to any antimicrobials. Additionally, Bcc has an amino arabinose biosynthesis operon which synthesize 4-amino-4-deoxy-L-arabinose (Ara4N) responsible for modifications of lipid A, resulting in intrinsic resistant to polymyxin. Target modification has also been observed among the species which are associated with the resistance to fluoroquinolones and trimethoprim (Rhodes and Schweizer, 2016).

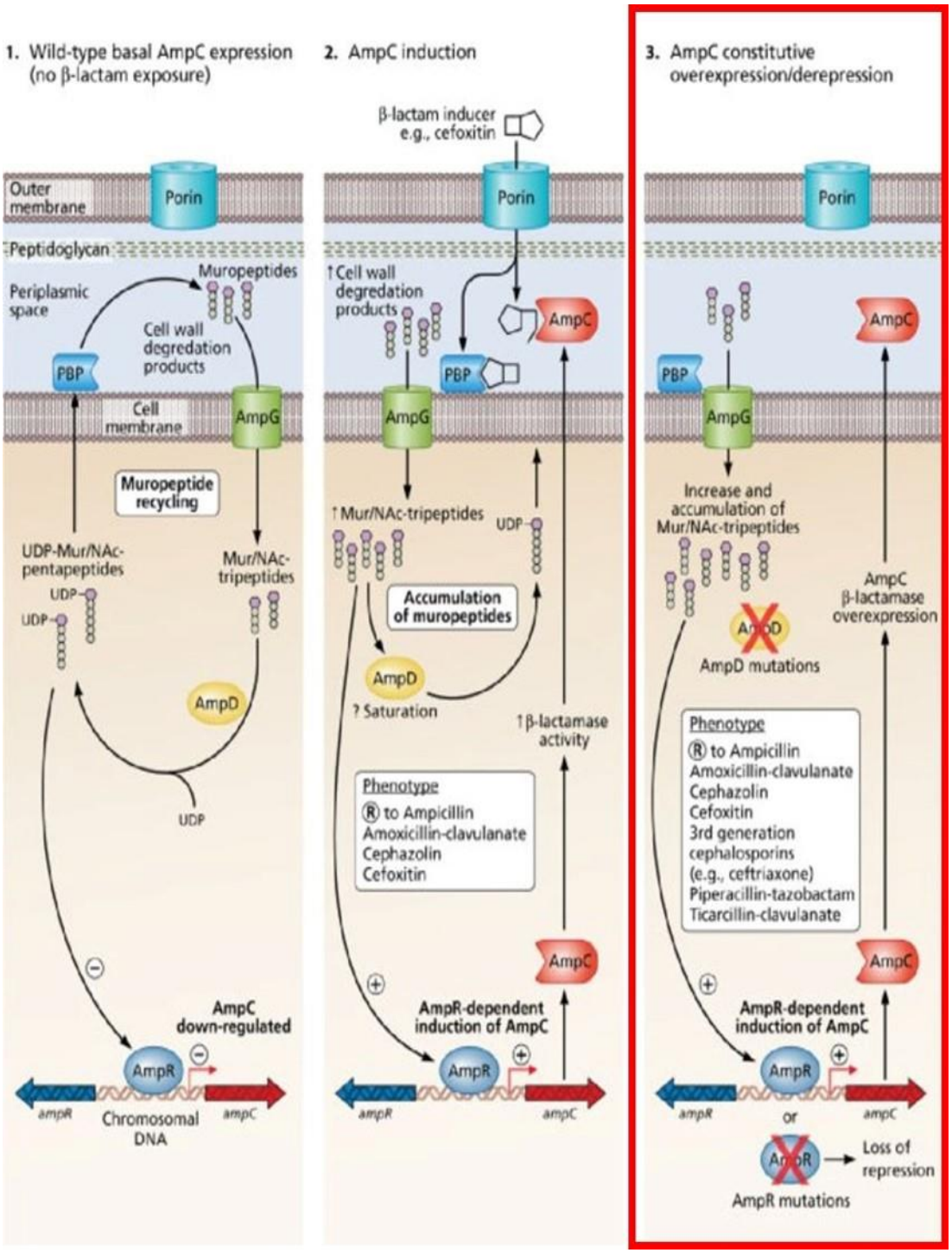


Figure 9.2 β-lactam resistance mediated by *ampC* overexpression. Reproduced from (Harris, 2015). Bcc show reduced susceptibilities to β-lactams due to *ampD* mutation.

Table 9.1 Efflux pumps of RND family in *B. cenocepacia* causing antimicrobial resistance (Podnecky *et al.,* 2015).

Efflux pumps	**Gene name**	**Efflux of antimicrobials**
RND-1	NA[1]	Non-detectable
RND-3	NA[1]	Nalidixic acid, ciprofloxacin, tobramycin, chlorhexidine
RND-4	NA[1]	Aztreonam, chloramphenicol, fluoroquinolones, tobramycin
RND-8	NA[1]	Tobramycin
RND-9	NA[1]	Tobramycin, chlorhexidine
RND-10	*ceoAB-opcM*	Chloramphenicol, fluoroquinolones, trimethoprim

[1]NA, not applicable

9.1.4.1.2 Colonisation factors

Bcc colonizes the respiratory tract in CF by means of their motility, adhesion, tissue damage and biofilm formation. *B. cenocepacia* with flagella-gene mutants are capable to invade respiratory epithelial cells and survive in murine models of infection. Pilli are the determinants for adhesion and different lipases and proteases produced by the bacteria causing tissue damage. Once colonization has been established at the site of infection, bacteria invariably form biofilms. While more abundant biofilms are formed by *B. cenocepacia* and *B. multivorans,* all the members of Bcc can produce biofilms, enhancing both pathogenesis and antimicrobial resistance (Mahenthiralingam and Vandamme, 2005; Shommu *et al.,* 2015; Sfeir, 2018).

9.1.4.1.3 Cell surface properties

LPS of Bcc mediates intrinsic resistance to cationic antibiotics such as polymixin (discussed above) and antimicrobial peptides such as human defensins and five times more endotoxic than other CF pathogens. Moreover, Bcc LPS by possessing an O-antigen sugar side chains mediating rough phenotypes, are more resistant to killing by human serum than Bcc with smooth phenotypes lacking sugar linkage (Mahenthiralingam and Vandamme, 2005; Shommu *et al.,* 2015; Sfeir, 2018).

9.1.4.1.4 Cellular invasion

Bcc is capable to invade and survive in eukaryotic cells and yield bacteraemia in CF. *B. cenocepacia* gathers on the apical surface of cell and invade into the cell *via* a biofilm-dependent pathway. Flagella and proteins homologous to type III secretion system help in invasion (Mahenthiralingam and Vandamme, 2005; Shommu *et al.,* 2015; Sfeir, 2018).

This chapter describes an outbreak of *B. cep acia* bacteraemia in a burn's critical care unit at DMCH supported by WGS. The population structure and genetic relationship of *B. cepacia* isolated in this study were compared with 91 global *B. cepacia* deposited previously in the '*Burkholderia* Genome Database' (Burkholderia Genome DB, 2020). Although the objectives of this PhD focused on Enterobacterales infections, this study attempted to explore this outbreak to inform the importance of standard IPC policies in Bangladesh.

9.2 Result

9.2.1 Study population and overview of *B. cepacia* cases

Among 528 blood specimens, 45% (237/528) were culture positive and 3% (15/528) were *B. cepacia* BSI. The study outline is shown in Figure 9.3. Of the 15 *B. cepacia* infected patients, 7 (46.7%) were male and 8 (53.3%) were female; the mean (±SD) age was 14 years (±12.57). All patients in this cohort belonged to a low socio-economic group (lower middle to below the national poverty level). The mean (±SD) hospital stay of *B. cepacia* cases was 43.9 days (±27.1). Antibiotic usage among the cases with *B. cepacia* septicaemia was: ceftriaxone, 80.0%; amikacin, 53.3%; levofloxacin, 33.3%; ceftazidime, 20%; meropenem, 13.3%, colistin, 13.3%; flucloxacillin, 6.7%; azithromycin, 6.7%; gentamicin, 6.7%; clindamycin, 6.7%; cefuroxime, 6.7% (Table 9.2). Two of the *B. cepacia* cases took discharge against medical advice (DAMA). Not including DAMA, the mortality rate was 31% (4/13) (Table 9.2; Figure 9.4). Patient characteristics are illustrated in Table 9.2. The first case was admitted to paediatrics department of DMCH on the 23rd of October 2016. According to hospital records, the patient was a suspected case of sepsis, and blood was sent for culture and sensitivity on the 2nd of November 2016 which was positive for *B. cepacia*. The rest of the cases were confined to the burn HDU or the burn paediatric HDU or the burn ICU (Table 9.2; Figure 9.4). Excluding DAMA (5/135), in the burn unit, the mortality rate for patients with other bacterial sepsis was higher (60.17%, 71/118) compared to *B. cepacia* sepsis (33.33%, 4/12) (p=0.073).

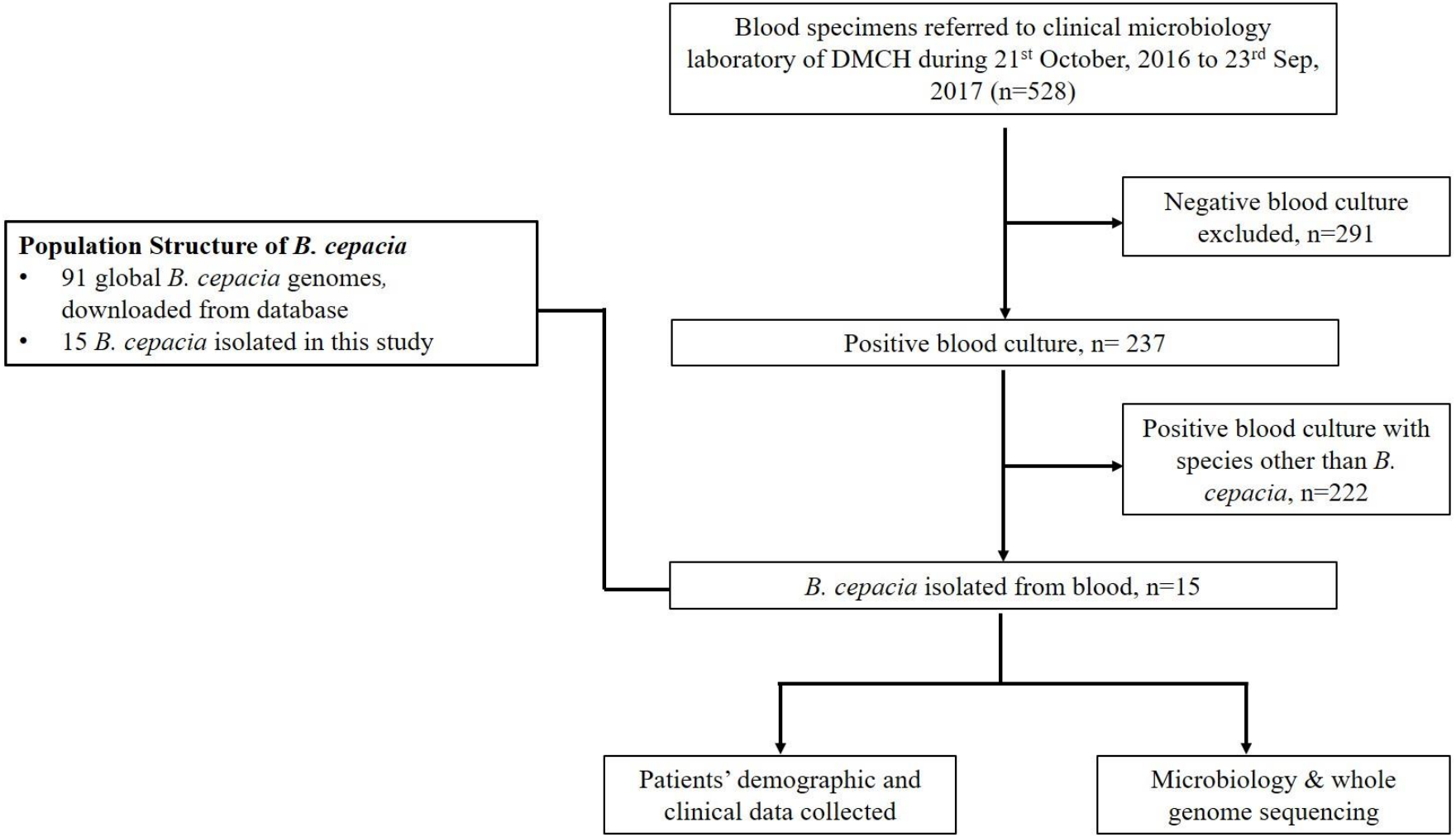


Figure 9.3 Study flowchart**.**

Table 9.2 Clinical characteristics of patients infected with *B. cepacia*.

Strain ID	Cases	Ward	Sample	Age[a]	Sex	SEC	DOA	DOSC	DOD	THS	Underlying disease	Antibiotic history	Outcome
dm93	Case 1	Pae	Blood	2.5	M	BPL	23.10.16	02.11.16	7.11.16	16	Unknown[b]	CRO, GEN	Discharge
b19	Case 2	Burn HDU	Blood	30	F	BPL	25.10.16	22.11.16	21.1.17	58	35% FB	CRO	DAMA
b06	Case 3	Burn HDU	Blood	22	M	Poor	10.11.16	22.11.16	30.11.16	21	35% FB	CXM, CLI	Discharge
b13	Case 4	Burn HDU	Blood	45	F	BPL	13.11.16	22.11.16	26.11.16	14	25% FB	AMK, CAZ	Died
b64	Case 5	Burn Pae. HDU	Blood	3.5	F	Poor	14.12.16	02.01.17	2.2.17	51	15% FB	AZM, CST, CRO	Discharge
b84	Case 6	Burn HDU	Blood	15	M	BPL	14.01.17	26.01.17	23.3.17	69	40% EB	AMK, CAZ, MEM	Discharge
b98	Case 7	Burn ICU	Blood	5	F	BPL	04.02.17	09.02.17	10.2.17	7	45% FB with II	AMK, CRO, FLU	Died
b99	Case 8	Burn ICU	Blood	10	F	LM	03.02.17	09.02.17	09.02.17	7	30% MB[c]	CRO, LVX	DAMA
b100	Case 9	Burn Pae. HDU	Blood	2.5	F	LM	28.01.17	09.02.17	8.4.17	71	22% SB	AMK, CRO, LVX	Discharge
b101	Case 10	Burn Pae. HDU	Blood	7	M	BPL	03.02.17	09.02.17	17.2.17	15	43% FB	AMK, CRO	Died
b111	Case 11	Burn HDU	Blood	19	M	Poor	16.01.17	23.02.17	7.4.17	82	30% FB	AMK, CRO, MEM	Discharge
b124	Case 12	Burn Pae. HDU	Blood	3.5	F	BPL	25.02.17	05.03.17	23.4.17	58	30% FB	CAZ, CST, CRO	Died
b163	Case 13	Burn ICU	Blood	1.5	M	Poor	10.4.17	17.4.17	26.5.17	46	40% SB	CRO, LVX	Discharge
b212	Case 14	Burn HDU	Blood	23	F	Poor	20.5.17	31.5.17	22.6.17	34	35% FB	AMK, CRO, LVX	Discharge
b219	Case 15	Burn HDU	Blood	20	M	Poor	15.6.17	19.6.17	30.8.17	76	35% EB with II	AMK, CRO, LVX	Discharge

M, male; F, female; SEC, socio-economic condition; BPL, below poverty level, LM, lower middle; DOA, date of admission; DOSC, date of sample collection; DOD, date of discharge/DAMA/death; THS, total hospital stay; Pae, paediatrics; FB, flame burn, EB, electric burn; II, inhalation injury; MB, mixed burn; SC, scald burn; DAMA, discharge against medical advice. AMK, amikacin; AZM, azithromycin; CAZ, ceftazidime; CXM, cefuroxime; CST, colistin; CLI, clindamycin; CRO, ceftriaxone; FLU, flucloxacillin; GEN, gentamicin; LVX, levofloxacin; MEM, meropenem.

[a]Age are given in years; [b]Underlying disease was not available in hospital record; however, this case was clinically suspected as sepsis and therefore, blood was referred for culture; [c]Combination of chemical and flame burn.

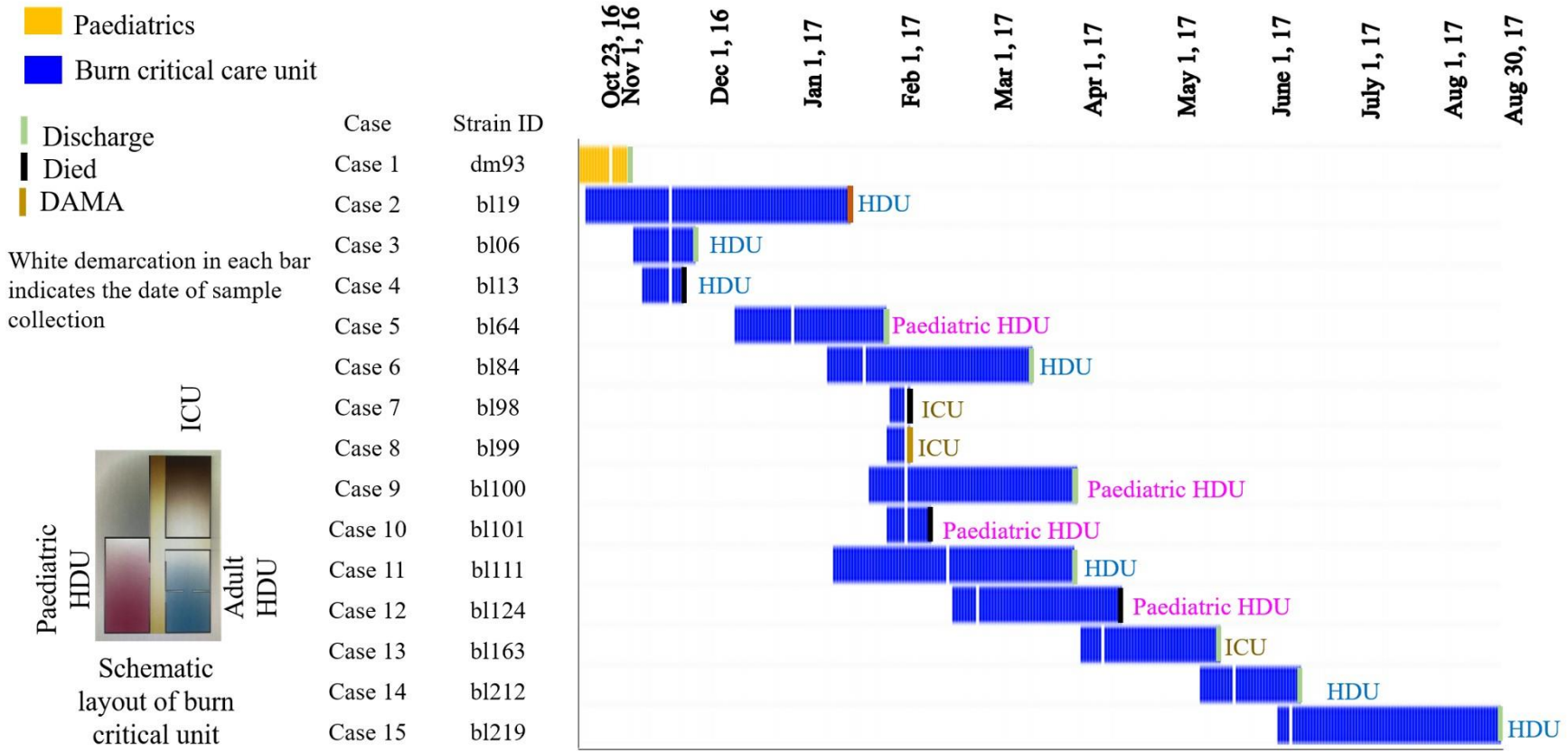


Figure 9.4 Length of hospital stay of the patients with *B. cepacia* bacteraemia.

9.2.2 Investigating possible outbreak by *B. cepacia*

Data extracted from patients' clinical history and WGS data of the outbreak strains were evaluated to investigate possible outbreaks. Likely epidemiological links on transmission (patient to patient connectivity) was predicted if there is overlapping of hospital stay among the outbreak cases (Figure 9.4). The outbreak by *B. cepacia* was identified retrospectively.

We documented this outbreak between October 2016 and August 2017 at DMCH (Figure 9.4). All outbreak cases had clinical signs of sepsis at the time of sample collection and patients in burn critical units were undergoing artificial ventilation and had central venous catheter lines and urinary catheters at the time of diagnosis. There was a continuous overlapping of patients at DMCH during the outbreak period (Table 9.2; Figure 9.4). Core genome SNPs-based phylogeny showed that the outbreak strains were confined as a single clade (Figure 9.5), corresponded to a common novel clone (ST1578) (Figure 9.6).

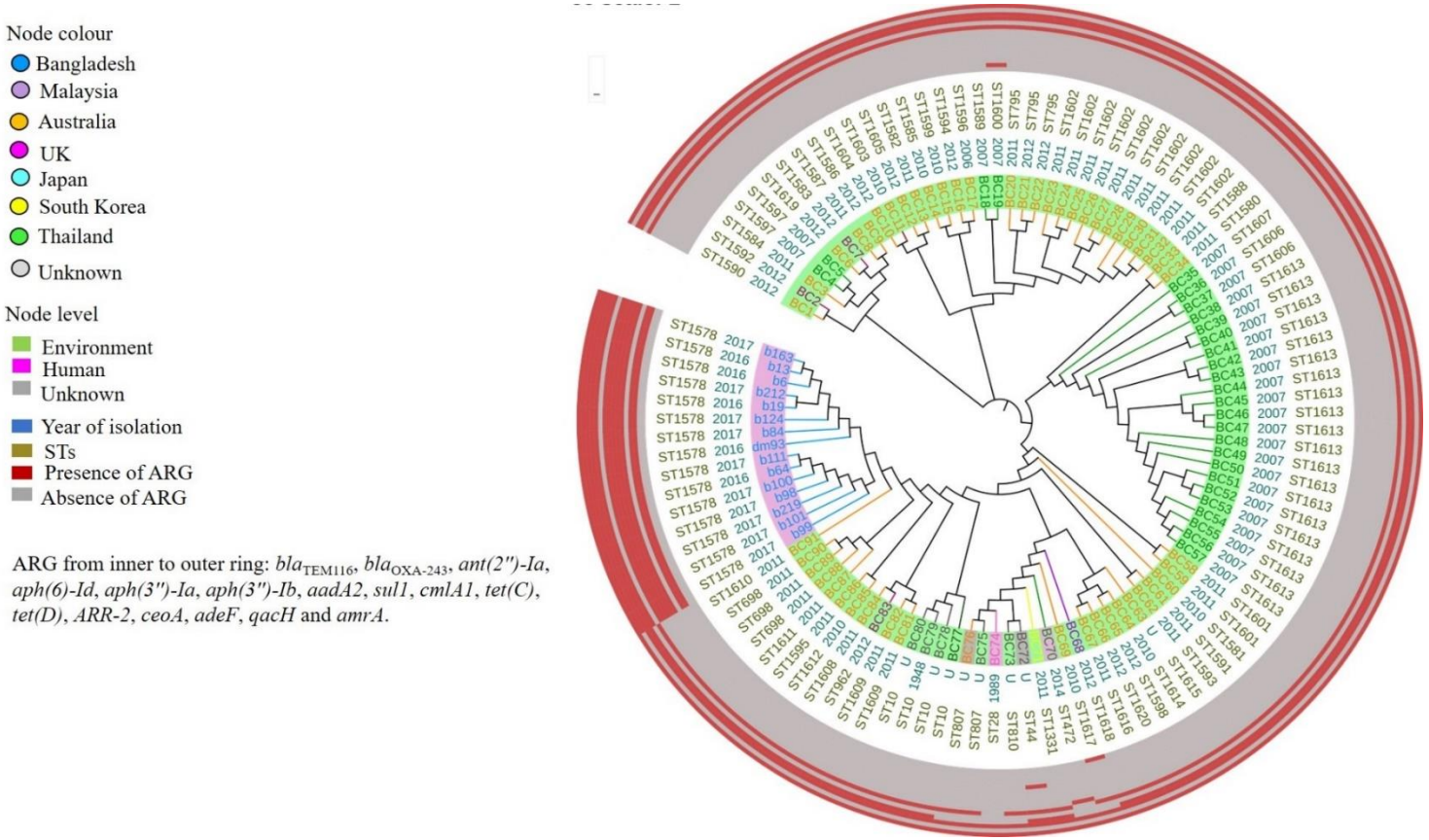


Figure 9.5 A maximum likelihood tree of *B. cepacia* by core genome SNPs with epidemiological data and ARGs. Country of origin is represented by specific colour of node. Node level are highlighted according to source of sample. Global strains are stated with specific codes. Genomes' (retrieved from '*Burkholderia* Genome Database') attributes with corresponding codes are compiled in appendix F.

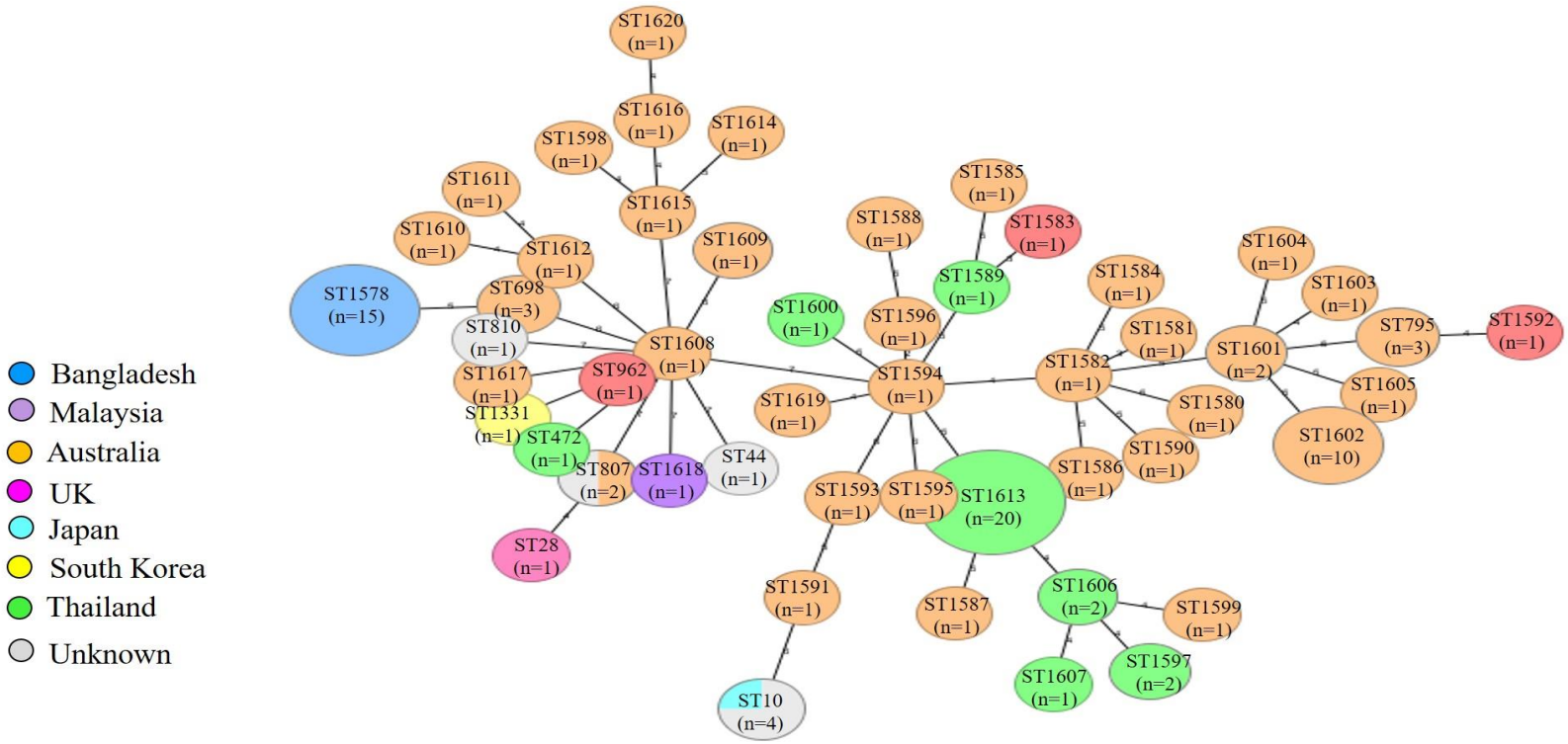


Figure 9.6 Minimum spanning tree of *B. cepacia* by MLST type. Each node within the tree represents a single ST. The size of the nodes is proportional to the number of isolates represented by corresponding node. Selected nodes are labelled with corresponding STs, and number of isolates represented. All global strains including Bangladeshi outbreak strains mentioned in this diagram were assigned as corresponding STs in this study except ST10, ST44, ST807 and ST810. Genomes' (retrieved from '*Burkholderia* Genome Database') attributes with corresponding codes are compiled in appendix F.

9.2.3 Clonal relationship of Bangladeshi outbreak strains of *B. cepacia* with the global *B. cepacia*

Global *B. cepacia* (n=91) genomes were downloaded from '*Burkholderia* Genome Database' of which 82 strains could not be matched with previously described STs which were also submitted to pubMLST for ST assignment (*Burkholderia* Genome DB, 2020; PubMLST, 2020). All strains (n=106) were grouped into 9 clusters (Figure 9.6). The Bangladeshi strains shared ST clusters with strains from Australia, USA, South Korea, Thailand, UK and Malaysia. Interestingly, STs differed according to geographical area (Figure 9.6). Core genome alignment also suggested that the Bangladeshi *B. cepacia* are genetically closer to environmental strains of Australian origin rather than human strains isolated from cystic fibrosis patients in the UK (LMG16656.fsa nt), Thailand (LO6.fasta), and other Asian strains (Figure 9.5).

9.2.4 Background on resistance

B. cepacia isolated in this study were analysed for antibiotic resistance profiles which were performed in triplicate and all repetitions showed consistent findings, however, few repeats had MIC of one-dilution difference. There are no MIC breakpoints for *B. cepacia* recommended by EUCAST (EUCAST, 2020). According to CLSI breakpoints, 20% of *B. cepacia* (in this study) were resistant to ceftazidime and 93.33% intermediate resistant to levofloxacin. All the outbreak strains had high MIC value for amoxicillin-clavulanate, amikacin, gentamicin, fosfomycin, trimethoprim-sulfamethoxazole and colistin. All were sensitive to meropenem (Table 9.3) (CLSI, 2020). According to their MIC profiles, antibiotics deployed as empirical therapy to treat outbreak cases were invariably deemed to be inappropriate (Table 9.2). The antimicrobial resistance genes identified in the outbreak strains were identical (Figure 9.5). Likewise, *ampD* for all strains was homogenous; however, compared to *ampD* (BCAL3430) of *B. cenocepacia* strain J2315, we found 10 substitutions of amino acid in *ampD* (Figure 9.7). The resistance genes present in the outbreak strains of *B. cepacia* were *ant(2'')-Ia*, *aph(6)-Id*, *aph(3'')-Ib*, *aadA2*, *amrA*, *ARR-2*, *sul1*, *qacH*, *cmlA1*, *tet(C)*, *ceoA*, and *adeF*. The outbreak strains were shown to possess more resistant genes than other global strains (Figure 9.5).

Table 9.3 MIC values of *B. cepacia* in this study against relevant antibiotics (n=15).

Strain ID	AMC[a]	TZP[a]	CRO[a]	CAZ[a]	CTX[a]	[a]FEP	IPM[a]	MEM[a]	CIP[a]	LVX[a]	AMK[a]	GEN[a]	FOF[a]	SXT[a]	TGC[a]	CST[a]
dm93	>256	4	8	4	16	8	8	4	2	4	64	>256	>256	32	16	>256
b19	>256	8	16	8	32	16	8	4	1	4	256	>256	>256	64	4	>256
b06	>256	8	16	8	32	16	8	4	1	4	256	>256	>256	32	16	>256
b13	>256	8	16	8	32	16	8	4	2	4	256	>256	>256	32	16	>256
b64	>256	2	4	2	8	8	8	2	2	4	64	>256	>256	64	8	>256
b84	>256	8	16	4	16	16	8	4	2	4	256	>256	>256	64	16	>256
b98	>256	2	4	2	8	8	8	2	2	4	64	>256	>256	64	8	>256
b99	>256	16	32	8	32	32	8	4	2	4	256	>256	>256	32	4	>256
b100	>256	8	16	4	32	16	8	4	2	4	256	>256	>256	64	8	>256
b101	>256	2	4	2	8	8	8	2	2	4	64	>256	>256	32	8	>256
b111	>256	2	4	2	8	8	8	2	1	2	256	>256	>256	64	4	>256
b124	>256	2	16	8	16	16	8	4	2	4	256	>256	>256	64	16	>256
b163	>256	16	32	16	32	8	8	4	2	4	128	>256	>256	32	16	>256
b212	>256	8	16	16	16	16	8	4	2	4	128	>256	>256	64	16	>256
b219	>256	16	32	32	32	32	8	4	2	4	64	>256	>256	64	16	>256

[a]MIC values are indicated by mg/l; MIC values were determined in triplicate. AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; TGC, tigecycline; CST, colistin.

Figure 9.7 Substitutions of amino acid in *ampD* compared to *ampD* (BCAL3430) of *B. cenocepacia* strain J2315. Substitutions are underlined by red.

9.2.5 Analysis of Bcc virulence

The Bangladeshi outbreak strains shared some common virulence genes with the global strains (*bimA*, *boaAB*, *pilABCDNOQRSTV*, *gmhA*, *manC*, *wcbABCDEFGHIJKLMNOPQRST*, *wzm/wzt2*, *cheABDRWYZ*, *flgABCDEFGHIJKLMN*, *fliACDEFGHIJKLMNOPQRS*, *motAB*, *tsr*, *bspI2/bspI3*, *bspR2/bspR3/bspR4/bspR5*, *pmlI/bspI1*, *pmlR/bspR1*, *bapABC*, *basJ*, *bicAP*, *bipBCD*, *bopACE*, *bprABCDPQ*, *bsaKLMNOPQRSTUVXYZ*, *orgAB*, *spaP*, gene for T6SS), although two of the global strains (MSMB1338WGS.fsa_nt and DWS16B-4.fsa_nt) were negative for all genes from VFDB (Figure 9.8). There was variation in the presence of virulence genes between our clinical outbreak strains and previous clinical *B. cepacia* (LMG16656.fsa nt and LO6.fasta) isolated from CF patients (Figure 9.8).

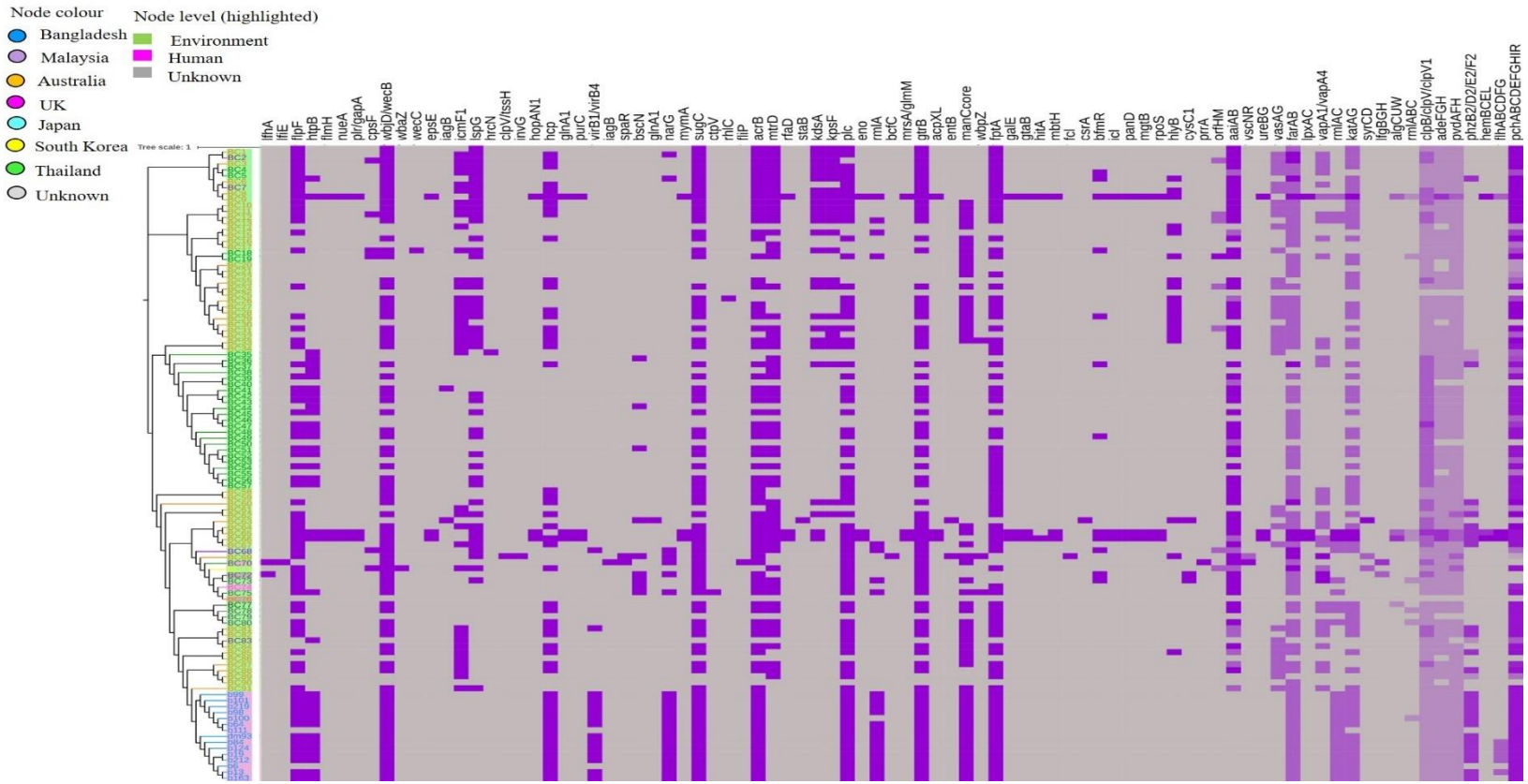


Figure 9.8 Heatmap showing the presence of virulence genes in *B. cepacia*. Original strain IDs with corresponding codes are compiled with appendix F. Common VFs shared by the strains were not included in heatmap. Violet indicates the presence and grey indicates the absence of VF in the respective strain. The increase in intensity is proportional to number of genes.

9.3 Discussion

Although *B. cepacia* is considered almost exclusively a pathogen for CF patients (Kenna *et al.,* 2017), our study reported *B. cepacia* bacteraemia predominantly from burns patients. The findings of this chapter have been published (Farzana *et al.,* 2020b). The laboratory works, and analysis related to the work have been completely performed by me as a part of this PhD. Only the sequencing services (Illumina MiSeq) were provided by Prof. Walsh's genomic laboratory at CU.

B. cepacia are considered opportunistic human pathogens and can be transmitted to patients via environmental contamination or person-to-person contact (Wiener-Well *et al.,* 2014; Ko *et al.,* 2015; Exner *et al.,* 2017; Becker *et al.,* 2018). Burns patients are generally more susceptible to infection due to impaired immune function. Risk factors for sepsis in burns include >20% of TBSA, inhalation injury, delayed burn wound excision, increased length of hospital stay, use of artificial medical devices and ICU admission (Dolp *et al.,* 2018; Yan *et al.,* 2018). Patients in burn ICUs are more vulnerable to septicaemia than general ICU patients (Snell *et al.,* 2013). Our findings demonstrate an outbreak of bacteraemia in DMCH caused by a single clone of *B. cepacia* ST1578, mostly confined to patients admitted in burn critical care units (Figure 9.4). The outbreak cases had a mean hospital stay of 43.9 days and straddled each other implying transmission via direct patient contact and/or patient to hospital environment and *vice versa*. Clinically, this outbreak was associated with a mortality rate of 31% (Table 9.2; Figure 9.4).

Previous studies show that burn sepsis accounts for 50-60% of deaths in burn patients (Manning, 2018). In this study, mortality due to burn sepsis with bacteria other than *B. cepacia* (60.17%) was higher than *B. cepacia* sepsis (33.33%) (p=0.073). A limitation of the current study was the lacked sufficient clinical information to access and to investigate why the mortality rate was significantly lower for *B. cepacia* cases. At the time of enrolment many of these patients were on “inadequate” antibiotics based on our susceptibility data. However, there are limitations to the interpretation of susceptibility results for *B. cepacia* and we lack data on whether antibiotics were changed later during hospital stay. It is possible there were other factors favouring survival in the group infected with *B. cepacia*, including possibly relatively lower pathogenicity of *B. cepacia* as a cause of bacteraemia. *B. cepacia* has been described

as a cause of pseudo-outbreaks (Ko *et al.,* 2015). Although this possibility should be considered here the timing of infections suggest this was not the case, and a persistent environmental source of cross contamination on the burn unit was a more likely cause. Unsurprisingly, radical infection control programs can mitigate the spread of infections and improve patient outcomes in burn units (Coban, 2012). No IPC intervention was undertaken here because the outbreak was identified retrospectively.

MDR Gram-negative bacteria infections have become a serious challenge in health care settings as a result of both intrinsic and acquired resistance mechanisms, limiting therapeutic options (Exner, 2017). *B. cepacia* is of concern due to their intrinsic resistance to clinically relevant antibiotics such as aminoglycosides and polymyxins (Rhodes and Schweizer, 2016). In this study, all *B. cepacia* showed very high MIC levels to amikacin and gentamicin mediated by resistance genes *ant*, *aph* and *aadA2* and/or overexpression of efflux pump such as ArmA, CoeA (Figure 9.5; Table 9.3) (Sfeir, 2018). *Burkholderia* spp. are typically resistance to colistin due to a unique intrinsic amino arabinose biosynthesis operon (Rhodes and Schweizer, 2016). Genome sequencing also identified resistance genes such as *adeF*, *tetC*, *sul1*, and *qacH* (Figure 9.5) which contribute to phenotypic resistance of *B. cepacia* tetracycline, fluoroquinolones, sulphonamides, and antiseptics (Alcock *et al.,* 2020) (Table 9.3). Mutations in *ampD* are associated with the upregulation of β-lactam degrading enzymes, PenB and AmpC. This mechanism has been found to be one of the causes of β-lactam resistance in *B. cepacia* (Hwang *et al.,* 2015; Rhodes and Schweizer, 2016). Mutations in *ampD* were identical in all *B. cepacia* analysed in our study (Figure 9.7) and all the isolates had high MIC value for amoxicillin-clavulanate; however, MICs for piperacillin-tazobactam and cephalosporins were variable (Table 9.3). Perhaps enzymatic degradation was mainly responsible for putative ß-lactam resistance in this study, however, we did not evaluate the expression of ß-lactamase such as PenB, AmpC or PenA in relation to AmpD mutations (Rhodes and Schweizer, 2016). Although the empirical antibiotics were shown to be ineffective (Table 9.3), the relationship between patients' morbidity/mortality and antibiotics prescription cannot be fully explored as datasets are incomplete.

Virulence of *B. cepacia* is typically related to adhesins, invasins, intracellular pathogenicity, antiphagocytic factors, secretory and signalling systems (Liu *et al.,*

2019c). A set of virulence genes in relation to all steps in pathogenesis were identified in Bangladeshi outbreak strains *B. cepacia* (Figure 9.8). However, whether other virulence determinants are associated with the ability to cause bacteraemia, and the role of patient factors, could not be determined by this current study.

B. cepacia identified in this study belonged to a novel clonal type ST1578 (Figure 9.6). The genetic background of the outbreak strains was very similar; however, a few variations were found regarding the presence of virulence genes (Figure 9.5; Figure 9.8). Compared to global strains from the database, resistance genes for aminoglycosides (*ant(2'')-Ia, aph(6)-Id, aph(3'')-Ib, aadA2*), fluoroquinolones (*qacH*), tetracycline (*tet(C)*) and sulphonamide (*sul1*) were found only among the strains isolated in this study (Figure 9.5). Virulence gene, *aai* was absent in our outbreak strain, which was common in both human strains, LMG16656.fsa nt and LO6.fasta, (from *Burkholderia* Genome Database), but the overall virulence pattern of the outbreak strains was similar to LMG16656.fsa nt (human strain isolated from the UK) than LO6.fasta (human strain isolated from Thailand). Compared to *B. cepacia* LO6.fasta, flagellar protein (*lfg, lfh, lfi*), secretory system (*clpV1, vsc, iagB, spaR*), and regulatory protein (*prrA*) were absent in the Bangladeshi strains (Figure 9.8). Thus, it can be inferred that the Bangladeshi outbreak strains identified in this study are genetically distinct from global strains retrieved from the database.

Although we did not evaluate the epidemiological link between patients and the environment, epidemiological and molecular data suggest that the outbreak clone was circulating within DMCH over a protracted period (Figure 9.4). It is disconcerting that antibiotic pressure might have enhanced the elevation in AMR during the outbreak (Hughes and Andersson, 2017). Identification of environmental sources of the outbreak followed by patient management can reduce the risk of infections in vulnerable populations (Coban, 2012; Wiener-Well *et al.*, 2014; Fernando *et al.*, 2017).

Section Ten

General Discussion

10.1 A general overview on Bangladeshi health system and AMR

Bangladesh has a population of about 165 million (Bangladesh Population [LIVE], 2020); ranked #35th (nominal, 2020) by gross domestic product (GDP) and 29th by purchasing power parity (PPP) (nominal, 2020). Bangladesh was the world's seventh fastest growing economy with a rate of 7.3% real GDP annual growth (nominal, 2019). (Wikipedia, 2020g). The fifth Bangladesh National Health Accounts (BNHA) 1997-2015 data accounts that Bangladesh Government spends 3% of GDP to public health (WHO, 2015). A report on the proportion of total health expenditure in different sectors in Bangladesh between 1997 to 2012 is described in Figure 10.1. A sizeable proportion of this commitment is implementing and maintaining health services across the public Medical College Hospitals (MCHs) which are free to the general public. MCHs are grossly oversubscribed (typically 4-5 times the #inpatient/bed). World Bank Development Indicator (2008-2015) implies an inadequate health facility in public health sectors of Bangladesh (1 physician per 2,500 population, 1 nurse per 5,000 population, and 1 bed per 1,670 individuals) (CDDEP, 2018). Antibiotics are usually free in the public hospitals but delivered empirically with limited variety. Antibiotics are prescribed usually based on availability in hospital supply and certainly do not address serious MDR or extensively drug-resistant (XDR) infections (Chowdhury *et al.*, 2009; The Fleming Fund, 2019; Essential Drugs Company Limited, 2020). IEDCR has commenced an AMR surveillance in 2017 in the major MCHs in Bangladesh (WHO, 2020a). On 22nd April 2019, the Government of Bangladesh formally banned the sales of antibiotics without prescription (bdnews24.com, 25 April 2019). However, the impact of AMR is grossly under reported, and infection control is suboptimal in the public hospitals of Bangladesh (Shahida *et al.*, 2016; Hsu *et al.*, 2017; Hoque *et al.*, 2020). During the time-period of the PhD, I was involved to undertake scoping exercise in Bangladesh with T. R. Walsh to bid for the FF Country Grant (CG) application (The Fleming Fund, 2019). The brief description of microbiology capacity of the major MCHs is mentioned in Table 10.1. Our survey also noted the lack of functional IPC committee in the tertiary heath care settings of Bangladesh.

National Strategy for AMR Containment in Bangladesh, 2017-2021 has been established with an aim to develop multisectoral approach for Antimicrobial stewardship. The strategies include coordination down to the subdistrict level, and

involvement of both the Ministry of Health and Family Welfare, and the Ministry of Fisheries and Livestock, however, there is little evidence of working jointly, and an operational systematic AMR surveillance. Table 10.2 illustrates the updates on national AMR programmes (BLRI, 2019; Department of Livestock Service, n. d.; Rahman *et al.*, 2017; WHO, 2018; The Fleming Fund, 2019; WHO, 2020d).

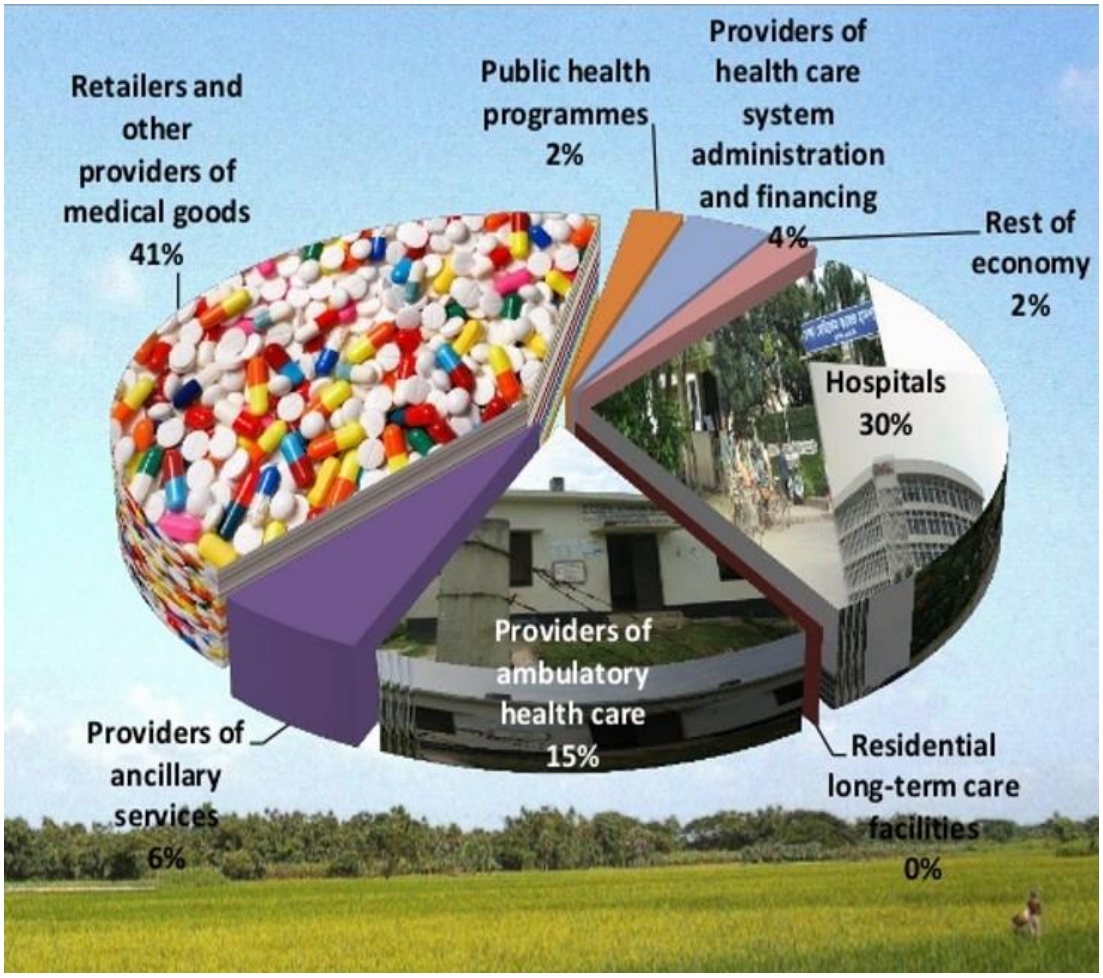


Figure 10.1 Bangladesh National Account 1997 to 2012. Reproduced from: MOHFW, 2002.

Table 10.1 Brief description of microbiology capacity of the major MCHs of Bangladesh.

	CMCH	DMCH	KMCH	MMCH	RMCH	RpMCH	SOMCH
# Core staff	7	2	2	2	2	2	5
# Rotating staff	14	14	5	15	15	11	14
# Beds	1326	2600	500	1000	1200	1000	500
# Inpatients	3000	7000	980	3000	3000	2100	2300
# Out-patients	5500	5000	3500	5000	2500	4000	4500
Automated blood culture	-	√√√	√*	-	-	-	√√
Sputum culture	-	√	-	√	√	-	√
Wound swab culture	-	√	-	√	√	√	√
Urine culture	-	√	-	√	√	√	√
⊗AST – disc	√	√√√	√	√	√√√	√	√√√
AST – other	-	-	-	-	-		-
Digital reporting	-	-	-	-	-	-	-

CMCH, Chittagong Medical College Hospital; DMCH, Dhaka Medical College Hospital; KMCH, Khulna Medical College Hospital; MMCH, Mymensingh Medical College Hospital; RpMCH, Rangpur Medical College Hospital; RMCH, Rajshahi Medical College Hospital; SOMCH, Sylhet M.A.G. Osmani Medical College Hospital.

*Dysfunctional. ⊗Number of ticks reflects the degree of understanding and correct methodology.

Table 10.2 AMR programmes at the national level in Bangladesh.

Surveillance sector	Time-period	Organisation involved	Key information
Human	2016-2020	IEDCR	• Involved 10 sites • Culture and sensitivity on WHO recommended priority pathogens • All sites do not do blood culture • Number and types of specimens collected varies among the sites • No data on number of tested patients • No data on patients 'clinical progress, antibiotic prescription for the certain patients, and outcome • Number of reports submitted to GLASS: [*E. coli* from blood (n=21); *E. coli* from urine (n=317); *K. pneumoniae* from blood (n=10); *K. pneumoniae* from urine (n=95); *Salmonella* spp. from blood (n=74); *Shigella* spp. from stool (n=11)]
Poultry	2018-present	CDIL	• 164 chicken caecal samples have been collected 24 live bird markets so far • Surveillance focused on isolated *E. coli*
Human and animal	2016-2020	FAO, OIE, WHO	• Aims of the project were to develop new guideline for use of antimicrobials for human and animal, to support farmers for the use of appropriate antibiotics • FAO are working with the BARA to develop a mobile app to be used by vets in the field to improve prescription practices for poultry
Animal	2016-2017	BLRI, IEDCR	AMR surveillance on 399 faecal samples from *Rhesus macaque*
Animal	2018-present	BLRI	Phenotypic and genotypic profiling of AMR in enteric bacterial communities in finisher livestock poultry in Bangladesh
Human	2018-2019	DGDA	Aims to estimate the consumption of antimicrobials based on production, import, export, distribution, and destruction of antimicrobials
Human	2018-2019	DGDA	Point prevalence study on the of the use of antimicrobials in selective hospital and community clusters (pharmacy based)

BARA, Bangladesh AMR Response Alliance; BLRI, Bangladesh Livestock Research Institute; CDIL, Central Disease Investigation Laboratory; DGDA, Directorate General of Drug Administration; FAO, Food and Agriculture Organization; IEDCR, Institute of Epidemiology, Disease Control and Research; OIE, World Organisation for Animal Health; WHO, World Health Organization.

Though this PhD project, I attempted to document the precise scenario of AMR prevalence, burden, drivers, and transmission in a Bangladeshi public heath setting. Nevertheless, this data represents one hospital only, the overall prevalence of all clinically relevant antimicrobials including the detailed epidemiology of one of WHO classified '**Watch'** group of antibiotics (carbapenem), and one of the '**Reserve**' antibiotics (colistin) were recoded (*Chapter 3*; *Chapter 5*; *Chapter 6*; *Chapter 8*) (WHO, 2019). The prevalence of CRE from the clinical specimens at DMCH was alarmingly high (11.1%, 210/1893). This study found the connections between CRE infections and usage of certain antimicrobials such as levofloxacin, amikacin, clindamycin, and meropenem (p<0.05) (*Chapter 3*). The findings in '*Chapter 6*' evinced the associations of MDR determinants (resistance to aminoglycosides, sulphonamide, trimethoprim, macrolides, rifampin, quinolones, chloramphenicol, and also colistin) with bla_{NDM} which signifies the dissemination of resistance genes against multiple antibiotics due to selective pressure of antibiotics pertinent to resistance plasmids which might include either carbapenems or non-carbapenems (Chereau *et al.,* 2017). It was certainly worrying that CRE infections ensue a significant high mortality (27.8%) than CSE (p<0.05). The relation between mortality and effective antimicrobial therapy was evaluated based on data available which showed decease probability of mortality with the patients without any effective antimicrobials (*Chapter 3*). However, this study did not record antibiotics' dosage and duration of treatment among the participants. Whether the ineffective antibiotics therapy either resulted high death rate or not among the patients with CRE infections was impossible to conclude. Nevertheless, these data highlights intransient practice of empirical therapy (*Chapter 3*) and thus necessitates an urgent need to improve the current clinical microbiology facilities at DMCH and to establish an appropriate standard antibiotic stewardship program in the hospital (Table 10.1) (Hoque *et al.,* 2020).

10.2 The reasons behind the indiscriminate antimicrobial usage in Bangladesh

Clinical microbiology services at the MCHs are not free, however, it has been operated at low cost for the hospitalised patients (The Fleming Fund, 2019). Patients attending at the public hospitals are usually from low socio-economic group (*Chapter 3*; *Chapter 4*), most often are unable to afford the minimal cost (Joarder *et al.*, 2019).

There is discrepancy of working hours between hospital services and clinical laboratory services. Government hospitals in Bangladesh are dedicated to providing 24 hours/7 days services to public; however, working hours for the laboratory staff are six and half hours only (0800hrs-1430hrs). Hitherto, there is no legislation by the Government to increase laboratory capacity for 24 hours services. Moreover, clinicians are not always convinced with the quality of report provided by the laboratories of public medical colleges. Therefore, a limited number of infection cases are managed according to sensitivity reports (The Fleming Fund, 2019). The patients are often advised to have microbiology testing performed at private laboratories. Given that microbiology costs are considerably higher in private laboratories than the public hospitals, most patients attending public hospitals will barely be able to afford the private costs (Rahman, personal communication).

All the factors lead the physicians to prescribe antimicrobials without any microbiological testing (The Fleming Fund, 2019). Moreover, when the AST is in place, essential antimicrobials are often not available in the hospital supply. Therefore, patients have to pay for the drug from outside pharmacy themselves as there is no health insurance policy available in the country which can support low socio-economic group. Unable to complete the full courses of antimicrobials due to unaffordability is common among the patients attending in the public hospitals (Thomson [manuscript in press]).

The overall usage of empirical antimicrobials among the inpatients enrolled in this study was >85% (*Chapter 3; Chapter 4*). AMU in each hospital needs to be monitored on a regular basis under proper legislation. Till now no data is available on AMU at the national level (WHO, 2018). Rational use of antimicrobials can be an effective option to reduce the trends of AMR in Bangladesh (Chandra *et al.*, 2020).

10.3 Hygiene baselines in Bangladesh and spread of AMR

The proportion of population served by the different types of sanitation system in different parts of the globe is depicted in Figure 10.2. WHO and UNICEF jointly reported the data on pre-COVID-19 hygiene baselines of each WHO region in 2020. Data showed that only 57% of household, and 53% of schools have access to basic hygiene in SA. No data is available on basic hygiene facilities in the healthcare in SA. In Bangladesh, only 35% of households and 50% of schools have hand-washing facilities. Basic washing facilities in households of the country are comparatively lower in rural areas (26%) than urban (51%) (nominal, 2017). Washing practices following patients' care in the hospital are very poor in the country (Figure 10.3) (WHO-UNICEF JMP, 2020). One survey report on hand washing facilities in 875 Bangladeshi health care facilities in 2013 showed that there was large discrepancy of availabilities of washing agents between hospital staffs and patients/caregivers. Although more than 85% of hospitals had available washing agents for doctors/nurses/other staffs, caregivers or patents had access to washing agents just 23% of hospital. However, patients' daily care in the public hospitals is mostly performed by the family members/caregivers rather than the hospital staffs (GOV.UK, 2015). The caregivers presumably have less education and less likely to follow appropriate hygiene practices (Khan *et al.*, 2008). More research on washing habits and sanitation in Bangladesh are required to understand the level of hygiene practices. Improving hand hygiene, and sanitation are basic steps to fight against infectious diseases and subsequent AMR (Lee *et al.,* 2013; O'Neill, 2016; Cheng *et al.,* 2018).

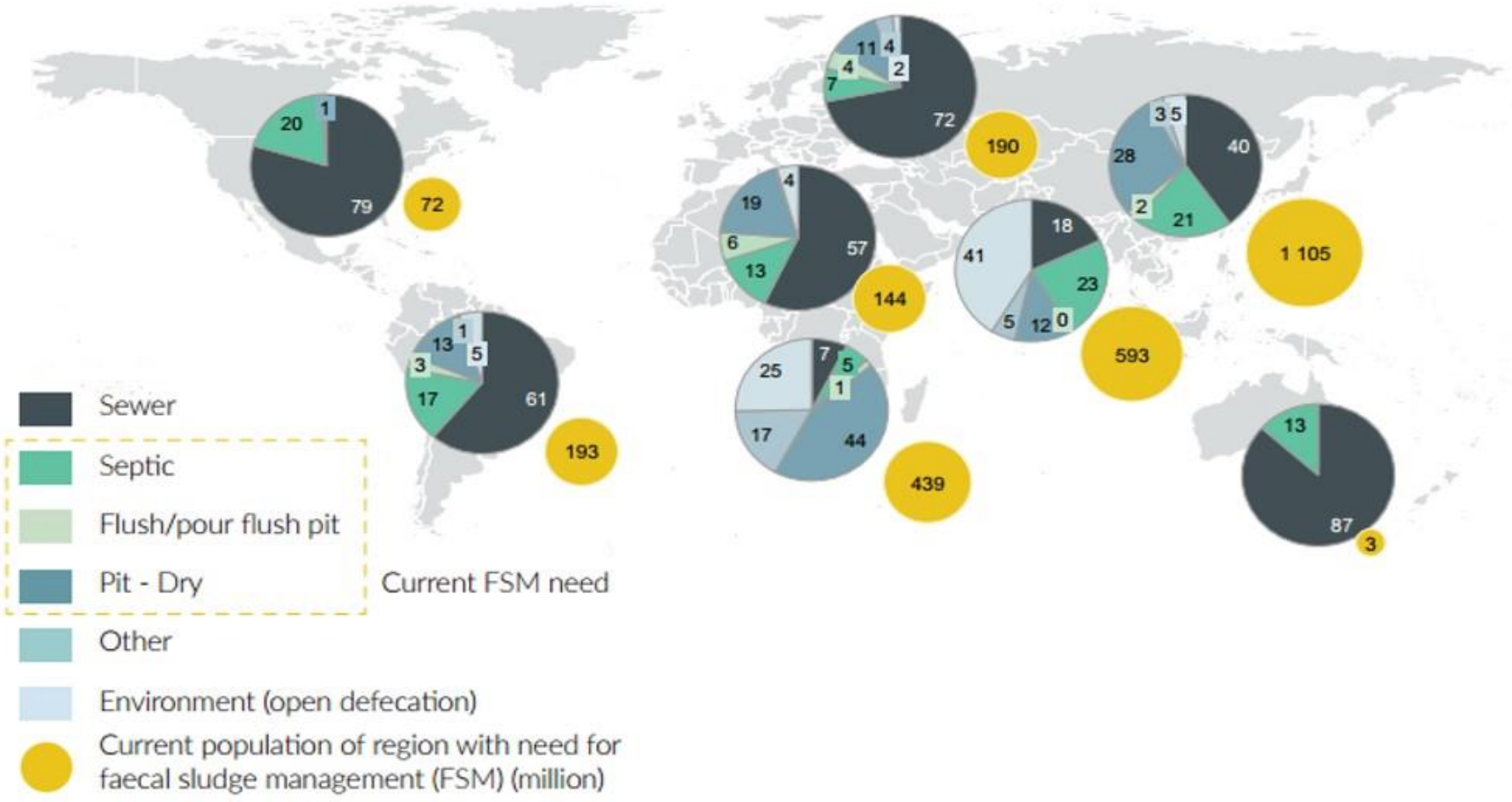


Figure 10.2 Percentage of population served by different type of sanitation system across the world (Nominal, 2014). Reproduced from: Wikimedia Commons, 2020.

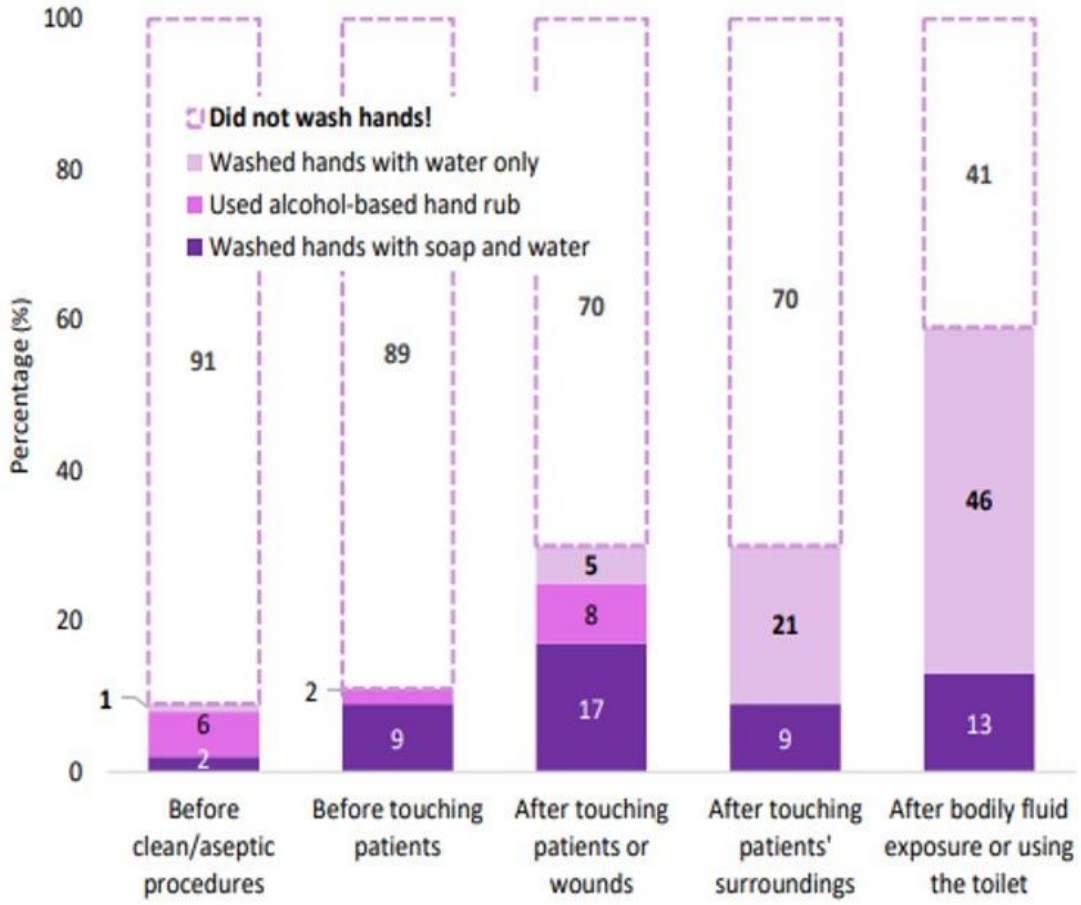


Figure 10.3 Hand hygiene practices in the hospitals of Bangladesh. Reproduced from: WHO-UNICEF JMP, 2020.

10.4 The magnitude of hospital outbreak in Bangladesh

A hospital outbreak is defined as an increase in the frequency of any disease event above the baseline level for a geographical area during a given period of time. The hospital outbreaks are the consequences of IPC failure (Sood and Perl, 2016). A fundamental part of effective IPC policy in a hospital involves the proper management of hospital waste (Khan *et al.,* 2019; WHO, 2020e). Previous reports calculated that healthcare associated waste from several districts in Bangladesh ranged from 0.19 kg to 1.99 kg per bed per day. PRISM, Bangladesh, a non-governmental organisation deals with the medical waste from the whole of Dhaka city. A quantitative assessment of waste in Dhaka city by PRISM in 2013 showed that 24% of the total waste were classified as 'hazardous' according to WHO guidelines. PRISM's survey also showed that a significant proportion of medical hazardous waste were disposed to open bin without any treatment. Contemplating the situation in Bangladesh, the waste is most likely to be collected by scavengers for recycling, and often simple washing is only applied before recycling (PRISM, 2013). There is no specific Governmental policy/guideline available in Bangladesh for the medical waste disposal. All the regulatory documents such as 'Environmental Conservation Act 1995', 'the Medical Waste Management and Administration Act 2010', and 'the Medical Waste Management Rules 2010' on medical waste management formulated by the Government so far only outlined the key components without any detailed instructions, and devoid of monitoring activities (CDDEP, 2018). In the hospital setting, inadequately managed hazardous waste is the reservoir of infectious agents which can be spread to the hospital environment through the staffs dealing with the waste, by vectors such as flies or insects, or in the air. Patients with underlying disease, neonates, and the elderly are prone to develop infection due to hospital contamination.

Taken together, overcrowding in Bangladeshi public health setting, insufficient hygiene baselines, poorly managed hospital waste and inadequate decontamination of hospital environment might lead to develop outbreaks in Bangladeshi healthcare facilities (GOV.UK, 2015; Shahida *et al.,* 2016; WHO-UNICEF JMP, 2020). Given the numbers of putative clinical outbreaks among the limited microbiological sampling population at DMCH in this study are worrying indicators of the impact of very poor IPC and antimicrobial stewardship practices (*Chapter 5*; *Chapter 7*; *Chapter 9*). Bangladesh is the endemic zone for certain AMR

determinants such *bla*$_{\text{CTX-15}}$, and *bla*$_{\text{NDM}}$ (Molton *et al.*, 2013; Lai *et al.*, 2014; Bevan *et al.*, 2017; Chen *et al.*, 2019; Nordmann and Poirel, 2019). This PhD is the first study in Bangladesh to undertake a detailed epidemiological and molecular assessment on an outbreak associated with AMR (*Chapter 5*). An institutional body of Bangladesh, the One Health Secretariat, was created in 2017 with an aim to establish One Health outbreak management team. The body incorporated the issues of AMR into their strategic plans (The Fleming Fund, 2019). Till now, no report by the management team has been found implying that they have not delt with any AMR related hospital outbreak.

Outbreaks might act as aggravators to expand the endemicity of AMR in a setting when there are no facilities for source identification, outbreak cases isolation, and radical decontamination. Outbreaks are also accountable for epidemic expansion of pathogens and/ or AMR determinants. Therefore, outbreaks in this setting might have serious global impact if the events would not be managed radically, and when AMR is a matter of concern (Koornhof *et al.*, 2001; Zahar *et al.*, 2014; Hassing *et al.*, 2015; Hawkey, 2015; Cimmino *et al.*, 2016; Sood and Perl, 2016).

10.5 Significance of faecal AMR screening in Bangladesh

Chapter 4 describes the prevalence of CRE in faecal flora among the hospitalised patients in Bangladesh and represents the prevalence in the community to lesser extent. The overall prevalence of CRE in faecal carriage was 34.8% (244/700) while a higher prevalence was found among inpatients (206/383, 53.8%) than the outpatients (38/317, 12%) ($p<0.05$). Perhaps the findings resulted from multifactorial combined involvement such as increase burden of AMR in the gut due to selective antibiotic pressure, very high rate of cross-transmission between patients to patients, and/or hospital staff to patients, inappropriate management/decontamination of hospital waste, and spread of MDR pathogens through fomites, flies or insects (Carlet, 2012; van Schaik, 2015; Gupta *et al.,* 2019). The possible transmission cycle of faecal carriage of MDR pathogens is illustrated in Figure 10.4.

Faecal AMR screening in the health setting is particularly important as high concentration of bacteria (10^8 per gram of faeces or more) is present in faeces which can be transmitted via faecal-oral route (Carlet, 2012). Bangladesh has a very poor sanitation, about 107 million population in the country do not have basic facilities of handwashing at home (Figure 10.2; Figure 10.3) (WHO-UNICEF JMP, 2020). Our scoping findings from the meetings with national stakeholders and visit at different MCHs suggest health facilities are embryonic in terms of advocating AMR screening program for human gut flora. Studies in the neighbouring country, India reported 1.6% to 81% prevalence of CRE in the RSs of hospitalised patients, and 30% in the community (Rai *et al.,* 2014; Datta *et al.,* 2015; Mohan *et al.,* 2017; Antony *et al.,* 2018; Bharadwaj *et al.,* 2018; Kumar *et al.,* 2018; Ramanathan *et al.,* 2018; Singh *et al.,* 2018; Smith *et al.,* 2020). Data from India and our findings indicate the significance of establishing periodic systematic screening of AMR in Bangladesh among healthy volunteers in both hospitals, and community settings, in hospitalised patients, and during the outbreak events.

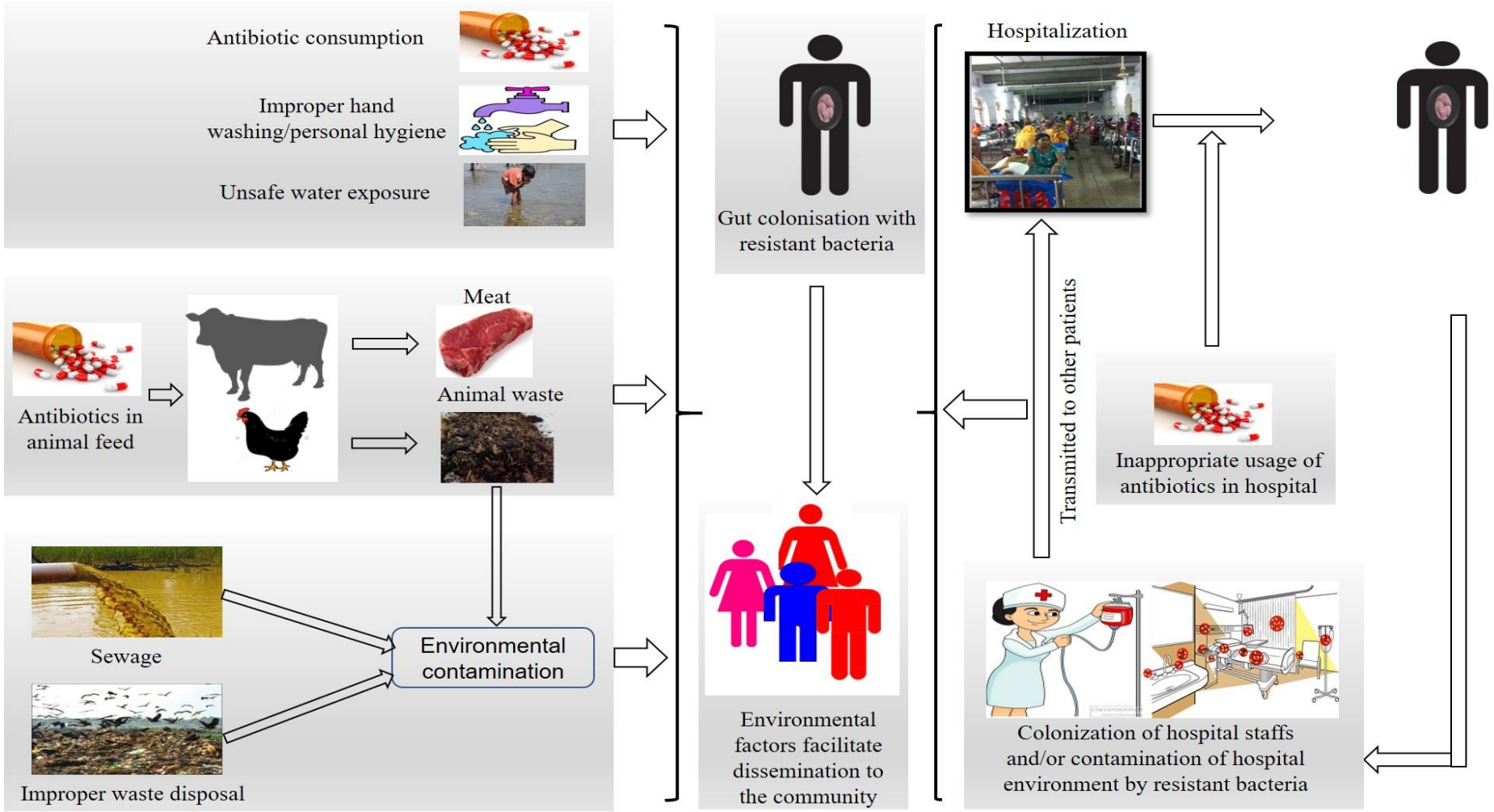


Figure 10.4 Factors facilitate the dissemination of AMR.

10.6 Emergence of colistin resistance in Bangladesh: situation analysis

Antibiotics have been used widely in farming therapeutically, metaphylactically, or for fostering growth (animal feed) for human consumption. AMU in agriculture has even exceed the usage in the clinical practice in recent years (Kirchhelle, 2018). Non-human antibiotic usage of colistin engendered a serious global effect in the recent widespread development of *mcr* in agriculture, and the emergence of *mcr* in human infections limit the treatment options for MDR pathogens (Liu *et al.,* 2016; Shen *et al.,* 2016; Davies and Walsh, 2018). A growing incidence of *mcr* has been observed in Bangladesh, although data mostly derived from small-scale study only. Consecutive reports of *mcr* from Bangladesh including the data from this PhD are demonstrated in Figure 10.5 which clearly demonstrate an increasing trend of *mcr* in 2018 than 2017 (Islam *et al.,* 2017; Sobur *et al.,* 2019a; Sobur *et al.,* 2019b; Akter *et al.,* 2020; Amin *et al.,* 2020; Johura *et al.,* 2020).

Literature reviews suggested that the agricultural use of antibiotics to prevent disease in Bangladesh is similar to the other parts of the world in spite of National Livestock Development Policy (2007) which documented the strict law for antibiotics sales without prescription (Chowdhury *et al.,* 2009; Islam *et al.,* 2016; Hoque *et al.,* 2020). A report on AMU in poultry farms by Bangladesh Department of Livestock Services (DLS) showed that 68% of the farms utilised Watch antibiotics, and 24% used Reserve antibiotic 'colistin'. Tetracyclines and fluoroquinolones are the common antibiotics used in farming (Department of Livestock Service, n.d.). Data on Bangladeshi shrimp hatcheries indicates the use of up to 80 kg antibiotics per production cycle in a single hatchery (Thornber *et al.,* 2020). An assessment of 73 Bangladeshi poultry farms by Islam *et al.,* (2016) recorded that AMU for treatment purposes is 43.8%, 31.5% for prophylaxis, 47.9% for dual purposes (treatment and prevention), and 8.2% as a growth promotor. Colistin is being used widely in Bangladeshi poultry farms. Higher concentration of colistin was reported in meat from the middle and late growth stage of chicken, indicating that meat to be sold in the market are rich with colistin. Farmers have significant lack of knowledge about aseptic management of poultry and poultry products, leading to usage of antimicrobials to control disease and to enhance production (Bristy *et al.,* 2019; Ferdous *et al.,* 2019). Small-scale farmers are often financially dependent on poultry dealers to run their business. Farmers are mostly influenced by the poultry dealers to use antibiotics

(Masud *et al.*, 2020). 'Animal feed Act 2010' banned the usage of antibiotics, growth hormones etc. in poultry feed (Hoque *et al.*, 2020). Therefore, traditionally farmers in the setting are adding colistin in the poultry's drinking water (Saha, personal communication). Colistin is available in liquid form for the point-of and online sales in the Bangladeshi market of colistin specifically for veterinary usage (myHealthbox, n.d.). This study recorded 3.2% usage of colistin in clinical practice (*Chapter 3*) and found the emergence of colistin resistance (*mcr-1.1* and *mcr-8.1*) in clinical infection/colonisation (*Chapter 8*). Non-human colistin usage was assumed to be responsible for the *mcr* emergence in Bangladesh. Colistin usage in the country should be monitored and regulated under strict laws, banned from animal use and treated singularly for human MDR infections.

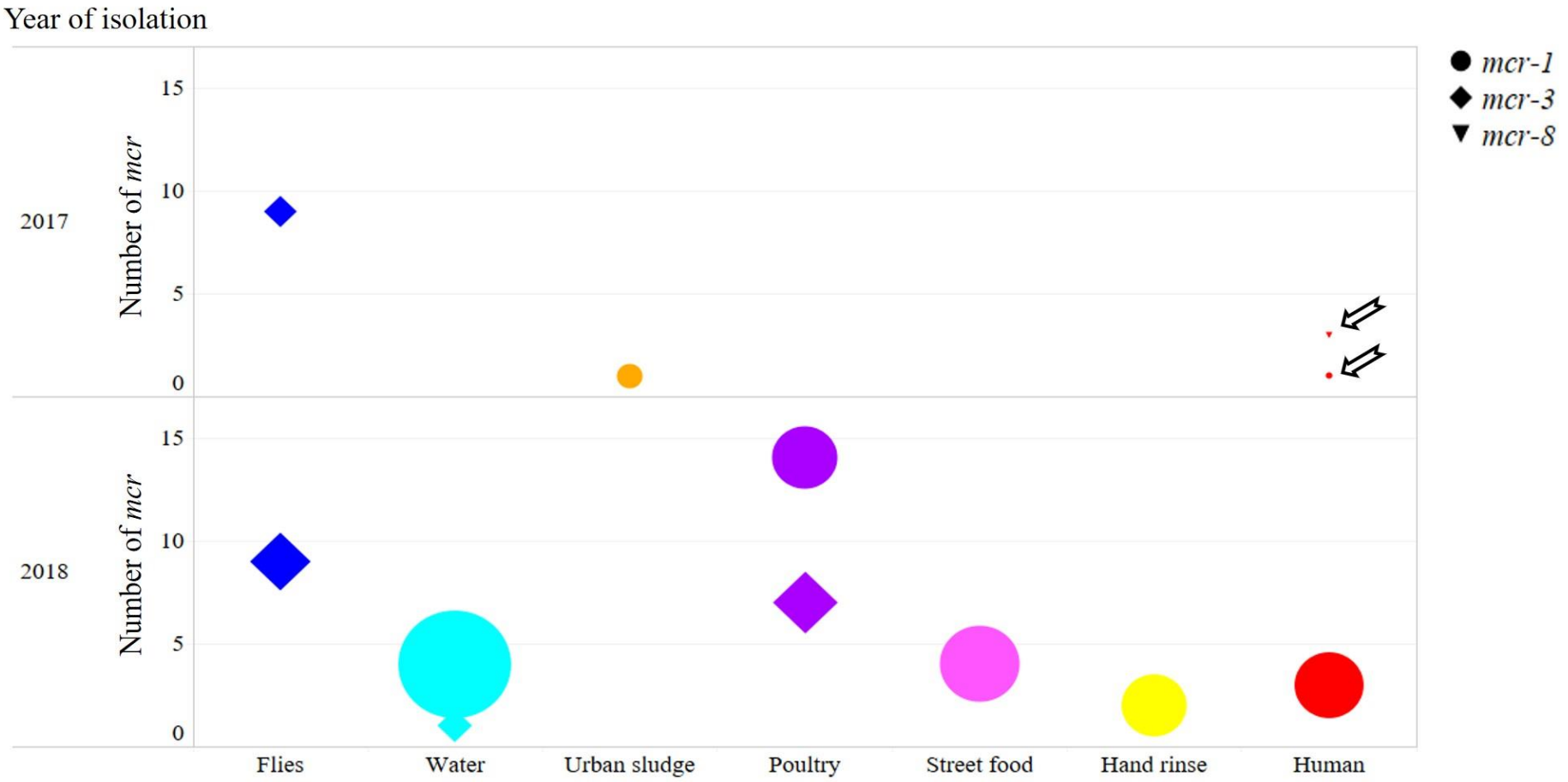


Figure 10.5 Report of *mcr* from Bangladesh from different sources. The size of symbol is proportional to prevalence of *mcr* from the respective sources. Arrows indicate the data from this PhD.

10.7 Limitation of the study

- This study only included clinical specimens referred to clinical microbiology of DMCH. As I have already discussed, a limited number of specimens at DMCH is selected for microbiology testing and a proportion of samples also referred to private laboratory. Despite the fact, this study enrolled 1892 clinical specimens. The main focus of the study was to assess the burden of carbapenem resistance in Bangladesh by compiling epidemiological, clinical and molecular data. As I have already discussed, no electronic data capturing system has been developed in Bangladeshi public hospital. Patients' information is documented in paper which lacked detail comorbid state of the patients. Only the basic information about the patients such as malignant condition or history of DM could be retrieved in this study. In this study the relation between mortality and carbapenem resistance was statistically adjusted with the patients' demographic and clinical data; however, the study was unable to interpret how the comorbid state can affect patients' mortality in association with carbapenem resistance (*Chapter 3*).
- Whether the ineffective antimicrobial therapy either resulted patients' death due to carbapenem resistance or not in this study was unrevealed in this study as data on antibiotics' course and duration was not recorded. The challenges behind the limitations on patients' data collection has been discussed with the respective chapter (*Chapter 3*).
- The presumptive outbreaks during the study period were identified retrospectively, this study did not have direct role the eradication of the outbreak during the sampling period. There was lack of data to identify index patients in the outbreak. As sampling from hospital environment, or hospital staffs beyond the scope of the study, source of outbreak, or sequential transmission event was not evaluated (*Chapter 5*; *Chapter 7*; *Chapter 9*).
- Given the high prevalence of CRE in clinical infections, a carriage study was undertaken after a year of clinical study to investigate the extent of any putative outbreak. The data would be more reproducible if clinical and faecal specimens were taken simultaneously from the same study population (*Chapter 4*).

10.8 Research prospects accomplished following this PhD and concluding remark

A few successfully funded research projects on Bangladeshi AMR were designed based on the findings of my PhD. I designed a project on burn sepsis through a network involving Cardiff University (CU), National Institute of Burn and Plastic Surgery, Bangladesh with an aim to investigate the health and economic burden of AMR in burn patients including the role of environment in the acquisition of MDR infections where our attempts were to record a complete profile of patients' treatment during their hospital stay including the outcome data. This project was funded by Global Challenges Research Fund (GCRF).

GCRF also funded us for the facilitation activities in Bangladesh for a project titled '*Establishing a network to monitor the emergence and impact of Klebsiella variicola, a hypervirulent and extensively drug-resistant pathogen, in South-Asia'*. Our research data *K. variicola* (Farzana *et al.,* 2019a; Chapter 7) and subsequent GCRF project, provided a comprehensive framework to understand the pathogenicity factors contributing *K. variicola* virulence. A large multi-centre study on the topic was built and submitted to the Biotechnology and Biological Sciences Research Council (BBSRC).

Finally, Prof. Walsh has been awarded by Welcome Trust for a large grant on the impact of COVID-19 bacterial sepsis, antibiotic consumption and stewardship, and resistance involving twelve countries. It has been proposed to include this PhD data to compare the effect of pre-COVID and ongoing COVID state on AMR crisis in Bangladesh. I will continue my research career on the grant after completion of my PhD.

Bangladesh is a high-risk zone for the emergence, and dissemination of AMR based on geography, population density, climates, heath practices, and infrastructure. Exploring the epidemiological aspects of AMR at the tertiary level along with district, and sub-district level is required for AMR control in the country, further, to reduce the global burden.

Section Eleven

Appendices

Appendix A

Antimicrobial classes according to mechanism of action and their spectrum of activity (Duplessis *et al.*, 2011; Bialvaei and Samadi, 2015; Falagas *et al.*, 2016; Grossman, 2016; Kapoor *et al.*, 2017; Percival, 2017; Thamban and Garneau-Tsodikova, 2018; McCarthy, 2019; Wikipedia, 2020c).

Cell wall synthesis inhibitors (β-lactams)			
Class	**Drugs**	**Indications (**Drug of Choice)**	**Effect on bacteria**
Penicillins	• Penicillin G • Aqueous penicillin G • Procaine penicillin G • Benzathine penicillin G • Penicillin V	*Strep. pyogenes*** *Step. agalactiae*** *C. perfringens* **	Bactericidal
	• Ampicillin • Amoxicillin	Above + Increase activity against Gram-negative: *E. faecalis*** *E. Coli***	Bactericidal
	• Methicillin • Nafcillin • Oxacillin • Cloxacillin • Dicloxacillin	Above + penicillinase-producing *Staph. aureus*	Bactericidal
	• Carbenicillin • Ticarcillin • Piperacillin	Above + *Pseudomonas aeruginosa***	Bactericidal
Cephalosporins	1st generation • Cefazolin • Cephalexin	*Staph. aureus*** *Staph. epidermidis*** Some Gram-negatives: *E. Coli* *Klebsiella*	Bactericidal
	2nd generation • Cefoxitin • Cefaclor • Cefuroxime	Above + increase activity against Gram-negative	Bactericidal
	3rd generation • Ceftriaxone • Cefotaxime • Ceftazidime	Above + increase activity against Gram-negative *Pseudomonas*	Bactericidal
	4th generation • Cefepime	Above + increase activity against Gram-negative *Pseudomonas*	Bactericidal
	5th generation • Ceftaroline	MRSA Vancomycin intermediate *Staph. aureus* (VISA) Vancomycin resistant *Staph. aureus* (VRSA)	Bactericidal

		Streptococcus pneumoniae *Haemophilus influenzae* *Moraxella catarrhalis*	
β-lactamase Inhibitors	• Clavulanic Acid • Sulbactam • Tazobactam	*S aureus*** *S epidermis*** *E.Coli*** *Klebsiella***	Bactericidal
Carbapenems	• Imipenem • Meropenem • Doripenem • Ertapenem	Broadest activity [except methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycoplasma*]	Bactericidal
Monobactams	Aztreonam	Gram-negative rods Aerobes	Bactericidal
Cell wall synthesis inhibitors (non-beta-lactams)			
Vancomycin	Vancomycin	MRSA** Penicillin or cephalosporin allergies *S. aureus* *S. epidermidis*	Bactericidal
Bacitracin	Bacitracin	Gram-positive infections	Bactericidal
Fosfomycin	Fosfomycin	Clinically significant Gram-positives and Gram-negatives form biofilm	Bactericidal
Teixobactin	Teixobactin	MRSA *Enterococcus* *Streptococcus* *Clostridium difficile* *Bacillus anthracis*	Bactericidal
Protein synthesis inhibitors (Anti-30S ribosomal subunit)			
Aminoglycosides	• Gentamicin • Neomycin • Amikacin • Tobramycin • Streptomycin • Neamine • Neomycin B • Paromomycin • Paromamine • Ribostamycin • Arbekacin • Amikacin • Dibekacin • Gentamicin • Geneticin • Isepamicin • Kanamycin A and B • Nebramine • Netilmicin	Aerobic Gram-negatives *Enterobacteriaceae* *Pseudomonas*	Bactericidal

	• Plazomicin • Sisomicin • Tobramycin • Apramycin		
Tetracyclines	• Tetracycline • Doxycycline • Minocycline • Demeclocycline • Methacycline, • Rolitetracycline • Lymecycline • Tigecycline	*Rickettsia* *Mycoplasma* *Spirochetes* (Lyme's disease)	Bacteriostatic
Protein synthesis inhibitors (Anti-50S ribosomal subunit)			
Macrolides	• Erythromycin • Azithromycin • Clarithromycin	*Streptococcus* *H. influenzae* *Mycoplasma pneumonia*	Bacteriostatic
Chloramphenicol	Chloramphenicol	*H influenzae* Bacterial Meningitis Brain abscess	Bacteriostatic
Lincosamide	Clindamycin	*Bacteroides fragilis* *S aureus* Coagulase-negative *Staph. & Strep.* Excellent Bone Penetration	Bacteriostatic
Oxazolidinone	Linezolid	Resistant Gram-positives	Variable
Streptogramins	• Quinupristin • Dalfopristin	Vancomycin resistant *Enterococcus* (VRE) Group A *Streptococcus* (GAS) *S. aureus* skin infections	Bactericidal
DNA synthesis inhibitors			
Fluoroquinolones	1st generation • Nalidixic acid	*Streptococcus* *Mycoplasma* Aerobic Gram positives	Bactericidal
	2nd generation • Ciprofloxacin • Norfloxacin • Enoxacin • Ofloxacin • Levofloxacin	Above +*Pseudomonas*	Bactericidal
	3rd generation • Gatifloxacin	Above + Gram-positives	Bactericidal
	4th generation • Moxifloxacin • Gemifloxacin	Above + Gram-positives + Anaerobes	Bactericidal
Other DNA inhibitors			
Metronidazole	Metronidazole	Anaerobes	Bactericidal
RNA synthesis inhibitors			

Rifampin	Rifampin	*Staphylococcus* *Mycobacterium*	Bactericidal
Mycolic acid synthesis inhibitor			
Isoniazid	Isoniazid	Mycobacterium	Bactericidal
Folic acid synthesis inhibitors			
Trimethoprim/ Sulfonamides	Trimethoprim/ Sulfamethoxazole	Urinary tract infections (UTI) *Proteus* *Enterobacter*	Bacteriostatic
Alteration of cell membrane			
Polymyxins	Colistin	Gram-negative bacteria	Bactericidal
Daptomycin	Daptomycin	Gram-positive bacteria	Bactericidal

Appendix B

Classification of β-lactamase (Bush and Jacoby, 2010; Bush, 2018).

Bush-Jacoby-Medeiros group (Functional classification)	Molecular classification	Preferred substrates	Inhibition by β-lactamase inhibitors			Enzymes
			AV	CA	EDTA	
1	C	Cephalosporins	+	-	-	AmpC, CMY, ACT-1, FOX-1, MIR-1
1e	C	Extended-spectrum cephalosporins	+	-	-	GC1, CMY-37
2a	A	Penicillins	+	+	-	PC1
2b	A	Penicillins, early cephalosporins	+	+	-	TEM-1, TEM-2, SHV-1
2br	A	Penicillins	+	-	-	TEM-30, SHV-10
2be	A	Extended-spectrum cephalosporins, monobactams	+	+	-	TEM-3, SHV-2, CTX-M ESBLs, PER-1, VEB-1
2ber	A	Extended-spectrum cephalosporins, monobactams	-	-	-	TEM-50
2c	A	Carbenicillins	+	+	-	PSE-1, CARB-3
2ce	A	Carbenicillins, cefepime	+	+	-	RTG-4
2d	D	Cloxacillins	+	±	-	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	+	±	-	OXA-11, OXA-15
2df	D	Carbapenems	+	-	-	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	+	+	-	CepA
2f	A	Carbapenems	+	±	-	KPC, IMI, SME
3a	B	Carbapenems	-	-	+	IMP, VIM, NDM
3b	B	Carbapenems	-	-	+	CphA, Sfh-1

AV, avibactam; CA, clavulanic acid.

Classification of metallo-β-lactamase (Bebrone, 2007; Sawa *et al.*, 2020).

Subclass	Enzyme	Discovery	Structure
B1 (chromosomal)	BcII	1966	Mono-zinc, Di-zinc, Apo-form
	CcrA	1990	Di-zinc
	BlaB	1998	Di-zinc
	IND-1	1999	
	EBR-1	2002	
	SFB-1	2005	
	SLB-1	2005	
B1 (acquired)	IMP-1	1994	Di-zinc
	VIM-1	1999	
	VIM-2	2000	Mono-zinc, Di-zinc
	IMP-2	2000	
	SPM-1	2002	Mono-zinc
	VIM-4	2003	
	GIM-1	2004	
	SIM-1	2005	
	NDM-1	2009	Di-zinc
B2	CphA	1991	Mono-zinc
	ImiS	1996	
	Sfh-1	2003	
	CAU-1		Di-zinc
	FEZ-1		Di-zinc
	GOB-1		Di-zinc
B3	L1	1991	Di-zinc, Mono-zinc, Apo-form
	GOB-1	2000	
	FEZ-1	2000	Di-zinc
	THIN-B	2001	
	Mbl1b	2001	
	CAU-1	2002	
	BJP-1	2006	Di-zinc

Appendix C

Genome attributes of *bla*$_{NDM}$-positive *E. coli* and *K. pneumoniae* retrieved from NCBI

BioSample	Species
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SAMN11399731	*K. pneumoniae*
SAMN11399732	*K. pneumoniae*
SAMN11399729	*K. pneumoniae*
SAMN11399740	*K. pneumoniae*
SAMN11419359	*K. pneumoniae*
SAMN11399443	*K. pneumoniae*
SAMN11399721	*K. pneumoniae*
SAMN11399481	*K. pneumoniae*
SAMN11399713	*K. pneumoniae*
SAMN11399502	*K. pneumoniae*
SAMN11399496	*K. pneumoniae*
SAMN11399497	*K. pneumoniae*
SAMN11399733	*K. pneumoniae*
SAMN11399499	*K. pneumoniae*
SAMN11399741	*K. pneumoniae*
SAMN11399493	*K. pneumoniae*
SAMN11399323	*K. pneumoniae*
SAMN11399415	*K. pneumoniae*

SAMN11399364	*K. pneumoniae*
SAMN11399354	*K. pneumoniae*
SAMN11399450	*K. pneumoniae*
SAMN11360905	*K. pneumoniae*
SAMN10516706	*K. pneumoniae*
SAMN10516702	*K. pneumoniae*
SAMN10516701	*K. pneumoniae*
SAMN10516700	*K. pneumoniae*
SAMN10516699	*K. pneumoniae*
SAMN10516694	*K. pneumoniae*
SAMN10516693	*K. pneumoniae*
SAMN10516689	*K. pneumoniae*
SAMN10516698	*K. pneumoniae*
SAMN10516697	*K. pneumoniae*
SAMN10516688	*K. pneumoniae*
SAMN10516703	*K. pneumoniae*
SAMN11793480	*K. pneumoniae*
SAMN10600576	*K. pneumoniae*
SAMN11961995	*K. pneumoniae*
SAMN11974893	*K. pneumoniae*
SAMN12098371	*K. pneumoniae*
SAMN12285710	*K. pneumoniae*
SAMN12285738	*K. pneumoniae*
SAMN12285730	*K. pneumoniae*
SAMN12431272	*K. pneumoniae*
SAMN12431303	*K. pneumoniae*
SAMN12627831	*K. pneumoniae*
SAMN12676103	*K. pneumoniae*
SAMN12714270	*K. pneumoniae*
SAMN12714271	*K. pneumoniae*
SAMN12714267	*K. pneumoniae*
SAMN12714266	*K. pneumoniae*
SAMN12714261	*K. pneumoniae*
SAMN12714264	*K. pneumoniae*
SAMN12714262	*K. pneumoniae*
SAMN12714257	*K. pneumoniae*
SAMN12714265	*K. pneumoniae*
SAMN12714256	*K. pneumoniae*
SAMN12714268	*K. pneumoniae*
SAMN12714263	*K. pneumoniae*
SAMN12738928	*K. pneumoniae*
SAMN12738926	*K. pneumoniae*
SAMN12738924	*K. pneumoniae*
SAMN12868504	*K. pneumoniae*
SAMN13091586	*K. pneumoniae*
SAMN13091613	*K. pneumoniae*
SAMN13151936	*K. pneumoniae*
SAMN13153751	*K. pneumoniae*

SAMN13153742	*K. pneumoniae*
SAMN13262230	*K. pneumoniae*
SAMN13315560	*K. pneumoniae*
SAMN13229306	*K. pneumoniae*
SAMN13229293	*K. pneumoniae*
SAMN13229286	*K. pneumoniae*
SAMN13229283	*K. pneumoniae*
SAMN13229282	*K. pneumoniae*
SAMN12572245	*K. pneumoniae*
SAMN12572246	*K. pneumoniae*
SAMN13679093	*K. pneumoniae*
SAMN13675337	*K. pneumoniae*
SAMN13675338	*K. pneumoniae*
SAMN13639765	*K. pneumoniae*
SAMN13735565	*K. pneumoniae*
SAMN13623932	*K. pneumoniae*
SAMN13623933	*K. pneumoniae*
SAMN13623931	*K. pneumoniae*
SAMN12126279	*K. pneumoniae*
SAMN12126189	*K. pneumoniae*
SAMN10371624	*K. pneumoniae*
SAMN11479264	*K. pneumoniae*
SAMN11479262	*K. pneumoniae*
SAMN11479261	*K. pneumoniae*
SAMN12250576	*K. pneumoniae*
SAMN11811878	*K. pneumoniae*
SAMN11811877	*K. pneumoniae*
SAMN11811876	*K. pneumoniae*
SAMEA2742597	*K. pneumoniae*
SAMEA104168729	*K. pneumoniae*
SAMEA104168728	*K. pneumoniae*
SAMEA104168727	*K. pneumoniae*
SAMEA3649640	*K. pneumoniae*
SAMEA3649637	*K. pneumoniae*
SAMEA3649616	*K. pneumoniae*
SAMEA3649693	*K. pneumoniae*
SAMEA3649587	*K. pneumoniae*
SAMEA3649754	*K. pneumoniae*
SAMEA3649601	*K. pneumoniae*
SAMEA3649604	*K. pneumoniae*
SAMEA3649819	*K. pneumoniae*
SAMEA3649740	*K. pneumoniae*
SAMEA3649752	*K. pneumoniae*
SAMEA3649753	*K. pneumoniae*
SAMEA3649802	*K. pneumoniae*
SAMEA3649809	*K. pneumoniae*
SAMEA3649816	*K. pneumoniae*
SAMEA3649713	*K. pneumoniae*

SAMEA3673019	*K. pneumoniae*
SAMEA3673042	*K. pneumoniae*
SAMEA3720946	*K. pneumoniae*
SAMEA3720962	*K. pneumoniae*
SAMEA3721167	*K. pneumoniae*
SAMEA3721166	*K. pneumoniae*
SAMEA3721175	*K. pneumoniae*
SAMEA3721192	*K. pneumoniae*
SAMEA3721201	*K. pneumoniae*
SAMEA3721220	*K. pneumoniae*
SAMEA3720956	*K. pneumoniae*
SAMEA3720964	*K. pneumoniae*
SAMEA3720963	*K. pneumoniae*
SAMEA3720925	*K. pneumoniae*
SAMEA3721138	*K. pneumoniae*
SAMEA3721144	*K. pneumoniae*
SAMEA3721140	*K. pneumoniae*
SAMEA3721153	*K. pneumoniae*
SAMEA3721149	*K. pneumoniae*
SAMEA3721161	*K. pneumoniae*
SAMEA3721170	*K. pneumoniae*
SAMEA3721173	*K. pneumoniae*
SAMEA3721174	*K. pneumoniae*
SAMEA3721178	*K. pneumoniae*
SAMEA3721184	*K. pneumoniae*
SAMEA3721197	*K. pneumoniae*
SAMEA3721194	*K. pneumoniae*
SAMEA3721218	*K. pneumoniae*
SAMEA3721221	*K. pneumoniae*
SAMEA3721223	*K. pneumoniae*
SAMEA3727569	*K. pneumoniae*
SAMEA3727558	*K. pneumoniae*
SAMEA3727640	*K. pneumoniae*
SAMEA3729827	*K. pneumoniae*
SAMEA3729837	*K. pneumoniae*
SAMEA3729654	*K. pneumoniae*
SAMEA3729743	*K. pneumoniae*
SAMEA3729770	*K. pneumoniae*
SAMEA3729774	*K. pneumoniae*
SAMEA3729777	*K. pneumoniae*
SAMEA3729781	*K. pneumoniae*
SAMEA3729748	*K. pneumoniae*
SAMEA3729872	*K. pneumoniae*
SAMEA3499895	*K. pneumoniae*
SAMEA3499939	*K. pneumoniae*
SAMEA3499003	*K. pneumoniae*
SAMEA3499901	*K. pneumoniae*
SAMEA3499943	*K. pneumoniae*

SAMEA3512046	*K. pneumoniae*
SAMEA3512122	*K. pneumoniae*
SAMEA3515090	*K. pneumoniae*
SAMEA3538532	*K. pneumoniae*
SAMEA3538546	*K. pneumoniae*
SAMEA3538547	*K. pneumoniae*
SAMEA3538550	*K. pneumoniae*
SAMEA3538553	*K. pneumoniae*
SAMEA3538552	*K. pneumoniae*
SAMEA3538558	*K. pneumoniae*
SAMEA3538555	*K. pneumoniae*
SAMEA3538562	*K. pneumoniae*
SAMEA3538965	*K. pneumoniae*
SAMEA3538743	*K. pneumoniae*
SAMEA4916044	*K. pneumoniae*
SAMEA4916106	*K. pneumoniae*
SAMEA4916116	*K. pneumoniae*
SAMEA4916118	*K. pneumoniae*
SAMEA4916119	*K. pneumoniae*
SAME4916132	*K. pneumoniae*

Appendix D

ঢাকা মেডিকেল কলেজ
DHAKA MEDICAL COLLEGE
Dhaka, Bangladesh

Ref.
Memo No. MEU-DMC/ECC/2017/122 Date: 30/05/2017

Ethical Clearance Certificate

The Ethical Committee of Dhaka Medical College Approved the Following Research Protocol in time.

Title of the Research Work:	**"A deep sequencing approach to understanding the risks and clinical burden of Multi-drug Resistant Enterobacteriaceae infections in a Hospital Setting of Bangladesh".**
Principal Investigator:	**Dr. Refath Farzana** **PhD** student, Department of Medical Microbiology & Infectious Diseases, Institute of Infection & Immunity, School of Medicine, Cardiff University, UK.
Supervisor:	**Professor Timothy R. Walsh** Head of the Microbiology Research, Department of Medical Microbiology & Infectious Diseases, Cardiff University, UK.
Place of Study:	**Department of Virology** Dhaka Medical College Hospital, Dhaka.
Duration:	**October 2016 to September 2020.**

Prof. Dr. Nasima Sultana
Head of the Department of Biochemistry &
Chairman, Ethical Review Committee
Dhaka Medical College, Dhaka.

Dhaka Medical College, Dhaka-1000, Bangladesh. Phone: 880-2-55165088, Fax : 880-2-55165006
E-mail : principal@dmc.gov.bd, dmc_principal@yahoo.com, web: http://www.dmc.gov.bd

Consent form (English)

I am Mr. / Mrs. …… hereby giving well informed consent for my participation in the study conducted by Dr. Refath Farzana. I fully understand that my participation in the study will bring fruitful medical information to be useful for me and for the others in the future. I am convinced that during participation in the study I shall not be exposed to any physical, social, psychological, and legal risk. My privacy and confidentiality will be safeguarded, and my anonymity will be protected. I will preserve the right to withdraw myself from the study whenever I want.

I would not like to be compensated because of my loss of work time.

Signature/Thumb impression of the patient/ patient's attendant

Date:

Signature of the investigator:

Date:

Appendix E

Recipes for reagents prepared locally

Ethidium bromide

Stock solution of ethidium bromide (Sigma-Aldrich, Missouri, USA) (10 mg/ml) was prepared. The stock was transferred to a dark bottle and was stored at room temperature. Working concentration was achieved by 1:20 dilution of the stock.

TBE buffer (10X)

108 g of tris (Thermo Fisher Scientific, Waltham, USA), 54 g of boric acid (Sigma-Aldrich, Missouri, USA), 7.44 g of NaEDTA (Thermo Fisher Scientific, Waltham, USA) were added in 1 L distilled water.

Cell Suspension Buffer

100 ml of 1 M Tris (pH 8.0), 200 ml of 0.5 M EDTA (pH 8.0) were diluted in 1 L of CLRW.

Cell Lysis Buffer

50 ml of 1 M Tris (pH 8.0), 100 ml of 0.5 M EDTA (pH 8.0), 100 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt) (Sigma-Aldrich, Missouri, USA) were diluted in 1 L of CLRW.

Tris-EDTA (TE)

10 ml of 1 M Tris (pH 8.0) and 2 ml of 0.5 M EDTA (pH 8.0) were diluted in 1 L of CLRW.

S1 buffer (10X)

S1 buffer was prepared by adding 12.3 g of Sodium acetate (30 mM, pH 4.6) (Thermo Fisher Scientific, Waltham, USA) and 0.92 g Zinc acetate (1 mM) (Thermo Fisher Scientific, Waltham, USA) to 200 ml of water. Then 250 ml of glycerol (5%) (Sigma-Aldrich, Missouri, USA) was added and pH was adjusted to 4.6. Finally, 500 ml water was added, and the mixture was stored at -20^{o} C.

Denaturing solution

20 g NaOH (0.5 M) (Sigma-Aldrich, Missouri, USA) and 87.66 g NaCl (1.5 M) were added to 1 L water.

Neutralising solution

60.5 g Tris base (0.5 M, pH 7.5) and 87.6 g NaCl (1.5 M) were dissolved in 800 ml of water and pH was adjusted to 7.5 with concentrated HCl (Sigma-Aldrich, Missouri, USA).

Saline-sodium citrate (SSC) buffer (20X)

175.3 g NaCl and 77.4 g sodium citrate (Thermo Fisher Scientific, Waltham, USA) were added to 1 L of water and pH was adjusted to 7.

Pre-hybridisation solution

The solution consists of 5% Polyvinylpyrrolidone (0.4 ml) (Sigma-Aldrich, Missouri, USA), 5% ficoll (0.4 ml) (Thermo Fisher Scientific, Waltham, USA), 10 mg/ml herring testes DNA (0.3 ml) (Sigma-Aldrich, Missouri, USA), 10% SDS (1 ml) (Thermo Fisher Scientific, Waltham, USA), 20X SSC (6 ml) and full cream UHT milk (1 ml). All the components were mixed and finally water was added up to 2 L.

Appendix F

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.1.

BioSample	Species	ST
SAMN03263950	*E. coli*	ST10
SAMN03294311	*E. coli*	ST10
SAMN03252431	*E. coli*	ST101
SAMN02673556	*E. coli*	ST1011
SAMN00016779	*E. coli*	ST1079
SAMN08993917	*E. coli*	ST117
SAMN09462202	*E. coli*	ST1193
SAMN08382661	*E. coli*	ST131
SAMN04481707	*E. coli*	ST131
SAMN03287565	*E. coli*	ST131
SAMN08161275	*E. coli*	ST1312
SAMN03963241	*E. coli*	ST155
SAMN06198938	*E. coli*	ST156
SAMD00061087	*E. coli*	ST156
SAMN12214763	*E. coli*	ST162
SAMN06198937	*E. coli*	ST167
SAMN11283108	*E. coli*	ST167
SAMD00059754	*E. coli*	ST167
SAMN10956393	*E. coli*	ST167
SAMN04625459	*E. coli*	ST182
SAMN08579566	*E. coli*	ST205
SAMN10250173	*E. coli*	ST206
SAMN10219252	*E. coli*	ST2161
SAMN03252425	*E. coli*	ST224
SAMN07759721	*E. coli*	ST2705
SAMN03252413	*E. coli*	ST295
SAMN07618121	*E. coli*	ST349
SAMN02604066	*E. coli*	ST354
SAMN10691120	*E. coli*	ST354
SAMN03252409	*E. coli*	ST38
SAMN05194390	*E. coli*	ST405
SAMN04158294	*E. coli*	ST405
SAMN06909153	*E. coli*	ST405
SAMN12569951	*E. coli*	ST410
SAMN06909178	*E. coli*	ST410
SAMEA2272277	*E. coli*	ST414
SAMN05511168	*E. coli*	ST44
SAMN03252454	*E. coli*	ST442

SAMN07503734	*E. coli*	ST443
SAMN07594018	*E. coli*	ST446
SAMN08663441	*E. coli*	ST448
SAMN10531954	*E. coli*	ST448
SAMN06219550	*E. coli*	ST4542
SAMD00056131	*E. coli*	ST457
SAMD00179457	*E. coli*	ST4577
SAMN03252430	*E. coli*	ST46
SAMN02604037	*E. coli*	ST46
SAMN03252453	*E. coli*	ST48
SAMN08668598	*E. coli*	ST5030
SAMN03252442	*E. coli*	ST5082
SAMN11087649	*E. coli*	ST515
SAMN03252457	*E. coli*	ST517
SAMN08579578	*E. coli*	ST5350
SAMN03996288	*E. coli*	ST538
SAMN07618127	*E. coli*	ST539
SAMN02647163	*E. coli*	ST540
SAMD00168435	*E. coli*	ST542
SAMN03963234	*E. coli*	ST56
SAMEA3529258	*E. coli*	ST57
SAMN03252423	*E. coli*	ST58
SAMN11266496	*E. coli*	ST583
SAMN06219548	*E. coli*	ST602
SAMN03960341	*E. coli*	ST607
SAMN03081526	*E. coli*	ST6130
SAMN02666696	*E. coli*	ST6131
SAMN04448503	*E. coli*	ST6140
SAMN06029894	*E. coli*	ST617
SAMN06029895	*E. coli*	ST617
SAMN06029893	*E. coli*	ST617
SAMEA3138234	*E. coli*	ST62
SAMN07260764	*E. coli*	ST635
SAMN10163231	*E. coli*	ST636
SAMN13242665	*E. coli*	ST641
SAMN03252419	*E. coli*	ST642
SAMN00017915	*E. coli*	ST643
SAMN08579587	*E. coli*	ST646
SAMN05511150	*E. coli*	ST648
SAMN07765387	*E. coli*	ST648
SAMEA5128442	*E. coli*	ST648
SAMN02800875	*E. coli*	ST648
SAMN08579558	*E. coli*	ST655
SAMN13951922	*E. coli*	ST656
SAMN06928086	*E. coli*	ST69

SAMN09704974	*E. coli*	ST73
SAMN12233489	*E. coli*	ST761
SAMN08040561	*E. coli*	ST764
SAMN08663435	*E. coli*	ST7786
SAMN07792806	*E. coli*	ST793
SAMN05210898	*E. coli*	ST795
SAMN09710898	*E. coli*	ST80
SAMEA104093909	*E. coli*	ST8217
SAMEA4916090	*E. coli*	ST8346
SAMN02949644	*E. coli*	ST847
SAMEA3368339	*E. coli*	ST8740
SAMN07807402	*E. coli*	ST88
SAMN03252421	*E. coli*	ST90
SAMN07618120	*E. coli*	ST929
SAMN08450093	*E. coli*	ST93
SAMN08165042	*E. coli*	ST937
SAMN08161312	*E. coli*	ST94
SAMN07974445	*E. coli*	ST964
SAMN08579581	*E. coli*	ST967
SAMN03612246	*E. coli*	ST99

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.6.

BioSample	Species	ST
SAMN05774083	*K. pneumoniae*	ST983
SAMN05231873	*K. pneumoniae*	ST37
SAMN08391417	*K. pneumoniae*	ST307
SAMN07638733	*K. pneumoniae*	ST54
SAMN02603641	*K. pneumoniae*	ST23
SAMN03097203	*K. pneumoniae*	ST65
SAMN10343201	*K. pneumoniae*	ST1685
SAMN08366843	*K. pneumoniae*	ST1265
SAMN08366844	*K. pneumoniae*	ST23
SAMN09476115	*K. pneumoniae*	ST29
SAMN07187263	*K. pneumoniae*	ST15
SAMN03076168	*K. pneumoniae*	ST48
SAMN03076171	*K. pneumoniae*	ST395
SAMN08212048	*K. pneumoniae*	ST147
SAMN08222595	*K. pneumoniae*	ST101
SAMN03196972	*K. pneumoniae*	ST846
SAMN03076172	*K. pneumoniae*	ST11
SAMN03197141	*K. pneumoniae*	ST327
SAMN03197209	*K. pneumoniae*	ST70
SAMN04868736	*K. pneumoniae*	ST11
SAMN12264830	*K. pneumoniae*	ST16
SAMN10678732	*K. pneumoniae*	ST86
SAMN04868740	*K. pneumoniae*	ST258
SAMN03197492	*K. pneumoniae*	ST34
SAMN08222601	*K. pneumoniae*	ST111
SAMN03197549	*K. pneumoniae*	ST60
SAMN07760932	*K. pneumoniae*	ST147
SAMN03197962	*K. pneumoniae*	ST234
SAMN03197968	*K. pneumoniae*	ST528
SAMN03455989	*K. pneumoniae*	ST45
SAMN10790857	*K. pneumoniae*	ST23
SAMN04014907	*K. pneumoniae*	ST14
SAMN04014916	*K. pneumoniae*	ST16
SAMN04014981	*K. pneumoniae*	ST34
SAMN07291519	*K. pneumoniae*	ST15
SAMN10432823	*K. pneumoniae*	ST231
SAMEA3141919	*K. pneumoniae*	ST91
SAMN10910564	*K. pneumoniae*	ST15
SAMN02138655	*K. pneumoniae*	ST186
SAMN02138664	*K. pneumoniae*	ST15
SAMN02138667	*K. pneumoniae*	ST661
SAMN02356586	*K. pneumoniae*	ST540
SAMN02581290	*K. pneumoniae*	ST17
SAMN03280412	*K. pneumoniae*	ST23

SAMN03280413	*K. pneumoniae*	ST23
SAMN02138593	*K. pneumoniae*	ST133
SAMN02581256	*K. pneumoniae*	ST592
SAMN02581302	*K. pneumoniae*	ST17
SAMN03280382	*K. pneumoniae*	ST16
SAMN12289282	*K. pneumoniae*	ST12
SAMN10537194	*K. pneumoniae*	ST11
SAMN03733725	*K. pneumoniae*	ST941
SAMN02581304	*K. pneumoniae*	ST1199
SAMN03280255	*K. pneumoniae*	ST15
SAMN03280300	*K. pneumoniae*	ST158
SAMN03280347	*K. pneumoniae*	ST2121
SAMN02581368	*K. pneumoniae*	ST14
SAMN09811763	*K. pneumoniae*	ST726
SAMN02603941	*K. pneumoniae*	ST38
SAMD00171892	*K. pneumoniae*	ST668

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.11.

BioSample	Species
SAMN08222607	*P. hauseri*
SAMN03197609	*P. mirabilis*
SAMN12217607	*P. mirabilis*
SAMN04014870	*P. mirabilis*
SAMN04014900	*P. mirabilis*
SAMN04014996	*P. mirabilis*
SAMN03418023	*P. mirabilis*
SAMN06674010	*P. vulgaris*
SAMN13082492	*P. mirabilis*
SAMN12258067	*P. mirabilis*
SAMN12636071	*P. mirabilis*
SAMN04395118	*P. mirabilis*
SAMEA1705945	*P. mirabilis*
SAMN03140307	*P. mirabilis*
SAMN10458171	*P. mirabilis*
SAMN09976767	*P. mirabilis*
SAMN10614391	*P. vulgaris*
SAMN05715220	*P. mirabilis*
SAMN08020255	*P. vulgaris*

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.12.

BioSample	Species	ST
SAMN04014891	*E. cloacae*	ST78
SAMN04014977	*E. cloacae*	ST171
SAMN02603901	*E. cloacae*	ST1
SAMEA2273209	*E. cloacae*	ST133
SAMEA2273351	*E. cloacae*	ST544
SAMEA2273372	*E. cloacae*	ST795
SAMEA2273398	*E. cloacae*	ST797
SAMEA2273399	*E. cloacae*	ST346
SAMN10359476	*E. cloacae*	ST813
SAMN02713682	*E. cloacae*	ST93
SAMEA4785257	*E. cloacae*	ST89
SAMN12258091	*E. hormaechei*	ST116
SAMN03996283	*E. aerogenes*	-
SAMN07312407	*E. aerogenes*	-
SAMN11031503	*E. cloacae*	ST261
SAMN10856232	*E. hormaechei*	ST418

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.13.

BioSample	Species
SAMN03197842	*C. braakii*
SAMN07452765	*C. farmeri*
SAMN11055879	*C. freundii*
SAMN03455986	*C. freundii*
SAMN04287052	*C. freundii*
SAMN03996309	*C. amalonaticus*
SAMN06173303	*C. braakii*
SAMN07312408	*C. werkmanii*
SAMN10163242	*C. freundii*
SAMN10163243	*C. freundii*
SAMN11056361	*C. koseri*
SAMN09428653	*C. koseri*
SAMN07729552	*C. freundii*
SAMEA2272524	*C. rodentium*
SAMN07206908	*C. youngae*
SAMD00019865	*C. sedlakii*
SAMN05570461	*C. freundii*
SAMEA4830818	*C. freundii*

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.14.

BioSample	Species
SAMN10252231	*P. stuartii*
SAMN04014867	*P. stuartii*
SAMN05629093	*P. stuartii*
SAMN14048635	*P. stuartii*
SAMEA2665135	*P. stuartii*
SAMN11523820	*P. rettgeri*
SAMN11982199	*P. rettgeri*
SAMN06159664	*P. stuartii*
SAMN07974484	*P. stuartii*

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.17.

BioSample	Species	ST
SAMN06218054	*E. coli*	ST167
SAMN06284178	*E. coli*	ST167
SAMN04361562	*E. coli*	Unknown ST
SAMN04157977	*E. coli*	ST167
SAMN04157978	*E. coli*	ST167
SAMN04157979	*E. coli*	ST167
SAMN04157980	*E. coli*	ST167
SAMN07344983	*E. coli*	ST167
SAMN04157981	*E. coli*	ST167
SAMN07344982	*E. coli*	ST167
SAMN09985619	*E. coli*	ST167
SAMD00059754	*E. coli*	ST167
SAMN08281024	*E. coli*	ST167
SAMN03220397	*E. coli*	ST167
SAMN06909181	*E. coli*	ST167

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.18.

BioSample	Species	ST
SAMN05977366	*E. coli*	ST448
SAMN06311279	*E. coli*	ST448
SAMN06311280	*E. coli*	ST448
SAMN08637775	*E. coli*	Unknown ST
SAMN10531472	*E. coli*	ST448
SAMEA4672938	*E. coli*	ST2083
SAMEA4916063	*E. coli*	ST448
SAMEA4916089	*E. coli*	ST448
SAMN06973353	*E. coli*	ST448
SAMN04992597	*E. coli*	ST448
SAMN10105866	*E. coli*	ST448
SAMN12822948	*E. coli*	ST205
SAMN10531954	*E. coli*	ST448
SAMN06909173	*E. coli*	ST448
SAMN10620088	*E. coli*	ST448

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.19.

BioSample	Species	ST
SAMN08637787	*E. coli*	ST8346
SAMEA4916096	*E. coli*	ST8346
SAMEA4916090	*E. coli*	ST8346

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.20.

BioSample	Species	ST
SAMN13829989	*E. coli*	ST405
SAMN06311269	*E. coli*	ST405
SAMN06311274	*E. coli*	ST405
SAMN06311283	*E. coli*	ST405
SAMN08637779	*E. coli*	ST405
SAMN08637780	*E. coli*	ST405
SAMN08637778	*E. coli*	ST405
SAMN02801869	*E. coli*	ST405
SAMN02801870	*E. coli*	ST405
SAMN08637781	*E. coli*	ST405
SAMN10249152	*E. coli*	ST405
SAMN10248954	*E. coli*	ST405
SAMN04046665	*E. coli*	ST405
SAMD00076990	*E. coli*	ST405
SAMN06973348	*E. coli*	ST405
SAMN07312492	*E. coli*	ST405
SAMEA4643524	*E. coli*	ST405
SAMN11232786	*E. coli*	ST405
SAMN05604797	*E. coli*	ST405
SAMN03145051	*E. coli*	ST405
SAMN05786429	*E. coli*	ST405
SAMN07760941	*E. coli*	ST405
SAMN11233046	*E. coli*	ST405
SAMN11233061	*E. coli*	ST405
SAMN11233063	*E. coli*	ST405
SAMN11233068	*E. coli*	ST405
SAMN11233092	*E. coli*	ST405
SAMN11233094	*E. coli*	ST405
SAMN06924979	*E. coli*	ST405

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.21.

BioSample	Species	ST
SAMN06311264	*E. coli*	ST648
SAMN06806389	*E. coli*	ST648
SAMN02709589	*E. coli*	ST648
SAMN08103372	*E. coli*	ST648
SAMN08103377	*E. coli*	ST648
SAMN08519241	*E. coli*	ST648
SAMN08519242	*E. coli*	ST648
SAMN08519249	*E. coli*	ST648
SAMN03922986	*E. coli*	ST648
SAMD00052661	*E. coli*	ST648
SAMN05440277	*E. coli*	ST648
SAMN05440276	*E. coli*	ST648
SAMN11232909	*E. coli*	ST648
SAMN05511150	*E. coli*	ST648
SAMN11233027	*E. coli*	ST648

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.27.

BioSample	Species	ST
SAMEA4364586	*K. pneumoniae*	ST15
SAMEA4364670	*K. pneumoniae*	ST15
SAMN02138664	*K. pneumoniae*	ST15
SAMN10963497	*K. pneumoniae*	ST15
SAMEA3721057	*K. pneumoniae*	ST15
SAMEA3512047	*K. pneumoniae*	ST15
SAMEA3649536	*K. pneumoniae*	ST15
SAMEA3721053	*K. pneumoniae*	ST15
SAMEA3721059	*K. pneumoniae*	ST15
SAMEA3515136	*K. pneumoniae*	ST15
SAMEA3538544	*K. pneumoniae*	ST15
SAMEA3729663	*K. pneumoniae*	ST15
SAMN10600498	*K. pneumoniae*	ST15
SAMEA2273810	*K. pneumoniae*	ST15
SAMN03281077	*K. pneumoniae*	ST15

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 5.28.

BioSample	Species	ST
SAMEA4364585	*K. pneumoniae*	ST16
SAMEA4394736	*K. pneumoniae*	ST16
SAMEA23996668	*K. pneumoniae*	ST16
SAMEA23998168	*K. pneumoniae*	ST16
SAMN07173931	*K. pneumoniae*	ST16
SAMN07173932	*K. pneumoniae*	ST16
SAMEA104569902	*K. pneumoniae*	ST16
SAMEA3499992	*K. pneumoniae*	ST16
SAMN10592664	*K. pneumoniae*	ST16
SAMEA2273664	*K. pneumoniae*	ST16
SAMEA2273689	*K. pneumoniae*	ST16
SAMEA2273709	*K. pneumoniae*	ST16
SAMEA2273723	*K. pneumoniae*	ST16
SAMEA2273726	*K. pneumoniae*	ST16
SAMN07450698	*K. pneumoniae*	ST16
SAMEA3345126	*K. pneumoniae*	ST16
SAMEA3531804	*K. pneumoniae*	ST16
SAMEA3531868	*K. pneumoniae*	ST16
SAMN04008891	*K. pneumoniae*	ST16

Genome attributes of the isolates (retrieved from NCBI) used in the phylogenetic analysis of Figure 9.5, 9.6, and 9.8.

Codes for global strains	BioSample	ST type
BC1	SAMN03449377	ST1590
BC2	SAMN03449230	ST1592
BC3	SAMN03449286	ST1584
BC4	SAMN03449156	ST1597
BC5	SAMN03449157	ST1597
BC6	SAMN03449379	ST1619
BC7	SAMN03449246	ST1583
BC8	SAMN03449420	ST1587
BC9	SAMN03449517	ST1586
BC10	SAMN03449534	ST1604
BC11	SAMN03449675	ST1603
BC12	SAMN03449532	ST1605
BC13	SAMN03449474	ST1582
BC14	SAMN03449608	ST1585
BC15	SAMN03449614	ST1599
BC16	SAMN03449400	ST1594
BC17	SAMN03449633	ST1596
BC18	SAMN03449130	ST1589
BC19	SAMN03449179	ST1600
BC20	SAMN03449505	ST795
BC21	SAMN03449378	ST795
BC22	SAMN03449298	ST795
BC23	SAMN03449466	ST1602
BC24	SAMN03449469	ST1602
BC25	SAMN03449473	ST1602
BC26	SAMN03449467	ST1602
BC27	SAMN03449468	ST1602
BC28	SAMN03449480	ST1602
BC29	SAMN03449470	ST1602
BC30	SAMN03449472	ST1602
BC31	SAMN03449471	ST1602
BC32	SAMN03449479	ST1602
BC33	SAMN03449460	ST1588
BC34	SAMN03449557	ST1580
BC35	SAMN03449128	ST1607
BC36	SAMN03449159	ST1606
BC37	SAMN03449182	ST1606
BC38	SAMN03449174	ST1613
BC39	SAMN03449169	ST1613

BC40	SAMN03449172	ST1613
BC41	SAMN03449173	ST1613
BC42	SAMN03449175	ST1613
BC43	SAMN03449176	ST1613
BC44	SAMN03449161	ST1613
BC45	SAMN03449160	ST1613
BC46	SAMN03449165	ST1613
BC47	SAMN03449162	ST1613
BC48	SAMN03449166	ST1613
BC49	SAMN03449170	ST1613
BC50	SAMN03449163	ST1613
BC51	SAMN03449180	ST1613
BC52	SAMN03449164	ST1613
BC53	SAMN03449177	ST1613
BC54	SAMN03449167	ST1613
BC55	SAMN03449171	ST1613
BC56	SAMN03449168	ST1613
BC57	SAMN03449178	ST1613
BC58	SAMN03449418	ST1601
BC59	SAMN03449419	ST1601
BC60	SAMN03449607	ST1581
BC61	SAMN03449415	ST1591
BC62	SAMN03449288	ST1593
BC63	SAMN03449447	ST1615
BC64	SAMN03449282	ST1614
BC65	SAMN03449304	ST1598
BC66	SAMN03449294	ST1620
BC67	SAMN03449290	ST1616
BC68	SAMN02892978	ST1618
BC69	SAMN03751789	ST1617
BC70	SAMN03486590	ST472
BC71	SAMN02680268	ST1331
BC72	SAMN02597170	ST44
BC73	SAMN02796019	ST810
BC74	SAMN03144973	ST28
BC75	SAMN02797514	ST807
BC76	SAMN02796046	ST807
BC77	SAMD00018762	ST10
BC78	SAMN06173358	ST10
BC79	SAMN02440719	ST10
BC80	SAMN07312432	ST10
BC81	SAMN03449485	ST1609
BC82	SAMN03449496	ST1609
BC83	SAMN03449237	ST962

BC84	SAMN03449558	ST1608
BC85	SAMN03449618	ST1612
BC86	SAMN03449578	ST1595
BC87	SAMN03449556	ST1611
BC88	SAMN03449494	ST698
BC89	SAMN03449570	ST698
BC90	SAMN03449571	ST698
BC91	SAMN03449452	ST1610

Section Twelve

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