THE MOLECULAR EPIDEMIOLOGY OF BLOODSTREAM INFECTIONS CAUSED BY NON-SALMONELLA GRAM-NEGATIVE BACTERIA IN A TERTIARY HOSPITAL IN NEPAL WITH FOCUS ON NEONATAL SEPSIS AND ENTEROBACTER SPECIES

by

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Abstract

The molecular epidemiology of bloodstream infections caused by non-*Salmonella* Gram-negative bacteria in a tertiary hospital in Nepal with focus on neonatal sepsis and *Enterobacter* species

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Bloodstream infections (BSIs) are common cause of morbidity and mortality among patients of all age groups. Of particular concern is the increasing emergence and spread of antimicrobial resistance (AMR) in many Gram-negative bacteria (GNB) that cause BSIs. The aim of this thesis was to determine some of the epidemiological features of BSIs in a tertiary hospital in Nepal providing a specific focus on the molecular aspects of AMR, virulence, and variations in *Enterobacter* spp., a clinically important GNB associated with BSIs.

To better understand BSIs-causing non-*Salmonella* GNB and their AMR determinants in this setting, I conducted a retrospective surveillance study, accessing data from 2012 to 2018. I identified a high prevalence of multi-drug resistance (MDR) and several key AMR genes associated with predominant GNB pathogens. The neonatal intensive care unit (NICU) and the emergency room (ER) were the most prevalent hospital departments for BSIs. To gain an insight into the epidemiology of hospital-acquired (HA) BSIs and identify potential preventive measures, I conducted a further prospective study in the NICU. An increased isolation of *Enterobacter* spp. in the NICU during the study period suggested an outbreak, prompting an outbreak investigation of *Enterobacter* spp. isolates using whole genome sequencing (WGS). Identification of ER to having the highest burden of BSIs due to *Enterobacter* spp. demanded for an epidemiological research to understand the genomic determinants of virulence, AMR, and variations of *Enterobacter* spp.. To address this, I performed a comprehensive WGS analysis of *Enterobacter* spp. isolated in the ER.

The conclusion of my work is that BSIs due to antimicrobial resistant non-*Salmonella* GNB is an expanding problem in our hospital, highlighting the need for a continued AMR surveillance. I found that *Enterobacter* spp. was an emerging MDR pathogen causing BSIs in this hospital. The complex transmission dynamics of *Enterobacter* spp. observed in ER warranted for a more comprehensive genomic investigation complemented with demographic and clinical data of patients to better disaggregate the epidemiology of BSIs in ER.

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Abbreviations

Abbreviations	Full forms
AMR	Antimicrobial resistance
AMS	Antimicrobial stewardship
ANC	Antenatal care
ARGs	Antimicrobial resistance genes
AST	Antimicrobial susceptibility test
bp	Base pair
BRIG	BLAST Ring Image Generator
BSI	Bloodstream infection
CA-BSI	Community-acquired bloodstream infection
CANS	Community-acquired neonatal sepsis
CBC	Complete blood cell count
CFR	Case fatality rate
CHW	Community health worker
CI	Confidence interval
CLABSI	Central line-associated bloodstream infections
CLSI	Clinical laboratory standards institute
CNS	Central nervous system
CO-BSI	Community-onset bloodstream infections
conc.	Concentration
CoNS	Coagulase negative staphylococci
CPAP	Continuous positive airway pressure
CRF	Case report form
CRP	C-reactive protein

CSF	Cerebrospinal fluid
CVC	Central venous catheterization
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy nucleotide tri-phosphates
ELBW	Extremely low birth weight
EOS	Early onset sepsis
ER	Emergency department
ESBL	Extended spectrum beta-lactamase
ESR	Erythrocyte sedimentation rate
ET	Endo-tracheal tube
GA	Gestational age
GBS	Group-B streptococci
gm	Grams
GNB	Gram-negative bacilli
GPC	Gram-positive cocci
HAIs	Hospital-acquired infections
HANS	Hospital-acquired neonatal sepsis
HCW	Health care workers
HGT	Horizontal gene transfer
HICs	High income countries
HPF	High power field of microscope
IAP	Intrapartum antibiotic prophylaxis
IPC	Infection prevention and control
ICU	Intensive care unit
IL	Interleukin
IMCI	Intergrated manual for childeren infections

IQR	Intra-quartile range
IR	Inverted repeats
IS	Insertion sequence
IUGR	Intra uterine growth retardation
IV	Intravenous
LB	Luria-Bertani
LBW	Low birth weight
LGT	Lateral gene transfer
LMICs	Low and middle income countries
LOS	Late onset sepsis
LP	Lumbar puncture
MBL	Metallo beta-lactamase
MDR	Multi drug resistance
MDRO	Multi drug resistant organisms
MgCl2	Magnesium chloride
MGEs	Mobile genetic elements
MHA	Mueller Hinton Agar
MLST	Multi locus sequence typing
NC	Negative control
NG tube	Noso-gastric tube
NGS	Next generation sequencing
NHRC	Nepal health research council
NICU	Neonatal intensive care unit
NMR	Neonatal mortality rate
NS	Neonatal sepsis
NTC	No template control

OG	Oro-gastric
OPD	Outpatient department
OR	Odds ratio
OxTREC	Oxford Tropical Research Ethics Committee
PC	Positive control
PCR	Polymerase chain reaction
PCT	Procalcitonin
PFGE	Pulsed-field gel electrophoresis
PICU	Paediatric intensive care unit
PNC	Post-natal care
PPE	Personal protective equipment
PPL	Priority pathogen list
PPROM	Prolonged premature rupture of membrane
PROM	Premature rupture of membrane
PSBI	Possible severe bacterial infections
PT	Pre-term
RDS	Respiratory distress syndrome
rpm	Rotations per minute
rxn	Reaction
SD	Standard deviation
SNP	Single nucleotide polymorphism
ST	Sequence type
STI	Sexually transmitted infections
TBE	Tris-Borate-EDTA
Temp.	Temperature
Tn	Transposons

TORCH	Toxoplasma Rubella Cytomegalovirus
U/rxn	Unit/reaction
UAC	Umbilical artery catheter
UTI	Urinary tract infection
UV	Ultraviolet
UVC	Umbilical vein catheter
VAP	Ventilator associated pneumoniae
VLBW	Very low birth weight
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensive drug resistance

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Chapter 1

Introduction

1.1 Bloodstream infection (BSI)

Acute undifferentiated febrile illness is a common presentation among patients seeking healthcare in low- and middle-income countries (LMICs). Bacterial BSI is one of the reasons of such febrile illnesses. BSI is a clinical condition characterized by the presence of viable microorganisms in the bloodstream that elicits an inflammatory immunological response resulting into changes of clinical, laboratory and haemodynamic parameters [1]. BSI is an important cause of increased morbidity and mortality among people across the world [1–3].

1.1.1 Stages of BSI and definitions of bacteraemia, septicaemia, severe inflammatory response syndrome, sepsis, severe sepsis, and septic shock

Blood is normally sterile; however, infectious agents can enter the bloodstream such as during surgical procedures, exacerbation of focal infections, or via contaminated foreign bodies invading arteries or veins [4]. The clinical condition showing the presence of viable bacteria in the blood circulation is known as “bacteraemia” [4]. Bacteraemia can be transient, intermittent or persistent depending on the timing of bacterial presence in the bloodstream [4]. The brief presence of bacteria in blood for few minutes to hours before being cleared by the body’s immune system is called ‘transient bacteraemia’ [4]. This is relatively harmless in healthy people and may occur after manipulation of body parts that are heavily colonized by bacteria, such as mucosal surfaces of mouth, bladder, and colon. The clinical condition when bacteria are periodically or sporadically introduced into the bloodstream by an existing infection such as wound abscesses, dental caries, pneumonia etc. is referred to as ‘intermittent bacteraemia’ [4]. The condition where viable bacteria are continuously present in the blood circulation is called ‘persistent bacteraemia’ [4]. It may occur

due to the presence of infected implants or prosthetic devices, or during infections such as typhoid, brucellosis, endocarditis etc. [4]. The consequence of persistent bacteraemia may be fatal if not treated timely.

When bacteria multiply and spread in the bloodstream producing bacterial toxins, the clinical condition is referred to as ‘septicaemia’ [4]. The immune system efficiently recognizes infectious agents such as bacteria and bacterial toxins as foreign invaders and strongly elicits a defensive immunological response. Among immuno-compromised individuals, such as young infants, elderly people, or those with underlying comorbidities and infections, it may lead to a prolonged exacerbated immune response. It may result in an uncontrolled systemic inflammatory response with a cytokine storm, which continues and progresses despite successful eradication of bacterial infection [5]. Such a clinical condition is called ‘systemic inflammatory response syndrome’ (SIRS) [5]. SIRS is clinically manifested as an abnormal body temperature (>38.5°C or <36°C), tachycardia (mean heart rate >2 standard deviations(SD) above normal for age), tachypnea (mean respiratory rate >2 SD above normal for age) and/or abnormal leucocytes count for given age or more than 10% immature neutrophils [5].

The SIRS originating due to infection caused by microorganisms is called sepsis [5]. Positive blood culture sample proves the presence of infection and thus diagnoses sepsis. However, not in all instances do the blood samples yield culture positive results. False negative culture results can arise due to several reasons: for example, the limitation of routinely utilized microbiological procedures for the growth of fastidious and anaerobic bacteria, virus and fungi; recent administration of antimicrobials before taking culture sample etc. Specific clinical and pathological findings may compensate the diagnosis of sepsis in case of negative culture results.

Once triggered, sepsis can rapidly worsen and become a life-threatening condition called ‘severe sepsis’ [5]. During severe sepsis, there is a cascade of inflammatory response leading to the secretion of pro- and anti-inflammatory cytokines; vasodilation and mobilization of leukocytes leading to leaky blood vessels; activation of coagulation and inhibition of fibrinolysis resulting in the deposition of fibrin clots in microvasculature and increased apoptosis [5]. This process impairs the body’s ability to efficiently pump blood to tissues and organs. In the lack of sufficient nutrients, oxygenation and increasingly accumulating wastes, multiple organs start showing signs of end stage damage leading to multiple organ dysfunction syndromes (MODS) [6,7]. Severe sepsis is clinically manifested as hypotension, metabolic acidosis, thrombocytopenia, leucocytosis or decreased urine output [5]. Early diagnosis and prompt intensive care treatment with intravenous fluid and antimicrobial therapy are required to manage severe sepsis. However, in immuno-compromised conditions such as among young infants, despite prompt treatment, severe sepsis persists and rapidly deteriorates to a deadly condition called ‘septic shock’. Here, blood pressure drops persistently despite the administration of intravenous fluids, eventually leading to the death. Figure 1.1 shows the stages of sepsis [7].

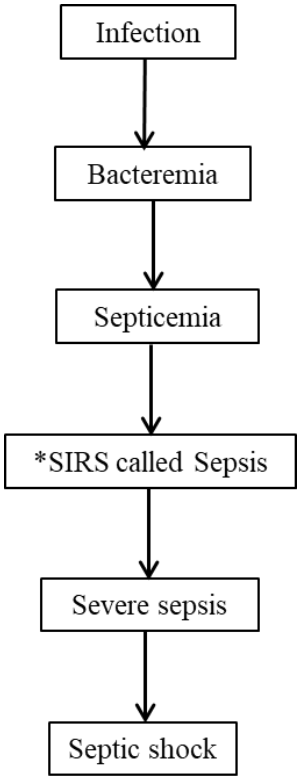


Figure 1.1. The progression and stages of sepsis.

Bacterial invasion of bloodstream leads to septicaemia, which elicits an immunological response leading to sepsis. In the absence of timely therapeutic management, the condition progresses to severe sepsis to eventually deteriorate to a life-threatening condition called septic shock.

1.1.2 Types of BSI

1.1.2.1 BSI by focus of infection

BSI may be defined as primary BSI when focus of infection other than bloodstream is not involved. Such undifferentiated BSI where other infection sources could not be localized both on clinical judgement and microbiological investigation is referred to as primary BSI. Typhoid is one of the most common primary undifferentiated bacterial BSIs, especially in southern Asia [8]. Alternatively, in secondary BSI, the main focus of infection lies elsewhere other than bloodstream and it can be microbiologically or clinically identified [9]. Urinary tract infection, pneumonia, and meningitis that are concomitantly associated with positive blood culture results are examples of secondary BSI.

1.1.2.2 BSI by immune-competency of patients

BSI can occur in different groups of people based on their immuno-competency. BSI caused by *Salmonella enterica* Typhi, *Streptococcus pyogenes*, and viridans streptococci may occur in immunologically competent individuals and is usually community-acquired [1]. On the other hand, young infants and elderly who have impaired immunity due to physiological conditions may be prone to BSIs due to pathogens like *Listeria monocytogenes*, *Streptococcus agalactiae*, and GNB. Such infections can be community- or hospital- acquired. Third, people with immuno-deficiencies, such as those undergoing immunosuppressive therapy and those with underlying chronic diseases may be susceptible to BSIs by several Gram-positive and –negative pathogens that may be acquired both from hospital as well as community [1].

1.1.2.3 BSI by time and origin of infection

Traditionally, BSI is classified as either hospital-onset (nosocomial) or community-onset BSI. Further, owing to the changes in the demographics and modes of healthcare service delivery to the community patients, the third category of BSI called healthcare-associated BSI has been introduced.

1.1.2.3.1 Community-onset BSI (CO-BSI):

BSI occurring among outpatients or present or incubating 48 hours prior to hospital admission is referred to as CO-BSI [2]. However, given dramatic alternations in the healthcare delivery services within local communities, this definition of CO-BSI is insufficient. To accommodate, CO-BSI has been further sub-classified into two categories: healthcare-associated BSI and community-acquired BSI based on whether or not the patient had recently received any healthcare services [10].

1.1.2.3.1.1 Healthcare-associated BSIs (HCA-BSIs):

BSIs occurring within 48 hours of hospital admission among the patients who received invasive medical care at home or nursing home, or received frequent invasive healthcare services such as haemodialysis or chemotherapy, or have been recently discharged from the healthcare institution before the current admission are called healthcare associated BSIs. As such, healthcare-associated BSIs are more common among elderly and people with underlying clinical conditions or chronic diseases [2].

1.1.2.3.1.2 Community-acquired BSIs (CA-BSIs):

BSIs occurring among outpatients or within 48 hours of hospital admission among patients without any recent history of significant healthcare contact are termed CA-BSIs. As such, CA-BSIs are acquired in the community environment and are not associated with healthcare interventions [11]. BSIs such as typhoid, typhus, dengue, and malaria may arise in immunologically competent individuals in community.

1.1.2.3.2 Hospital-onset or hospital-acquired BSIs (HA-BSIs):

BSIs that are first identified at or beyond 48 hours following hospital admission or within 48 hours of discharge from the hospital are referred to as HA-BSIs or nosocomial BSIs [2]. As patients often do not have an apparent infection at the time of hospital admission, such infections are presumably acquired from the hospital environment. Like other nosocomial infections, HA-BSIs often lead to increased morbidity, mortality, hospital stay and hospital cost, which are often preventable. HA-BSIs account for nearly 15% of all hospital-acquired infections [12].
HA-BSIs are important cause of infectious complications, mostly among critically ill patients admitted to intensive care units (ICU) who are heavily reliant on invasive medical support. Elderly, preterm (PT) and low birth weight neonates, patients with underlying chronic diseases

such as malignant tumors, diabetes, renal failure, those under immunosuppressive medications, and those treated with prolong use of invasive devices are most at risk of HA-BSIs [12–14].

1.1.3 The epidemiology of BSIs

1.1.3.1 The epidemiology of CO-BSIs

Studies reporting the incidence rates of CO-BSIs at a population level are severely lacking. The majority of studies do not distinguish CO-BSI into HCA-BSI or CA-BSI. Further, because of methodological variations among studies, the global epidemiology of CO-BSI is difficult to assess [1]. Based on stark differences in quality of life and healthcare systems, the incidence and specific aetiologies of CO-BSI can be different between high-income countries (HICs) and LMICs. A review estimated that the annual incidence rate of CO-BSI in HICs in Europe and America ranged from 43-154/100,000 population, with *E. coli*, *Staphylococcus aureus*, *S. pneumoniae,* and *Klebsiella* spp. being the most common aetiological agents [2]. In Africa [15] and South Asia[3], *Salmonella enterica* Typhi was reported to be the most common bacterial aetiology of CA-BSI.

1.1.3.2 The epidemiology of HA-BSIs

The incidence of HA-BSIs is variable by time and setting depending on several determinants of the host (age, immune-competency, medical interventions, and underlying diseases), pathogen (virulence, antimicrobial resistance, and inoculum size), and environment (infection control measures of hospital) [14]. It has been estimated that there are nearly 250,000 episodes of HA-BSIs per year among hospitalized patients in the United States [13]. The incidence rate of HA-BSI has been estimated to be 5 per 1,000 central-line days [12]. Gram-positive cocci (GPC), particularly *S. aureus*, enterococci, enteric GNB such as *K. pneumoniae, E. coli, Enterobacter* spp., non-enteric GNB such as *Acinetobacter* spp., *P. aeruginosa* are frequent causes of HA-BSIs [13,16].

1.2 Neonatal sepsis

Neonatal sepsis is a serious and life-threatening medical condition arising in response to BSI in neonates [7,17–19]. The immune system of neonates, especially those born prematurely, is immature. Furthermore, as trans-placental transfer of maternal antibodies occurs most efficiently in the third trimester, PT babies are deficient in humoral immune responses [20]. Consequently, neonates, particularly PTs, are immune-compromised and therefore more susceptible to infection by a wide variety of pathogens [21].

1.2.1 The epidemiology of neonatal sepsis

Despite advances in healthcare systems globally, neonatal death is still a major cause of childhood deaths. More than 47% (2.7 million) of global deaths in under-5-year-olds occur in the neonatal period. Of 2.7 million global neonatal deaths, neonatal sepsis is responsible for one fifth of the fatalities [22,23]. The incidence of neonatal sepsis ranges from 1-5/1,000 live births in HICs. The incidence of clinical sepsis in LMICs is 40 times greater than in HICs, with estimates ranging from 49–170 per 1,000 live births [20].

1.2.2 The classification of neonatal sepsis

Neonatal sepsis has been classified on the basis of various factors: the time of clinical presentation, mode of transmission, origin of acquisition etc. [7]. Classifications that are based on such differences can aid in the management of disease by guiding appropriate antimicrobial therapy.

1.2.2.1 Early onset neonatal sepsis (EOS)

Based on the time of occurrence after birth, neonatal sepsis can be divided into early- and late-onset sepsis. Sepsis that appears at or before 72 hours of life is called early onset sepsis (EOS) [24]. Most of newborns have clinical manifestation of EOS within 6 to 48 hours of birth [25,26].

EOS results from infections proceeding birth (antepartum period) or during birth (intrapartum period). EOS usually results from vertical transmission of microorganisms (colonizers or pathogens) from the maternal birth canal to the newborn during the natural birthing process. Infection of the foetus also may occur *in-utero*. During maternal chorioamnionitis, the foetus may acquire infection through aspiration or ingestion of the contaminated amniotic fluid. During prolonged premature rupture of the membrane (PPROM), the bacterial flora from the maternal vaginal vault may ascend to gain access to and proliferate in the foetus, thereby infecting it. Pathogens may also be acquired intrapartum in the early hours of birthing due to unhygienic practices such as improper cord care [27].

EOS is a major cause of neonatal morbidity and mortality in HICs and LMICs. The estimated incidence rate of culture positive EOS among normal birth weight neonates (birth weight ranging 2,500- 4,200 grams [28]) in HICs is <1/1,000 live births, with a case fatality rate (CFR) of 10%-15% [29]. Lower birth weight significantly increases the risk for EOS. The incidence of EOS is estimated to be 8/1,000 live births among very low birth weight (VLBW) neonates (birth weight ranging 1,000- 1,500 grams) and is approximated to be as high 26/1,000 live births among extremely low birth weight (ELBW) neonates (birth weight <1,000 grams) in HICs [30]. The incidence of EOS is higher in LMICs. In Africa and South Asia, the rates of EOS among normal birth weight neonates are estimated to be 39.3 and 9.8/1,000 live births respectively [31,32]. Mortality due to EOS in LMICs is estimated to be as high as 60% [25].

1.2.2.2 Late onset sepsis (LOS)

Sepsis that manifests after 72 hours of birth in a neonatal period is called late onset sepsis (LOS) [17]. Most of newborns show clinical manifestation of LOS between day 10 and 22 of life [25,26]. LOS results from infection with pathogens from within the surrounding environment and/or

immediate care givers of the community or hospital setting during the postpartum period (after childbirth).

The incidence of LOS is high in HICs and LMICs. In HICs, the incidence of LOS is 3–3.7 per 1,000 live births [20], while that in LMICs is 18/1,000 live births. Low birth weight (LBW) and premature birth are major risk factors for LOS. The incidence of LOS is inversely proportional to birth weight [33]. In a study conducted in England, the proportion of LOS among normal birth weight neonates from 541 neonatal infections was 1.6%, while the proportions of LOS were 2.2% and 10.2% among LBW and VLBW neonates, respectively [34].

1.2.2.3 Community-acquired neonatal sepsis (CANS)

Based on the origin of acquisition, LOS can be either community acquired or hospital acquired. LOS that is acquired by the neonates from surrounding environment of community including immediate care givers is called community-acquired neonatal sepsis (CANS). For post-natal hospital admissions, CANS is identified as the neonatal infection with clinical onset starting within 48 hours of hospital admission [29].

In the rural areas in LMICs, the majority of child births take place at home. It has been estimated that 80-90% of child deliveries in very poor communities in LMICs, e.g., in Pakistan, take place in homes via unskilled birth attendants [35]. Poor sanitary conditions during home deliveries, such as use of unclean bedding like hay, soiled hands, non-sterile cord cutting etc. greatly put the newborns at risk of acquiring CANS [36]. The inability to identify and treat early danger signs of newborn infections along with the failure to refer such cases to higher healthcare facilities also contribute to the higher morbidity and mortality associated with CANS in LMICs [36,37].

There is an ever widening gap in the incidence rate of CANS between HICs and LMICs. In Switzerland, the national incidence rate of culture positive CANS has been estimated to be 0.28/1,000 live births [29]. In contrast, in LMICs, the incidence rate of culture confirmed CANS was reported to have ranged between 2.9 to 24 per 1,000 live births [38]. In South Asia, the incidence of culture proven CANS has been estimated to be 12.3/1,000 live births.

1.2.2.4 Hospital-acquired neonatal sepsis (HANS)

Nosocomial infections that are acquired by neonates from the hospital environment after 72 hours of life and after at least 48 hours of hospitalization are categorized as hospital-acquired LOS. The incidence of neonatal sepsis and resulting mortality is particularly high in NICUs. In LMICs, it has been estimated that approximately half of the NICU admitted neonates acquire HANS, with CFR as high as 52% [39].

With medical advances in neonatology, there have been improvements in the survival rates of critical neonates such as those born extremely premature (<28 weeks of gestation), born with VLBW and ELBW [40]. Such neonates require admission to hospitals, mostly NICUs, where they usually require invasive medical interventions, often immediately after birth. For example, newborns having breathing difficulties may require immediate resuscitation or even mechanical ventilation to avoid serious consequences of hypoxia; neonates in whom enteral feeding is impossible may require parenteral nutrition. Such invasive procedures are essential to maintain and/or evaluate lives of the neonates. Ironically, despite being life-saving, such procedures may pose substantial risk of nosocomial infections such as primary BSI by acting as portals of entry for pathogens upon compromised infection prevention and control (IPC) measures [41,42]. The resulting HANS may substantially increase the clinical complications, risk of mortality, use antimicrobials, hospital stay, and hospital cost [35,41].

1.2.3 Definitions of clinical sepsis, culture proven sepsis, assumed sepsis (blood culture negative sepsis)

The condition where sepsis is suspected based on the clinical signs presented by neonates is called 'clinical sepsis' [18]. The clinical signs of neonatal sepsis are often subtle, non-specific and overlapping with several other non-infectious clinical syndromes. This imposes a significant challenge in diagnosis and subsequent management of clinical sepsis [18,20]. When sepsis is clinically suspected, a series of tests including a blood culture are conducted. For a microbiological report of blood culture to arrive, it takes at least 24 hours after collection of a blood sample. Therefore, an empirical antimicrobial therapy is often initiated in the neonates clinically suspected of sepsis before a microbiological culture based definite diagnosis could be made.

When microbiological culture results of blood samples are positive for bacteria of known clinical significance, such clinical condition of sepsis is regarded as 'culture-confirmed sepsis' [18]. Neonates are treated with targeted antimicrobial therapy, making clinical management of sepsis direct and easier [20]. It is common that many infectious agents of sepsis, including several fastidious and anaerobic bacteria, fail to be cultured by routine microbiological techniques. Under such instances, other laboratory results such as abnormal blood cell count may suggest a probable case of sepsis. The clinical condition where bacterial infection is assumed based on clinical and pathological grounds, and the neonate is empirically treated with antimicrobials in the absence of a positive blood culture result is referred to as 'assumed sepsis' or 'blood culture negative sepsis' [18].

1.2.4 Risk factors of neonatal sepsis

The naïve immune status of neonates puts them at increased risk of acquiring infections, which can occur at any of antenatal, intrapartum or postnatal periods. The risk factors can be of maternal, neonatal or environmental (community- or hospital-based) origin.

1.2.4.1 Maternal and neonatal risk factors

Several mother-related factors may increase the risk of development of EOS in neonates (Table 1.1). Intra-uterine infections such as, chorioamnionitis, can lead to a fatal infection of the foetus. Maternal infections such as urinary tract infection (UTI), sexually transmitted infections (STI) occurring during the last trimester of pregnancy may lead to vertical transmission of infection to the neonate. Further, symptomatic or asymptomatic colonization of the birth canal may lead to infection of the newborn during child birth. The premature rupture of the membranes for a prolonged period of more than 18 hours may increase the risk of sepsis development in the neonate in the absence of antimicrobial prophylaxis [43]. Additionally, several neonatal characteristics, such as low gestational age, low birth weight, VLBW, ELBW, presence of congenital abnormalities etc. may increase the risk of sepsis development in neonates (Table 1.1).

Table 1.1. The maternal and neonatal risk factors for sepsis development in neonates.

Maternal risk factors	Neonatal risk factors
PPROM	Prematurity
Fever in third trimester of pregnancy	Low APGAR score
Chorioamnionitis	Sustained foetal tachycardia
Multiple aseptic obstetric procedures	Male gender
Multiple gestation	Birth asphyxia
Low socioeconomic status	Meconium aspiration
Poor antenatal care	Congenital anomalies
Poor nutrition	Galactosemia
Substance abuse	Malnourishment
Maternal UTI during late pregnancy	IUGR
TORCH infections	Foetal hypoxia
Perineal colonization	Immunological defects
Maternal STI during late pregnancy	

IUGR: Intra-uterine growth retardation, PPROM: Prolonged premature rupture of membrane, STI: sexually transmitted infections, TORCH: Toxoplasmosis, Others (Syphilis, Varicella Zoster virus, Parvovirus B19, HIV), Rubella virus, Cytomegalovirus, and Herpes Simplex virus infections, UTI: urinary tract infection

1.2.4.2 Antenatal and Intrapartum risk factors

Neonatal sepsis may occur as a result of infection acquired before the birth (antepartum period). Several factors, particularly maternal infections, serve as major antepartum factors for increased risk of development of neonatal sepsis (Table 1.2). Additionally, the birth process related factors, such as duration of labor exceeding 20 hours, traumatic delivery, use of unclean birthing process, septic cord care etc. may also increase the odds of sepsis development [43].

Table 1.2. The antenatal and intrapartum risk factors for sepsis development in neonates.

Antenatal risk factors	**Intrapartum risk factors**
PPROM	Foul smelling amniotic fluid
Multiple aseptic obstetric procedures	Premature (<37 weeks of gestation) labour
Multiple gestation	Foetal hypoxia
Low socioeconomic status	Traumatic delivery
Poor antenatal care	Unclean delivery
Poor nutrition	Delivery by unskilled personal
Substance abuse	Septic cord care
Maternal UTI during late pregnancy	
TORCH infections	
Perineal colonization	
Maternal STI during late pregnancy	
Chorioamnionitis	

PPROM: Prolonged premature rupture of membrane, STI: sexually transmitted infections, TORCH: Toxoplasmosis, Others (Syphilis, Varicella Zoster virus, Parvovirus B19, HIV), Rubella virus, Cytomegalovirus, and Herpes Simplex virus infections, UTI: urinary tract infection

1.2.4.3 Risk factors for development of EOS and LOS in neonates

The antenatal risk factors, particularly maternal infections and birth canal colonization during later stages of pregnancy, are mostly associated with the development of EOS (Table 1.3) [43,44]. Failure to prompt administration of intrapartum antimicrobial prophylaxis during maternal

infections and PPROM may also attribute to the increased risk for sepsis development. Alternatively, postnatal factors such as admission to NICU, prolong hospitalization of the newborn, prolong use of invasive procedures, poor hygiene, bottle feeding etc. may increase the odds for development of LOS [29,44].

Table 1.3. The risk factors for the development of early- and late-onset sepsis in neonates.

Risk factors for EOS	**Risk factors for LOS**
PPROM	Prolonged hospitalization
Maternal fever in third trimester of pregnancy	LBW/VLBW
Chorioamnionitis	Prolonged antimicrobial use
Maternal UTI in third trimester of pregnancy	Prolonged insertion of invasive devices
Multiple aseptic obstetric procedures	Overcrowding
Maternal vaginal colonization with pathogens	PT
Inadequate IAP	Formula feeding
TORCH infections	Neonatal resuscitation and suctioning
Perineal colonization	Multiple invasive procedures
Maternal STI in third trimester of pregnancy	Extended parenteral nutrition

IAP**:** Intrapartum Antimicrobial Prophylaxis, LBW: low birth weight, PPROM: Prolonged premature rupture of membrane, PT: preterm, STI: sexually transmitted infections, TORCH: Toxoplasmosis, Others (Syphilis, Varicella Zoster virus, Parvovirus B19, HIV), Rubella virus, Cytomegalovirus, and Herpes Simplex virus infections, UTI: urinary tract infection, VLBW: very low birth weight

1.2.5 The diagnosis of neonatal sepsis

An early suspicion of neonatal sepsis is based on clinical manifestations and underlying risk factors. Laboratory tests results can aid in confirming the diagnosis of sepsis [45].

1.2.5.1 Clinical manifestations of neonatal sepsis

Clinical manifestations are invaluable initial clues for suspicion of sepsis. If not treated promptly, sepsis may rapidly progress into severe sepsis, thereby increasing the risk of complications and

mortality [46]. It is therefore necessary that clinicians be vigilant to identify any subtle signs of sepsis and respond immediately by initiating an empirical antimicrobial therapy after taking biological samples for further laboratory evaluation [20].

1.2.5.1.1 General clinical manifestations of neonatal sepsis

General clinical manifestations of neonatal sepsis could be poor feeding behaviour, irregular body temperature (hypothermia (<35.5 °C) or fever (>37.5 °C)), lethargy, poor cry, irregular blood sugar (hypoglycaemia or hyperglycaemia), oedema, sick look, or movement only when stimulated [47,48].

1.2.5.1.2 Body system specific symptoms

Cardiovascular symptoms mainly result from poor perfusion, hypoxia, and hypotension. The signs include hypotension, prolonged capillary refill time (>2 seconds), irregular heart rate (bradycardia or tachycardia), cold body, bleeding, petechia, purpura or mottled skin [47,48]. Respiratory symptoms are common and could range from apnoea, dyspnoea, tachypnea, retractions, nose flaring, grunting to cyanosis [47,48]. Gastrointestinal (GI) symptoms may include abdominal distention, diarrhoea, vomiting, feeding intolerance, poor sucking, necrotising enterocolitis, and blood in stool [47,48]. Renal symptoms, such as oliguria and acute renal failure may occur in some neonates [47,48]. Skin related symptoms, such as sclerema (firm, waxy skin extending throughout the body) are rare and associated with poor outcome [49,50]. Neonatal sepsis may also manifest with central nervous system related symptoms, such as bulging anterior fontanel, high-pitched cry, excessive irritability, coma, seizures or neck retraction. These symptoms could suggest conditions, such as meningitis or encephalitis [47,48].

1.2.5.2 **Laboratory tests for diagnosis of neonatal sepsis**

There are several laboratory tests that are carried out for screening neonates who are suspected of sepsis [45].

1.2.5.2.1 Blood culture

The gold standard for diagnosis of neonatal sepsis is the isolation of pathogen from sterile biological samples such as blood or cerebrospinal fluid (CSF) [20]. Blood cultures provide the clinicians with a definite diagnosis and acts as a guide to switch to targeted antimicrobial therapy. Though considered as the gold standard, blood culture suffers from several limitations [20,51]. Blood culture takes nearly 24 hours to generate a presumptive culture results based on microscopic examination of content of positively flagged BACTEC culture vial. Neonatal sepsis can be fulminant and delayed treatment can have serious and irreversible implications. Therefore, clinicians rely on clinical judgement and initiate antimicrobial therapy empirically before culture results are available. The sensitivity of blood culture tends to be compromised by various factors such as low blood volume (0.5-1 ml), low bacteraemia, or prior use of antimicrobial agents [20,51]. Such limitations may lead to false negative results. Moreover, conventional culture and bacterial identification methods routinely practiced in many laboratories are not optimal for retrieval of anaerobic and fastidious bacteria, fungi, and viruses. Hence, blood culture negative results do not always exclude the possibility of neonatal sepsis [20]. The clinical presentation and other laboratory test results are required for careful evaluation of suspected sepsis [51].

1.2.5.2.2 Haematological laboratory tests

Haematological tests for screening sepsis in neonates include complete blood cell count (CBC) and micro-erythrocyte sedimentation rates (μ-ESR). CBC includes total leucocytes count (TLC), absolute neutrophil count (ANC), immature to total neutrophil ratio (I: T), and platelets count. CBC is difficult to interpret in neonates because the count rapidly varies with the gestational age of

the neonate [51]. Leukopenia (<5,000-7,500 WBC/mm^3), leucocytosis (>40,000 WBC/mm^3), low ANC (<1,750/mm^3), low I:T neutrophil ratio (>0.20), low platelets count (<100,000/mm^3) or, elevated μ-ESR (>15 mm in the first hour) may be indicative of sepsis [20,45]. The limitation of the haematological tests is that none of these markers have high sensitivity and specificity to be solely used for precise diagnosis of neonatal sepsis [20,21,47,52].

1.2.5.2.3 Inflammatory biomarkers

Several potential biological markers of sepsis are under investigation. Acute phase reactants, such as C-reactive protein (CRP) and procalcitonin **(**PCT) are two of the most promising and commonly used biomarkers [51].

CRP is the most commonly used test for sepsis diagnosis. CRP is a protein synthesized by liver cells when elicited by cytokines, such as interleukin (IL)-6, IL-1 and tumour necrosis factor (TNF)-α [52]. The concentration of serum CRP of newborns varies significantly with their physiological development [45]. The cut off values for serum CRP indicating inflammation may differ with institutional guidelines. Infection is usually indicated when serum CRP is >1 mg/dl [20]. Production of CRP is however a non-specific response of body to diseases, and therefore it cannot be solely used as a diagnostic test for sepsis. When used with other laboratory test results and clinical indications, serial measurement of CRP helps in establishing an early diagnosis and progress of sepsis [53]. PCT is an acute phase reactant protein produced by macrophages and liver cells. A rapid rise in serum PCT level following bacterial invasion makes PCT a good marker for the early detection of neonatal septicaemia. A serum PCT level of >8 mg/dl is indicative of neonatal septicaemia [7]. Unlike other markers, increase in the level of PCT during bacterial sepsis is not affected by the gestational age of neonate [52]. However, false increase in serum PCT has been observed in several non-specific conditions such as preterm, birth asphyxia, neonatal

hypoxemia, intracranial hemorrhage, PPROM, post-natal use of antimicrobials, thus underscoring the need of proper assessment of PCT results in neonates [52].

1.2.5.2.4 Molecular diagnostic tests

Polymerase Chain Reaction (PCR) is one of the promising molecular diagnostic tools for rapid detection of neonatal sepsis. PCR has high sensitivity, specificity, and shorter processing time compared to microbiological culture, thereby assisting clinicians in prompt patient management [51]. Further, PCR can identify pathogens beyond bacteria and can also detect the presence of AMR genes. Unlike microbiological cultures, PCR does not rely on viability of pathogens. Therefore, the PCR results are not affected by prior antimicrobial therapy, thereby potentially improving diagnosis and management of sepsis. However, the use of molecular tests demand experienced staffs, expensive equipment, and consumables [51].

1.2.6 The microbiology of neonatal sepsis

The spectrum of aetiological agents of neonatal sepsis varies by setting and time. The causative agents of neonatal sepsis can also differ by origin of acquisition and time of disease onset.

1.2.6.1 The microbiology of neonatal sepsis in HICs

Group-B streptococci (GBS) (*S. agalactiae*) are the predominant aetiological agents of EOS in HICs [17,30]. Though pre-natal screening and IAP have greatly reduced their incidence, GBS are still the leading cause of EOS in HICs, constituting of 38% to 43% of all aetiologies in the United States [54]. *E. coli* is the second leading cause of EOS, constituting of nearly 24% of all EOS episodes [30,55]. The remaining minor aetiologies are comprised of pneumococci, viridans streptococci, enterococci, *S. aureus, Listeria monocytogenes* and GNB such as *Haemophilus influenzae* [55]. Common aetiological agents of neonatal LOS in HICs are coagulase negative

staphylococci (CoNS), *S. aureus*, and GNB [56]. The most common GNB include *E. coli, Klebsiella* spp., *Pseudomonas* spp., *Enterobacter* spp., *and Serratia* spp. [17].

1.2.6.2 The microbiology of neonatal sepsis in LMICs

The aetiological scenario in LMICs is different from HICs. GNB such as *K. pneumoniae*, *Enterobacter* spp., *E. coli*, *Acinetobacter baumannii,* and *P. aeruginosa* are the most common causes of neonatal EOS and LOS in LMICs [32,57,58]. Among GPC, CoNS and *S. aureus* are the predominant causes of sepsis in LMICs [32]. The role of GBS in neonatal sepsis, as observed in HICs, is insignificantly reported in LMICs of South Asia [32,58]. Most published data regarding neonatal sepsis from LMICs are hospital-based studies. Few large community based studies from South Asian countries such as Bangladesh and India have suggested that the aetiology of CANS follows the pattern of hospital-acquired sepsis [50,57,59].

1.2.7 Bacterial pathogens causing neonatal sepsis

1.2.7.1 Group-B streptococci (GBS)

GBS (*S. agalactiae)* are Gram-positive facultatively anaerobic cocci belonging to the bacterial family 'Streptococcaceae' [60]. Asymptomatic vaginal colonization by GBS has been estimated to occur in 10-30% of women [30]. During pregnancy and natural childbirth, the colonizing GBS may serve as source of infection to their babies [30]. Most of GBS neonatal infections lead to systemic BSI without localizing findings. Despite timely antimicrobial treatment, the mortality rate of EOS caused by GBS may reach as high as 20% [61].

1.2.7.2 *Staphylococcus aureus*

Staphylococci are facultatively anaerobic GPC bacteria belonging to the bacterial family 'Staphylococcaceae' [62]. *S. aureus* is the most virulent and medically relevant species of staphylococci. *S. aureus* is one of the members of '*E. coli* and the ESKAPE' group of pathogens, which are a group of bacterial pathogens associated with nosocomial drug resistant infections [63]. In Africa and South Asia, the prevalence of HANS due to *S. aureus* has been estimated to be 14.3% (224/1,563) and 20.2% (1,206/5,984), respectively [35]. It has been observed that >90% of NICU admitted neonates get colonized with *S. aureus* on their umbilical stump, nasal mucosa, skin, and GI tract by first week of their NICU stay [64]. Asymptomatic colonization of pathogens may serve as important reservoir of infection for newborns.

1.2.7.3 Coagulase negative Staphylococci (CoNS)

All species of staphylococci, except *S. aureus*, belong to a broader group of less virulent species called CoNS. Though less virulent, CoNS play important clinical role in causing HA-BSIs among VLBW and PT newborns, immuno-compromised individuals, and patients with implanted medical devices [21]. The two most medically relevant species are *S. epidermidis* and *S. saprophyticus*. *S. epidermidis* is responsible for 60 to 93% of CoNS sepsis episodes in neonates [30].

CoNS are frequently isolated in blood cultures. However, being a normal commensal flora of skin, the isolation of CoNS in blood culture is often considered as contaminant. Distinguishing CoNS as a causative agent of true infection from a contaminant is often challenging. Several criteria have been set to distinguish the role of CoNS between a pathogen and a contaminant. One of the proposed criteria for determining the clinical significance of CoNS was to obtain at least two independent blood cultures positive for CoNS within 5 days or, one positive blood culture with clinical signs suggestive of an infection [65]. In addition to this, other criteria such as absence of another likely infection at the time of blood collection, improved symptoms after therapy, and

shorter time (<16 hours) to blood culture positivity have also been taken as further criteria to consider significance of CoNS in blood culture [66,67].

1.2.7.4 Enteric Gram-negative bacilli (GNB)

The Enterobacteriaceae are a family of diverse facultatively anaerobic GNB. The most medically relevant members are *E. coli, Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Salmonella* spp., and *Shigella* spp. [68]. Most of the members of Enterobacteriaceae comprise a part of normal flora of human GI tract. The enteric GNB are also transient flora of human skin and play a major role in horizontal transmission of infections in poor hygienic conditions. The enteric GNB are opportunistic pathogens and pose serious threat of sepsis among immuno-compromised individuals such as neonates [61]. Neonatal sepsis caused by enteric GNB is usually associated with a higher CFR than the CFR inflicted by GPC [32,59].

1.2.7.4.1 *Escherichia coli (E. coli)*

E. coli is a major causative agent of neonatal sepsis and meningitis. PT and VLBW neonates are the most at risk subpopulation for infections by *E. coli*, contributing >80% of EOS episodes [21,30]. The neonatal infections caused by *E. coli* are associated with higher mortality and increased long term complications, such as neurodevelopmental impairments among survivors [69].

1.2.7.4.2 *Klebsiella pneumoniae*

K. pneumoniae is one of the members of '*E. coli* and the ESKAPE' group of pathogens [63]. In HICs, K. *pneumoniae* is responsible for 20–30% of neonatal LOS [21]. In South Asia, *K. pneumoniae* has been reported to be the most frequently isolated pathogen of neonatal sepsis in

hospital (23% of total 24,273 isolates) and community (25% of total 703 isolates) [32]. *K. pneumoniae* is also a well-known pathogen of sepsis outbreaks in NICUs worldwide [70–75].

1.2.7.4.3 *Enterobacter* spp.

Seven species of *Enterobacter*, namely, *E. cloacae* subsp. *cloacae*, *E. asburiae*, *E. kobei*, *E. hormaechei*, *E. ludwigii*, *E. mori* and *E. nimipressuralis* are grouped as *E. cloacae* complex (ECC) [76]. The ECC members are one of the pathogens of '*E. coli* and the ESKAPE' group and are frequently associated with nosocomial drug resistant infections [63]. Since past decade, the ECC group members have increasingly emerged as third major enteric GNB causing BSIs in hospital ICUs and communities [63,77–80]. In LMICs, *Enterobacter* spp. were reported in 5% of culture positive neonatal sepsis episodes [35,57]. In HICs, *Enterobacter* spp. has been estimated to cause <1% of neonatal sepsis [30].

The taxonomy of *Enterobacter* spp. is complex [81]. Phenotypic tests have low resolution to precisely identify the species of *Enterobacter* [81]. Molecular tests, such as heat-shock-protein 60 (hsp60) typing and 16S rRNA gene sequencing also have insufficient discriminatory power for accurate species assignation [82]. The comparison of bacterial genome at nucleotide level using pairwise average nucleotide identity (ANI) score of > 96% provides an ultimate resolution for precise identification of bacterial species [81].

1.2.7.5 Non-enteric Gram-negative bacilli (GNB)

A. baumannii and *P. aeruginosa*, two of the members of '*E. coli* and the ESKAPE' group of pathogens, are major non-enteric GNB causing opportunistic HAIs [63]. *A. baumannii* and *P. aeruginosa* have been frequently associated with colonization of hospitalized patients and diverse hospital surfaces [83]. *A. baumannii* and *P. aeruginosa* are emerging nosocomial pathogens of hospital-acquired LOS in neonates. In LMICs, *Acinetobacter* spp. and *P. aeruginosa* were

reported in 5% (576/11,471) and 8% (904/11,471) of culture-positive neonatal LOS episodes, respectively [35]. The clinical outcome of HANS caused by *A. baumannii* and *P. aeruginosa* are usually life-threatening, and associated with poor prognosis. *A. baumannii* and *P. aeruginosa* have been reported to cause several sepsis outbreaks in NICUs worldwide [84–88] .

1.3 Antimicrobial resistance (AMR)

AMR is an ability of microorganisms to resist inhibitory or lethal effect of antimicrobial agents to which they were previously susceptible [89]. Infection with AMR pathogens, therefore, imposes therapeutic difficulties and can result into increased hospital stay, healthcare cost, and higher mortalities [90]. Bacteria continue to be selected and evolve to become increasingly resistant to a growing number of antimicrobial agents. Several AMR genes encode for specific phenotypic changes in bacteria, resulting into AMR to specific antimicrobial agents. Further, AMR genes can be shared between and across bacterial genus through mobile genetic elements (MGEs), such as plasmid, transposons, and insertion sequence, which leads to a rapid spread of AMR across the globe [89].

1.3.1 Extended-spectrum beta-lactamases (ESBLs)

The β-lactam antimicrobials are one of the most widely clinically used antimicrobial agents. The cleavage of β-lactam ring of β-lactam antimicrobials by bacterial enzymes, called β-lactamases is one of the robust AMR mechanisms against β-lactam agents [91]. Extended spectrum β-lactamases (ESBLs) and carbapenemases are the two most clinically relevant and rapidly evolving versatile β-lactamases [92] . ESBLs, such as TEM, SHV, CTX-M, FOX, and ACT are serine β-lactamases which can hydrolyze third-generation cephalosporins and aztreonams, but not carbapenems [93]. Most of the ESBLs have been derived from parental narrow-spectrum penicillinases TEM-1, TEM-2, SHV-1 and SHV-11 by fewer mutations around active sites [93].

CTX-M is the most rapidly evolved and extensively spread ESBL [92]. CTX-M is mostly distributed in enteric GNB, specifically isolated from South and South East Asia [91,93]. The CTX-M-1 is one of five clusters of CTX-M ESBL and includes the most clinical relevant variants, such as CTX-M-15 [94].

1.3.2 Carbapenemases

The carbapenemases are the most versatile β-lactamase enzymes which can efficiently hydrolyse all available β-lactam antimicrobials including carbapenems [95]. Most of the clinically relevant Gram-negative bacterial pathogens acquire carbapenemases by horizontal transfer from other bacteria via MGEs [96]. Infection by carbapenem resistant bacteria exerts a severe therapeutic challenge because several AMR genes for diverse antimicrobial classes often coexist in the same conjugative plasmid, resulting into multiple drug resistance [97].

The serine carbapenemases such as KPC, IMI, and SME have serine at their active sites [95,96,98]. KPC is the most efficient serine carbapenemase frequently associated with mobile genetic transporter structures leading to enhanced inter-species horizontal transmission and rapid global dissemination [99]. Though initially isolated from *K. pneumoniae*, KPC has now been isolated from diverse enteric and non-enteric GNB [96,98]. The metallo-β-lactamases (MBLs) are the carbapenemases with zinc at their active site and are inhibited by metal chelators such as ethylene-diamine-tetra-acetic acid (EDTA) [95]. The most clinically significant MBLs are VIM, IMP, and NDM [96]. MBLs are usually associated with integron structures that are embedded in MGEs, thereby facilitating an efficient inter-species bacterial gene transfer [95]. Acquired MBLs have been reported from all over the world specifically in enteric and non-enteric GNB.

1.4 The epidemiology of multidrug resistant Gram-negative bacilli

AMR, specifically in clinically relevant GNB, is one of the biggest global public health problems. An excessive and inappropriate use of broad-spectrum antimicrobials in human, livestocks, and environment have been considered to be the main drivers for an accelerated evolution, selection and spread of drug resistant bacteria [100]. According to CDC, in the United States, > 2 million infections every year are caused by antimicrobial resistant pathogens, resulting into > 23,000 deaths attributable to drug resistant organisms [101]. It has been estimated that by 2050, the annual infections by antimicrobial resistant pathogens will increase by 10 folds [102].

E. coli and a group of six specific bacterial pathogens have been designated as '*E. coli* and the ESKAPE' organisms [63]. ESKAPE is an acronym for pathogens *Enterococcus faecium, S. aureus, K pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* spp. [63]. Antimicrobial resistant isolates of '*E. coli* and the ESKAPE' pathogens can resist the effect of most of the currently available antimicrobial agents and are the most important causes of life-threatening HAIs in critically ill and immuno-compromised individuals [63,103]. Infection with drug resistant bacterial pathogens, specifically '*E. coli* and the ESKAPE' pathogens, results in increased health complications, diagnostic uncertainties, extended hospital stay, increased treatment costs, and elevated mortality [104]. An increasing AMR to existing antimicrobials and the unavailability of new clinically useful antimicrobials have made the therapeutic management of patients infected with drug resistant organisms extremely challenging. Considering such difficulty, WHO has recently included the '*E. coli* and the ESKAPE' bacteria in the priority pathogen list (PPL), for which the discovery of new antimicrobial agents is critically urgent. Carbapenem resistant *A. baumannii* and *P. aeruginosa*, and ESBL positive or carbapenem resistant *K. pneumoniae* and *Enterobacter* spp. are listed in the critical priority list [105].

1.5 The therapeutic management of neonatal sepsis

In LMICs, neonatal sepsis has been estimated to attribute up to 70% of neonates deaths [106]. Further, delayed treatment can result into severe long-term sequels such as cognitive impairment in the survivors [107]. Therefore, it is imperative to promptly manage and treat the neonates who are suspected of sepsis.

The bacterial infections being the most common cause of sepsis, antimicrobial therapy remains the cornerstone for sepsis management. Several international guidelines, such as by National Institute for Health and Clinical Excellence (NICE) and Surviving Sepsis Campaign (endorsed by Infectious Diseases Society of America) recommend that the treatment should be initiated as soon as possible [108,109]. As an antimicrobial therapy often needs to be initiated before the availability of microbiological culture reports, the empirical treatment is guided by several factors. The factors include prior knowledge on the potential spectrum of pathogens and antimicrobial susceptibility, neonatal age at the sepsis onset, and potential origin of infection acquisition [110,111]. In general, the empirical antimicrobial therapy should be of broad spectrum to cover most of the possible pathogens of a local setting. The β-lactams and aminoglycosides are the most common combination of antimicrobials used as first-line therapy for the treatment of neonatal sepsis [108,111–113]. An actual empirical antimicrobial protocol varies by institution depending on the local AMR profile of the most common isolates. Once the culture reports are obtained, the empirical therapy is adjusted to a targeted therapy as directed by the antimicrobial susceptibility test (AST) results.

1.6 Infection prevention and control (IPC) measures in NICU

NICUs have high prevalence of HANS, resulting into increased morbidity and mortality in admitted neonates [114]. An immunological immaturity and the need of increased use of invasive procedures elevate the risk of nosocomial infections in NICUs. A stringent implementation of IPC

measures remains essential to minimize the incidence of HANS in NICUs. The IPC measures include effective preventive strategies such as compliance to hand hygiene, a rigorous cleaning practice, aseptic preparation of supplies and equipment, an appropriate hospital waste management protocol, a longitudinal monitoring of infection rates and AMR surveillance, effective measures to minimize catheter-associated BSIs, an implementation of antimicrobial stewardship policies, and proper structural design of hospital units [114,115]. Additional preventive measures to limit the transmission of infection between patients include the isolation and cohorting of infected neonates, and screening for colonization with drug resistant bacterial pathogens [116].

1.6.1 Hand hygiene

Hands of healthcare workers (HCWs) may carry 3.9×10^4 to 4.6×10^6 of colony forming units (CFU)/cm2 of bacteria which may include potential pathogens such as *K. pneumoniae*, *Enterobacter* spp., *A. baumannii*, and *S. aureus* [117]. Personal unhygienic behaviour and direct contact with infected patients, biological sample, and contaminated surfaces can lead to a transient colonization of hands by potential pathogens. In the absence of proper cleaning, the contaminated hands of HCWs and immediate care givers can serve as possible vectors of infection. Therefore, strict adherence to hand hygiene is an essential interventional approach to prevent HAIs. HCWs must perform the recommended procedures of hand hygiene with stipulated preparations before and after contact with potentially contaminated sources [114].

1.6.2 Prevention of catheter-associated BSIs

Catheter-associated BSIs are major HAIs in the neonates admitted to NICUs, with the infection rates being 2-3 fold higher in LMICs than in HICs [118]. Catheter-associated BSI may result from the migration of skin flora at the insertion site resulting into the colonization of the tip, contamination of the catheter hub leading to an intra-luminal colonization of catheter, or rarely, by

the injection of contaminated infusions resulting into intra-luminal spread of infection (Figure 1.2). Sometimes, catheter tips may be contaminated haematogenously because of existing distant infections [119].

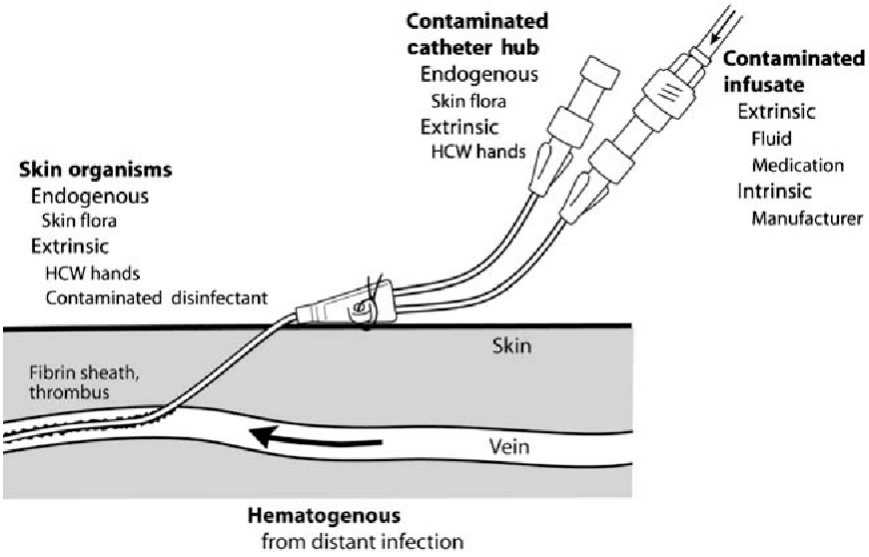


Figure 1.2. The pathophysiology of catheter-associated bloodstream infection.

The catheter tip may be contaminated by the skin colonizers of patient or HCWs during catheter insertion leading to the colonization of catheter tip and development of BSI. Alternatively, the catheter hub may be contaminated by the skin flora mostly during prolong catheter stay. The use of contaminated infusions also leads to BSI by direct intra-luminal spread of infection.

Inadequate aseptic procedures during insertion and maintenance of catheters increase the risk of development of catheter-associated BSIs. The Centers for Disease Control and Prevention (CDC) has recommended a guideline for the prevention of catheter-associated BSI. The guideline stipulates for the use of maximum sterile barrier precaution during catheter insertion, the use of skin disinfectant containing >0.5% chlorhexidine, prompt removal of catheters when no longer needed, avoiding routine replacement of central venous catheters, the use of chlorhexidine impregnated dressings, and training of concerned HCWs [40,120].

1.6.3 Antimicrobial stewardship (AMS) in NICUs

A considerable overlap in clinical manifestations between infectious and non-infectious illnesses in neonates causes an accurate diagnosis of neonatal sepsis challenging. Further, higher incidence of culture negative results and greater risk of rapid deterioration of septic neonates lead to increased inappropriate use of antimicrobials in NICUs [115]. An excessive use of antimicrobials has several consequences such as toxic effect in neonates, alteration of gut normal flora, rapid emergence of AMR, higher risk of fungal infections, and death [40].

Antimicrobial stewardship (AMS) ensures and promotes an optimal use of antimicrobials in healthcare settings. The IPC practices and AMS are integral components of healthcare systems to ensure an improved quality of patients' care and outcomes [121]. The IPC measures primarily intend at reducing the overall prevalence of HAIs and therefore, aim at reducing the use of antimicrobials. The AMS measures, on the other hand, aim to achieve favorable clinical outcomes by ensuring an optimal use of antimicrobials [115]. The main principles of AMS in NICUs are an accurate identification of patients requiring antimicrobial therapy, an optimal selection of empiric antimicrobial therapy based on updated antibiogram, avoiding the antimicrobial with overlapping mechanisms, and continued education of prescribers on adverse consequences of inappropriate use of antimicrobials [40,116,122].

1.6.4 Management of infection outbreaks in NICUs

NICUs have high prevalence of HAIs and are often affected by infection outbreaks. It has been reported that worldwide, NICUs account for 38% of all ICU-related outbreaks [40]. Bacteria such as *K. pneumoniae*, *S. aureus*, *Serratia* spp., *A. baumannii*, and *Enterobacter* spp. are the most commonly reported outbreak-related microorganism in NICUs [40,74,86,123,124]. The outbreaks caused by the ESBL and carbapenemase producing GNB are of specific concern because of an increased risk of treatment failures and poor outcomes. Further, outbreaks also impose significant

challenge to the hospital management system by incurring an increased operational- and economic cost to the hospital [40].

In outbreaks, the infected and colonized neonates may serve as important reservoirs of infection. On the other hand, in the healthcare settings with compromised IPC measures, the hands of the HCWs, the shared medical devices, and the invasive medical procedures often serve the mode of infection transmission [114]. The multiple events of horizontal transmission of pathogens from a common source to the susceptible neonates or direct cross-transmission from infected/colonized to uninfected neonate via contaminated hands of HCWs lead to infection outbreaks [125]. An environmental surveillance for an early identification of the source and transmission mode of infection assists in devising specific preventive measures so as to help in early management of the outbreak. Several animate or inanimate objects that are frequently used in NICU can be the source of infection. The frequently touched hospital surfaces, such as door handle, patients' monitor, telephone, trolley, sink, or tap may often be the source of infection. Other sources of infection may be water, intravenous solutions, disinfectant containers, or air conditioning system [40,125].

1.6.5 The use of molecular epidemiology in an outbreak investigation

The molecular epidemiology refers to an application of several molecular disciplines, such as molecular taxonomy, phylogeny, or population genetics to solve an epidemiological problem of an infectious disease [126]. With advances in molecular techniques that can provide highly resolved molecular data, the epidemiological investigation of infectious diseases has been revolutionized [127].

For infectious disease outbreak investigation, the evolutionary history and genetic similarity of bacterial isolates is investigated by analyzing the molecular typing data and using the clustering techniques known as phylogenetics [127]. A phylogenetic analysis helps answer several outbreak-

related epidemiological questions. In a phylogenetic reconstruction, the grouping of a majority of bacterial clones into a single tight cluster provides an evidence of a monoclonal outbreak resulting from the patient-to-patient transmission of the clone. Alternatively, if the bacterial isolates fail to form a single cluster, and show multiple clusters, it infers to a poly-clonal outbreak involving several independent events of disease acquisition and transmission [74]. In the phylogenetic analysis, if isolate cultured from an environmental sample is closely nested within a cluster of outbreak causing clinical isolates, the result would confirm the source of infection, thereby aiding in timely control of an outbreak [124].

1.7 Rationale and research questions of the thesis

In Nepal, bacterial BSIs leading to an acute undifferentiated febrile illness is a common public health problem in adult and paediatric population [128]. The incidence of BSIs caused by drug resistant organisms is high in hospitals and communities in Nepal [129,130]. Further, there is a rampant use of antimicrobials in hospitals and communities in Nepal [131]. The continued irrational use of antimicrobials may further increase the burden of AMR in Nepal. Therefore, similar to other South Asian countries, Nepal potentially contributes a substantial share in the global burden of AMR.

Patan hospital is the third largest tertiary referral hospital in Nepal, thereby representing the healthcare system of urban Nepal. An earlier study conducted in Patan hospital by Zellweger et al. is the most comprehensive microbiological study on bacteraemia in Nepal as it included a 23 years (1992-2014) of microbiological data constituting of >220,000 bacterial isolates from blood samples [132]. Therefore, the microbiological results of the study potentially reflect the spectrum of aetiology of bacteraemia in Nepal. An increasing trend in the proportions of drug resistant non-*Salmonella* GNB causing bacteraemia, particularly originating from the patients attending emergency (ER) and outpatient department (OPD) was reported in the study [132]. Another

relevant finding reported by the study was *Enterobacter* spp. being identified as the most prevalent non-*Salmonella* GNB causing bacteraemia in Nepali population [132]. The findings established in this earlier study provided an important prelude on an emerging role of non-*Salmonella* GNB in the causation of BSIs in Nepal.

Salmonella enterica Typhi had been the most prevalent cause of BSIs among outpatients at Patan hospital since 1992 [133]. A large number of demographic and genomic epidemiological studies, clinical trials, and vaccine-efficacy studies on *Salmonella enterica* Typhi had been conducted at Patan hospital [134–139]. Immediately after *Salmonella enterica* Typhi and Paratyphi, four non-*Salmonella* GNB namely, *Enterobacter* spp., *Acinetobacter* spp., *E. coli*, and *Klebsiella* spp. were identified to be the most common non-*Salmonella* GNB among bacteraemic patient attending at Patan hospital between 1992 to 2014 [132]. However, studies on BSIs causing non-*Salmonella* GNB were scarce at Patan hospital. The findings of an increasing trend in the proportion of drug resistant GNB as demonstrated by Zellweger et al. suggested for a need of further investigation so as to characterize the phenotypic and genetic determinants of AMR in bacteraemia causing GNB. Aiming to answer this research question, I conducted a longitudinal retrospective study (chapter 3) on blood culture results of four most prevalent GNB except *Salmonella enterica* isolated from patients of all age groups in past six years between 2012 and 2018. A study on six years of comprehensive microbiological data originating from patient of all age groups when stratified by hospital departments and bacterial aetiologies would identify key problems and opportunities to investigate.

Next, it was important to specifically identify the most at risk population for sepsis, where the burden of BSI was the highest with the most severe outcomes. At Patan hospital, among HA-BSIs caused by non-*Salmonella* GNB, the neonatal infections in NICU had been a major problem associated with high morbidity and mortality. Interestingly, of other neonatal units such as,

nurseries and paediatric ICU, the problem of infection outbreak was specifically and predominantly concentrated in NICU only. The other neonatal units had occasional sporadic infections, but no outbreak of infections. Further, my retrospective study (chapter 3) also identified that the NICU admitted neonates had the highest burden of culture-positive sepsis. It was therefore important to understand the epidemiological determinants of neonatal infections in NICU, particularly the potential risk factors of acquiring infections in NICU so that preventive measures could be identified and implemented. Addressing this research question was one of the objectives of this thesis. Based on this, a prospective longitudinal study (chapter 4) was conducted in NICU admitted neonates to identify the risks of sepsis development, and understand the bacterial microbiology of neonatal sepsis.

Enterobacter spp. had been identified as a predominant bacterial aetiological agent of BSIs in both neonatal and adult population at Patan hospital since 1992. This necessitated for understanding the potential transmission dynamics along with the determinants of AMR and virulence of *Enterobacter* spp. This was another research question of my thesis.The fulminant neonatal sepsis outbreaks caused by GNB were recurring in NICU at Patan hospital, requiring for a careful outbreak investigation. Several studies on neonatal sepsis outbreaks caused by *K. pneumoniae* had been reported in NICU at Patan hospital [73,74,140]. The prospective NICU study of my thesis showed that after *K. pneumoniae*, *Enterobacter* spp. was the second most common aetiological agent of neonatal sepsis. However, there were limited neonatal sepsis outbreak studies reported on *Enterobacter* spp. [124]. In order to fulfill the knowledge gap, a high-resolution genomic epidemiological analysis was conducted on *Enterobacter* spp. causing potential neonatal sepsis outbreak in NICU at Patan hospital (chapter 5).

Finally, the results of chapter 3 and earlier 23 years of retrospective study by Zellweger et al. indicated that *Enterobacter* spp. was potentially an emerging non-*Salmonella* bacterial pathogen

among patients attending ER with acute undifferentiated febrile illness at Patan hospital. To address this, in chapter 6, a genomic epidemiological analysis was conducted on clinical isolates of *Enterobacter* spp. originating from the ER to specifically understand the AMR- and virulence-genes, and reconstruct potential transmission dynamics of the pathogen.

1.8 The aims and objectives of the thesis

The overarching aims of the thesis were:

1. To understand the epidemiological features of BSIs caused by the most prevalent non-*Salmonella* GNB at Patan hospital in Kathmandu, Nepal, with a specific focus on risk factors for neonatal sepsis.
2. To understand the molecular determinants of AMR, virulence, variation, and transmission of *Enterobacter* spp., causing BSIs among patients in NICU and ER at Patan hospital.

The specific aims of the thesis were:

1. To characterize the phenotypic and genetic AMR profile of the most prevalent non-*Salmonella* GNB causing bacteraemia among patients of all age group undergoing microbiological investigation for suspected BSIs at Patan hospital.
2. To identify the hospital departments at Patan hospital with the highest prevalence of BSIs caused by non-*Salmonella* GNB so as to investigate further in the subsequent research chapters.
3. To understand the prevalence, aetiology of, and risk factors for sepsis among the inborne neonates admitted to NICU at Patan hospital.
4. To describe the molecular epidemiology and possible transmission routes of outbreak of HO-BSIs caused by *Enterobacter* spp. among inborne neonates admitted to NICU at Patan hospital.

5. To describe the molecular epidemiology of BSIs caused by *Enterobacter* spp. among the patients attending to the ER at Patan hospital for microbiological investigation of suspected BSIs.

Chapter 2

Methods

2.1 The study setting

The research for this thesis was conducted at Patan hospital, which is a tertiary care hospital affiliated to the Patan Academy of Health Sciences, located in Lalitpur metropolitan city in Kathmandu, Nepal. Patan hospital is the third largest public hospital in Kathmandu, Nepal with >500 beds, and provides healthcare service to >320,000 outpatients and >20,000 inpatients annually. The ER provides an acute healthcare service to >100 patients per day and >36,500 patients annually. A majority of patients coming to the ER are permanent or temporary residents of the sub-urban areas of Lalitpur metropolitan city. The patients from Kathmandu and Bhaktapur metropolitan cities also seek healthcare services at Patan Hospital.

At Patan hospital, there are approximately 9,500 livebirths per year with perinatal mortality rate of 6.3/1,000 births. The neonatal care is provided in two nurseries designated as a clean nursery and a septic nursery. Among the neonates requiring a clinical observation and non-intensive medical care, those without at any risk factors for sepsis are admitted to a clean nursery, while the neonates having underlying risk factors for, or indication of infection are admitted to the septic nursery. An average nursery admission rate at Patan hospital is 8.3% of total livebirth.The ICU-care requiring inborne neonates are admitted to NICU. The NICU at Patan hospital is a level-three unit with eight beds, of which two beds are dedicated for culture-positive neonates. An average NICU admission rate at Patan hospital is 1.9% of total livebirth. Adjacent to NICU is a six-bedded paediatric intensive care unit (PICU), where ICU care requiring out-borne neonates along with children up to 14 years are admitted. Rarely, when all beds of NICU are occupied and when patient party refuses a referral care, the ICU care requiring inborne neonates are also admitted to the PICU.

The microbiology laboratory at Patan hospital works under the department of laboratory medicine and pathology of the hospital. The microbiology laboratory is also one of the participating laboratories for national AMR surveillance program that is being held by National Public Health Laboratory (NPHL), a government national reference laboratory under the department of health services (DoHS) and ministry of health and population (MoHP) Nepal. The adherence to laboratory standards and quality assurance of laboratory results are ensured by the microbiology laboratory by undertaking multiple External Quality Assessment (EQA) programs. The laboratory undergoes EQA for bacteriological techniques, such as Gram's stain, bacteria identification, and antimicrobial susceptibility testing from NPHL six times a year. The microbiology laboratory also undertakes EQAs twice a year, each from CMC microbiology EQA scheme under the department of clinical microbiology, Christian Medical College, Vellore, India, and from UK NEQAS for microbiology, public health England, London, UK.

The microbiology laboratory serves to provide routine bacteriological and serological investigations. The laboratory works closely with hospital IPC committee to maintain an active infection control program in the hospital. It also works in association with antimicrobial policy and stewardship program in the hospital. An average number of blood culture investigations carried out in microbiology laboratory at Patan hospital is 8,000 blood samples per year. An average blood culture negativity rate is 83%, and blood culture contamination rate is 7.2%. The isolation of *Bacillus* spp., *Micrococcus* spp., and diptheroids is considered as contaminants. The isolation of CoNS from blood samples of adults are reported as contaminant. However, for the neonates, the isolation of CoNS from blood sample is considered clinically significant, and the results are reported. Under the AMR surveillance program of the hospital, all pure cultures of bacterial isolates, except for CoNS, obtained from blood culture are routinely stored. The bacterial isolates have been archived since 2012.

2.2 The study design and study population

Chapter 3 was a retrospective study on routine microbiological blood culture results originating from patients of all age groups attending to any hospital departments at Patan hospital for microbiological investigations of possible BSIs. The eligibility criteria for selecting the bacterial isolates for chapter 3 included the non-replicated stored isolates of four most prevalent non-*Salmonella* GNB (*E. coli*, *Enterobacter* spp., *Klebsiella* spp., and *Acinetobacter* spp.) cultured from the blood samples in last six years between 2012 and 2018. The unique patient identification number in microbiology database was used in identifying and excluding the duplicate isolates.

Chapter 4 was a prospective observational cohort study conducted primarily to understand the risk factors for sepsis development among inborne neonates requiring admission to level-three NICU at Patan hospital between April 2016 and October 2017. Upon admission of a neonate in an NICU, the parent/guardian of the corresponding neonate was approached in order to obtain an informed consent so as to take part in the study. The neonates whose parents/guardians provided a written informed consent were enrolled in the study. The enrolled neonates were followed up on a daily basis during their entire stay in NICU until one of the final outcomes (discharge, transfer to other department, death, or left against medical advice) was attained. Required medical care along with diagnostic and therapeutic management to the enrolled neonates were provided by the attending paediatrician as a part of routine clinical care, and as per the NICU protocol at Patan hospital. Relevant data on clinical, therapeutic, and laboratory investigations were recorded for the research purpose in a pre-tested case report form (CRF) by referring to the clinical notes marked by the paediatrician.

Chapter 5 was a genomic outbreak investigation study on *Enterobacter* spp. causing neonatal sepsis among NICU admitted inborne neonates enrolled in chapter 4. The *Enterobacter* spp. isolated from blood samples of enrolled neonates along a time of potential outbreak in NICU

during the study period of chapter 4 formed the bacterial isolates for genomic investigation in chapter 5.

Chapter 6 was a genomic epidemiological study on *Enterobacter* spp. isolated and archived from blood samples of ER- admitted patients undergoing microbiological investigation for clinically suspected BSIs between April 2016 and October 2017. The isolates of *Enterobacter* spp. investigated in chapter 6 were a subset of *Enterobacter* spp. studied in chapter 3.

2.3 Attributions

In chapter 3, all diagnostic and therapeutic management of patients suspected of BSIs and attending to various departments at Patan hospital during the study period were carried out as a part of routine clinical care of the hospital. This included the request for blood culture investigation, microbiological blood culture tests, bacterial identification, phenotypic AST, and storage of bacterial isolates. The confirmatory phenotypic test for ESBL positivity, and PCR test for ESBL and carbapenemase encoding genes were performed as a part of research for chapter 3 of my thesis. The Sanger sequencing of positive PCR products of ESBL and carbapenemase genes of representative samples was also performed for my thesis research to confirm the identity of the PCR products.

In chapter 4, all diagnostic, therapeutic and clinical management of suspected BSIs among enrolled neonates admitted to NICU at Patan hospital during the study period were performed as a part of routine clinical care of the hospital and were carried out as per the protocol of NICU. This included the clinical suspicion of sepsis, administration of antimicrobials, use of various modalities of intensive care, request for blood culture investigation, microbiological blood culture tests, bacterial identification, phenotypic AST, and storage of bacterial isolates. For my thesis research, the reconfirmation of hospital derived results on bacterial identification and phenotypic AST was

performed for the bacterial isolates derived from enrolled neonates of chapters 4. Further, for research purpose only, the confirmatory phenotypic test for ESBL positivity, and PCR test for ESBL and carbapenemase encoding genes were also performed.

In chapter 5, for research purpose, the reconfirmation of hospital derived routine results on bacterial identification and phenotypic AST was performed on NICU outbreak associated *Enterobacter* spp. isolates derived from chapter 4. Minimum inhibitory concentration (MIC) for colistin was also determined for isolates by the E-test method for the thesis chapter. Additionally, *Enterobacter* spp. isolates were also subjected to WGS and genomic analysis as a part of research.

In chapter 6, the reconfirmation of hospital derived routine results on bacterial identification and phenotypic AST was performed for the *Enterobacter* spp. isolated from ER admitted patients. The WGS and genomic analysis of *Enterobacter* spp. constituting chapter 6 were also performed as a part of the research.

2.4 An ethical approval

An ethical approval for conducting chapter 3 was obtained from Nepal Health Research Council (NHRC) under the approval registration number of 120/2012 for the research entitled “Prevalence of extended spectrum beta-lactamase (ESBL) pathogens and factors responsible for ESBL associated infections within Patan Hospital”. The *Enterobacter* spp. isolates investigated in chapter 6 were a subset of archived isolates of chapter 3, and therefore the study for chapter 6 was conducted under the same approval of chapter 3.

An ethical approval for conducting chapter 4 was obtained from NHRC under the approval registration number of 278/2015, and from Oxford Tropical Research Ethics Committee

(OxTREC) under the approval registration number of 24-16 for the research entitled “Prevalence of ESBL producing Enterobacteriaceae and the probable risk factors of hospital acquired neonatal infections in the NICU of Patan hospital”. The isolates investigated in chapter 5 were a subset of isolates of chapter 4, and therefore the study of chapter 5 was conducted under the same approval of chapter 4.

2.5 Collection, recording, and verification of clinical data

All relevant clinical research findings on the enrolled neonates of chapter 4 were recorded in the case report form (CRF) by a study nurse by referring to the clinical notes recorded by the paediatrician. The data were then entered in a designated online database called CLIRES Data Management System on a daily basis. Integrity of the recorded data was validated on a regular basis by double entry of data and verification by two different individuals.

2.6 Collection and microbiological processing of blood samples

The blood samples were collected from the patients with suspected septicaemia for microbiological investigations before administration of an antimicrobial as per the hospital protocol. For the adults, 8-10 ml of peripheral venous blood was collected by venepuncture in BD BACTEC™ Plus aerobic culture vial (Becton Dickinson, UK), while for the neonates 1-2 ml of peripheral blood sample was collected in BD-BACTEC Peds plus/F culture vials (Becton Dickinson, UK). The inoculated culture bottles were incubated in an automated BD BACTEC FX40 culture system (Becton Dickinson, UK). The microbiological processing of blood samples for isolation of bacterial pathogens was performed as per the hospital protocol.

2.6.1 Identification of bacterial isolates

Bacterial identification was conducted as per standard microbiological methods based on the colony characteristics on primary culture plates, Gram staining, and conventional biochemical tests [141] . One set of un-inoculated biochemical media were used as negative control while *E. coli* ATCC strain 25922 and *K. pneumoniae* ATCC strain 700603 were used as positive controls for the biochemical tests of GNB. The biochemical tests for bacterial identification of GNB constituted of indole test, motility test, and urease test in the MIU medium (HiMedia Laboratories Pvt. Ltd., India), the tests for utilization of lactose, glucose, and sucrose with production of hydrogen sulphide and fermentative gases in the TSI medium (Mast group Ltd., UK), and citrate utilization test on the Simmons citrate agar (Mast group Ltd., UK) [141]. Based on limited number of biochemical tests that were routinely used for bacterial identification of GNB, the species level identification could not be performed for *Enterobacter* spp. and *Acinetobacter* spp. Bacterial identification of GPC was conducted based on the haemolytic characteristics on blood agar, microscopic finding, catalase test using hydrogen peroxide, oxidase test (Mast group Ltd., UK), and/or test for mannitol fermentation using Mannitol Salt Agar (Mast group Ltd., UK) [141].

The isolation of CoNS from blood samples of adults was reported as contaminant. However, for the neonates, the isolation of CoNS from blood sample was considered clinically significant, and was reported.

2.6.2 Antimicrobial susceptibility testing (AST) by disc diffusion method

The AST was performed using modified Kirby-Bauer disc diffusion method [142]. A suspension of bacterial inoculum was prepared by the direct colony suspension method, where few isolated bacterial colonies from an overnight culture on the nutrient agar (Mast group Ltd., UK) were suspended in a glass tube containing sterile normal saline. The bacterial turbidity was measured using a McFarland densitometer (DEN-1, Grant bio, Grant instruments, UK) and was adjusted to

the reading of 0.5. The 0.5 McFarland bacterial suspension was then plated on the Muller Hinton Agar (MHA) (Becton Dickinson and Company, USA) with a sterile cotton swab with an aim to produce a confluent bacterial lawn. The antimicrobial discs were aseptically placed on the MHA plate such that a minimum of 24 mm space between the centres of the discs was maintained. The antimicrobial discs (Mast group Ltd., UK) as shown in the table 2.1 were applied on the MHA plate for the bacterial AST. Not all antimicrobials were tested initially for the bacterial isolates. The isolates were firstly tested for the antimicrobials of the first-line panel (Table 2.1). Only for the isolates that tested non-susceptible to all agents of the first-line panel were tested for the antimicrobial agents of the second-line panel (Table 2.1). The MHA plates were incubated at 35±2°C for 18 hours. In each batch of AST, the MHA plates inoculated with *E. coli* ATCC strain 259220 and *K. pneumoniae* ATCC strain 700603 were used as positive controls. After specified incubation, the sizes of zone of inhibition around each antimicrobial disc were measured, and the results were interpreted referring to the zone size breakpoints as recommended by the Clinical Laboratory Standards Institute (CLSI) [143]. The most recent CLSI guidelines on zone size breakpoints available in the respective years were followed by the hospital for interpreting AST results of bacterial isolates recovered between 2012 and 2018.

Table 2.1. The antimicrobial agents with symbols and specific disc contents (µg) that were applied for antimicrobial susceptibility testing of the bacteria relevant in the study.

Antimicrobial agent	**Symbol**	**Content (µg)**	**Enterobac**	***P. aeruginosa***	***Acinetobacter* spp.**	**Staphy**
Ampicillin	AP	10	Y			
Amp/Sul	SAM	10/10	Y*		Y	
Pip/Taz	PTZ	100/10	Y*	Y	Y	
Cefotaxime	CTX	30	Y		Y	
Imipenem	IMI	10	Y*	Y	Y	
Meropenem	MEM	10	Y*	Y	Y	
Amikacin	AK	30	Y	Y	Y	Y
Gentamycin	GM	10	Y	Y	Y	Y
Ciprofloxacin	CIP	5	Y	Y	Y	Y
Cotrim	TS	1.25/23.75	Y		Y	Y
Chloram	C	30	Y			Y
Cefepime	CPM	30			Y*	
Colistin[#]	CS				Y*	
Aztreonam	ATM	30		Y		
Cefoxitin	FOX	30				Y
Clindamycin	CD	2				Y

Amp/Sul: Ampicillin-sulbactam, Chloram: Chloramphenicol, Cotrim: Trimethoprim-sulphamethoxazole, Enterobac: Enterobacteriaceae except *Salmonella* spp. and *Shigella* spp, Pip/Taz: Piperacillin-tazobactam, Staph: Staphylococci

'Y'indicates the antimicrobial agent tested for given bacterial genus

*indicates the antimicrobial agents of the second-line AST panel, that were tested only when the test organism showed resistance to all antimicrobial agents in the first-line panel during routine AST in hospital

[#] During the study period of 2012 till 2017, microbiology laboratory performed colistin testing as a second-line AST agent by agar disc diffusion method for all GNB. Since 2018, test for colistin as a second-line AST agent was performed by E-test method for determining the MIC value for *Acinetobacter* spp. and *Pseudomonas aeruginosa* only.

2.7 Confirmatory tests for the presence of extended-spectrum beta-lactamase and carbapenemase

GNB isolates showing resistant (zone of inhibition ≤22 mm for Enterobacteriaceae, and ≤14 mm for *Acinetobacter* spp.) or intermediate (zone of inhibition 23-25 mm for Enterobacteriaceae, and 15-22 mm for *Acinetobacter* spp.) responses towards cefotaxime (30 µg) were suspected of

producing the ESBLs. The potential ESBL producing isolates were further confirmed by the phenotypic and genetic (PCR) confirmatory tests.

2.7.1 Confirmatory phenotypic tests

A phenotypic confirmatory test for the presence of ESBL was conducted by the combination disc agar diffusion method [144]. A bacterial inoculum measuring a turbidity of 0.5 McFarland was prepared and inoculated on two MHA plates (Becton Dickinson and Company, USA) to obtain a semi-confluent bacterial lawn, following the protocol as described in the methods section 2.6.2. On one MHA plate, four antimicrobial discs, namely, ceftazidime (30 µg), ceftazidime (30 µg) with clavulanate (10 µg), cefotaxime (30 µg), and cefotaxime (30 µg) with clavulanate (10 µg) (D62C, Mast Group Ltd., UK) were applied for the ESBL test. On the second inoculated plate, four antimicrobial discs, namely, cefpodoxime (10 µg), cefpodoxime (10 µg) with ESBL-inhibitor, cefpodoxime (10 µg) with AmpC-inhibitor, and cefpodoxime (10 µg) with ESBL-AmpC-inhibitors (D68C, Mast Group Ltd., UK) were applied for the detection of ESBL and/or AmpC production.*E. coli* ATCC strain 259220 and *K. pneumoniae* ATCC strain 700603 were also included. The MHA plates were incubated at 35±2˚C for 18 hours. The sizes of zone of inhibition were measured. The isolate was considered as ESBL positive when the size of zone of inhibition in β-lactamase inhibitor supplemented disc was ≥ 5 mm as compared to size of respective β-lactam alone. The results interpretation criteria are depicted in the table 2.2. The representative photographs showing the results of the confirmatory phenotypic tests for ESBL positivity are given in appendix 9.1 (Figure 9.1).

Table 2.2. The results interpretation criteria of phenotypic confirmatory tests for ESBL and AmpC positivity.

Name of antimicrobi-al disc sets	Antimicrobial disc content	Symbol for respective zones	Results interpretation criteria	Confirmatory results
D62C	CTX (30µg)	Z1	If Z2-Z1 ≥ 5mm and/or Z4-Z3≥ 5mm	ESBL positive
	CTX (30µg) + CV (10 µg)	Z2		
	CAZ (30µg)	Z3	If all zones (Z1,2,3,4) differ by ≤ 2 mm	ESBL negative
	CAZ (30µg) + CV (10 µg)	Z4		
D68C	CDP (10 µg)	ZA	If ZB – ZA, and ZD - ZC ≥ 5mm and the differences between each of ZB and ZD, and ZA and ZC are < 4mm	Only ESBL positive
	CPD (10 µg) + ESBL inhibitor	ZB	If ZD – ZB, and ZC - ZA ≥ 5mm, and the differences of each of ZA and ZB, and ZC - ZD are < 4mm	Only AmpC positive
	CPD (10 µg) + AmpC inhibitor	ZC	If ZD - ZC ≥ 5mm, and the difference between ZA and ZB is < 4mm	ESBL and AmpC positive
	CPD (10 µg) + ESBL inhibitor +AmpC inhibitor	ZD	All zones (ZA,B,C,D) differ by ≤ 2mm	ESBL and AmpC negative

CTX: Cefotaxime, CLA: Clavulanic acid, CAZ: Ceftazidime, CPD: Cefpodoxime, ESBL: Extended-spectrum beta-lactamase[143]

2.7.2 Confirmatory genetic tests

The presence of bacterial AMR genes conferring resistance to β-lactam antimicrobials was assessed by the PCR test.

2.7.2.1 The extraction of bacterial DNA by boiling method

For the PCR test, bacterial DNA was extracted by the boiling method. The GNB isolates were sub-cultured on the Nutrient agar (HiMedia Laboratories Pvt. Ltd., India), and incubated over night at 35±2°C. Two to five isolated colonies were suspended in an eppendorf tube (Eppendorf AG, Germany) containing 150 µl of sterile, molecular-grade distilled water. The bacterial suspension was boiled at 95°C for 10 minutes and vortexed for 15 seconds. The suspension was centrifuged at 8,000 rpm for 10 minutes. Eighty to 100 µl of clear supernatant was carefully aspirated and transferred to a sterile eppendorf tube to be used as a template DNA for the PCR test.

2.7.2.2 The detection of AMR genes by PCR test

The GNB isolates were tested for the ESBL genes, namely, bla_{TEM} [145], bla_{SHV} [146], $bla_{\text{CTXM-1}}$ [147], $bla_{\text{CTXM-2}}$ [147], $bla_{\text{CTXM-9}}$ [147], $bla_{\text{CTXM-8}}$ [147], $bla_{\text{CTXM-25}}$ [147], and bla_{OXA} [146] ; and carbapenemase genes, namely, bla_{KPC} [148], $bla_{\text{NDM-1}}$ [149], $bla_{\text{OXA-48}}$ [148], $bla_{\text{OXA-23}}$ [150], $bla_{\text{OXA-24}}$ [150], $bla_{\text{OXA-51}}$ [150], $bla_{\text{OXA-58}}$ [150], bla_{IMP} [151], and bla_{VIM} [151].

A 25 to 50 µl of PCR master mix was prepared containing specific final concentrations of the PCR reagents, namely, 1X PCR buffer (Qiagen, Germany), 1 unit HotStar Taq DNA Polymerase (Qiagen, Germany), 1.5 mM $MgCl_2$ (Qiagen, Germany), 0.2 mM dNTPs mix (Roche Diagnostics GmbH Mannheim, Germany), 0.1-0.2 µM of each primers (Sigma-Aldrich Pvt. Ltd., Singapore), and 3 µl of template DNA. The PCR was performed in a Bio-Rad C-1000 thermocycler (Bio-Rad laboratories Inc., USA) using the thermal-cycling program of initial enzyme activation at 95°C/15 min, followed by 35 cycles at 95°C/40 sec, annealing at 52-61°C/40 sec (Table 2.3), and extension at 72°C/50 sec, with final extension at 72°C for 7 min.

In each batch of PCR test for the AMR genes, the PCR positive control tubes added with bacterial DNA of *E. coli* ATCC strain 25922 and *K. pneumoniae* ATCC strain 700603 were included. Additionally, a no template control tube added with sterile distilled water was also included in each batch of PCR as a negative control.

The details of each multiplex PCR for the detection of specific ESBL and carbapenemase encoding AMR gene are given in the table 2.3. The calculations for the preparation of master-mix reactions and corresponding thermo-cycling parameters for the multiplex PCR assays, namely, TSO, CTX-M, Carba-2, Carba-1, A-Carba-1, and A-Carba-2 are given in the tables 2.4, 2.5, 2.6, 2.7, 2.8, and 2.9, respectively.

Table 2.3. The primer sequences, final primer concentrations (μM), annealing conditions, and PCR product sizes (bp) for the PCR tests of each AMR genes.

AMR genes	Primer sequence (5' - 3')	Multiplex PCR	Final primer conc.	Annealing parameter °C/sec	PCR product size (bp)
bla$_{\text{CTXM-1}}$	AAAAATCACTGCGCCAGTTC	CTX-M	0.1	60°C/ 40 sec	415
	AGCTTATTCATCGCCACGTT		0.1		
bla$_{\text{CTXM-2}}$	CGACGCTACCCCTGCTATT		0.1		552
	CCAGCGTCAGATTTTTCAGG		0.1		
bla$_{\text{CTXM-9}}$	CAAAGAGAGTGCAACGGATG		0.1		205
	ATTGGAAAGCGTTCATCACC		0.1		
bla$_{\text{CTXM-8/25}}$	TCGCGTTAAGCGGATGATGC		0.1		666/327
	GCACGATGACATTCGGG		0.1		
	AACCCACGATGTGGGTAGC		0.2		
bla$_{\text{TEM}}$	TGCGGTATTATCCCGTGTTG	TSO	0.1	57°C/ 40 sec	300
	TCGTCGTTTGGTATGGCTTC		0.1		
bla$_{\text{SHV}}$	AGCCGCTTGAGCAAATTAAAC		0.2		713
	ATCCCGCAGATAAATCACCAC		0.2		
bla$_{\text{OXA}}$	GGCACCAGATTCAACTTTCAAG		0.2		564
	GACCCCAAGTTTCCTGTAAGTG		0.2		
bla$_{\text{IMP}}$	GGAATAGAGTGGCTTAAYTCTC	Carba1	0.2	52°C/ 40 sec	232
	GGTTTAAYAAAACAACCACC		0.2		
bla$_{\text{VIM}}$	GATGGTGTTTGGTCGCATA		0.2		390
	CGAATGCGCAGCACCAG		0.2		
bla$_{\text{KPC}}$	CGTCTAGTTCTGCTGTCTTG	Carba2	0.2	52°C/ 40 sec	798
	CTTGTCATCCTTGTTAGGCG		0.2		
bla$_{\text{NDM-1}}$	GGTTTGGCGATCTGGTTTTC		0.2		621
	CGGAATGGCTCATCACGATC		0.2		
bla$_{\text{OXA-48}}$	GCGTGGTTAAGGATGAACAC		0.2		438
	CATCAAGTTCAACCCAACCG		0.2		
bla$_{\text{OXA-23}}$	GATCGGATTGGAGAACCAGA	A-Carba1	0.2	52°C/ 40 sec	501
	ATTTCTGACCGCATTTCCAT		0.2		
bla$_{\text{OXA-24}}$	GGTTAGTTGGCCCCCTTAAA		0.2		246
	AGTTGAGCGAAAAGGGGATT		0.2		
bla$_{\text{OXA-51}}$	TAATGCTTTGATCGGCCTTG		0.2		353
	TGGATTGCACTTCATCTTGG		0.2		
bla$_{\text{OXA-58}}$	AAGTATTGGGGCTTGTGCTG		0.2		599
	CCCCTCTGCGCTCTACATAC		0.2		
bla$_{\text{NDM-1}}$	GGTTTGGCGATCTGGTTTTC	A-Carba 2	0.2	61°C/ 40 sec	621
	CGGAATGGCTCATCACGATC		0.2		

bp: basepair, conc.: concentration, sec: seconds

Table 2.4. The calculation for the preparation of PCR reactions for TSO (TEM, SHV, and OXA) multiplex PCR assay.

PCR reagents	Initial concentration	Final concentration	µl/per reaction
PCR Buffer	10X	1X	5
MgCl2	25 mM	1.5 mM	3
dNTPs mix	10 mM	0.2mM	1
TEM-F	10 µM	0.1 µM	0.5
TEM-R	10 µM	0.1 µM	0.5
SHV-F	10 µM	0.2 µM	1
SHV-R	10 µM	0.2 µM	1
OXA-F	10 µM	0.2 µM	1
OXA-R	10 µM	0.2 µM	1
Taq Polymerase	5U/µl	1U/rxn	0.2
Distil water			32.8
DNA template			3
Final volume			50

Table 2.5. The calculation for the preparation of PCR reactions for CTX-M (CTX-M-1, 2, 8, 9, and 25) multiplex PCR assay.

PCR reagents	Initial concentration	Final concentration	µl/per reaction
PCR Buffer	10X	1X	5
$MgCl_2$	25 mM	1.5 mM	3
dNTPs mix	10 mM	0.2mM	1
CTXM1-F	10 µM	0.1 µM	0.5
CTXM1-R	10 µM	0.1 µM	0.5
CTXM2-F	10 µM	0.1 µM	0.5
CTXM2-R	10 µM	0.1 µM	0.5
CTXM8-F	10 µM	0.1 µM	0.5
CTXM25-F	10 µM	0.1 µM	0.5
CTXM8/25-R	10 µM	0.2 µM	1
CTXM9-F	10 µM	0.1 µM	0.5
CTXM9-R	10 µM	0.1 µM	0.5
Taq Polymerase	5U/µl	1U/rxn	0.2
Distil water			32.8
DNA template			3
Final volume			50

Table 2.6. The calculation for the preparation of PCR reactions for Carba-2 (KPC, NDM-1, and OXA-48) multiplex PCR assay.

PCR reagents	Initial concentration	Final concentration	µl/reaction
PCR Buffer	10X	1X	5
$MgCl_2$	25 mM	1.5 mM	3
dNTPs mix	10 mM	0.2 mM	1
KPC-F	10 µM	0.2 µM	1
KPC-R	10 µM	0.2 µM	1
NDM-1 F	10 µM	0.2 µM	1
NDM-1 R	10 µM	0.2 µM	1
OXA-48-F	10 µM	0.2 µM	1
OXA-48-R	10 µM	0.2 µM	1
Taq Polymerase	5U/µl	1U/rxn	0.2
Distil water			31.8
DNA template			3
Final volume			50

Table 2.7. The calculation for the preparation of PCR reactions for Carba-1 (IMP and VIM genes) multiplex PCR assay.

PCR reagents	Initial concentration	Final concentration	µl/reactions
10X PCR Buffer	10X	1X	2.5
MgCl2	25 mM	1.5 mM	1.5
dNTPs mix	10 mM	0.2 mM	0.5
IMP-F	10 µM	0.2 µM	0.5
IMP-R	10 µM	0.2 µM	0.5
VIM-F	10 µM	0.2 µM	0.5
VIM-R	10 µM	0.2 µM	0.5
Taq Polymerase	5U/µl	0.5 U/rxn	0.1
Distill water			15.4
Total			22
DNA template			3
Final volume			25

Table 2.8. The calculations for the preparation of PCR reactions for A-Carba 1 (OXA-51, 23, 24, and 58 AMR genes) multiplex PCR assay.

PCR reagents	Initial concentration	Final concentration	µl/per reaction
PCR Buffer	10X	1X	2.5
$MgCl_2$	25 mM	1.5 µM	1.5
dNTPs mix	10 mM	0.2 µM	0.5
OXA-23-like F	10 µM	0.1 µM	0.5
OXA-23-like R	10 µM	0.2 µM	0.5
OXA-24-like F	10 µM	0.2 µM	0.5
OXA-24-like R	10 µM	0.2 µM	0.5
OXA-51-like F	10 µM	0.2 µM	0.5
OXA-51-like R	10 µM	0.2 µM	0.5
OXA-58-like F	10 µM	0.2 µM	0.5
OXA-58-like R	10 µM	0.2 µM	0.5
Taq Polymerase	5U/µl	1U/rxn	0.2
Distill water			13.3
DNA template			3
Final volume			25

Table 2.9. The calculation for the preparation of PCR reactions for A-Carba 2 (NDM-1 gene) multiplex PCR assay.

PCR reagents	Initial concentration	Final concentration	µl/reaction
10X PCR Buffer	10X	1X	2.5
$MgCl_2$	25 mM	1.5 mM	1.5
dNTPs mix	10 mM	0.2 mM	0.5
NDM1-F	10 µM	0.2 µM	0.5
NDM1-R	10 µM	0.2 µM	0.5
Taq Polymerase	5U/µl	0.5 U/rxn	0.1
Distill water			16.4
Total			22
DNA template			3
Final volume			25

2.7.2.3 Agarose gel electrophoresis

The PCR products were electrophoresed in 1.5% agarose gel and the results were interpreted based on the detection of expected sizes of the PCR products, as guided by the PCR positive controls that were co-electrophoresed with the samples. Briefly, 1.5% of agarose gel was prepared by suspending 1.5 grams of molecular grade agarose (Bio-Rad Laboratories, Inc., USA) in 100 ml of 1X Tris borate EDTA (TBE) buffer (Sigma-Aldrich Pvt. Ltd, USA). Agarose was dissolved by boiling in the microwave oven for 3-4 minutes. When cooled to 45-50°C, 3 µl of Nancy-520 dye (Sigma-Aldrich Pvt. Ltd, USA) was added, swirled to mix the contents, and the slurry was poured on the gel casting tray set fitted with a required number of combs. When solidified, the tray was carefully submerged in a horizontal gel electrophoresis buffer tank (Bio-Rad Laboratories Inc., USA) filled with 1X TBE buffer. The combs were carefully removed from the gel so that the PCR products could be loaded into the agrose gel.

The PCR products of the samples were mixed with 6X gel loading dye (Invitrogen Corporation, USA) in a ratio of 10 µl: 2 µl, respectively. Then, 10 µl of the dye mixed PCR product was loaded into the wells of the submerged gel in a predetermined sequence. The first well of each lane of the gel was loaded with 5 µl of 100 bp DNA ladder (Invitrogen Corporation, USA). The positive controls for each AMR marker gene and the no template control were also included in each gel run. The gel was electrophoresed at 90 volts for an hour. The gel was examined under ultra violet light at 250 nm in a UV-transilluminator (Bio-Rad Laboratories, Inc., USA). The results of the samples were evaluated, only when the expected results of the controls were achieved. The results of the samples were scored, a photograph of the gel was taken, the gel image was properly annotated, and archived. Representative agarose gel electrophoresis images of each multiplex PCR are shown in appendix 9.2 (Figure 9.2).

2.7.2.4 Sanger sequencing of the AMR gene amplicons

In order to confirm the identity of the amplified PCR products targeting specific AMR genes, the representative positive PCR products were subjected to the Sanger sequencing.

2.7.2.4.1 Purification of PCR products

The PCR product of a sample that was confirmed to be positive for a specific AMR gene was purified by an enzymatic removal of residual primers and nucleotides by using ExoSAP-IT™ PCR Product Clean-up Reagent (Affymetrix Inc., USA). The reagents were firstly thawed on ice. Two µL of ExoSAP IT reagent was added to the tube containing 5 µL of positive PCR product. The mixture was incubated at 37°C for 15 minutes for the enzymatic degradation of the impurities. Next, the reaction was heat inactivated by incubating at 80°C for 15 minutes. The resulting purified PCR product was stored at 4°C until used.

2.7.2.4.2 Cycle sequencing PCR

The cycle sequencing was performed on purified PCR products by using BigDye™ Terminator v1.1 Cycle Sequencing Kit (Life Technologies Corporation, USA). The reagents were thawed on ice, and vortexed to mix. The reagent mix for cycle sequencing was prepared as per the calculation give in the table 2.10.

After adding the template PCR product for each forward and reverse reaction, the PCR tubes were briefly vortexed, spun down, and placed in the thermocycler as per the cycle sequencing parameters shown in the table 2.11.

Table 2.10. The calculation for preparation of reagent mix for Sanger sequencing of *bla*$_{NDM}$ PCR product.

Sequencing reagents	**Forward sequencing**	**Reverse sequencing**
	µl/per reaction	**µl/per reaction**
BigDye Terminator Ready Reaction Mix	4	4
NDM_F (10 µM)	3	-
NDM_R (10 µM)	-	3
Nuclease-free water	1	1
Purified NDM PCR product	2	2
Final volume	10	10

Table 2.11. The thermo-cycle parameters for Sanger cycle sequencing of *bla*$_{NDM}$ PCR product.

PCR steps	**Temperature**	**Time**	**PCR Cycles**
Denaturation	96°C	1:00 min	1x
Denaturation	96°C	0:10 sec	25x
Anneal*	61°C*	0:05 sec	
Extension	60°C	4:00 min	
Hold	4°C	30:00 min	1x

*The annealing temperature varies for each specific PCR product as shown in the table 2.3

2.7.2.4.3 Purification of cycle sequencing products

The amplified cycle sequencing products were purified to remove the salts, unincorporated fluorescent dye terminators, and dNTPs from the sequencing reactions by using the BigDye XTerminator™ Purification Kit (Life Technologies Corporation, USA). First, 5 µL of amplified cycle sequencing product was transferred to a new PCR plate, to which 22.5 µL of SAM solution, and 5 µL of BigDye XTerminator™ solution were added after vigourous mixing. The PCR plate was tightly sealed, firmly attached to a vortexer, and vortexed at 1,800 rpm for 20 minutes. After briefly centrifuging the PCR plate, 10 µL of purified sequencing product was transferred to a

MicroAmp™ 8-tube strip (Applied Biosystems, USA). The tube strips were capped with the septa, and a capillary electrophoresis was performed on the ABI-310 Genetic Analyzer (Applied Biosystems, USA).

2.7.2.4.4 Capillary electrophoresis, base calling, sequence alignment, and sequence blasting for the sequence identity

Firstly, the ABI-310 instrument was prepared for the capillary electrophoresis by filling the capillary and gel block with POP-6 polymer (Applied Biosystems, USA). The buffer vials were filled with freshly diluted EDTA running buffer (Applied Biosystems, USA). The second position tube and the waste tube were filled with de-ionsed water. The signal collection window and the laser bulb were cleaned with a Kim-wipe issue soaked with methanol (Fisher biotech reagents, USA). Once the instrument was prepared, an auto-sampler calibration was performed. Then, the sample PCR plate was loaded in the instrument, and the capillary electrophoresis was performed under the run module of Seq POP6 Rapid (1 ml) E. md4.

Upon completion of the capillary electrophoresis of the samples, the raw sequence data were analysed using the Sequencing Analysis Software of Applied Biosystem. The base calling was conducted using the module KB_310_POP6_BDTv3_36Rapid.mob. The quality of the reads was manually verified by examining the electropherograms. The electropherograms of the representative PCR products are shown in the appendix 9.3 (Figure 9.3). A consensus sequence was constructed by aligning the forward- and reverse- reads of each sample. Finally, the sequence was identified by blasting the sample query sequence against the sequences available in the NCBI database.

2.8 Whole genome sequencing (WGS) of the bacterial isolates

In order to obtain the highest resolution of sequence data of the bacterial isolates, the WGS was performed as per the given methods.

2.8.1 Extraction of bacterial genomic DNA by using a commercial DNA extraction kit

The genomic DNA was extracted from GNB using a commercial DNA extraction kit, named Wizard® Genomic DNA Purification Kit (Promega Corporation, USA), following the kit protocol. Initially, 5 isolated bacterial colonies were selected from an overnight pure culture of bacteria on the nutrient agar. The colonies were added into a 1.5 ml Eppendorf tube containing a 600 µl of nuclei lysis solution. The bacterial cells were re-suspended by gentle pipetting for 30 seconds. The lysis of bacterial cells was enhanced by incubating the tube at 80°C for 5 minutes. After cooling, 3 µl of RNase solution was added to the cell lysate and mixed by inverting the tube for 2-5 times. Next, it was incubated at 37°C for an hour. After incubation, the tube was cooled to room temperature, 200 µl of protein precipitation solution was added, and the lysate was vigorously vortexed for 30 seconds. The protein precipitation was facilitated by incubating the bacterial lysate in ice for 5 minutes. The coarse bacterial debris was separated by centrifugation at 14,000 x g for 5 minutes. The supernatant was carefully aspirated and transferred to a new eppendorf tube containing 600 µl of isopropanol (Merck, Germany). The content was mixed by inverting the tube for 5-6 times until a visible precipitate of DNA was observed. Then, the DNA was pelleted by centrifugation at 14,000 x g for 5 minutes. The supernatant was discarded by decantation. The DNA pellet was washed by adding 600 µl of 70% ethanol (Merck, Germany), mixed by inverting the tube for few times, and centrifugation at 14,000 x g for 5 minutes. The supernatant was discarded by decantation, and drained on clean absorbent paper. To ensure a complete evaporation of residual ethanol, the DNA pellet was air dried by leaving the tubes with their lids open at room

temperature for half an hour. Finally, the DNA was rehydrated by adding 100 µl of DNA rehydration solution and stored at 4°C.

2.8.2 Quantitation of bacterial DNA

The extracted bacterial DNA was quantitated in the Qubit 3.0 fluorometer using the Qubit® dsDNA HS kit (Invitrogen, USA), following the instructions provided by the manufacturer. Initially, the Qubit working solution was prepared in a clean tube by diluting Qubit® dsDNA HS reagent in Qubit® dsDNA HS Buffer so as to maintain a final ratio of 1:200, respectively for the total number of samples. Two 0.5 ml Qubit™ assay tubes (Invitrogen, USA) were labelled standard S1 and S2, and were added with 190 µl of Qubit working solution. To the appropriate tubes, 10 µl of Qubit standard S1 and S2 were added. The tubes were mixed by vortexing for 2–3 seconds. In rest of the 0.5 ml Qubit™ assay tubes (Invitrogen, USA) labelled with the samples numbers, each 199 µl of Qubit working solution was transferred, followed by 1 µl of sample DNA. The sample tubes were mixed by vortexing for 2–3 seconds. All the tubes were incubated at room temperature for 2 minutes. On Qubit 3.0 (Invitrogen, USA) home screen, the assay type 'dsDNA High Sensitivity' was selected. After 2 minutes of incubation, the absorbance readings of the standards S1 and S2 were taken by inserting the tubes into the sample chamber, one at a time. Next, the sample tubes were sequentially inserted in the sample holder, and the DNA concentrations (ng/µl) of the samples were subsequently measured and recorded.

2.8.3 Library preparation of genomic DNA

The dual index-tagged pair-ended pooled DNA libraries were prepared for Illumina short read sequencing using Nextera XT DNA library preparation kit (Illumina, USA). Before preparation of the library, the extracted DNA was initially normalized to achieve the final concentration of 0.2 ng/µl.

2.8.3.1 Dilution and normalization of the extracted DNA

The DNA of each sample was diluted to obtain the final concentration of 10 ng/µl by adding a calculated volume of each stock DNA. The diluted sample DNA (10 ng/µl) were then transferred to the first lane of a sterile 96-well PCR plate. The DNA was then serially diluted from 10 ng/µl to 1 ng/µl (by adding 2 µl of 10 ng/µl of DNA to 18 µl of distilled water) along the second lane of the plate, and finally from 1 ng/µl to 0.2 ng/µl (by adding 2 µl of 1 ng/µl of DNA to 8 µl of distilled water) along the third lane of the plate. The concentration of the diluted DNA was measured in Qubit 3.0 to ensure that final concentration of 0.2 ng/µl was achieved.

2.8.3.2 Tagmentation of the input DNA

This is the first step of library preparation under the protocol using Nextera XT DNA library preparation kit (Illumina, USA). The principle of the kit was to enzymatically fragment the genomic DNA, and add the Illumina adapter sequences at the end of each DNA fragment. Before proceeding, the kit contents, namely ATM, TD and NT were thawed on ice, mixed by inverting, and centrifuged briefly. The thermocycler was powered on, and was set with the program of 55°C for 5 minutes, followed by hold at 10°C, under the name of “TAG”.

To each well of new PCR plate labelled as ‘TAG’, 10 µl of TD was added, followed by 5 µl of 0.2 ng/µl of sample DNA, resulting into a total input DNA of 1 ng. The content was mixed, and 5 µl of ATM was added. After mixing for 10 times by pipetting, the PCR plate was sealed with an adhesive sheet, briefly centrifuged, and transferred to the pre-heated thermocycler and ran under the thermocycler program ‘TAG’. Once complete, 5 µl of NT solution was added to each well and mixed by pipetting. The PCR plate was sealed again, briefly centrifuged, and incubated at room temperature for 5 minutes.

2.8.3.3 Amplification and indexing of the tagmented DNA

Initially, the Illumina Nextera XT index adapters (Illumina, USA), and NPM solution (Illumina, USA) were thawed in ice, mixed, and briefly spun down. The thermocycler was powered on, and set with the PCR program of 72°C/3 minutes, 95°C/30 seconds, 12 cycles of each 95°C/10 seconds, 55°C/30 seconds, and 72°C/30 seconds, followed by 72°C/5 minutes and hold at 10°C, under the name of 'NXT PCR'. The earlier PCR plate labelled as 'TAG' was taken, and 5 µl of each unique i5 and i7 Illumina index adapters were added to each sample wells. After mixing, 15 µl of NPM was added to the samples well, the plate was sealed, and finally, the PCR plate was transferred to a pre-heated thermocycler to run the 'NXT' PCR program.

2.8.3.4 Purification of the amplified library by magnetic beads based method

The main aim of this process was to purify, and concentrate the amplified indexed DNA library by using the magnetic beads of Agencourt AMPure XP kit (A63880, Beckman Coulter, USA). Initially, the kit was thawed to room temperature, and vigorously vortexed to evenly re-suspend the magnetic beads. The RSV buffer was also thawed to room temperature, and vortexed to mix. A fresh preparation of 80% ethanol was prepared from an absolute ethanol (Merck, Germany).

The purification of the amplified indexed library was performed following the instruction directed by Illumina. The PCR plate containing amplified indexed library was briefly centrifuged, and 50 µl of index reaction was transferred to the each wells of a new midi plate. After adding 30 µl of thoroughly vortexed AMPure XP beads to each well, the midi plate was sealed, and vortexed on a plate shaker at 1,800 rpm for 2 minutes. The plate was incubated at room temperature for 5 minutes, and placed on a magnetic stand until the supernatant turned clear. The supernatant was careful discarded by pipetting. While still on the magnetic stand, the reactions were washed by adding 200 µl of freshly prepared 80% ethanol to each well. After 30 seconds, the supernatant was

carefully discarded by the pipette. The washing procedure was repeated one more time. Any remaining ethanol was removed by air drying the plate for 10-15 minutes while the plate was still on the magnetic stand. The midi plate was removed from the stand, and 52.5 µl of RSB was added to the wells to rehydrate the beads. The plate was sealed, vortexed on a plate shaker at 1,800 rpm for 2 minutes, incubated at room temperature for 2 minutes, and transferred again on the magnetic stand till the supernatant was clear. Finally, 50 µl of the supernatant, which was the cleaned library, was transferred to a new PCR plate, and stored in freezer.

2.8.3.5 Quality check of the purified tagmented DNA library using Bioanalyzer

The size of cleaned up DNA library was tested by loading 1 µl of cleaned library onto the Agilent 2100 Bioanalyzer using the High sensitivity DNA kit (Integrated Sciences, Australia). The size of DNA library produced by Nextera XT library preparation protocol was expected to range from 250-1000 bp.

2.8.3.6 Quantitation, normalization, and pooling of the library

The concentration of amplified indexed cleaned-up DNA library was measured by Qubit using dsDNA High Sensitivity assay kit (Invitrogen, USA) following the protocol as described in the methods section 2.8.2. The concentration of the DNA library was converted from ng/µl to nM by multiplying the ng/µl value by a factor of 1.5. The individual DNA library was diluted in distilled water, and normalized to the final concentration of 2 nM, and the final volume of 50 µl. Considering the genome size of Enterobacter spp. to be approximately 5 MB, the samples were pooled together accordingly, denatured in sodium hydroxide, diluted to the final loading concentration, and ran in the HiSeq X Ten platform.

2.8.4 Bioinformatic analyses of the WGS data

Initially, basic quality of raw reads, such as adapter contamination, and read length distribution were assessed using FastQC v0.11.5 [152]. The reads were also examined for the presence of any Illumina transposase adapter. The adapter sequences were trimmed off the reads by using Trimmomatic v0.36 [153] when the adapter contamination constituted of > 10% of the total read length.

The SRST2 v0.2.0 [154] was used to identify the AMR genes, virulence factors, plasmid replicons, and to determine the multi-locus sequence types (MLST) in the sample reads by mapping against the databases, namely ARG-ANNOT [155], BIGSdb [156], PlasmidFinder [157], and *Enterobacter* MLST typing scheme (https://pubmlst.org/mlst/), respectively. The SRST2 used Bowtie2 to map the raw reads against the reference databases and SAMtools to detect the gene variants. Additionally, the plasmid structure of representative sequence types was reconstructed and characterized using the BRIG tool [158].

Next, the raw reads were *de novo* assembled with the Unicycler v0.48 [159] to generate the contigs. The assembled genome was then annotated using Prokka v1.5 [160]. Next, the genetic distances between the samples were sought. Roary [161] was used to construct a pan genome for a major representative sequence types (STs) with a blastp percentage identity of 99% and a core definition of 99%. SNP-sites v2.1.3 [162] was used to extract the single nucleotide polymorphisms (SNPs) from the core gene alignment. The pairwise genetic distances (the difference in the number of SNPs) within and between major STs were estimated by the SNP alignment using ape (v4.1) and adegenet (v2.0.1) packages in R (v3.3.2) [163]. The SNP alignment of each ST was used to construct a maximum likelihood (ML) phylogenetic tree using IQ-TREE v1.4.4 [164], with the

best-fit evolutionary model identified based on the Bayesian Information Criterion in jModelTest implemented in the software. The support for the ML tree was assessed via 100 pseudo-replicates.

The bioinformatics pipeline used for the analyses of WGS data in this study has been illustrated in figure 2.1.

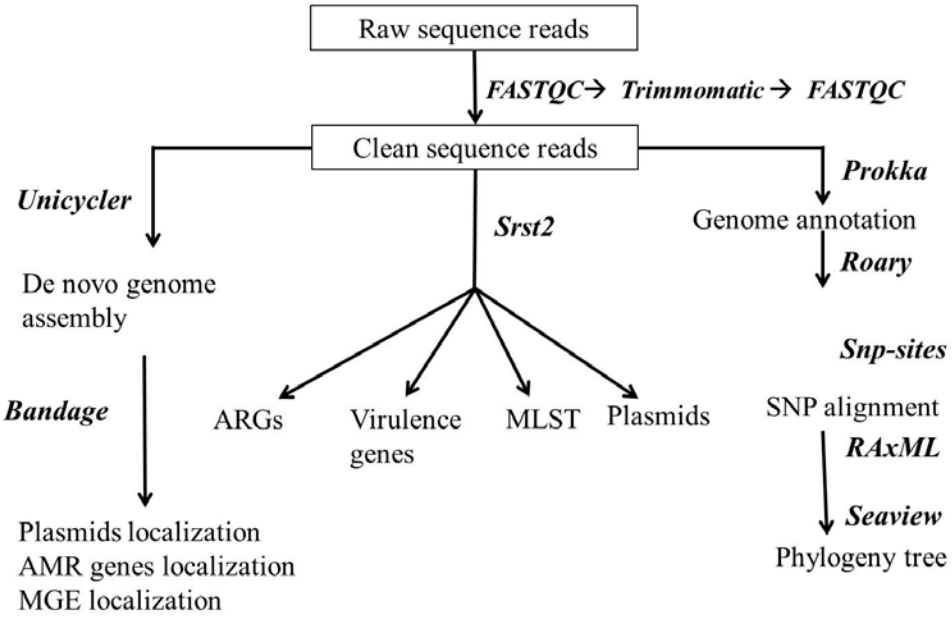


Figure 2.1. The bioinformatics pipeline for the analyses of WGS data of *Enterobacter* spp.

After initial quality check with FASTQC and Trimmomatic, SRST2 identified the AMR genes, virulence genes, plasmid replicons, and multi-locus sequence types in the sample reads. The raw reads were *denovo* assembled into contigs with Unicycler, and annotated with Prokka. A pan genome was constructed with Roary, and by core genome alignment, the SNPs were extracted using SNP-sites. With RAxML, the core SNPs were aligned to estimate the pairwise genetic distance and build a phylogenetic tree on Seaview.

Chapter 3

A high prevalence of multi-drug resistant non-*Salmonella* Gram-negative bacilli in a Nepali tertiary care hospital and associated widespread distribution of extended-spectrum beta-lactamase (ESBL) and carbapenemase-encoding genes

3.1 Abstract

MDR associated with ESBLs and carbapenemases in BSIs causing GNB is a global public health concern. Data on circulating AMR genes among GNB, and the correlation of the genes with MDR, and ESBL phenotypes are limited in Nepal. A retrospective study was performed investigating the distribution of ESBL, and carbapenemase genes, and the potential association of these genes with the ESBL, and MDR phenotypes in *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Acinetobacter* spp. isolated in a tertiary hospital in Nepal between 2012 and 2018. During the study period, 719 *E. coli*, 532 *Klebsiella* spp., 520 *Enterobacter* spp., and 382 *Acinetobacter* spp. were isolated as four prevalent non-*Salmonella* bacterial pathogens originating from the blood samples. It was found that 90% (1,955/2,153) of the isolates were MDR (resistant to ≥3 of following antimicrobials: cefotaxime, cotrimoxazole, ciprofloxacin, amikacin, chloramphenicol, meropenem, piperacillin/tazobactam, and colistin), and 50% (1,080/2,153) of isolates tested ESBL positive. In PCR test, bla_{TEM} (72%; 1,281/1,771) and $bla_{\text{CTXM-1}}$ (53%; 930/1,771) were the most prevalent ESBL genes in the enteric bacilli. Sixteen percent (342/2,153) of all isolates and 20% (357/1,771) of enteric bacilli tested positive for $bla_{\text{NDM-1}}$ and bla_{KPC} carbapenemase genes, respectively. The presence of each bla_{TEM}, bla_{CTXM}, and bla_{OXA} genes in tested GNB were significantly associated with the ESBL positivity (OR 2.96, $p<0.001$; OR 14.2, $p<0.001$, and OR 1.3, $p<0.05$, respectively) and being MDR (OR 1.96, $p<0.001$; OR 5.9, $p<0.001$, and OR 2.3, $p<0.001$, respectively). The

presence of bla_{CTXM} in *Klebsiella* spp. and *E. coli* was associated with non-susceptibility to third/fourth generation cephalosporin (OR 27.3, $p<0.001$). My study documented an alarming level of AMR with high prevalence of MDR ESBL, and carbapenemase positive GNB in the clinical setting. The results suggested a condition where the clinical management of infected patients was increasingly difficult, and required the use of last-resort antimicrobials, which in turn was likely to intensify the magnitude of global AMR crisis.

3.2 Introduction

GNB, such as *E. coli, Klebsiella* spp., *Enterobacter* spp., and *Acinetobacter* spp. are common causes of CO-BSIs and HA-BSIs. These four specific GNB are also the members of '*E. coli* and the ESKAPE' group of pathogens, and often associated with multi-drug resistance (MDR) in nosocomial infections [63,103]. The definition of MDR has been variably used in literature. The status of acquired non-susceptibility of bacterial isolates excluding their intrinsic resistance to at least one agent in ≥3 classes of antimicrobials has been the widely used definition of MDR [165]. However, several definitions of MDR often lack the standard approach for defining relevant classes of antimicrobial agents. Magiorakos et al., proposed a definition of MDR to be an acquired non-susceptibility to at least one agent in ≥3 classes of antimicrobials by taking consideration of organism-specific agents of antimicrobial classes as listed in CLSI table of 'suggested agents with FDA clinical indications that should be considered for routine testing and reporting by clinical microbiological laboratories'[166]. Primarily chosen to harmonize the epidemiological surveillances, this definition has been the clinically relevant and widely applied definition of MDR [118,132,167–170].

The production of ESBLs and carbapenemases is an important mechanism by which GNB exhibit resistance against beta-lactam antimicrobials. The presence of ESBLs increases the risk of treatment failure with third generation cephalosporins. Therefore, it is often recommended to

report ESBL-positive bacteria as resistant to all agents of third generation cephalosporins even when the isolate shows susceptible response as per the standard interpretative cut-off values [171]. Additionally, the carriage of ESBLs in bacterial isolates may contribute to the spread of such AMR genes to other nosocomial bacteria in the hospital [172,173]. Therefore, continued detection and reporting for the presence of ESBLs in bacterial pathogens are also important for developing efficient IPC measures in hospital by prompting the isolation procedures of ESBL positive patients so as to prevent potential cross-infection events to other patients [172].

South Asia is considered as a global epicentre for drug resistant nosocomial GNB [174,175]. However, little was known about various ESBL and carbapenemase genes in GNB causing BSIs in Nepal, one of the South Asian countries. Furthermore, there was little data originating from this region on the association of AMR genotypes with the observed phenotypes. Such data are important for assessing the contribution of combined phenotypic and genetic tests on clinical care. Several phenotypic methods based on synergy between a third generation cephalosporin and clavulanate, an ESBL inhibitor, have been designed to detect the presence of an ESBL [171]. Of these, the combination disc diffusion based tests are routinely used [176]. Though usually useful, the detection of an ESBL by phenotypic methods is sometimes difficult in practice. Some beta-lactamases such as OXA-type ESBLs, AmpC cephalosporinases, and metallo-beta-lactamases though having activity against the third generation cephalosporins, they are resistant to inhibition by clavulanate, rendering a diagnostic challenge by phenotypic methods [171]. The applicability of phenotypic methods is more often compromised due to the increasing co-existence of ESBLs with such other beta-lactamases. Further, resistance to the third generation cephalosporins may be partially attributed by other non-enzymatic mechanisms such as, reduced outer membrane permeability or efflux mechanisms, making the interpretation of phenotypic methods further difficult [171]. Alternatively, the molecular tests, such as PCR detect the presence of specific AMR genes and therefore, can be used to complement the phenotypic tests to generate an

additional epidemiological data on the distribution of key resistance genes in the sentinel locations [172] .

Here, I aimed to determine AMR profile, and phenotypic presence of ESBLs in *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Acinetobacter* spp. isolated from blood samples of the patients at a major tertiary hospital in Nepal between June 2012 and December 2018. Additionally, I performed PCR tests to detect any ESBL and carbapenemase associated AMR genes. Lastly, I investigated the relationship between the presence of $bla_{\text{CTX-M}}$, $bla_{\text{TEM,}}$ and bla_{OXA} genes with an ESBL or MDR phenotype.

3.3 Methods

3.3.1 The study design, and population

This was a retrospective study on routine microbiology culture results originating from Patan hospital in Kathmandu, Nepal. Microbiological data on all *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Acinetobacter* spp. isolated from the blood samples of patients of all age groups attending to any hospital department between June 2012 and December 2018 were included in this study.

3.3.2 An ethical approval

An ethical approval for conducting the study was taken as described in the methods section 2.4. Data used in this retrospective study were derived from the microbiology database of Patan Hospital which was devoid of any patient identifying information. Further, the data used in the study were a part of routine AMR surveillance at Patan Hospital. Therefore, an individually written informed consent was not required.

3.3.3 Antimicrobial susceptibility testing (AST)

The AST of bacteria isolated between June 2012 and December 2018 was performed by the hospital as a part of routine clinical care as described in the methods section 2.6.2. A subset of isolates was retested for the confirmation of routine results for the purpose of research. Enterobacteriaceae bacteria (*E. coli*, *Klebsiella* spp., and *Enterobacter* spp.) and *Acinetobacter* spp. were tested against the following antimicrobial classes and antimicrobial agents: penicillin (ampicillin), $3^{rd}/4^{th}$ generation cephalosporin (cefotaxime/cefepime), carbapenem (imipenem/meropenem), fluroquinolone (ciprofloxacin), folate path inhibitor (cotrimoxazole), aminoglycoside (amikacin/gentamycin), phenicol (chloramphenicol), penicillin β-lactamase inhibitor combination (ampicillin-sulbactam/pipercillin-tazobactam), and polymyxin (colistin). Not all isolates were tested against all antimicrobial agents. The isolates were tested against specific antimicrobial agents of the second-line AST panel only when the isolates were resistant to all agents of the first-line antimicrobial panel as indicated in Table 2.1. The most recent CLSI guidelines on the zone size breakpoints available in the respective years during 2012 to 2018 were followed by the hospital for interpreting AST results. The bacteria producing a resistant, or an intermediate response against the tested antimicrobials were grouped as "non-susceptible" for the purposes of analysis.

3.3.4 Definition of multi-drug resistance (MDR) used in the thesis research

MDR was defined as an acquired non-susceptibility (without intrinsic resistance) to at least one agent of ≥ 3 relevant antimicrobial classes as specified above. An intrinsic resistance was defined according to the CLSI guideline of 2017 [143]. Following intrinsic resistances were ignored for the purpose of generating MDR profile: resistance to ampicillin and ampicillin-sulbactam for *Enterobacter* spp., resistance to ampicillin for *Klebsiella* spp., and resistance to ampicillin and chloramphenicol for *Acinetobacter* spp. As not all antimicrobial classes as recommended for *Acinetobacter* spp. and Enterobacteriaceae were tested, the isolates could not be defined as extremely-drug resistant (XDR), i.e., non-susceptible to at least one antimicrobial agent in all

except two relevant antimicrobial classes, or pan-drug resistant (PDR), i.e., non-susceptible to all antimicrobial agents in all relevant antimicrobial classes [166].

3.3.5 Phenotypic and genetic tests for the detection of ESBLs

The ESBL activity was phenotypically tested by the combination disc diffusion method as described in the methods section 2.7.1. The bacterial isolates were screened for the presence of the ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV,}$, *bla*$_{CTXM-1}$, *bla*$_{CTXM-2}$, *bla*$_{CTXM-9}$, *bla*$_{CTXM-8}$, *bla*$_{CTXM-25}$, and *bla*$_{OXA)}$ and carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{OXA-48}$, *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-51}$, *bla*$_{OXA-58}$, *bla*$_{IMP}$, and *bla*$_{VIM}$) by PCR tests as described in the methods section 2.7.2.

3.3.6 The statistical analysis

Fisher's exact test was used to test for an association between the detection of a resistance gene, and a specific resistance phenotype (ESBL positive, MDR, or resistance to third and fourth generation cephalosporins). For analysis purpose, the detection of any of *bla*$_{OXA}$, *bla*$_{OXA23}$, *bla*$_{OXA24}$, *bla*$_{OXA48}$, *bla*$_{OXA51,}$ or *bla*$_{OXA58}$ was designated as *bla*$_{OXA}$ positive. Similarly, the detection of any of *bla*$_{CTXM-1}$, *bla*$_{CTXM-2}$, *bla*$_{CTXM-8}$, *bla*$_{CTXM-9}$, or *bla*$_{CTXM-25}$ was designated as *bla*$_{CTXM}$ positive. All analyses were performed using the statistical software R version 3.5.3 and R Studio version 1.0.143. Venn diagrams were generated using the UpSetR R package [177].

3.4 Results

During the study period, a total of 719 *E. coli*, 532 *Klebsiella* spp., 520 *Enterobacter* spp., and 382 *Acinetobacter* spp., were isolated from blood samples in the microbiology laboratory at Patan hospital, totalling 2,153 isolates (Table 3.1).

Table 3.1. The profile of four prevalent non-*Salmonella* GNB isolated from blood samples, overall hospital departments of isolation, and the ESBL positivity status.

Profile of non-Salmonella GNB (N = 2,153)	**Count (n)**	**Percentage (%)**
E. coli	719	33.4
Klebsiella spp.	532	24.7
Enterobacter spp.	520	24.2
Acinetobacter spp.	382	17.7
Hospital departments of GNB isolation (N = 2,153)	**Count (n)**	**Percentage (%)**
Emergency	512	23.8
Paediatric ICU (PICU)*	295	13.7
Gynaecology	287	13.3
Medical	208	9.7
Adult ICU	154	7.2
Nursery	136	6.3
Surgery	116	5.4
Paediatric[#]	125	5.8
Not known	219	10.2
Outpatient	82	3.8
Orthopaedic	19	0.9
ESBL positivity status of GNB (Total = 2,153)	**Count (n)**	**Percentage (%)**
No	1,073	49.8
Yes	1,080	50.2

*PICU constituted of bacteria originated from PICU and NICU
[#]Pediatric constituted of bacteria originated from children's ward, and outpatient children of less than 18 years of age

3.4.1 Distribution of GNB by the hospital departments of isolation

A majority of GNB isolates originated from the blood samples taken from the patients attending the ER (24%, 512/2,153), and PICU (14%, 295/2,153) departments (Table 3.1). When the specific departmental origin of each four GNB were analysed, it was found that 59% (311/520) of *Enterobacter* spp. were isolated from the patients admitted to the ER. Similarly, 26% (138/532) of

K. pneumoniae were isolated from the neonates admitted to the PICU department. Figure 3.1 illustrates the distribution of each four GNB by the hospital departments of isolation.

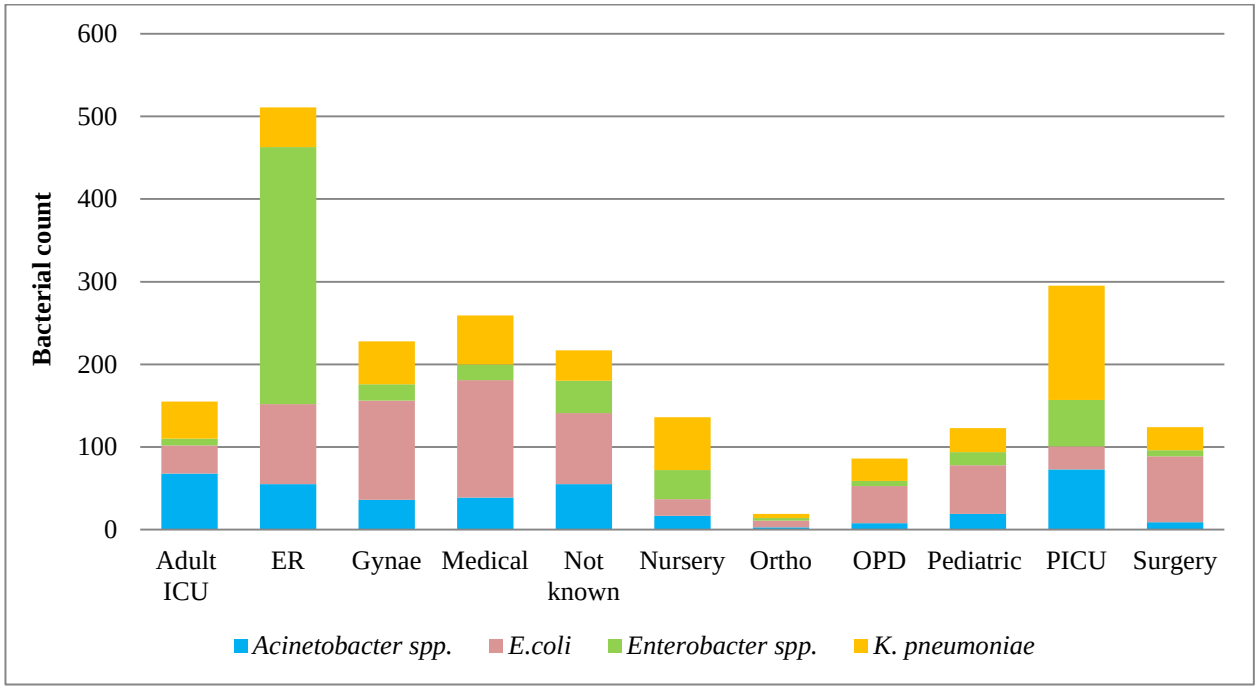


Figure 3.1. The distribution of each *Acinetobacter* spp., *E. coli*, *Enterobacter* spp., and *K. pneumoniae* by the hospital departments of isolation.

The Y-axis shows an absolute number of *Acinetobacter* spp., *E. coli*, *Enterobacter* spp., and *K. pneumoniae*, each indicated by specific colour as shown in the figure key. The X-axis shows different hospital departments from which the GNB were isolated.

3.4.2 Antimicrobial susceptibility test results of GNB

Overall, >50% of tested GNB isolates showed resistance to the antimicrobials of the first-line AST panel, though there was a variation in susceptibility status by specific bacterial genus and antimicrobial agent (Table 3.2). Of the first-line antimicrobial agents, <5% of the tested Enterobacteriaceae GNB were susceptible to cefotaxime, ≤15% were susceptible to ciprofloxacin, ≤ 31% were susceptible to cotrimoxazole, ≤48% were susceptible to amikacin, and ≤44% were susceptible to chloramphenicol except for *E. coli*. In case of *Acinetobacter* spp., only 1% (2/382) was susceptible to cefotaxime, whereas ≤27% of isolates was susceptible to ciprofloxacin,

cotrimoxazole, and amikacin. Of Enterobacteriaceae GNB tested for the second-line antimicrobials, ≥60% were susceptible to meropenem, ≥75% were susceptible to colistin, whereas only <20% were susceptible to piperacillin/tazobactam. In case of *Acinetobacter* spp., ≤16% of isolates was susceptible to meropenem and piperacillin/tazobactam, while 82% (160/195) were susceptible to colistin.

Table 3.2. The antimicrobial susceptibility test results of *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Acinetobacter* spp. isolated from the blood samples

	AST status	**Amp**	**Cefo**	**Cipro**	**Amik**	**Cotrim**	**Chloram**	**Mero**	**Pip/Taz**	**Colis**
E. coli	S	9 (1.3)	27 (3.8)	102 (14.3)	344 (48.2)	220 (31)	314 (72.4)	92 (69.2)	23 (29.9)	77 (80.2)
	R	703 (98.7)	688 (96.2)	611 (85.7)	370 (51.8)	490 (69)	120 (27.6)	41 (30.8)	54 (70.1)	19 (19.8)
	tested	712	715	713	714	710	434	133	77	96
Klebsiella spp.	S		17 (3.2)	82 (15.6)	173 (32.7)	102 (19.5)	179 (41.5)	176 (62.6)	35 (17)	161 (77)
	R		513 (96.8)	445 (84.4)	356 (67.3)	422 (80.5)	252 (58.5)	105 (37.4)	171 (83)	48 (23)
	tested	0	530	527	529	524	431	281	206	209
Enterobacter spp.	S		15 (2.9)	66 (12.9)	241 (47.6)	73 (14.1)	210 (43.6)	70 (60.3)	15 (18.1)	62 (74.7)
	R		504 (97.1)	445 (87.1)	265 (52.4)	444 (85.9)	272 (56.4)	46 (39.7)	68 (81.9)	21 (25.3)
	tested	0	519	511	506	517	482	116	83	83
Acinetobacter spp.	S		2 (0.5)	92 (24.3)	98 (25.9)	98 (26.7)		40 (16.5)	25 (14.1)	160 (82.1)
	R		380 (99.5)	286 (75.7)	281 (74.1)	269 (73.3)		203 (83.5)	152 (85.9)	35 (17.9)
	tested	0	382	378	379	367	0	243	177	195

Amik: Amikacin, Amp: ampicillin, AST: antimicrobial susceptibility test, Cefo: cefotaxime, Chloram: chloramphenicol, Cipro: ciprofloxacin, Colis: colistin, Cotrim: cotrimoxazole, Mero: meropenem, Pip/Taz: piperacillin/tazobactam, R: resistant, S: susceptible

3.4.3 MDR and ESBL positivity status of GNB by hospital department

The proportion of MDR phenotypes of GNB isolates ranged from 87% (75/86) in an OPD to 96% (150/155) in an adult ICU department. Similarly, the proportion of ESBL positive bacterial isolates ranged from 5% (1/19) in an orthopedic to 66% (335/511) in an ER department. The distribution of ESBL-positivity and MDR phenotype in the bacterial isolates by the hospital departments of isolation has been shown in the figure 3.2.

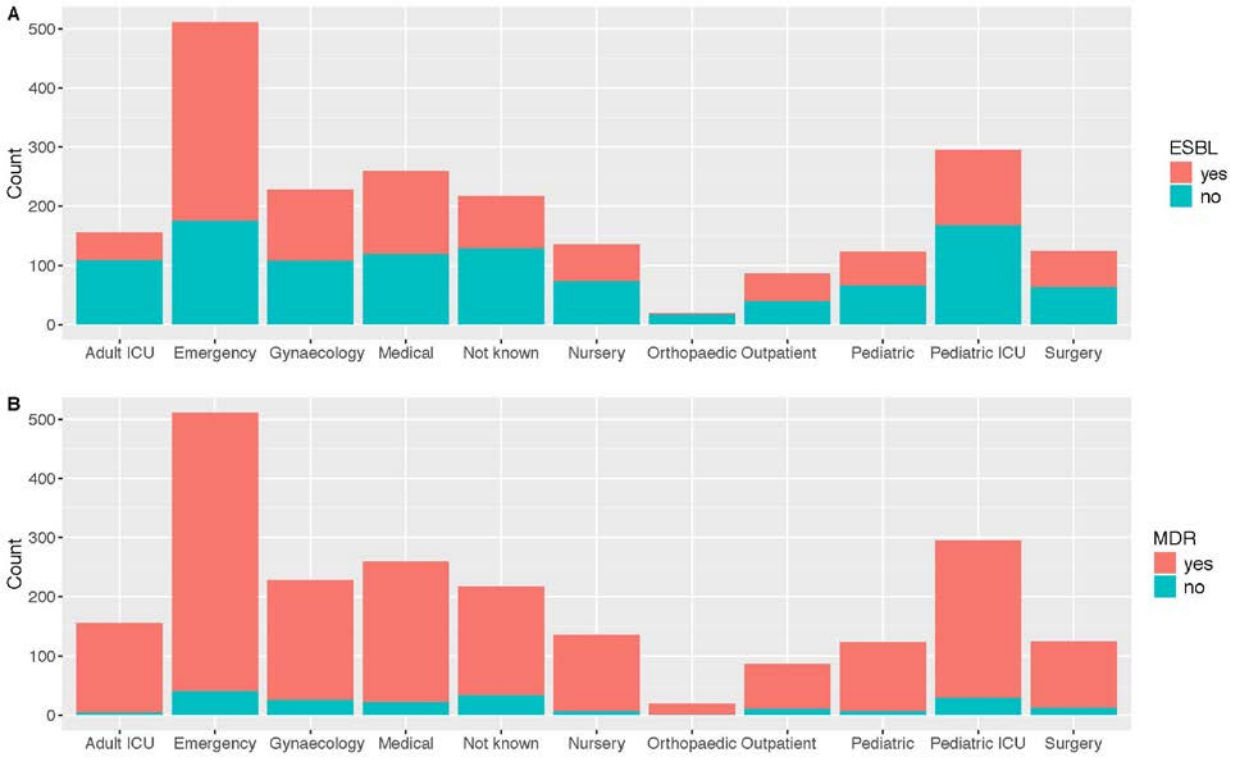


Figure 3.2. The ESBL and MDR positivity status of specific GNB isolates, stratified by the hospital departments from where the blood samples were taken.

The Y-axis shows an absolute count of (A) ESBL-positive and –negative, and (B) MDR and non-MDR status of specific GNB. The X-axis shows different hospital departments from which the GNB were isolated.

Fifty percent (1,080/2,153) of the GNB isolates were ESBL positive. The ESBL positivity was more common (>50%) in *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. isolates than in *Acinetobacter* spp. isolates (<1%) (Table 3.3). Similarly, ≥ 89% of *E. coli* (643/719), *Klebsiella* spp. (488/532), and *Enterobacter* spp. (510/520) were MDR, this proportion being relatively lower in *Acinetobacter* spp. (82%, 314/382).

Table 3.3. The phenotypic status of ESBL positivity and MDR in *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Acinetobacter* spp. isolated from the blood samples.

Bacterial pathogens (N = 2,153)	**ESBL-neg**		**ESBL-pos**		**Non-MDR**		**MDR**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
E. coli (N=719)	294	40.9	425	59.1	76	10.6	643	89.4
Klebsiella spp. (N=532)	210	39.5	322	60.5	44	8.3	488	91.7
Enterobacter spp. (N=520)	189	36.3	331	63.7	10	1.9	510	98.1
Acinetobacter spp. (N=382)	380	99.5	2	0.5	68	17.8	314	82.2

neg: negative, pos: positive

3.4.4 Distribution of AMR genes in specific GNB

In *E. coli* isolates tested for specific AMR genes, 90% (648/719) were positive for bla_{TEM}, 58% (417/719) were positive for $bla_{\text{CTXM-8}}$, and 37% (269/719) were positive for bla_{OXA}. In the *Enterobacter* spp., 56% (292/520) were positive for bla_{OXA}, 60% (201/520) were positive for bla_{KPC}, 80% (417/520) were positive for $bla_{\text{CTXM-1}}$, and 58% (301/520) were positive for bla_{TEM}. In *Klebsiella* spp., 77% (408/532) were positive for $bla_{\text{CTXM-1}}$, 67% (357/532) were positive for bla_{OXA}, 4% (22/532) were positive for bla_{KPC}, and 63% (332/532) of tested isolates were positive for bla_{TEM}. Similarly, in *Acinetobacter* spp., 59% (218/382) were positive for bla_{OXA51}, 36% (134/382) were positive for bla_{OXA23}, and 21% (79/382) were positive for $bla_{\text{NDM-1}}$. As bla_{OXA51} gene has been described to occur consistently and specifically in *A. baumannii* species [178,179], 59% (218/382) of *Acinetobacter* spp. isolated in this study could be considered to be *A. baumannii* species. The status of *E. coli*, *Klebsiella* spp., *Enterobacter* spp., and *Acinetobacter* spp. for the

presence of *bla*$_{CTXM}$ family genes (Table 3.4), *bla*$_{OXA}$ family genes (Table 3.5), and *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ (Table 3.6) has been summarized in the respective tables.

Table 3.4. The results on detection of *bla*$_{CTXM}$ family genes for *E. coli*, *Enterobacter* spp., *Klebsiella* spp., and *Acinetobacter* spp. shown by absolute number of bacteria and percentage.

GNB	**Detection status**	***bla*$_{CTXM-1}$**		***bla*$_{CTXM-2}$**		***bla*$_{CTXM-8}$**		***bla*$_{CTXM-9}$**		***bla*$_{CTXM-25}$**	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
E. coli	neg	612	85.4	717	100	302	42.0	714	99.3	710	98.7
	pos	105	14.6	0		417	58.0	5	0.7	9	1.3
	n.t.	2		2		0		0		0	
Enterobacter spp.	neg	102	19.7	0		0		518	99.8	0	
	pos	417	80.3	0		0		1	0.2	0	
	n.t.	1		520		520		1		520	
Klebsiella spp.	neg	119	22.6	527	100	525	99.6	524	99.4	521	98.9
	pos	408	77.4	0		2	0.4	3	0.6	6	1.1
	n.t.	5		5		5		5		5	
Acinetobacter spp.	neg	368	98.9	372	100	372	100	372	100.0	372	100
	pos	4	1.1	0		0		0		0	
	n.t.	10		10		10		10		10	

neg: negative, pos: positive, n.t.: not tested

Table 3.5. The results on detection of *bla*$_{OXA}$ family genes for *E. coli*, *Enterobacter* spp., *Klebsiella* spp., and *Acinetobacter* spp. shown by absolute number of bacteria and percentage.

GNB	Detection status	***bla*$_{OXA}$**		***bla*$_{OXA23}$**		***bla*$_{OXA24}$**		***bla*$_{OXA48}$**		***bla*$_{OXA51}$**		***bla*$_{OXA58}$**	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
E. coli	neg	450	62.6	0		0		715	99.4	0		0	
	pos	269	37.4	0		0		4	0.6	0		0	
	n.t.	0		719		719		0		719		719	
Enterobacter spp.	neg	228	43.8	0		0		221	65.6	0		0	
	pos	292	56.2	0		0		116	34.4	0		0	
	n.t.	0		520		520				520		520	
Klebsiella spp.	neg	174	32.8	0		0		475	89.5	0		0	
	pos	357	67.2	0		0		56	10.5	0		0	
	n.t.	1		532		532		1		532		532	
Acinetobacter spp.	neg	0		238	64.0	363	97.6	0		154	41.4	357	96.0
	pos	0		134	36.0	9	2.4	0		218	58.6	15	4.0
	n.t.	382		10		10		382		10		10	

neg: negative, pos: positive, n.t.: not tested

Table 3.6. The results on detection of *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{NDM-1}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ genes for *E. coli*, *Enterobacter* spp., *Klebsiella* spp., and *Acinetobacter* spp. shown by absolute number of bacteria and percentage .

GNB	Detection status	***bla*$_{IMP}$**		***bla*$_{KPC}$**		***bla*$_{NDM1}$**		***bla*$_{SHV}$**		***bla*$_{TEM}$**		***bla*$_{VIM}$**	
		n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
E. coli	neg	577	99.7	719	100	681	94.7	719	100	71	9.9	578	99.8
	pos	2	0.3	0		38	5.3	0		648	90.1	1	0.2
	n.t.	140		0		0		0		0		140	
Enterobacter spp.	neg	90	100	136	40.4	412	79.2	515	99.0	219	42.1	90	100
	pos	0		201	59.6	108	20.8	5	1.0	301	57.9	0	
	n.t.	430		183		0		0		0		430	
Klebsiella spp.	neg	100	100	509	95.9	414	78.0	415	78.2	199	37.5	100	100
	pos	0		22	4.1	117	22.0	116	21.8	332	62.5	0	
	n.t.	432		1		1		1		1		432	
Acinetobacter spp.	neg	0		0		303	79.3	361	97.0	300	78.5	0	
	pos	0		0		79	20.7	11	3.0	82	21.5	0	
	n.t.	382		382		90		10		90		382	

neg: negative, pos: positive, n.t.: not tested

3.4.5 The association between the presence of ESBL genes and an ESBL phenotype

The detection of each *bla*$_{OXA}$, *bla*$_{CTX-M}$, and *bla*$_{TEM}$ ESBL genes was significantly associated with an ESBL phenotypes (Table 3.7). The strength of association varied by the specific ESBL genes, with the odds ratio (OR) of 1.3 (95% CI: 1.1-1.5, $p<0.05$) for *bla*$_{OXA}$, 3.0 (95% CI: 2.5-3.6, $p<0.001$) for *bla*$_{TEM}$, and 14.2 (95% CI: 11.2-18.1, $p<0.001$) for *bla*$_{CTXM}$ in the Fisher's test.

Table 3.7. The association between presence of ESBL genes, and phenotypic status of being ESBL positivite, MDR, or non-susceptible to the third or fourth generation cephalosporins.

ESBL genes	**ESBL**	**non-ESBL**	**OR (95% CI)[(c)]**	**p-value[(d)]**
bla$_{OXA}$[(a)]				
OXA-neg	406	458	1.3 (1.1-1.5)	0.009
OXA-pos	674	604		
bla$_{CTXM}$[(b)]				
CTXM-neg	112	660	14.2 (11.2-18.1)	<0.001
CTXM-pos	968	401		
bla$_{TEM}$				
TEM-neg	262	517	3.0 (2.5-3.6)	<0.001
TEM-pos	818	545		
ESBL genes	**MDR**	**non-MDR**	**OR (95% CI)[(c)]**	**p-value[(d)]**
bla$_{OXA}$[(a)]				
OXA-neg	751	113	2.3 (1.7- 3.1)	<0.001
OXA-pos	1198	80		
bla$_{CTXM}$[(b)]				
CTXM-neg	629	143	6.0 (4.2- 8.6)	<0.001
CTXM-pos	1319	50		
bla$_{TEM}$				
TEM-neg	680	99	2.0 (1.4- 2.7)	<0.001
TEM-pos	1269	94		
ESBL genes	**Cephal. 3/4 non-susceptible [(e)]**	**Cephal. 3/4 susceptible**	**OR (95% CI)[(c)]**	**p-value[(d)]**
bla$_{OXA}$[(a)]				
OXA-neg	568	25	1.5 (0.8- 3.0)	0.21
OXA-pos	632	18		
bla$_{CTXM}$[(b)]				
CTXM-neg	261	38	27.3 (10.6- 89.6)	<0.001
CTXM-pos	939	5		
bla$_{TEM}$				
TEM-neg	254	13	1.6 (0.8- 3.2)	0.18
TEM-pos	946	30		

Cephal. 3/4: 3rd or 4th generation cephalosporin, (a) an isolate was considered as *bla*$_{OXA}$ positive if any of *bla*$_{OXA}$, *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-48}$, *bla*$_{OXA-51,}$ or *bla*$_{OXA-58}$ was detected, (b) an isolate was considered as *bla*$_{CTXM}$ positive if any of *bla*$_{CTXM-1}$, *bla*$_{CTXM-2}$, *bla*$_{CTXM-8}$, *bla*$_{CTXM-9,}$ or *bla*$_{CTXM-25}$ was detected, (c) odds ratio and 95% confidence interval, (d) p-value from the Fisher's exact test, (e) for finding the association between ESBL genes and non-susceptibility to

3rd or 4th generation cephalosporins, *Enterobacter* spp. and *Acinetobacter* spp. were disregarded as these bacterial genera are likely to be resistant to 3rd or 4th generation cephalosporins due to the expression of AmpC gene.

A distribution of different combinations of bla_{OXA}, bla_{CTXM}, and bla_{TEM} resistance genes with the phenotypic ESBL positivity is shown in figure 3.3.

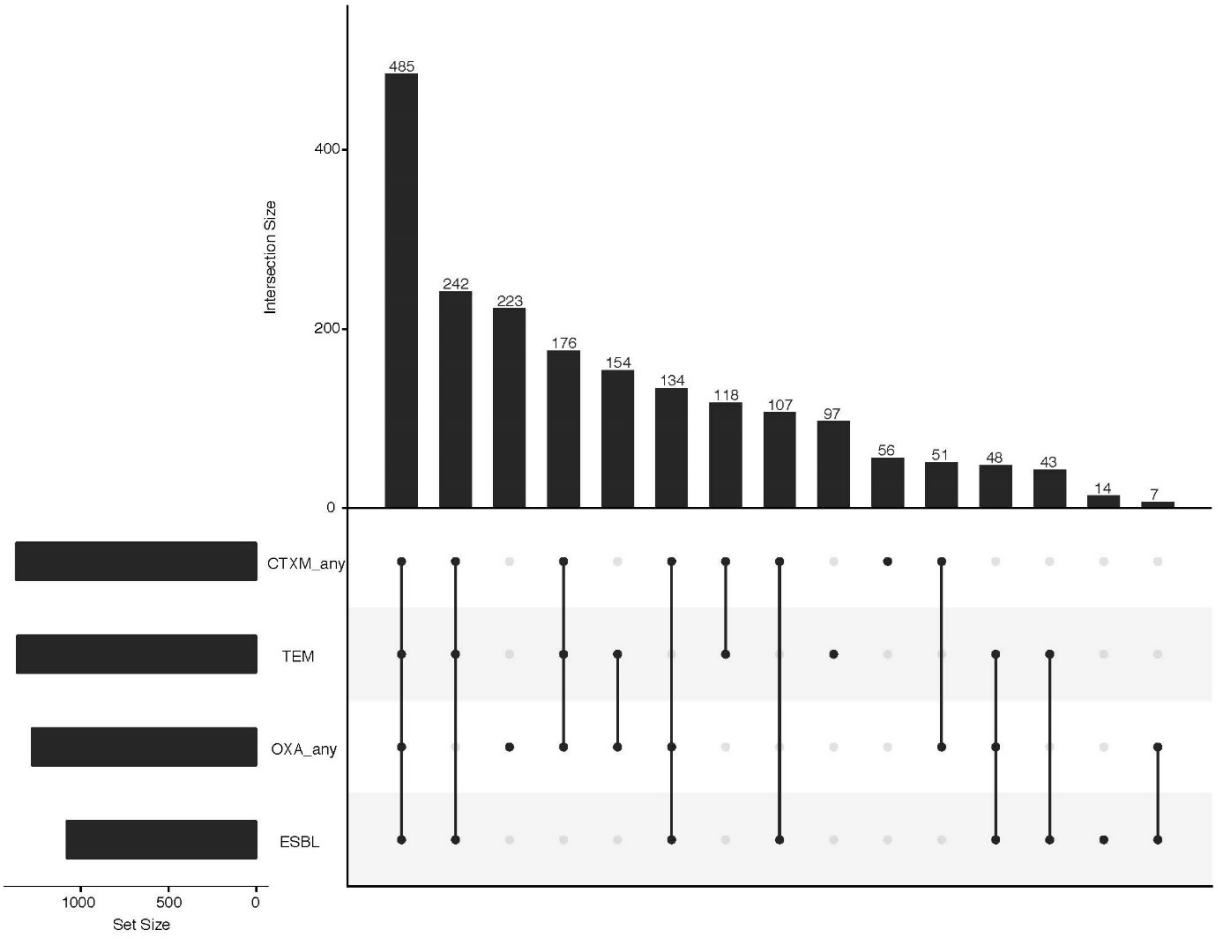


Figure 3.3. The association between phenotypic ESBL positivity, and the combinations of ESBL genes bla_{OXA}, bla_{CTXM} and bla_{TEM}.

The black dots represent ESBL positivity and/or the detection of resistance genes of bla_{OXA} family, bla_{CTXM} family or bla_{TEM}. A grey dot indicates that the characteristic was not detected. The vertical bars indicate the number of isolates in the group summarized directly below. The horizontal bars indicate the total number of isolates positive for resistance genes of bla_{OXA} family, bla_{CTXM} family, bla_{TEM} or for ESBL positivity. An isolate was classified as bla_{OXA} positive if any gene of bla_{OXA} family was detected. The same is true for bla_{CTXM} genes. Only the isolates that were tested for ESBL positivity, at least one member of bla_{OXA} family, at least one member of bla_{CTXM} family and bla_{TEM} were included.

A majority (25%, 485/1,955) of isolates were ESBL positive, and carried the bla_{OXA}, bla_{CTXM} and bla_{TEM} ESBL genes. Nine percent (176/1,955) of isolates were PCR positive for bla_{OXA}, bla_{CTXM} and bla_{TEM}, but was phenotypically ESBL negative. The second most common (12%, 242/1,955) gene combination associated with ESBL positivity was bla_{CTXM} and bla_{TEM}. The proportion of isolates that were phenotypically ESBL positive, but PCR amplification negative for bla_{OXA}, bla_{CTXM} and bla_{TEM} was 0.7% (14/1,955).

3.4.6 The association between the presence of ESBL genes and the non-susceptibility to third or fourth generation cephalosporins

The association between the non-susceptibility to third or fourth generation cephalosporins (cefotaxime, cefepime, ceftriaxone or cefixime), and the presence of bla_{OXA}, $bla_{CTXM,}$ or bla_{TEM} for *E. coli* and *Klebsiella pneumoniae* was also sought. The presence of bla_{CTXM} gene was significantly associated with the non-susceptibility to third or fourth generation cephalosporins with OR of 27.3 (95% CI: 10.6-89.6, p<0.001) (Table 3.7).

The most frequent combination (32%, 409/1,250) associated with the non-susceptibility against third or fourth generation cephalosporins was the presence of all three (bla_{OXA}, bla_{CTXM}, and $bla_{TEM)}$ genes, followed by the presence of bla_{CTXM} and bla_{TEM} only (27%; 346/1,250). Only 4% (53/1,250) of isolates phenotypically non-susceptible to third or fourth generation cephalosporins were PCR amplification negative for bla_{OXA}, $bla_{CTXM,}$ and bla_{TEM} (Figure 3.4).

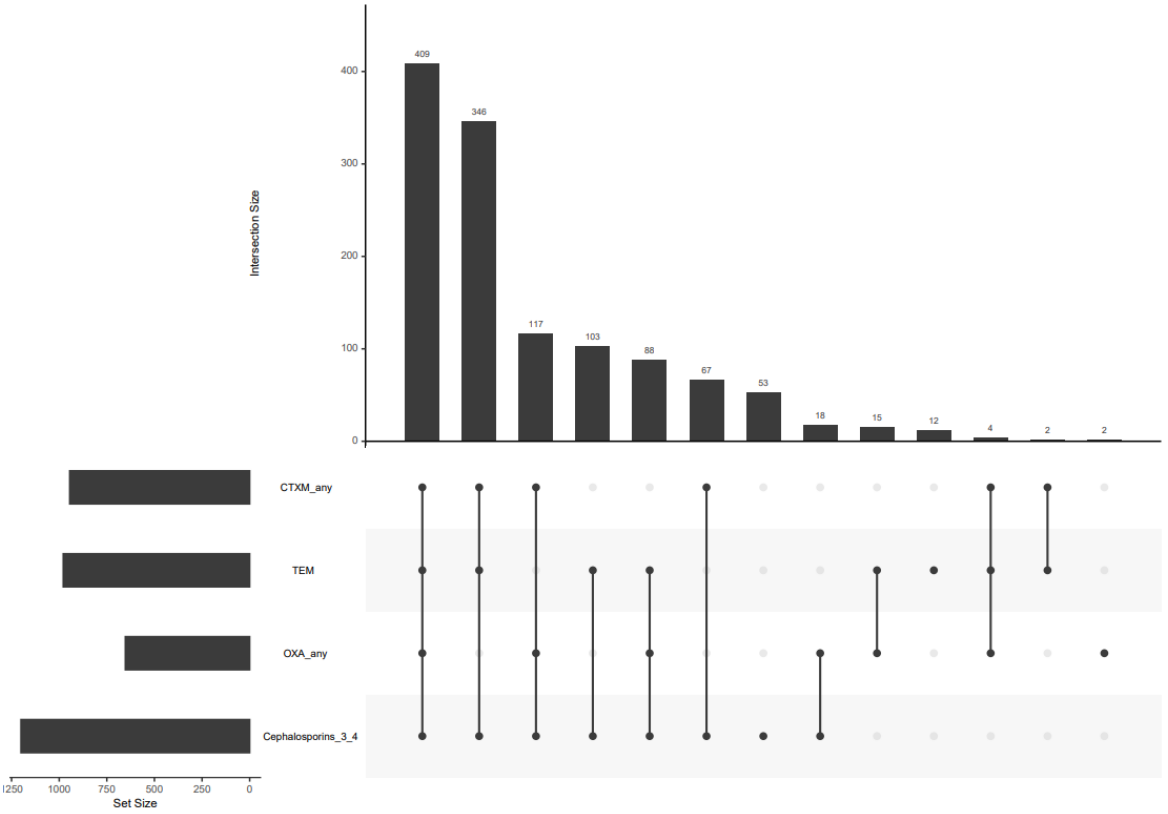


Figure 3.4. The association between the non-susceptibility to 3rd or 4th generation cephalosporins, and the presence of *bla*$_{OXA}$, *bla*$_{CTXM}$, and *bla*$_{TEM}$ ESBL genes for *E. coli* and *K. pneumoniae*.

The black dots represent the non-susceptibility to cephalosporins (3rd/4th gen.) and/or the detection of ESL genes of *bla*$_{OXA}$ family, *bla*$_{CTXM}$ family or *bla*$_{TEM}$. A grey dot indicates that the characteristic was not detected. The vertical bars indicate the number of isolates in the group summarized directly below. The horizontal bars indicate the total number of isolates positive for the ESBL genes of *bla*$_{OXA}$ family, *bla*$_{CTXM}$ family or *bla*$_{TEM,}$ or for the non-susceptibility to the 3rd or 4th generation cephalosporins. An isolate was considered as *bla*$_{OXA}$ positive if any gene of *bla*$_{OXA}$ family was detected. The same is true for *bla*$_{CTXM}$ genes. Only isolates that had a known 3rd or 4th generation cephalosporin resistance profile and were tested for at least one member of *bla*$_{OXA}$ family, at least one member of *bla*$_{CTXM}$ family and *bla*$_{TEM}$ were included.

3.4.7 The association between the presence of ESBL genes and an MDR phenotype

Any potential association between the presence of *bla*$_{OXA}$, *bla*$_{CTXM}$ or *bla*$_{TEM}$ genes and an MDR was investigated. The MDR phenotype was significantly associated with the presence of the ESBL genes *bla*$_{CTXM}$ (OR 5.99, 95% CI: 4.2-8.6, p<0.001), *bla*$_{OXA}$ (OR 2.3, 95% CI: 1.7-3.1, p<0.001), and *bla*$_{TEM}$ (OR 1.96, 95% CI: 1.4-2.7, p<0.001) (Table 3.7). Thirty one percent (648/2,097) of

GNB showing MDR phenotype were PCR positive for all three ESBL genes (*bla*$_{OXA}$, *bla*$_{CTXM}$, and *bla*$_{TEM}$), followed by 16% (338/2,097) of MDR isolated testing positive for a combination of *bla*$_{CTXM,}$ and *bla*$_{TEM}$. A small fraction of isolates (7%, 156/2,097) were PCR amplification negative for *bla*$_{OXA}$, *bla*$_{CTXM,}$ and *bla*$_{TEM,}$ but were MDR phenotypes (Figure 3.5).

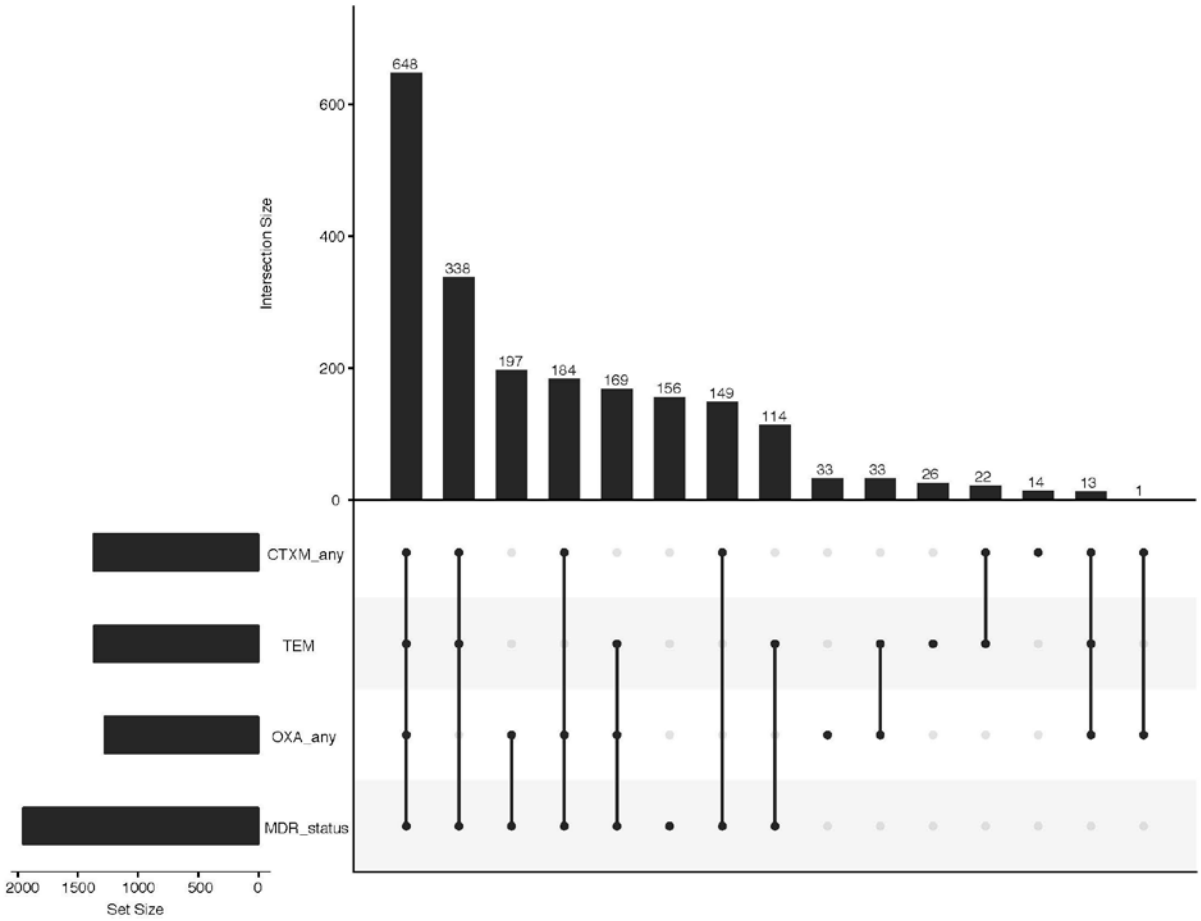


Figure 3.5. The association between an MDR phenotype, and the presence of *bla*$_{OXA}$, *bla*$_{CTXM,}$ and *bla*$_{TEM}$ ESBL gens.

The black dots represent an MDR status, and/or the detection of *bla*$_{OXA}$, *bla*$_{CTXM}$, or *bla*$_{TEM}$ ESBL genes. A grey dot indicates that the characteristic was not detected. The vertical bars indicate the number of isolates in the group summarized directly below. The horizontal bars indicate the total number of isolates positive for the *bla*$_{OXA,}$ *bla*$_{CTXM}$, or *bla*$_{TEM}$ ESBL genes, or for the MDR phenotype. An isolate was considered as *bla*$_{OXA}$ positive if any gene of *bla*$_{OXA}$ family was detected. The same is true for *bla*$_{CTXM}$ genes. Only the isolates that had an AMR profile, and were tested for at least one member of *bla*$_{OXA}$ family, at least one member of *bla*$_{CTXM}$ family, and *bla*$_{TEM}$ were included.

As MDR was defined as an acquired non-susceptibility to at least one agent in three different classes of antimicrobials, a MDR phenotype could have originated from a multitude of individual AMR phenotypes. Therefore, I compared the pattern of resistance for each bacterial genus originating from the outpatients (patients attending ER and OPD), and that from inpatients (patients attending other inpatient wards). Figure 3.6, 3.7, 3.8 and 3.9 depict the AMR combinations for MDR status for *Acinetobacter* spp., *Enterobacter* spp., *Klebsiella* spp., and *E. coli* respectively, each stratified by isolation from the inpatients (A) and outpatients (B). Overall, the diversity of AMR combination phenotypes for each four bacterial genus was broader for the bacterial isolates obtained from the inpatients than from outpatients.

For *Acinetobacter* spp. isolated from inpatients, the most prevalent (34%, 89/264) AMR profile for MDR status was antimicrobial non-susceptibility to all tested antimicrobial classes (3^{rd} or 4^{th} generation cephalosporin, carbapenem, penicillin/beta-lactamase inhibitor combination, fluoroquinolone, folate-path inhibitor, and aminoglycoside) except for polymyxin (Figure 3.6 (A)). In contrary, among *Acinetobacter* spp. isolated from outpatients, the most common combination (20%, 13/63) was non-MDR status with mono-resistance to 3^{rd} or 4^{th} generation cephalosporin, followed by 12% (8/63) of isolates being MDR with non-susceptibility to all tested antimicrobial classes except for polymyxin.

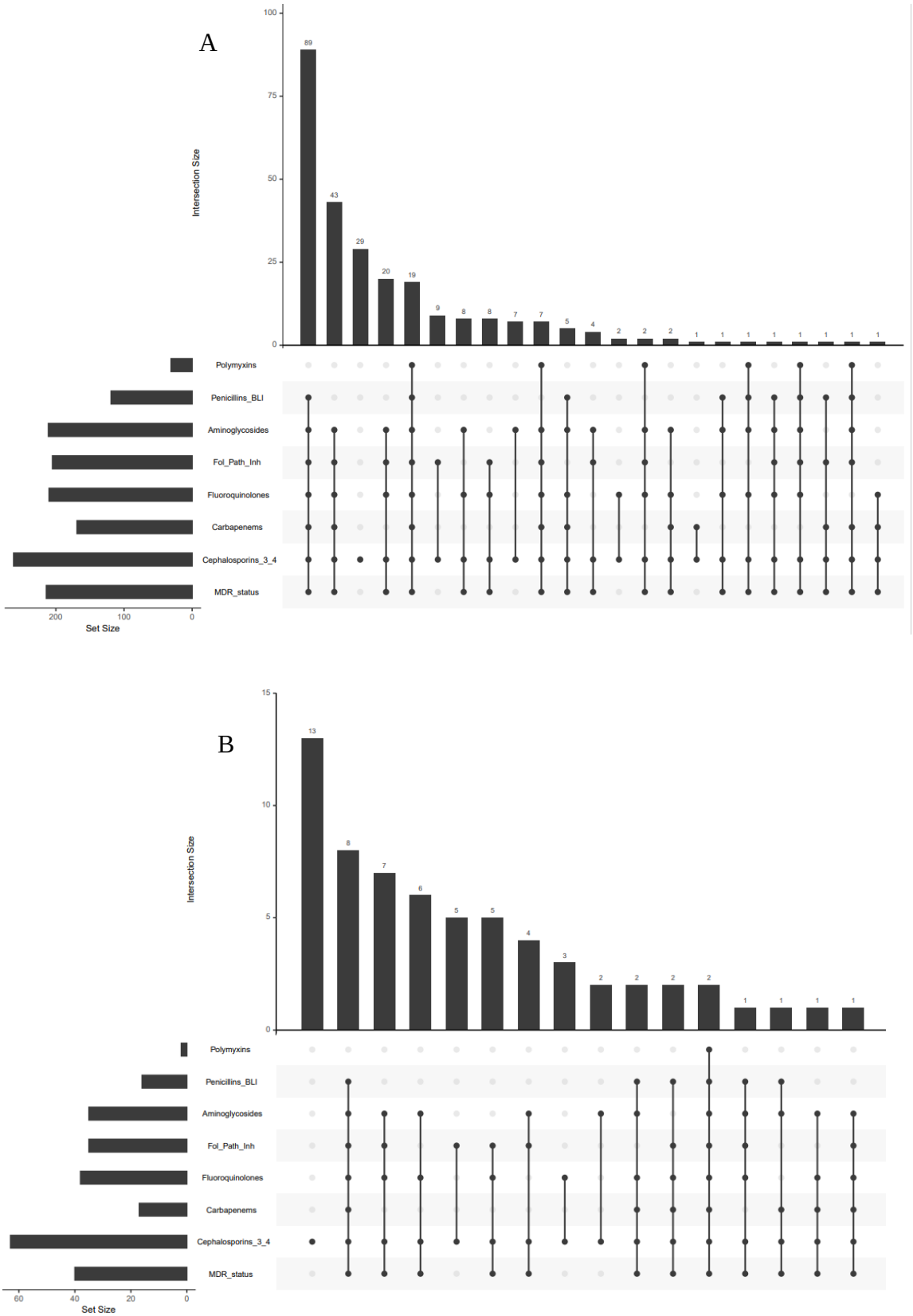
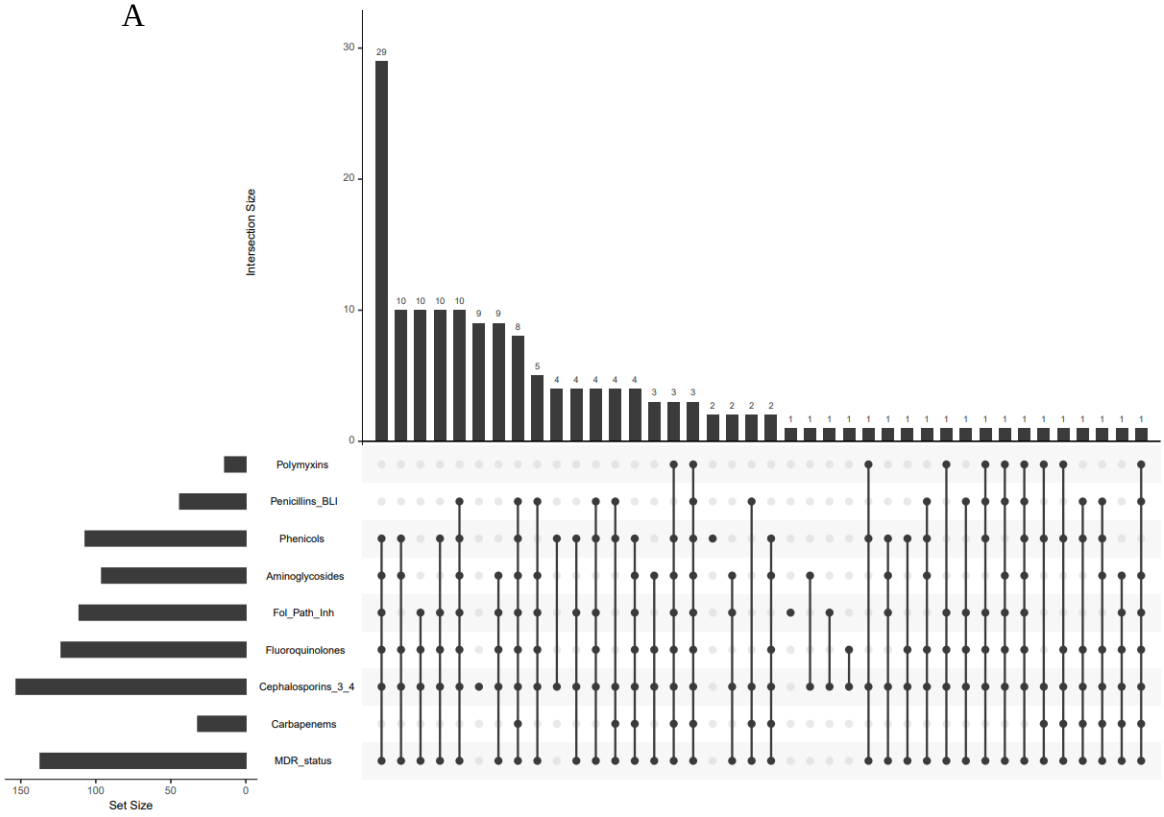


Figure 3.6. The phenotypic AMR pattern and MDR status of *Acinetobacter* spp. isolated from A) inpatients and B) outpatients.

Only the 42 most frequent combinations are shown. The black dots represent the phenotypic resistance to the given antimicrobial classes and/or an MDR phenotype. The vertical bars represent the number of isolates characterized by the AMR pattern described under the bar. The horizontal bars indicate the total number of isolates resistant to the antimicrobial classes, or MDR status.

For *Enterobacter* spp., isolated from inpatients, the most prevalent (17%, 29/164) AMR profile for MDR status was antimicrobial non-susceptibility to all tested antimicrobial classes (3rd or 4th generation cephalosporin, fluoroquinolone, folate-path inhibitor, aminoglycoside, and phenicol) except carbapenem, penicillin/beta-lactamase inhibitor, and polymyxin (Figure 3.7 (A)). In case of *Enterobacter* spp. isolated from outpatients, two AMR profiles for MDR status were prevalent and constituted of 77% (244/316) of isolates. The first AMR profile was antimicrobial non-susceptibility to 3rd or 4th generation cephalosporin, fluoroquinolone, and folate-path inhibitor and the second profile was antimicrobial non-susceptibility to 3rd or 4th generation cephalosporin, fluoroquinolone, folate-path inhibitor, aminoglycoside, and phenicol (Figure 3.7 (B)).

A



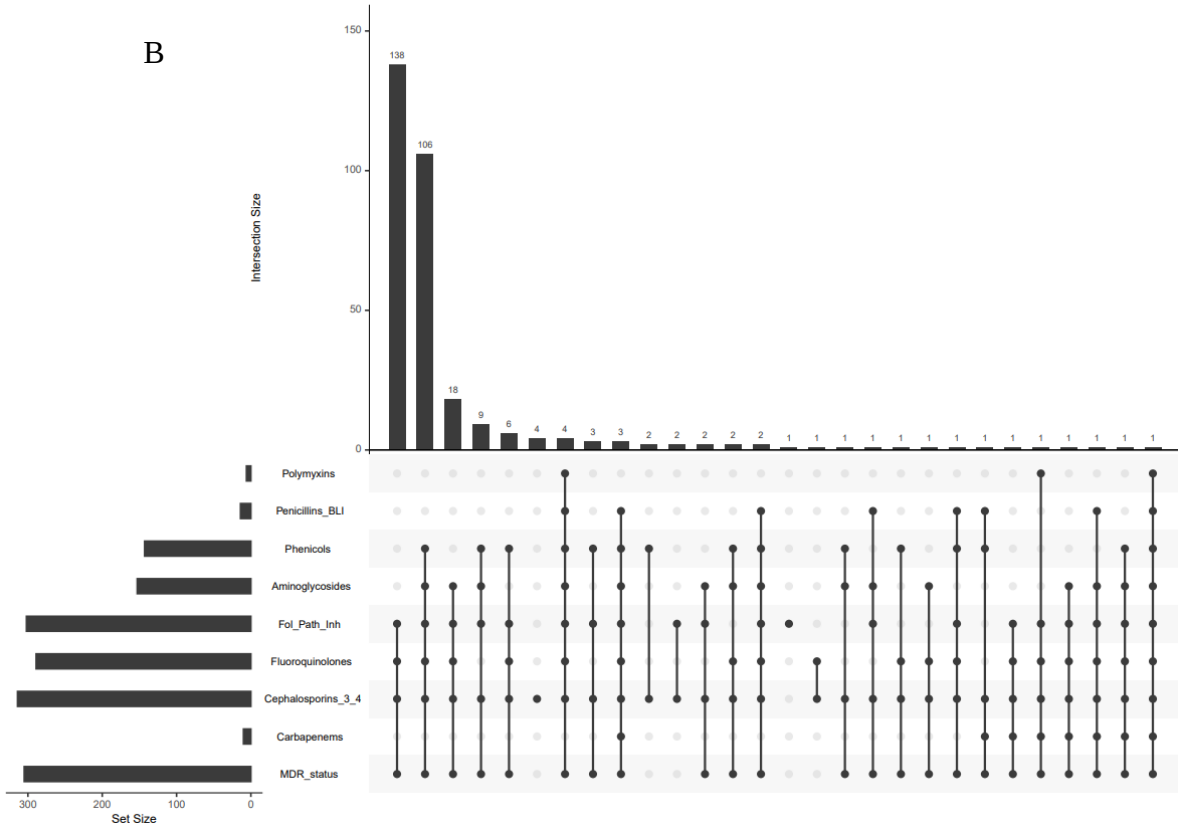


Figure 3.7. The phenotypic AMR pattern and MDR status of *Enterobacter* spp. isolated from A) inpatients and B) outpatients.

Only the 42 most frequent combinations are shown. The black dots represent the phenotypic resistance to the given antimicrobial classes and/or an MDR phenotype. The vertical bars represent the number of isolates characterized by the AMR pattern described under the bar. The horizontal bars indicate the total number of isolates resistant to the antimicrobial classes, or MDR status.

For *Klebsiella* spp., the most prevalent AMR profile for MDR status for both inpatients (16%, 68/419) (Figure 3.8 (A)) and outpatients (17%, 13/75) (Figure 3.8 (B)) was antimicrobial non-susceptibility to 3rd or 4th generation cephalosporin, fluoroquinolone, folate-path inhibitor, and aminoglycoside.

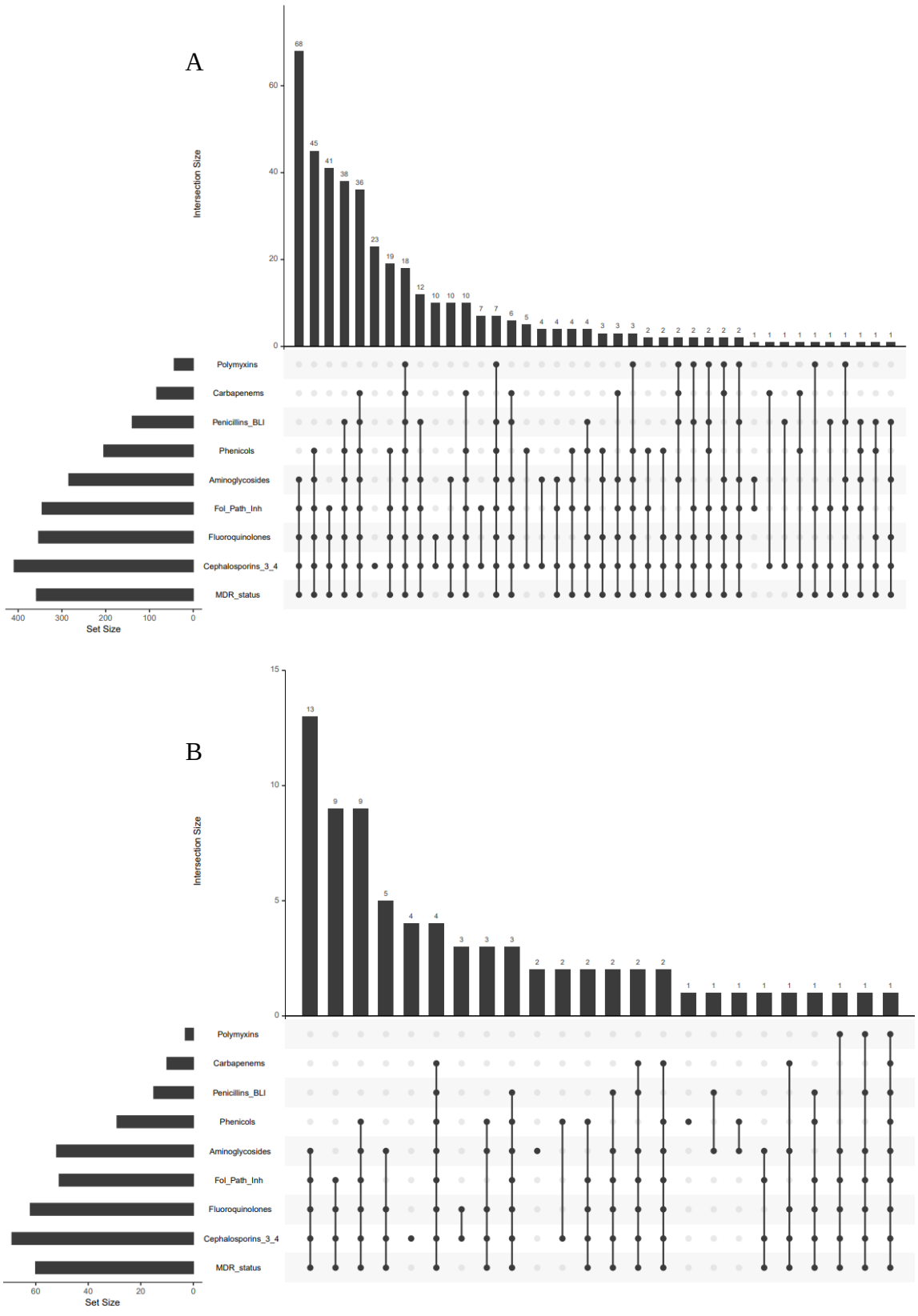
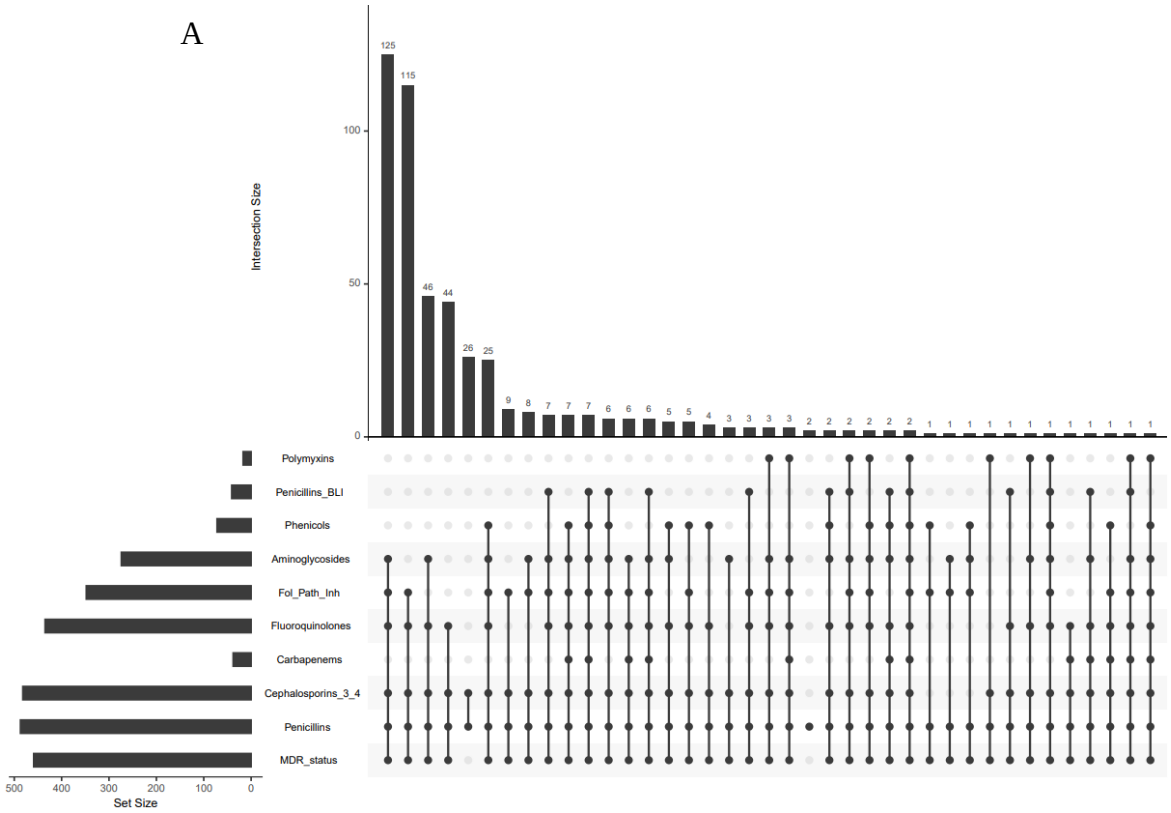


Figure 3.8. The phenotypic AMR pattern and MDR status of *Klebsiella* spp. isolated from A) inpatients and B) outpatients.

Only the 42 most frequent combinations are shown. The black dots represent the phenotypic resistance to the given antimicrobial classes and/or an MDR phenotype. The vertical bars represent the number of isolates characterized by the AMR pattern described under the bar. The horizontal bars indicate the total number of isolates resistant to the antimicrobial classes, or MDR status.

For *E. coli*, the antimicrobial non-susceptibility to penicillin, 3rd or 4th generation cephalosporin, fluoroquinolone, folate path inhibitor, and aminoglycoside was the most common AMR profile for being MDR among isolates from both inpatients (25%, 125/490) (Figure 3.9 (A)) and outpatients (22%, 31/142) (Figure 3.9 (B)).



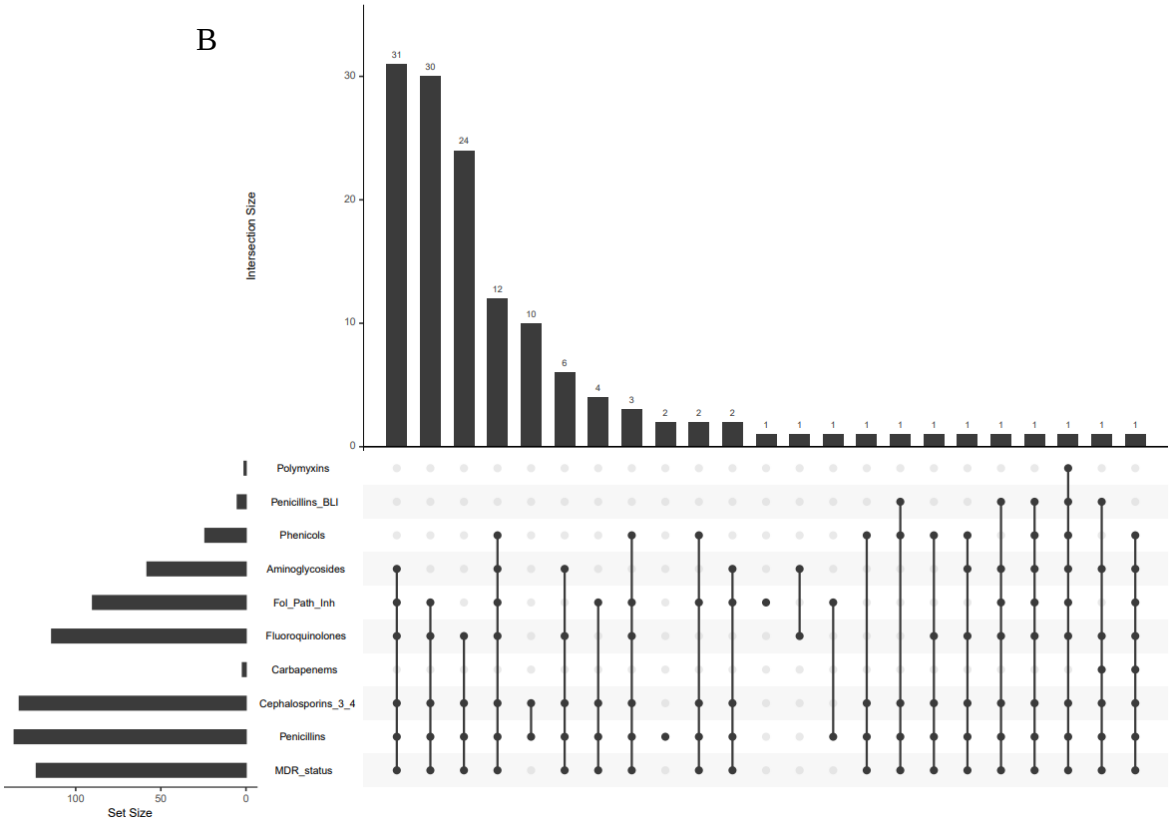


Figure 3.9. The phenotypic AMR pattern and MDR status of *E. coli* isolated from A) inpatients and B) outpatients.

Only the 42 most frequent combinations are shown. The black dots represent the phenotypic resistance to the given antimicrobial classes and/or an MDR phenotype. The vertical bars represent the number of isolates characterized by the AMR pattern described under the bar. The horizontal bars indicate the total number of isolates resistant to the antimicrobial classes, or MDR status.

3.5 Discussion

In this study, a high prevalence of ESBL positivity (≥50%) and MDR (≥89%) was found in the Enterobacteriaceae family (*E. coli*, *Klebsiella* spp., and *Enterobacter* spp.) isolated from the blood samples. A high prevalence of MDR in GNB was also reported in other studies from Nepal, however the ESBL positive prevalence that I observed in this study (50%; 1,080/2,153) was higher than the prevalence other studies have reported. A study from Nepal on *E. coli* causing potential community-acquired infections reported that 24% (48/200) of *E. coli* were ESBL positive, and 78% (156/200) were MDR [180]. Another hospital-based study from Nepal conducted on urine

isolates obtained from urinary tract infection (UTI) suspected patients undergoing haemodialysis in National Kidney Centre in 2014 showed that 27% (18/67) of *E. coli* were ESBL positive, and 97% (92/95) of Enterobacteriaceae bacteria (mostly *E. coli*, and *Klebsiella* spp.) were MDR [181]. For *Acinetobacter* spp., MDR proportion of 81% (314/382) was detected in this study. A high prevalence (>80%) of MDR in clinical isolates of *Acinetobacter* spp. has also been reported by other studies conducted in hospital ICUs in Nepal [182–184]. Specifically, of *Acinetobacter* spp. isolated from tracheal aspirates from ICU admitted patients admitted to a tertiary care neuro-hospital of Nepal between 2011 and 2012, 85% (47/55) were reported to be MDR [182]. Another study conducted on *Acinetobacter* spp. isolated from lower respiratory tract specimens obtained in microbiology laboratory of a tertiary hospital of Nepal reported that 95% (59/62) of isolates were MDR [183]. Alternatively, the ESBL prevalence of <1% that I found for *Acinetobacter* spp. (<1%) was much lower than the proportions ranging 12-13% reported by other studies on the isolates obtained from lower respiratory tract specimens of ICU admitted patients in tertiary hospitals of Nepal [182–184]. Corresponding to my study, a research conducted on *A. baumannii* isolated from tracheal aspirates of patients admitted to an ICU at a tertiary hospital of Dhaka, Bangladesh also reported that all 25 isolates tested negative for ESBL phenotypic confirmatory test [185]. In the context of South Asia, 79% (1,060/1,347) of *Acinetobacter* spp., 71% (3,048/4,312) of *Klebsiella* spp., and 54% (1,510/2,798) of *E. coli* isolated from the blood samples of young infants were estimated to be MDR [32]. ESBL positivity was confirmed in 42% (127/304) of *K. pneumoniae*, and 33% (98/298) of *E. coli* isolated from several hospitals in India [186], and 16% (179/1,114) in enteric and non-enteric GNB isolated from Bangladesh [187].

For the Enterobacteriaceae family bacteria, namely, *E. coli*, *Enterobacter* spp., and *Klebsiella* spp., the most frequently detected AMR genes were bla_{OXA}, bla_{TEM}, $bla_{\text{CTXM-1,}}$ and $bla_{\text{CTXM-8}}$. The finding was consistent with a study performed in a tertiary hospital in Nepal between 2012 and 2013, though the prevalence of ESBL genes was lower than I detected. A study conducted on

clinical isolates of *E. coli* obtained between June 2012 and January 2013 from ICU and OPD units of a hospital of Kathmandu, Nepal reported the detection of bla_{CTXM} gene in 24% (23/93), and bla_{TEM} in 8% (7/93) of isolates [188]. A study from Bangladesh reported $bla_{\text{CTXM-1}}$ (51%, 73/142) and bla_{SHV} (27%, 38/142) to be the most common ESBL genes in *Klebsiella pneumoniae* [189]. In Pakistan and India, bla_{OXA}, bla_{TEM}, bla_{CTXM}, and bla_{SHV} were detected to be common AMR genes in the Enterobacteriacea bacterial isolates, corresponding to my study findings [186,190]. For *Acinetobacter* spp., $bla_{\text{OXA-51}}$, $bla_{\text{OXA-23}}$, and $bla_{\text{NDM-1}}$were the most frequently detected AMR genes, as found by others studies reported from Nepal, India, and Bangladesh [185,191,192]. Specifically, of 44 *A. baumannii* isolated from various clinical specimens from a tertiary hospital of Nepal, 100% (44/44) of isolates tested positive for $bla_{\text{OXA-23}}$ gene and 14% (6/44) tested positive for $bla_{\text{NDM-1}}$ [191]. Similarly, a study conducted on clinical isolates of 75 *A. baumannii* obtained from five centres across India, 97% (73/75) isolates tested positive for $bla_{\text{OXA-23}}$ gene, while 17% (13/75) were positive for $bla_{\text{NDM-1}}$ [192]. While a study conducted on *A. baumannii* strains isolated from tracheal aspirates of patients admitted to ICU of a tertiary hospital in Dhaka, Bangladesh between July 2013 and June 2014 reported that 77% (17/22) and 18% (4/22) of isolates were positive for $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ genes respectively [185].

The phenotypic confirmatory tests used for the detection of ESBL positivity are labour intensive and often less sensitive when ESBLs with reduced susceptibility to ESBL inhibitors coexist in the isolates [171]. The automated systems exist, but the associated installation, running and, maintenance costs are often a barrier in the low resource settings. Alternatively, PCR can detect specific AMR genes with high sensitivity [172,193,194]. Further, the PCR assays can be multiplexed, decreasing the turnaround time. A better understanding of the association between the phenotypic and genotypic determinants of AMR is a prerequisite to evaluate the usefulness of genetic tests, such as PCR in clinical settings. In order to fulfil this gap, in this study, I sought to

assess the correlation between the detection of *bla*$_{CTXM}$, *bla*$_{TEM,}$ and *bla*$_{OXA}$ genes by PCR, and the ESBL positivity or MDR as detected phenotypically by the disc diffusion method.

The detection of *bla*$_{CTXM}$ gene was strongly associated with ESBL positivity (OR 14.2), MDR (OR 5.99), and resistance to 3rd and 4th generation cephalosporins (OR 27.3). The detection of *bla*$_{TEM}$ was strongly associated with ESBL positivity (OR 2.96), and MDR (OR 1.96). And finally, the detection of *bla*$_{OXA}$ was associated with ESBL (OR 1.3), and MDR phenotype (OR 2.3). The results suggested that the detection of *bla*$_{TEM}$, *bla*$_{CTXM}$ or *bla*$_{OXA}$ gene was an important index for MDR and ESBL phenotype in bacterial isolates.

In this study, the presence of each *bla*$_{CTXM,}$ *bla*$_{OXA}$, and *bla*$_{TEM}$ was associated with ESBL positivity. However, a significant number of isolates positive for each ESBL gene tested negative in phenotypic confirmatory test. Forty seven percent (604/1,278) of GNB positive for *bla*$_{OXA}$ were phenotypically ESBL negative. This is because of the reason that Ambler class D oxacillinase encoded by *bla*$_{OXA}$ is weakly inhibited by clavulanate, thus producing a negative result in the ESBL test. Similarly, 39% (545/1,363) of *bla*$_{TEM}$ positive GNB and 29% (401/1,369) of *bla*$_{CTXM}$ positive GNB isolates tested ESBL negative. It may be because of the reason that *bla*$_{TEM}$ gene detected by PCR encoded for a narrow spectrum TEM beta-lactamase, producing contrasting results between phenotypic and PCR tests. Further, other beta-lactamases and carbapenemases that are weakly inhibited or not inhibited by clavulanate may have been co-existed in the GNB isolates, thereby masking the detection of ESBL. Additionally, *Enterobacter* spp. and *Acinetobacter* spp., which together constituted of 41% (902/2,153) of all tested GNB are consistently associated with hyper-production of chromosomally encoded cephalosporinase (AmpC) which is weakly inhibited by clavulanate [171]. Also, *bla*$_{AmpC}$ gene has also been increasingly detected as plasmid-encoded gene in other GNB such *E. coli*, and *Klebsiella* spp. [195]. The coexistence of the ESBL genes and

bla$_{\text{AmpC}}$ in the bacterial isolates may have resulted into the observed discrepancy between genetic and phenotypic confirmatory test results for ESBL positivity.

In this study, I observed less diverse phenotypic AMR patterns among the isolates of each bacterial genus originating from ER and OPD (outpatients) compared to the isolates originating from the inpatients departments of the hospital. Such difference in the AMR pattern may reflect the differences in the use of antimicrobials between outpatients and inpatients. The inpatients may have been exposed to a wider array of antimicrobials in the hospital compared to the outpatients. As clinical-demographic information, such as prior exposure to hospital and/or health care institution, invasive medical devices, underlying comorbidities, and prior administration of antimicrobials were lacking, the possibility that the isolates originating from a subset of patients from ER/OPD could have been acquired from other hospitals could however not be excluded.

3.6 Conclusions

The MDR and the presence of ESBL, and carbapenemase encoding AMR genes in GNB pose a great problem in the therapeutic management of patients. The sub-optimal IPC practices and inadequate antimicrobial usage policies, together with a high burden of GNB possessing transferable AMR genes in resource limited settings set out an ideal scenario for the emergence and dissemination of MDR pathogens. In this extensive retrospective study, a high burden of MDR GNB possessing diverse ESBL, and carbapenemase resistance *bla* genes were observed. Further, this study signified that there was a high probability of GNB to be MDR and ESBL positive when any of *bla*$_{\text{TEM}}$, *bla*$_{\text{CTXM}}$ or *bla*$_{\text{OXA}}$ genes were detected. Additionally, the detection of *bla*$_{\text{CTXM}}$ genes strongly implied that the GNB were non-susceptible to the extended-spectrum beta-lactam antimicrobials.

Chapter 4

Risk factors for the development of neonatal sepsis in a neonatal intensive care unit of a tertiary care hospital of Nepal

4.1 Abstract

Sepsis is an overwhelming and life-threatening response to bacteria in the bloodstream, and a major cause of neonatal morbidity, and mortality. Understanding the aetiology, and potential risk factors for neonatal sepsis is urgently required, particularly in LMICs where the prevalence of infection is high, and the disease epidemiology is poorly understood. A prospective observational cohort study was conducted between April 2016 and October 2017 in a level three NICU at a tertiary care hospital in Nepal to determine the bacterial aetiology, and potential risk factors for neonatal sepsis. Fifteen percent (21/142) and 32% (46/142) of 142 NICU admitted neonates developed the blood culture-positive, and -negative sepsis, respectively. *K. pneumoniae* (34%, 15/44), and *Enterobacter* spp. (25%; 11/44) were the most prevalent isolates. The AMR of isolates to ampicillin (100%, 43/43), cefotaxime (74%, 31/42), and ampicillin-sulbactam (55%, 21/38) were the highest. Bla_{TEM} (53%,18/34), and bla_{KPC} (46%, 13/28) were the most common ESBL, and carbapenemase genes, respectively. In univariate logistic regression analysis, the odds of sepsis increased with each additional day of use of invasive procedures, such as mechanical ventilation (OR 1.086, 95% CI 1.008-1.170), umbilical artery catheter (OR 1.375, 95% CI 1.049-1.803), intravenous cannula (OR 1.140, 95% CI 1.062-1.225), blood transfusion events (OR 3.084, 95% CI 1.407-6.760), NICU stay (OR 1.109, 95% CI 1.040-1.182), and failure to breast feed (OR 1.130, 95% CI 1.060-1.205). Odds of developing sepsis also increased with leukopenia (OR 1.790, 95% CI 1.04-3.082), increase in C-reactive protein (OR 1.028, 95% CI 1.016-1.040), and thrombocytopenia (OR 0.992, 95% CI 0.989-0.994). In the multivariate analysis, increase in IV

cannula insertion days (OR 1.147, 95% CI 1.039 - 1.267), and increase in CRP level (OR 1.028, 95% CI 1.008 - 1.049) increased the odds of developing sepsis. My study indicated various nosocomial risk factors for neonatal sepsis, and underscored the need to improve local IPC measures so as to reduce the existing problem of sepsis. The findings also identified specific sepsis associated laboratory parameters, together with the identification of AMR genes, which can guide an early and optimal therapeutic management of sepsis. The findings of my study may be extrapolated to other LMIC settings in the region.

4.2 Introduction

Neonatal sepsis refers to the clinical syndrome of a systemic inflammatory response which develops secondarily to a proven or suspected infection in neonates [196]. While the incidence of sepsis ranges from 1-5 cases/1,000 live births in HICs, it is estimated to be at least 3 to 20 folds higher in LMICs [35]. In 2018, there were 2.5 million global neonatal deaths majorly occurring in Sub-Saharan Africa and South Asia; 15% of the deaths were associated with sepsis [197]. In Nepal, in 2015, 18.4% of 12,881 neonatal deaths were attributed to sepsis [198].

Immature immunity, PT birth, and VLBW make neonates vulnerable to infections. Majority of vulnerable newborns often require special clinical care, and admission to a NICU for improved survival. Ironically, the unique vulnerability of neonates, and hostile environmental factors of NICUs make the NICU admission an important risk factor for the development of HANS [41,199]. The studies have shown that NICUs have greater incidences of infection compared to the other neonatal units [41,199]. Various invasive modalities of therapeutic or supportive clinical care often used in NICUs, such as parenteral feeding, mechanical ventilation, and intravascular catheterization have been shown to be associated with increased risk of neonatal sepsis [41].

Most of the published studies from Nepal on neonatal sepsis are of cross-sectional and/or retrospective designs utilizing the routine microbiology or NICU data, and primarily addressing the bacterial and clinical features only. The longitudinal developmental studies aiming at rigorously observing the neonates throughout their stay in NICU provide unprecedented opportunities to collect real time data on the predisposing factors associated with neonatal sepsis, together with other valuable information, such as clinical-pathological characteristics, bacterial aetiology, and associated AMR. Understanding of such variables which are reflective of the local setting can provide crucial information required for an early diagnosis, and effective treatment, in addition to devising the best fitted preventive measures. Such longitudinal studies on neonatal sepsis have been scarcely reported from Nepal. Therefore, in this prospective cohort observational study, I aimed to fulfil the identified knowledge gaps by longitudinally observing the neonates admitted to the NICU in a tertiary care hospital in Nepal.

4.3 Methods

4.3.1 The study setting and study design

A prospective observational cohort study was conducted in a level three NICU at Patan Hospital. The eight bedded NICU had the provision of admitting the neonates who were born at Patan Hospital, and who required an intensive medical care. In the NICU unit, two beds were dedicated for providing an ICU care for the culture-positive neonates. The neonates who were born elsewhere were admitted to the PICU. The neonates without any risk factor for sepsis were admitted to a clean nursery, while the neonates having underlying risk factors for, or evidence of infection, were admitted to the septic nursery for an observation and non-intensive medical care.

4.3.2 An ethical approval

Before conducting the study, an ethical clearance was obtained from NHRC under the approval registration number of 278/2015, and from OxTREC under the approval registration number of 24-16 for the research entitled “Prevalence of ESBL producing Enterobacteriaceae and the probable risk factors of hospital acquired neonatal infections in the NICU of Patan hospital”.

4.3.3 The enrolment and follow up of the neonates

The parents/guardians of the neonates who were admitted to the NICU from April 2016 to October 2017 were approached for obtaining an informed consent so as to take part in the study. The neonates for whom written consents were obtained from their parents/guardians were enrolled in the study, and were followed up on a daily basis for the development of any signs of septicaemia during the stay in the NICU, or until one of the final outcomes (discharge, transfer to other department, death, or left against medical advice) was attained. The relevant data on clinical, therapeutic, and laboratory investigations were recorded in a pre-tested case report form (CRF) by referring to the clinical notes marked by the paediatrician. The data were recorded onto the CliRes Data Management System as described in methods section 2.5.

4.3.4 The inclusion and exclusion criteria

All neonates who were born at Patan hospital during a sampling period of 1.5 years from April 2016 to October 2017, requiring an admission to the NICU, and whose parents/guardians provided a written informed consent for participation were included in the study. At Patan hospital, among the neonatal units, the burden of infections had been specifically high only in NICU with recurrence of infection outbreaks with high case fatality rate. Therefore, only the NICU admitted inborne neonates were included in this study. The neonates presenting with gross congenital abnormalities, the neonates denying the consent, and the neonates who were readmitted to the NICU after being discharged from Patan hospital were excluded from the study.

4.3.5 The diagnosis of sepsis

The enrolled neonates were suspected of having sepsis based on the clinical signs, and/or the underlying risk factors as listed in the table 4.1. The relevant biological samples were collected from the sepsis suspected neonates to perform the sepsis screening tests as mentioned in the table 4.1 [200,201]. This was a part of routine clinical care of the hospital.

Table 4.1. The sepsis related clinical features, the sepsis risk factors, and the screening algorithm for neonatal sepsis (based on NICU protocol at Patan hospital).

The clinical features of neonatal sepsis	
Body temperature <35.5˚C or, >37.7˚C	Reduced digital capillary refill time
Respiratory rate >60 breaths/min	Movement only when stimulated
Difficulty in feeding or no feeding	Bulging fontanelle
Cyanosis	Mottled skin
Grunting	Lethargic
Severe chest in-drawing	
The risk factors for neonatal sepsis	
Maternal risk factors	**Foetal risk factors**
PPROM (>18 hours)	Low birth weight (<2.5 K.G)
Fever during late* pregnancy	Preterm (<37 weeks of gestation)
Urinary tract infection during late* pregnancy	Requiring instrumental delivery
Sepsis during late* pregnancy	Requiring perinatal suctioning
Multiple per vaginal examination (>3 times)	Requiring perinatal resuscitation
Chorioamnionitis	
Foul smelling vaginal discharge	
The sepsis screening algorithm	
Blood sample	**Abnormal value**
Total leukocyte count (TLC)	<7,000 /mm^3
Absolute neutrophil count (ANC)	Low counts as per Manroe chart [201]
Immature/total neutrophil	>0.2
C-reactive protein (CRP)	>6 mg/dl
Urine catheter sample	**Abnormal value**
WBC count (in centrifuged sample)	>5 cells/ high-power field

*late pregnancy refers to the third-trimester of pregnancy

The neonates who were clinically suspected of sepsis were treated with empirical antimicrobial therapy according to the neonatal treatment guidelines of Patan hospital (Table 4.2).

Table 4.2. The protocol on empirical antimicrobial therapy practiced in NICU at Patan hospital, and the duration of use of antimicrobials in the study.

Antimicrobials	**Protocol on empirical antimicrobial therapy**	**Neonates**		**Days of use**	
		Number	**%**	**Median**	**IQR**
Ampicillin	first line	129	90.8	6	4-8
Amikacin	first line	128	90.2	6	4-8
Cloxacillin	first line*	6	4.2	3.5	3-7.8
Cefotaxime	first line**	80	56.3	3	2-6.3
Chloramphenicol	second line	57	40.1	6	3-10
Ofloxacin	second line	60	42.3	6.5	3-10
Meropenem	third line[#]	37	26.1	7	7-11
Colistin	third line[#]	24	16.9	10	6-12.3
Clindamycin	third line[#]	19	13.4	6	3-9.5
Piperacillin-Tazobactam	third line[#]	18	12.7	7	4.8-9.8
Fluconazole	third line[#]	17	11.9	7	6-12
Vancomycin	third line[#]	12	8.5	7	5.3-8.5

*added when GPC were the suspected aetiology
**added when meningitis was suspected
[#] The third line empirical therapy constituted of a combination of meropenem and ofloxacin, or piperacillin/tazobacam and ofloxacin, or meropenem and vancomycin/linezolid, or cefepime and pipercillin/tazobactam, or meropenem and colistin.

According to the NICU protocol adopted by Patan hospital, the sepsis was diagnosed as culture-positive sepsis when the blood culture was positive in a patient with ≥1 clinical signs of sepsis as depicted in table 4.1. The culture-negative sepsis was diagnosed when ≥2 clinical signs of sepsis (Table 4.1) were present, but blood culture failed to produce positive culture results. The overall workflow for sepsis diagnosis has been outlined in the figure 4.1. The neonates who were not suspected of sepsis during the entire stay in the NICU were designated as the non-sepsis group.

The neonates who were earlier clinically suspected of sepsis, but eventually yielded the negative results for sepsis screen tests, and culture, together with clinical improvement, were ruled out of sepsis, and formed a part of the non-sepsis group. For the statistical analysis to identify the risk factors for sepsis, the non-sepsis group was taken as the control group against the blood culture-positive sepsis group.

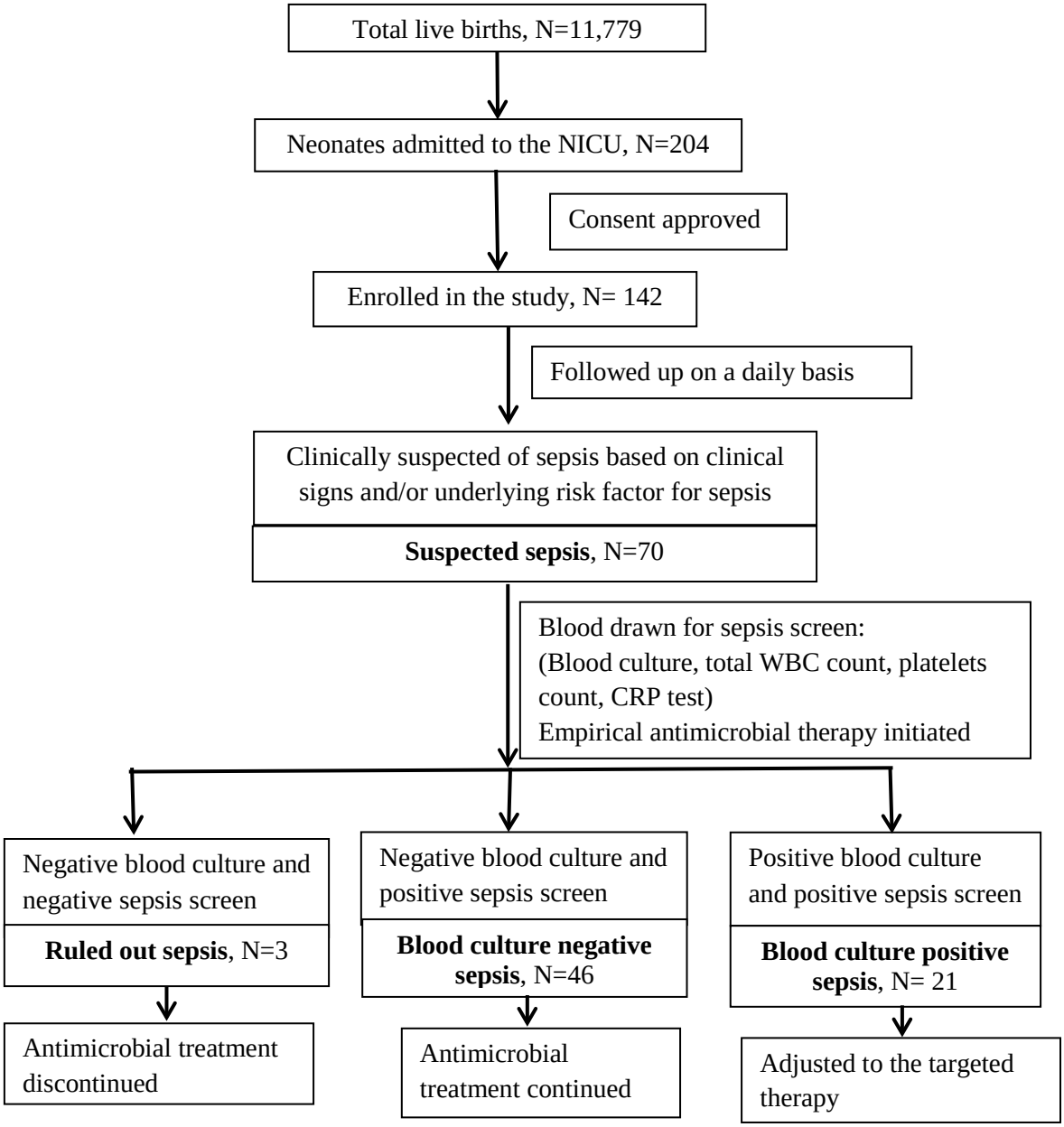


Figure 4.1. The workflow for the diagnosis and management of neonatal sepsis in the NICU.

The figure illustrates the course of events for the inborn neonates enrolled in the study for the identification of the risk factors of sepsis development at NICU.

4.3.6 The microbiological investigation of the blood sample

As a part of routine clinical care, 1-2 ml of peripheral blood samples were collected from the neonates clinically suspected of sepsis, and before the administration of antimicrobials, as per the protocol described in the methods section 2.6. The blood samples were microbiologically processed for the identification of bacterial pathogens as described in the methods section 2.6.1 and AST was performed as mentioned in the methods section 2.6.2, with the inhibition zone sizes interpreted according to the CLSI 2017 breakpoint guidelines [143]. For research, the AST was repeated for all bacterial isolates derived from the blood samples of enrolled neonates following the standard protocol as described in methods section 2.6.2. MDR was defined as an acquired non-susceptibility (without intrinsic resistance) to at least ≥ 3 of following antimicrobials (amikacin/gentamycin, ceftriaxone, ciprofloxacin, cotrimoxazole, meropenem, chloramphenicol, ampicillin-sulbactam/piperacillin-tazobactam). Additionally, I performed the phenotypic confirmatory test for ESBL positivity (methods section 2.7.1) and multiplex PCR for ESBL/carbapenemase AMR genes (methods section 2.7.2).

4.3.7 The statistical analyses

The statistical analyses were conducted in the SPSS (version 20.0). The descriptive statistics of qualitative variables were expressed in absolute frequency (N) with percentage (%). The descriptive statistics of quantitative variables was calculated as mean ± standard deviation (SD), or median with interquartile range (IQR). The OR with 95% confidence interval (CI) was calculated under univariate logistic regression analysis. The threshold of $p \leq 0.05$ was considered significant. The five independent variables which produced highly significant p-values (<0.001) in the univariate analysis were modelled using backward stepwise multiple logistic regression. In this process, only two variables remained significant at the final stage.

4.4 Results

4.4.1 The demographic features

From April 2016 to October 2017, there were a total of 11,779 livebirths at Patan hospital, 204 (2%) required an admission to the NICU, and 142 NICU-admitted neonates were enrolled in the study. The demographic features of the enrolled neonates are shown in the tables 4.3 and 4.4. Briefly, 59% (83/142) of 142 enrolled neonates were PT, 65% (93/142) were male, 16% (22/142) were of VLBW, and 15% (22/142) required an intubation immediately after birth. A majority (68%, 96/142) of deliveries were of caesarean type, and respiratory distress was the most common (55%, 78/142) clinical indication for admission. The use of various invasive and non-invasive devices of clinical care was frequent. The median duration of NICU stay and entire hospital stay were 7 days (IQR 4-12) and 16 days (IQR 10-26), respectively. The uses of antimicrobials during the study period are shown in table 4.2. At least 90% of 142 enrolled neonates received the first line antimicrobials (ampicillin and amikacin) while 56% and 26% of enrolled cases received cefotaxime and meropenem, respectively.

Table 4.3. The demographic summary of the neonatal and maternal features for the enrolled neonates shown by the absolute number and percentage, or median and inter-quartile range.

Neonatal features	**n or, median**	**% or, IQR**
Birth weight (grams)	2250	2300-2700
LBW	62	43.7
VLBW	22	15.5
Gestational age (weeks)	36	34.7-35
PT (<37 weeks of gestation)	83	58.5
Male	93	65.5
Obstetric and peri-partum features	**n or, median**	**% or, IQR**
PPROM	34	23.9
≥ 3 times of vaginal examination	10	7.0
Culture-positive UTI in the 3rd trimester	6	4.2

Culture positive high vaginal infection in the 3rd trimester	4	2.8
C-section delivery	96	67.6
Maternal post-partum fever	15	10.6
Meconium stained amniotic fluid	21	14.8
Intubation at birth	22	15.5
Admission indication	**n or, median**	**% or, IQR**
Respiratory distress	78	54.9
Grunting	55	38.7
Tachypnea	24	16.9
Respiratory acidosis	13	9.2
Birth asphyxia	11	7.7
Congenital pneumonia	3	2.1
Cyanosis	3	2.1
Persistent desaturation	2	1.4
Metabolic acidosis	2	1.4
Neonatal jaundice	2	1.4
Thrombocytopenia	2	1.4
Meconium aspiration syndrome	2	1.4
Congenital heart disease	2	1.4
Respiratory failure	1	0.7
Cardiovascular failure	1	0.7
Dehydration	1	0.7
Polycythaemia	1	0.7
Mottled skin	1	0.7
Pneumothorax	1	0.7

LBW: low birth weight, PPROM: prolonged premature rupture of the membrane, PT: preterm, VLBW: very low birth weight

Table 4.4. The summary of the laboratory investigations and the neonatal care for the enrolled neonates shown by the absolute number and percentage, or median and inter-quartile range.

Laboratory investigation features	**n or, median**	**% or, IQR**
Total WBC count in blood (X 10^3/µl)	10.9	7 - 15.9
Leukopenia (<7,000 WBC/µl)	92	24.3
Leukocytosis (>30,000 WBC /µl)	19	5.0
Total platelets count (X 10^3/µl)	191	124.5 - 265
Thrombocytopenia (<150,000 platelets/µl)	125	33.7
CRP level (mg/dl)	9	2.5 - 39
Elevated CRP (>6 mg/dl)	99	58.6
Insertion of invasive devices	**n or, median**	**% or, IQR**
Umbilical artery catheter	50	35.2
Umbilical vein catheter	59	41.5
Intravenous cannula	124	87.3
Mechanical ventilation	105	73.9
Duration of invasive device uses (days)	**n or, median**	**% or, IQR**
Umbilical artery catheter	6	5-8
Umbilical vein catheter	6	4-8
Intravenous cannula	11	7-16
Mechanical ventilation	5	3-10
Neonatal feeding features	**n or, median**	**% or, IQR**
Never breast fed	120	85
Never spoon fed	116	81.7
Enterally fed	117	82.4
Days not breastfed	7.5	4-12
Days not spoon-fed	8	4-12.2
Days enterally-fed	4	2-7
Outcome	**n or, median**	**% or, IQR**
NICU stay >7 days	65	45.8
Hospital stay >14 days	53	37.3
Total days of NICU stay	7	4-12
Total days of hospital stay	16	10-26
Dead	20	14.1

4.4.2 The prevalence and types of sepsis

Sepsis was clinically suspected in 49% (70/142) of the enrolled neonates; and the remaining 72 neonates didn't develop any signs of sepsis during their NICU stay. Fifteen percent (21/142) and 32% (46/142) of the enrolled neonates developed blood culture-positive and -negative sepsis, respectively. Three of the clinically suspected cases were later ruled out of sepsis, giving a total non-sepsis group of 75 neonates. The 21 culture positive neonates yielded a total of 44 blood culture-positive sepsis events. Nine cases developed a single sepsis episode, 3 neonates developed 2 culture-positive events, and rest 9 cases developed ≥3 culture-positive events during the study period. The multiple blood culture-positive events caused by the bacteria of different genus or, same genus but non-identical AST profile were considered as reinfection episode by new bacterial strains. Eighty percent (35/44) of culture-positive sepsis events were of LOS type. Fourteen percent (20/142) of the enrolled neonates died during the study period, 8 neonates who died had culture-positive sepsis.

4.4.3 The microbiological results

A total of 118 blood samples obtained from the 70 sepsis suspected neonates were microbiologically investigated during the study period as a part of routine clinical care at NICU. Forty four blood samples were culture positive, giving a blood culture-positivity proportion of 37% (44/118). The majority (89%, 39/44) of bacterial isolates were GNB, with *K. pneumoniae*, *Enterobacter* spp., and *Acinetobacter* spp. being the most common, constituting 34% (15/44), 25% (11/44), and 18% (8/44) of the total isolates, respectively. GPC were less common, with CoNS being the most prevalent (9%; 4/44) (Table 4.5). According to the NICU protocol at Patan hospital, the isolation of CoNS from neonatal blood samples was considered significant and the neonates were treated accordingly.

Table 4.5. The bacterial aetiology of neonatal sepsis isolated from blood culture shown by absolute number of isolation and percentage.

Bacterial isolates	**n**	**%**
GNB		
K. pneumoniae	15	34.1
Enterobacter spp.	11	25.0
Acinetobacter spp.	8	18.2
E. coli	3	6.8
K. oxytoca	1	2.3
Pseudomonas spp.	1	2.3
Total GNB	**39**	**88.6**
GPC		
CoNS	4	9.1
S. aureus	1	2.3
Total GPC	**5**	**11.4**
Total (All isolates)	**44**	

GNB: Gram-negative bacilli, GPC: Gram-positive cocci, CoNS: Coagulase-negative *Staphylococcus*

The results of AST and ESBL confirmatory tests for the bacteria isolated from the blood samples have been shown in the tables 4.6 and 4.7, respectively. In summary, 72% (31/43) of bacterial isolates were MDR. One hundred percent and 37% of isolates were resistant to ampicillin and amikacin, respectively. Further, 74% (31/42), 55% (21/38), and 34% (13/38) of the isolates were resistant to cefotaxime, ampicillin-sulbactam, and meropenem, respectively. In general, GNB were more susceptible to amikacin, ciprofloxacin, and cotrimoxazole, while less susceptible towards beta-lactam antimicrobials, except meropenem.

Table 4.6. The phenotypic MDR status and the AMR profile of the GNB and GPC bacteria isolated from the blood samples, shown by proportion (percent).

GNB	**MDR**	**AMK**	**GEN**	**CTX**	**CHL**	**CIP**	**TS**	**MEM**	**SAM**	**PTZ**
Klebsiella spp., 16	12/16 (75)	8/16 (50)	8/16 (50)	13/16 (81)	3/16 (19)	8/16 (50)	8/16 (50)	4/16 (25)	10/16 (63)	5/16 (31)
Enterobacter spp., 11	9/11 (82)	1/11 (9)	3/11 (27)	8/11 (73)	8/11 (73)	2/11 (18)	2/11 (18)	6/11 (55)	5/11 (45)	4/11 (36)
Acinetobacter spp., 8	5/7 (71)	3/7 (43)	3/7 (43)	6/7 (86)	6/7 (86)	3/7 (43)	4/7 (57)	3/7 (43)	4/7 (57)	2/7 (29)
E. coli, 3	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	1/3 (33)	0/3 (0)
P. aeruginosa, 1	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)	1/1 (100)	0/1 (0)
Total for GNB	**27/38 (71)**	**13/38 (34)**	**15/38 (39)**	**28/38 (74)**	**18/38 (47)**	**14/38 (37)**	**15/38 (39)**	**13/38 (34)**	**21/38 (55)**	**11/38 (29)**
GPC	**MDR**	**AMK**	**GEN**	**CTX**	**CHL**	**CIP**	**TS**	**ERY**	**CLI**	**OXA**
S. aureus, 1	1/1 (100)	1/1 (100)	1/1 (100)	1/1 (100)	0/1 (0)	1/1 (100)	0/1 (0)	1/1 (100)	1/1 (100)	1/1 (100)
CoNS, 4	3/4 (75)	2/4 (50)	3/4 (75)	2/3 (67)	1/4 (25)	3/4 (75)	1/1 (100)	3/3 (100)	0/3 (0)	2/3 (67)
Total for GPC	**4/5 (80)**	**3/5 (60)**	**4/5 (80)**	**3/4 (75)**	**1/5 (20)**	**4/5 (80)**	**1/2 (50)**	**4/4 (100)**	**1/4 (25)**	**3/4 (75)**
Total for GNB and GPC	**31/43 (72)**	**16/43 (37)**	**19/43 (44)**	**31/42 (74)**	**19/43 (44)**	**18/43 (42)**	**16/40 (40)**			

AMK: Amikacin, GEN: Gentamycin, CTX: Cefotaxime, CHL: Chloramphenicol, CIP: Ciprofloxacin, TS: Cotrimoxazole, MEM: Meropenem, SAM: Ampicillin-Sulbactam, PTZ: Piperacillin-Tazobactam, ERY: Erythromycin, CLI: Clindamycin, OXA: Oxacillin
All GNB and GPC isolates were resistant to ampicillin.

Twenty one percent (7/34) of GNB isolates were phenotypically confirmed to be ESBL positive, 40% (6/15) of ESBL positive isolates being *Klebsiella* spp. (Table 4.7). Overall, bla_{TEM} (53%, 18/34) and bla_{KPC} (46%, 13/28) were the most common ESBL, and carbapenemase genes, respectively in the

GNB isolates. Ninety four percent (14/15) and 47% (7/15) of *Klebsiella* spp. had bla_{TEM} and $bla_{\text{NDM-1}}$ respectively. Similarly, 73% (8/11) of *Enterobacter* spp. had $bla_{\text{KPC,}}$ and 83% (5/6) of *Acinetobacter* spp. had $bla_{\text{OXA-51}}$ resistance genes. All bacterial isolates of neonatal sepsis tested negative for bla_{IMP} and bla_{VIM} carbapenemase genes.

Table 4.7. The results of bacterial isolates of neonatal sepsis for the phenotypic and genetic confirmatory tests for ESBL and carbapenemase positivity, shown by proportions (percentage).

Bacterial isolates[d]	**Phenotypic test**		**PCR test**						
	ESBL	**AmpC**	***bla*$_{\text{TEM}}$**	***bla*$_{\text{CTXM}}$**[b]	***bla*$_{\text{SHV}}$**	***bla*$_{\text{NDM}}$**	***bla*$_{\text{OXA}}$**	***bla*$_{\text{KPC}}$**	***bla*$_{\text{OXA-48}}$**
Klebsiella spp.	6/15 (40)	1/15 (7)	14/15 (94)	7/15 (47)	5/15 (33.4)	7/15 (47)	6/15 (40)	5/15 (33)	7/15 (46.7)
Enterobacter spp.	1/11 (9)	1/11 (9)	4/11 (36)	2/11 (18)	0/11 (0)	0/11 (0)	4/11 (36)	8/11 (73)	3/11 (27.2)
Acinetobacter spp.[c]	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	NA	NA
Total	**7/34 (21)**	**`2/34 (6)**	**18/34 (53)**	**9/34 (26)**	**5/34 (15)**	**7/34 (21)**	**10/34 (29)**	**13/28 (46.4)**	**10/28 (36)**

[a]All tested isolates were negative for bla_{IMP} and $bla_{\text{VIM.}}$
[b]All tested isolates were negative for other bla_{CTXM} genes: $bla_{\text{CTXM-2}}$, $bla_{\text{CTXM-8}}$, $bla_{\text{CTXM-9}}$, and $bla_{\text{CTXM-25,}}$ except for $bla_{\text{CTXM-1}}$
[c] For *Acinetobacter* spp., a PCR tests for $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-58}}$ was also performed. 83% (5/6) Acinetobacter spp. isolates tested positive for $bla_{\text{OXA-51}}$, and for rest other bla_{OXA} genes, all isolates tested negative.
[d]Both *E. coli* isolates were negative for all AMR genes tested
NA, not tested

4.4.4 The risk factors for neonatal sepsis in NICU

The univariate logistic regression was used to estimate the odds of sepsis development for each potential exposure factors of neonatal and maternal origins (Table 4.7), and laboratory and clinical care origins (Table 4.8). The analysis was based on the specific variable values between the non-sepsis (N=75) and culture-proven sepsis cases (N=21).

The majority of factors for which the OR for sepsis occurrence was statistically significant were found to be related to the nosocomial exposures. Every single day of increased use of various invasive devices, such as mechanical ventilation (OR 1.086, 95% CI 1.008-1.170), UAC (OR 1.375, 95% CI 1.049-1.803), UVC (OR 1.325, 95% CI 1.047-1.676), IV cannula (OR 1.140, 95% CI 1.062-1.225), and every additional blood transfusion events (OR 3.084, 95% CI 1.407-6.760) were associated with an increased odds of sepsis development in NICU. Additionally, every additional day of stay in NICU, and hospital also increased the risk of sepsis development with OR of 1.109 (95% CI 1.040-1.182), and 1.097 (95% CI 1.031-1.167), respectively. Similarly, with every single day increase in failure to feed orally (breast and spoon feeding) increased the risk of neonatal sepsis with the OR of 1.130 (95% CI 1.060-1.205) and 1.140 (95% CI 1.064-1.222) respectively. In laboratory parameters, when compared to the normal WBC count range (7000-30,000/ µl), leukopenia (<7000 WBC/µl) was associated with an increased odds of sepsis (OR 1.790, 95% CI 1.04-3.082). Similarly, decrease in platelets count, and increase in CRP level also raised the odds of sepsis with OR of 0.992 (95% CI 0.989-0.994), and 1.028 (95% CI 1.016-1.040), respectively. In multivariate logistic regression analysis, the increase in IV cannula insertion days (OR 1.147, 95% CI 1.039 - 1.267), and CRP level (OR 1.028, 95% CI 1.008 - 1.049) increased the odds of sepsis.

Table 4.8. The univariate logistic regression analysis on obstetric and neonatal factors for determining the OR for neonatal sepsis.

Variables	Values*, mean (sd) or frequency (%)		Logistic regression analysis		
	Culture-positive neonates	**Non-Sepsis neonates**	**Odds ratio**	**95% CI**	**p-value**
Univariate analysis					
Obstetric-peripartum factors					
Maternal age, years	29.2 (sd 5.5)	27.6 (sd 4.9)	1.062	0.964-1.168	0.221
Vaginal test, times	0.7 (sd 0.5)	0.8 (sd 0.9)	0.955	0.595-1.53	0.848
PPROM:					
Absence	15 (70%)	60 (80%)	1	Reference	
Presence	6 (30%)	15 (20%)	1.714	0.564-5.208	0.342
Delivery mode:					
Normal	5 (24%)	17 (23%)	1	Reference	
Vaginal/assisted	2 (9%)	8 (11%)	0.85	0.135-5.366	0.863
C-section	14 (67%)	50 (67%)	0.952	0.298-3.037	0.934
Amniotic fluid color:					
Clear	20 (95%)	63 (84%)	1	Reference	
Meconium stained	1 (5%)	12 (16%)	0.263	0.032-2.158	0.214
Resuscitation at birth:					
No	16 (76%)	60 (80%)	1	Reference	
Yes	5 (24%)	15 (20%)	1.25	0.395-3.958	0.704
Neonatal factors					
Birth weight, grams:					
>2500 (Normal)	6 (29%)	35 (47%)	1	Reference	
1500-2500 (Low)	10 (47%)	31 (41%)	1.882	0.613-5.777	0.269
<1500 (Very low)	5 (24%)	9 (12%)	3.241	0.803-13.072	0.098
Gender:					
Female	9 (43%)	26 (35%)	1	Reference	
Male	12 (57%)	49 (65%)	0.707	0.264-1.897	0.492

* variable values for each group are shown, CI: confidence interval, sd: standard deviation, %: percentage

Table 4.9. The univariate logistic regression analysis on the factors related to laboratory parameter and clinical care for determining the OR for neonatal sepsis.

Variables	**Values*, mean (sd) or frequency (%)**		**Logistic regression analysis**		
	Culture-positive neonates	**Non-Sepsis neonates**	**OR**	**95% CI**	**p-value[#]**
Univariate analysis					
Laboratory factors					
WBC count (x10^3/µl):					
Normal (7-30)	12.5 (sd 6.2)	14.7 (sd 6.2)	1	Reference	
Leukopenia (<7)	4.8 (sd 6.2)	4.9 (sd 6.4)	1.79	1.040-3.082	**0.036**
Leukocytosis (>30)	32 (sd 13.8)	41.8 (sd 10.2)	0.154	0.019-1.216	0.076
Platelets (x10^3/µl)	129 (sd 130)	234 (sd 114.7)	0.992	0.989-0.994	**0.000**
CRP level (mg/dl)	72.0 (sd 72.5)	14.8 (sd 28.1)	1.028	1.016-1.040	**0.000**
Therapeutic/supportive care factors					
Transfusion, times	4.7 (sd 4.1)	1.5 (sd 0.6)	3.084	1.407-6.76	**0.005**
UAC insertion, days	26.5	17.6	1.375	1.049-1.803	**0.021**
UVC insertion, days	31.2	19.9	1.325	1.047-1.676	**0.019**
IV cannula, days	19.6 (sd 8.4)	11.1 (sd 6.8)	1.14	1.062-1.225	**0.000**
Intubation, days	11 (sd 8.1)	6.6 (sd 6.2)	1.086	1.008- 1.170	**0.030**
NG tube insertion, days	2.7	2.2	1.07	0.972-1.177	0.168
Feeding factors					
Not breast fed, days	17.1 (sd 9.6)	8.6 (sd 6.5)	1.13	1.060-1.205	**0.000**
Not spoon fed, days	16.7 (sd 8.5)	8.5 (sd 6.4)	1.14	1.064-1.222	**0.000**
Outcome					
NICU stay, days	16.1 (sd 9.3)	8.9 (sd 6.7)	1.109	1.040-1.182	**0.002**
Hospital stay, days	23.9 (sd 10.1)	14.9 (sd 9.1)	1.097	1.031-1.167	**0.004**
Final outcome					
Survived			1	Reference	
Dead	4 (22%)[##]	12 (18%)	1.333	0.369-4.817	0.661
Multivariate analysis**					
IV cannula insertion, days			1.147	1.039-1.267	**0.006**
CRP level (mg/dl)			1.028	1.008-1.049	**0.006**

* variable values for each group are shown, [#]significant p-values (<0.05) are shown in bold fonts, **the final significant results of multi-variate regression are also shown, [##]of four culture positive neonates died, three died as consequences of severe sepsis (such as, intraventricular haemorrhage with disseminated intravascular coagulation, septic shock with bilateral pneumothorax and purpura fulminans) while one neonate had necrotizing enterocolitis and surgical abdomen with malrotation as competing risk of death)**,** CI: confidence interval, sd: standard deviation, %: percentage,

4.5 Discussion

In this prospective longitudinal study of sepsis on the neonates admitted to NICU, I found a prevalence of culture-positive sepsis to be 15% (21/142), and that for culture-negative sepsis to be 32% (46/142). Eighty percent (35/44) of culture-positive sepsis events were of LOS type, and 89% (39/44) of culture-positive sepsis episodes were caused by GNB, with *K. pneumoniae* being the most common (34%, 15/44) isolates. Seventy two percent (31/43) of bacterial isolates were MDR, while 53% (18/34) and 46% (13/28) of GNB tested positive for bla_{TEM} and bla_{KPC} genes, respectively. Further, I found that every additional day of insertion of various invasive devices, failure to oral feeding, and stay in NICU and hospital increased the odds of sepsis development.

The prevalence of neonatal sepsis varies spatiotemporally, depending on differences in the local epidemiology of sepsis. The prevalence of culture-positive neonatal sepsis reported from various neonatal units in South Asia was found to vary widely ranging from 6% to 57%, compared to 15% in my study [202–206]. The underlying differences in the study design, and definitions might also have significant role in the reported differences in the sepsis prevalence. I found a majority (80%; 35/44) of culture-positive episodes to be of LOS type, suggesting the postnatal horizontal acquisition of infection from the surrounding environment being a main potential mode of infection in NICU, as corresponding to the results from other Asian countries [207,208]. Alternatively, few studies from Pakistan and Bangladesh have reported a higher prevalence of EOS compared to the LOS episodes. Such predominance of EOS events may suggest for the vertical transmissions, or early horizontal infections during the birthing, instead, being the major potential mode of disease transmission in the specific South Asian countries [203,209].

I found that GNB were the most common (87%, 39/44) causes of EOS and LOS in neonates admitted to the NICU, which corresponded to the findings in other LMICs [32,210]. *K. pneumonia*

was the most common isolate (34%, 15/44) in this study, similar to the observations reported in other NICUs [209,210]. In fact, *K. pneumoniae* had been a predominant and a persisting aetiological agent of sepsis in NICU at Patan hospital, being associated with several outbreak events [73,74]. An increasing AMR in clinical Enterobacteriaceae family isolates is a global public health threat [211]. Because of an extensive use of broad spectrum antimicrobials, the NICUs are likely to play a major role in the emergence and spread of MDR organisms [212]. In my study, 72% (31/43) of isolates were MDR, and a higher proportion of GNB isolates were resistant to third generation cephalosporin (74%; 31/42), and meropenem (34%, 13/38), as reported in the NICUs of LMICs [17,32,213]. The higher proportion of neonatal sepsis causing GNB being resistant to meropenem was in contrary to the results reported in the studies from Southeast Asia [207], Africa [214], and Latin America [215], where <10% of GNB were reported to be resistant to carbapenems, despite higher proportion of resistance to third generation cephalosporins. I found 53% (18/34) and 46% (13/28) of GNB isolates of neonatal sepsis testing positive for bla_{TEM} and bla_{KPC} genes, similar to the results reported in some Indian studies [213,216,217]. The β-lactam antimicrobials have been a mainstay for the therapeutic management of sepsis. A higher proportion of MDR phenotypes of GNB possessing AMR genes conferring potentially transferable resistance to all available β-lactams, portrayed a formidable therapeutic challenge for the treatment of neonatal sepsis.

In my study, ≥80% (35/44) of sepsis episodes were potentially acquired from the NICU at Patan hospital, because the neonates had no apparent signs of infection at the time of NICU admission, all clinical sepsis were detected > 48 hours of NICU admission, and only the inborne neonates who had never been discharged from Patan hospital after birth were enrolled in the study. Further, >80% of sepsis episodes being developed lately >72 hours of birth, these observations strongly suggested that the infections were acquired horizontally from the environment during their stay in

NICU. During the study period, because of the logistical constrains of the hospital, a physically separated NICU unit was not available to isolate the culture-positive cases. Although two beds in the NICU were dedicated for the care of culture-positive neonates, being within the same NICU unit, the sharing of the human and medical resources could not be avoided. An aseptic cleaning of the medical devices requiring sharing between the neonates was practiced as much as possible. However, an absolute seclusion of an infected neonate might not have achieved, leading to massive cross-infection events from infected to non-infected neonates. Therefore, during the study period, the prevalence of infection was high, and a majority of infections were potentially horizontally acquired. Such events of cross-infection between admitted neonates in NICU could be a common scenario in NICUs, specifically in several resource strapped hospitals of LMICs [218].

Every single day increase in the use of invasive devices, such as IV cannula, central vascular lines (UAC, UVC), and every additional event of blood transfusion were found the increase the odds of sepsis occurrences by the OR of 1.14, 1.37, 1.32, and 3.08, respectively. The use of umbilical catheterization [41,219,220], and mechanical ventilation [199,220] were significantly associated with sepsis in other studies also. While the invasive procedures are the integral components of neonatal care in NICUs, such life-sustaining devices may often simultaneously serve as portals of systemic infections, as observed in my study. My findings therefore emphasize the need to strengthen the local IPC measures, such as hand hygiene, and aseptic placement, and maintenance procedures for the invasive devices. Additionally, the removal of invasive devices whenever possible to reduce total dwelling time can be another preventive measure for reducing the prevalence of sepsis. I also found that every additional day of stay in the NICU, and hospital increased the risk of sepsis development with OR of 1.109, and 1.097, respectively, as observed in other studies [41,118]. An increased stay in NICU, and other hospital wards invariably raises the exposure to various nosocomial risk factors, such as handling, and device use, thereby increasing

the occurrence of sepsis leading to increased health complications and cost. Therefore, it is suggested that an unnecessarily protracted NICU or hospital stay for the neonates should be avoided as far as possible.

This study is one of few prospective longitudinal studies on neonatal sepsis from Nepal. Additionally, while majority of published studies lacked the results on the genetic characterization of AMR genes in the bacterial aetiology, which has been investigated in my study. Given an increasing prevalence of drug resistant nosocomial pathogens in NICUs, it becomes imperative to investigate pathogens for the presence of crucial AMR genes such as those encoding for ESBL and carbapenemase so as to aid the hospital unit in taking a precautionary care to limit the chances of cross-infection events to the other patients. Further, the ability to convince the hospital administration to build a physically separated isolation room with the NICU facility (NISO, Neonatal ICU isolation room) based on the results of this study was one of the greatest achievements of my study. Since after the operation of the NISO room, which was developed soon following this study for handling the culture-positive neonates only, the incidence of neonatal sepsis in NICU reduced significantly.

My study had few limitations. I might not have captured a complete picture on the epidemiology and transmission dynamics of neonatal sepsis, because the daily observation of the enrolled neonates was limited to the NICU only, irrespective of the further destiny of the neonates. A majority (86/142) of enrolled neonates were directly admitted to the NICU within few hours of birth. Depending on their prior clinical course, the remaining neonates were, however, admitted to the NICU after the stay in nursery or mother's ward. Although the neonates did not have any apparent signs of infection at the time of NICU admission, I could not test for any incubating infection, and therefore it may be difficult to confirm that all of the observed infections were

acquired solely from the NICU. Further, several studies have underscored the role of asymptomatic colonization of GNB on non-sterile body sites, including the gut of neonates in the causation of infection [221,222]. In this study, the neonates were not screened for the carriage of drug resistant nosocomial pathogens at NICU admission and therefore, some infections might have been occurred as a result of earlier colonization rather than from NICU acquisition.

Further, during this study, in a span of three-month period (July 2016 to September 2016), a fatal infection outbreak due to *Enterobacter* spp. was evidenced in the NICU. There was a frequent turnover of nursing staffs in NICU during this period, which may have resulted in breaches of existing IPC measures contributing to the observed infection outbreak. Immediately following an outbreak, a multi-disciplinary approach was followed to limit the cross-transmission of infection. The preventive strategy included staff training, an extensive environmental decontamination, strict implementation of preventive measures, such as reinforcement of standard hand hygiene practice followed by a periodic audit, a standard aseptic protocol for all invasive procedures, allocation of all daily care essentials such as thermometer, stethoscope, and measuring tape for individual neonates by the cot side, the use of disposable ventilator circuit, a proper decontamination of all equipment before being shared with others, a fortnightly high dusting, and a fumigation after any suspicious outbreak. The implementation of these preventive measures may have affected the results of risk factor analysis in the study. I found that the use of various invasive devices such as, UAC (OR 1.375), UVC (OR 1.325) central lines, IV cannula (OR 1.14), and NG tube (1.07) during the entire study period significantly increased the odds of sepsis development. However, the odds ratio values for the variables were small, indicating weak associations. The implementation of several IPC measures following an outbreak might have impacted the observed odds ratio values. Also, several factors such as, preterm birth[41,223,224], low birth weight [199,224], and PPROM[225] that were well established as significant risk factors of neonatal

sepsis in other studies failed to show significant association in my study. A smaller sample size in my study might have compromised the statistical power of the analysis. A study with larger sample size either by prolonging the study period or including multiple centres would have identified further risk factors with stronger odds ratio values.

4.6 Conclusions

The study determined the prevalence, demographic, clinical, and laboratory parameters, the bacterial aetiology, the phenotypic and genetic profile of AMR, and the risk factors for the NICU-linked neonatal sepsis in the low resource setting of Nepal. A high prevalence of neonatal sepsis, and associated AMR were documented. Further, it was evidenced that a majority of sepsis episodes were LOS, and were hospital acquired with the odds of sepsis being raised with a prolong insertion of several invasive devices of neonatal care. Such findings can aid in an early identification of high-risk neonates, and in selecting an optimal antimicrobial therapy in the similar settings. The findings also emphasized on the need to conduct infection surveillance, and improve IPC measures. The accommodation of an extensive environmental investigation to pinpoint the source of infection was warranted in the further studies.

Chapter 5

The molecular epidemiological investigation on the fatal outbreak of neonatal sepsis caused by colistin resistant *Enterobacter kobei* in a tertiary care in Nepal

5.1 Abstract

Enterobacter spp. is an emerging cause of nosocomial outbreaks in NICU. In mid-2016, over a period of three months, the neonatal units, primarily the NICU at a tertiary care hospital in Nepal encountered a fatal outbreak of neonatal sepsis, where 48% (11/23) of in-borne neonates were concomitantly infected of *Enterobacter kobei*, and 43% (10/23) were died, of which, 5 deaths were because of an exacerbated sepsis related to *E. kobei*. The core genome based phylogenetic analysis of WGS data revealed that of 10/11 non-duplicated *E. kobei* isolates originating during this period were clonal, and carried an identical array of limited AMR genes (bla_{AmpC}, *fosA2* and *mcr-10*), and virulence genes (*fliA* and *mrkA*). The subsequent reinforcement of strict IPC measures, including an establishment of a physically separated isolation unit for a secluded care of culture-positive cases, successfully terminated the outbreak. This study provided a highly resolved genomic evidence of an infection outbreak caused by *E. kobei*, and underscored the need to implement stringent infection control preventive measures to break the infection transmission chain so as to prevent further outbreak events. Additionally, for the first time, I report the detection of novel mobile colistin resistance genes *mcr-10* in neonatal sepsis causing *E. kobei*.

5.2 Introduction

Neonates with critical health conditions often require specialized hospital care in NICUs. The vulnerable nature of neonates, and the routine use of invasive medical procedures make NICUs prone to infection outbreaks, especially when IPC measures are comprised [40,84,226]. *Enterobacter* spp. is an important opportunistic pathogen commonly associated with outbreaks of infection in NICUs in HICs and LMICs [123,124,226–228]. The outbreaks are often associated with high morbidity and mortality in neonates [84,226].

There were several reports of recurrent NICU outbreaks with high mortality in Nepal [73,74,124,140]. The results of chapter 4 indicated that the NICU infections were potentially hospital-acquired, associated with increased invasive procedures, and driven by compromised IPC measures, including the lack of isolation of neonates with culture-positive sepsis [229]. Additionally, several studies have underscored the role of asymptomatic carriage of drug resistant nosocomial pathogens in various non-sterile body sites including the gut of neonates in the causation of sepsis [221,222]. The lack of screening of the neonates for such carriage at admission and periodically thereafter, followed by the lack of isolation or cohorting of the colonized neonates also serve an important role in the infection outbreaks [230,231]. WGS analysis plays an important role in outbreak investigation by providing the highest genetic resolution data to determine the transmission dynamics of infections. In the study, I aimed to perform WGS on a collection of *Enterobacter* spp. isolated from outbreak events that occurred between 2016 and 2017 in an NICU of a tertiary care hospital in Nepal to provide an evidence for the trajectory of the outbreak.

5.3 Methods

5.3.1 The study setting

I performed WGS on all *Enterobacter* spp. isolated from the blood of septic neonates admitted to the NICU at Patan hospital between May 2016 and December 2017. To obtain a complete overview of transmission of neonatal sepsis caused by *Enterobacter* spp., I further traced any additional isolation of *Enterobacter* spp. in the microbiology laboratory that were originated from blood culture of the enrolled neonates after the discharge from NICU to other neonatal units. The post NICU discharge isolates, if any, were also subjected to WGS.

5.3.2 An ethical approval

The *Enterobacter* spp. isolates recovered from the blood cultures of the NICU admitted neonates that were investigated in chapter 5 were a subset of isolates of chapter 4 of my thesis. Therefore, this study was conducted under the same ethical approval of chapter 4. An ethical approval for conducting chapter 4 was obtained from NHRC under the approval registration number of 278/2015, and from OxTREC under the approval registration number of 24-16 for the research entitled “Prevalence of ESBL producing Enterobacteriaceae and the probable risk factors of hospital acquired neonatal infections in the NICU of Patan hospital”.

5.3.3 The microbiological investigation

As a part routine clinical care, 1 to 2 ml of peripheral blood sample was collected from the sepsis suspected neonates for performing a blood culture, as described in the methods section 2.5. The microbiological processing for bacterial isolation, identification, and AST were performed, as described in the methods section 2.6. The GNB that were lactose-fermenting, motile, indole-negative, urease-negative, citrate-positive, hydrogen sulfide non-producing, and giving acid/acid reaction with gas production in the triple sugar iron test were presumptively identified as *Enterobacter* spp. The pure culture colonies of identified *Enterobacter* spp. were preserved at -80°C as a routine process.

Additionally, several environmental swab samples were collected routinely every fortnightly and additionally during the outbreak events. After moistening in sterile normal saline, a sterile cotton swab was horizontally moved over an environmental surface for 10 to 30 seconds. The swab was then transported to the microbiology laboratory at room temperature to be processed for routine microbiological investigation. If microbiologically significant results were reported, the given area was subjected to high dusting and was re-evaluated by resampling.

5.3.4 The whole genome sequencing (WGS)

The WGS of 18 *Enterobacter* spp. isolates was performed, as described in the method section 2.8. Briefly, the bacterial genomic DNA was extracted using the Wizard Genomic DNA Extraction Kit (Promega Corporation, Madison, USA), as described in the method section 2.8.1. The dual index-tagged pair-ended pooled DNA libraries were prepared, and WGS was performed on the HiSeq X Ten platform (Illumina, California, USA) to generate 150 bp paired-end reads, as described in methods section 2.8.3. The raw Illumina reads were deposited in European nucleotide archive (ENA) under the accession numbers as described in the Appendix table 9.5.

5.3.5 The bacterial conjugation experiment

The bacterial conjugation experiment was performed between *E. kobei* STNF isolates, and an *E. coli* J53 recipient (sodium azide resistant) by combining equal volumes (5 mL) of an overnight Luria-Bertani (LB) cultures. The bacteria were conjugated for 12 hours in LB broth at 37^{o}C, and the *E. coli* transconjugants were then selected on MacConkey agar containing 100 mg/l of sodium azide, and 6 mg/l of ceftriaxone. The conjugation frequency was calculated as the number of the transconjugants per recipient cell. The AST was performed on *E. coli* J53 trans-conjugants to confirm the transfer of plasmid-mediated AMR genes.

5.3.6 The genomic analysis

The genomic analysis was performed as per the bioinformatics pipeline, as described in the methods section 2.8.4.

5.3.6.1 The taxonomic assignment

A genomic-based average nucleotide identity (ANI) tool had been recently used to provide an updated taxonomy of *Enterobacter* genus. ANI was defined as mean nucleotide identity of all orthologous genes shared between two microbial genomes [232]. In this study, a fast approximate ANI tool, called MASH [232] was used to generate a pairwise ANI-based distance matrix between *Enterobacter* isolates of this study, and a recently published reference collection of 22 *Enterobacter* species [81]. The pairwise ANI with a cut-off of 96% was used to identify the *Enterobacter* species with precision.

5.3.7 The phylogenetic analysis

I sought to reconstruct the phylogenetic relationships between the potential NICUoutbreak causing *E. kobei* isolates. For this, all trimmed reads of 11 non-replicated *E. kobei* STNF isolates, potentially causing an outbreak in the NICU were mapped against the reference genome of *E. kobei* strain C16 (accession number: CP042578), using the RedDog pipeline v1.10b (https://github.com/katholt/RedDog). The RedDog used the Bowtie2 v2.2.3 to map all raw reads, and the SNPs were identified with SAMTools v1.3.1 [233]. The high-quality SNPs were then extracted using a standard approach, removing the low confidence alleles with base quality < 20, and < 5 supporting reads) [234]. Next, the Gubbins v1.4.5 was used to remove any recombinant regions from the resulting alignment file, and the SNPs identified in those regions were subsequently removed [233]. The final alignment resulted into 13 SNPs. Finally, the SNP alignment was used to construct a ML phylogenetic tree using IQ-TREE v1.4.4 [164], utilising the

best-fit evolutionary model model (F81+ASC) based on the Bayesian Information Criterion in jModelTest. The support for the ML tree was assessed via 100 pseudo-replicates.

5.4 Results

5.4.1 The temporal description of *Enterobacter* sepsis outbreak in NICU

During the study period of May 2016 to December 2017, a total of 18 *Enterobacter* spp. were isolated from the septic neonates admitted in the NICU. Fourteen isolates obtained from the eleven neonates were genomically identified to be *E. kobei*, whereas 2 isolates were found to be *E. cloacae*, and 1 each were identified to be *E. mori*, and *E. xiangfangensis* (Table 5.1).

Table 5.1. The species, sequence type, and antimicrobial susceptibility test results of *Enterobacter* spp. shown by the isolates recovered from individual neonates admitted at different neonatal units in the hospital.

Case ID	Isolate ID	Depart	Species	ST	Antimicrobial susceptibility test results							
					AMX	CTX	CIP	COT	GEN	AMK	IMI	CS^
N_{25}	54	NICU	*E. kobei*	NF	R	I	S	S	S	S	S	3
N_{25}	56*	PICU	*E. kobei*	NF	R	R	I	S	R	I	S	3
N_{SB}	57*	NICU	*E. kobei*	NF	R	R	I	S	R	I	S	3
N_{RPG}	60*	NICU	*E. kobei*	NF	R	R	S	S	S	S	I	3
N_{GMM}	61*	Nursery	*E. kobei*	NF	R	R	S	I	S	I	I	4
N_{26}	62*	NICU	*E. kobei*	NF	R	I	S	S	S	S	I	3
N_{28}	63*	NICU	*E. kobei*	NF	R	I	S	S	S	S	I	3
N_{NN}	66*	Nursery	*E. kobei*	NF	R	R	S	S	S	S	I	3
N_{31}	72	NICU	*E. kobei*	NF	R	S	S	S	S	S	I	2
N_{31}	73*	NICU	*E. kobei*	NF	R	S	S	S	S	S	R	2
N_{31}	83	NICU	*E. kobei*	NF	R	R	I	S	S	S	I	2
N_{30}	80*	NICU	*E. kobei*	NF	R	R	S	S	S	S	S	2
N_{33}	85*	NICU	*E. kobei*	NF	R	R	S	S	S	S	I	4
N_{ST}	84*	NICU	*E. kobei*	NF	R	R	S	S	S	S	I	3
N_{123}	152	NICU	*E. cloacae*	167	R	S	S	S	S	S	S	2
N_{134}	153	NICU	*E. cloacae*	167	R	R	R	R	R	S	S	2
N_{RK}	154	NICU	*E. xiangf*	NF[#]	R	R	R	R	R	S	S	3
N_{RK}	155	NICU	*E. mori*	NF[##]	S	S	S	S	S	S	S	2

AMK: amikacin, AMX: amoxicillin, CIP: ciprofloxacin, COT: cotrimoxazole, CS: colistin, CTX: cefotaxime, ER: emergency, GEN: gentamycin, I: intermediate, ID: identification, IMI: imipenem, NF: not found, NICU: neonatal intensive care unit, PICU: paediatric intensive care unit, R: resistant, S: sensitive, ST: sequence type

^For colistin, MIC (mg/L) values obtained by E-test are shown.

E.xiangf indicates for *E. xiangfangensis.*

* indicates 11 non-duplicate *E. kobei* isolates which were included in the phylogenetic analysis, NF, NF[#] and NF[##] had distinct, and unique allelic profiles which were not found in the reference database.

Between May and June 2016, *Enterobacter* spp. was not isolated in NICU. Notably, the blood isolation of *E. kobei* from the neonates with clinical sepsis increased sharply in the NICU between July and September of 2016, suggestive of an outbreak (Figure 5.1). The number of *E. kobei* infected cases peaked in August. During this three-month period, 11/23 neonates admitted to NICU developed sepsis caused by *E. kobei*, and 5/11 *E. kobei* infected neonates died. *Enterobacter* spp. were not isolated till next nine months. In July, August, and December 2017, 4 isolates of non-*kobei Enterobacter* spp. (*E. cloacae*, *E. xiangfangensis*, and *E. mori*) were cultured from the blood samples of septic neonates in NICU however *E. kobei* was no longer detected.

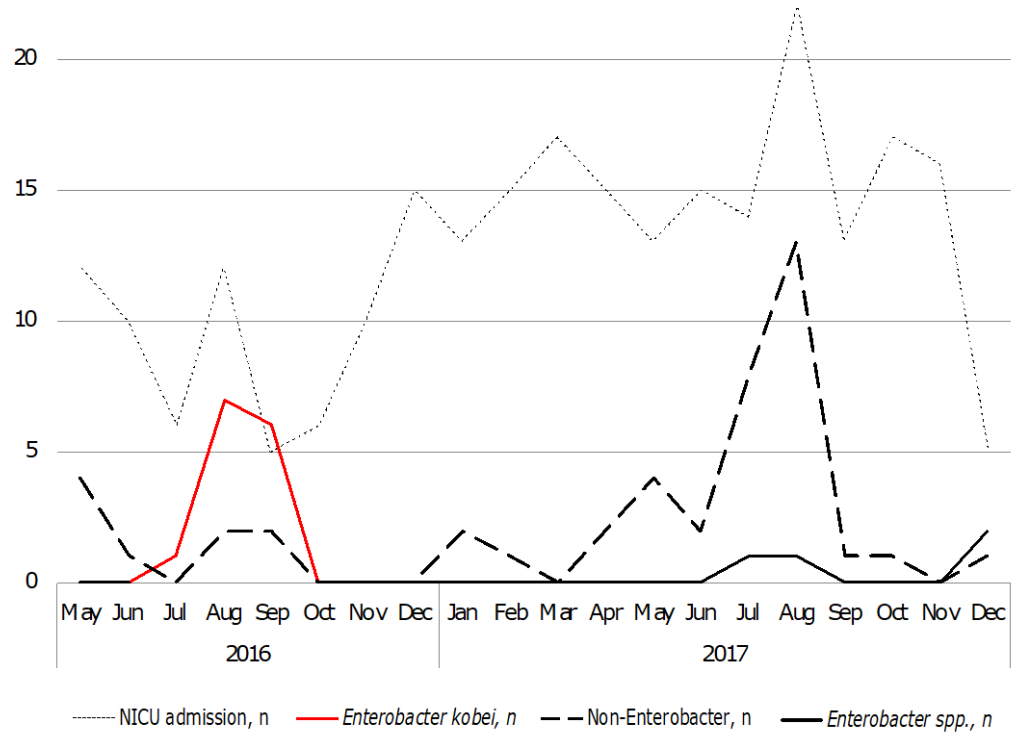


Figure 5.1. The temporal trend showing the number of *E. kobei*, *Enterobacter* spp. and non-*Enterobacter* isolated from the blood samples of neonates admitted to the neonatal units between May 2016 and December 2017.

The Y-axis shows an absolute bacterial count, and the X-axis shows the months of the year. The numbers of monthly NICU admissions and bacterial isolations are shown in the chart as the vertical peaks.

5.4.2 The clinical description of *E. kobei* outbreak between July and September of 2016

A total of 11 neonates admitted to the NICU were infected of *E. kobei* during the three month period of July 2016 to September 2016 (Figure 5.2).

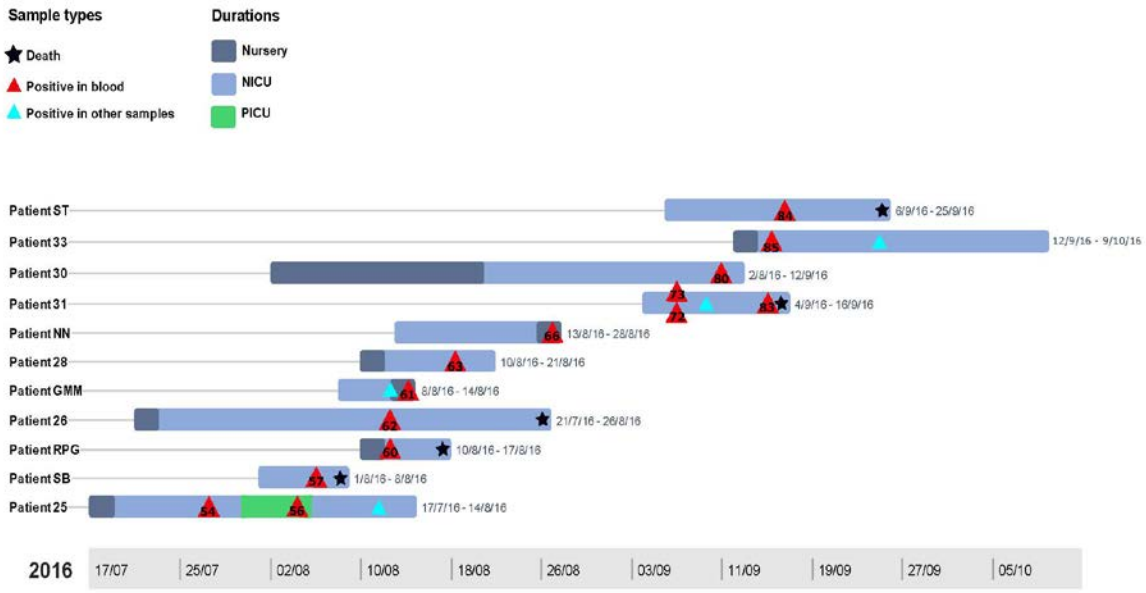


Figure 5.2. An illustrative depiction of the chronological development of neonatal sepsis outbreak caused by *E. kobei* in an NICU at Patan hospital between July and September 2016.

The Y-axis shows individual neonatal patients (N=11) admitted to the NICU, and developed neonatal sepsis caused by *E. kobei*. The X-axis indicates the timeline between July 2016 and October 2016. The specific dates indicating the dates of bacterial isolation are marked in the X-axis. The horizontal bars indicate the total time for the clinical observation for each neonate. The neonatal stay in various hospital departments have been coloured as indicated in the figure key. Important clinical events, such as bacterial isolation from biological samples, and death are indicated with specific signs, as shown in the figure key. Specific bacterial IDs for *E. kobei* isolated from the blood samples have also been marked.

A case by case description on epidemiological, laboratory, and clinical features including exposure to potential risk factors and the final outcome has been listed in table 5.2.

Table 5.2. The epidemiological, clinical, and laboratory features of patients infected with *E. kobei* during the outbreak.

Case ID	Gestational age group	Birth weight group	NICU admission indication
N_{25}	PT	LBW	PT/IUGR; symptomatic polycythaemia for possible exchange transfusion
N_{SB}	PT	VLBW	Severe respiratory distress
N_{RPG}	T	NBW	Persistent respiratory distress, congenital pneumonia
N_{26}	PT	LBW	Severe respiratory distress (tachypnoea> 90 breaths/min, nasal flaring, grunting), respiratory acidosis
N_{GMM}	T	NBW	Birth asphyxia
N_{28}	PT	LBW	Persistent respiratory distress
N_{NN}	PT	LBW	Severe respiratory distress
N_{31}	PT	LBW	Grunting with subcostal recession
N_{30}	PT	VLBW	Respiratory distress (occasional apnea, grunting, subcostal recession), congenital pneumonia
N_{33}	PT	LBW	Severe respiratory distress
N_{ST}	PT	VLBW	Severe respiratory distress with respiratory acidosis

Table 5.2 continued. The epidemiological, clinical, and laboratory features of patients infected with *E.kobei* during the outbreak

Case ID	Potential risk factors for sepsis							
	PPROM	UAC use, days	UVC use, days	IV cannula use, days	Intubation days	NG tube use, days	Not breast fed, days	NICU days
N_{25}	No	0	9	29	0	0	26	26
N_{SB}	No	7	7	7	6	3	7	7
N_{RPG}	No	6	6	7	4	3	7	7
N_{26}	No	0	0	33	24	10	33	33
N_{GMM}	No	4	4	4	4	3	4	4
N_{28}	Yes	8	0	12	9	12	12	12
N_{NN}	No	6	6	11	10	9	11	12
N_{31}	No	14	14	14	14	14	13	14
N_{30}	Yes	2	2	23	23	3	23	23
N_{33}	No	14	0	24	23	24	24	26
N_{ST}	Yes	15	15	17	19	12	19	19

Table 5.2 continued. The epidemiological, clinical, and laboratory features of patients infected with *E.kobei* during the outbreak

Case ID	Key laboratory features			Final outcome
	WBC count (x10^3/µl)	Platelets count (x10^3/µl)	CRP (mg/dl)	
N_{25}	9.9	72	42.5	Survived
N_{SB}	4.8	15	55	Dead
N_{RPG}	8.5	88.2	85	Dead
N_{26}	7.6	51.2	101.7	Dead
N_{GMM}	35.7	361	15	Survived
N_{28}	12.5	122	97.5	Survived
N_{NN}	106	28	35	Survived
N_{31}	6.5	64.5	61.5	Dead
N_{30}	7.9	108.8	49.7	Survived
N_{33}	8.3	81.6	111.6	Survived
N_{ST}	9	71	17	Dead

CRP: C-reactive protein; IUGR: intra-uterine growth retardation; IV: intra-vascular; LBW: low birth weight; NBW: normal birth weight; NG: nasogastric tube; PPROM: prolonged premature rupture of membrane; PT: pre-term; T: term; UAC: umbilical artery catheter; UVC: umbilical vein catheter; VLBW: very low birth weight

The first case (patient 25) was a PT, and LBW neonate, admitted to a nursery after few hours of birth on 17th July. At the 2nd day of life, the patient was transferred to the NICU for the management of symptomatic polycythaemia. On the 4th day of life (21st July), CoNS, and *Acinetobacter* spp. were isolated from umbilical pus, and blood culture samples, respectively. On the 8th day of life (25th July), *E. kobei* (ID 54) was isolated from the blood culture. Accordingly, the patient was treated with ofloxacin, meropenem, and fluconazole. On the 18th day of life (4th August) when the neonate was temporarily transferred to the PICU because of logistical issues of the unit, another isolate of *E. kobei* (ID 56) was isolated from the blood culture. On 1st August, the last line antimicrobial constituting colistin was initiated to be continued for 16 days. *Enterobacter* spp. was isolated from the femoral tip on the 26th day of life. Following clinical improvement, on the 28th day of life, the patient was discharged from the NICU, and transferred to the nursery.

The second neonate (Patient SB) was a PT and VLBW neonate who was transferred to the NICU at the 30th minute of its life because of severe respiratory distress. On the 1st day of life (1st August), meningitis was diagnosed, and 1st line antimicrobial treatment (ampicillin, amikacin, and cefotaxime) was initiated. On the 6th day of life (7th August), the blood culture results showed the growth of *E. kobei* (ID 57), and the antimicrobials were upgraded to include ofloxacin, meropenem, and fluconazole accordingly. The patient manifested a clinical deterioration, and on the 8th day of life (9th August), the patient expired because of the consequences of exacerbated neonatal sepsis.

The patient RPG was a term, and normal weight neonate, who was admitted to NICU from the nursery at 4 hours of life on 10th August because of congenital pneumonia. The 1st line empirical antimicrobials were initiated. On the 3rd day of life (13th August), *E. kobei* (ID 60) was isolated from the blood culture. The antimicrobial therapy was upgraded to the 2nd line (ofloxacin, and chloramphenicol), and later, on the 4th day of life, was promoted to add meropenem. The clinical condition deteriorated, and on the 7th day of life, the patient died of a refractory shock caused by the *Enterobacter* sepsis.

The patient 26 was a PT and LBW neonate who was admitted to the NICU for severe respiratory distress at 8 hours of life on 21st July. The patient was treated with the 1st line antimicrobials. In the absence of any clinical improvement, on 24th July, the antimicrobials were upgraded to the 2nd line empirical therapy. After 3 days, the therapy was further upgraded to the 3rd line that included meropenem, fluconazole, linezolid, and colistin which was continued for over two weeks. On the 22nd day of life, *E. kobei* (ID 62) was isolated from the blood culture, and piperacillin was added to the treatment accordingly. The neonatal health continued deterioration, and eventually on the 33rd days of life (26th August), the death occurred resulting from a septic shock.

The patient GMM was a term, and normal birth weight neonate who was admitted to the NICU on 8th August at the 30 minutes of its birth for the clinical management of birth asphyxia. At NICU, the patient was treated with the 1st line antimicrobials for 4 days, after which on 12th August, the neonate improved and was discharged from NICU to the nursery. On the 6th day of life (14th August), at nursery, the neonate developed a positive blood culture producing *E. kobei* (ID 61), and was treated as per the sensitivity report.

The patient 28 was a PT and LBW neonate who was moved to the nursery at the 15 minutes of life on 10th August because of the respiratory distress. After few hours of life, the patient was transferred to NICU for the management of congenital pneumonia, where the 1st line antimicrobial treatment was started. On the 3rd day, the antimicrobials were upgraded to the 2nd line. On the 8th day of life (18th August), the blood culture produced *E. kobei* (ID 63). The therapy was further upgraded to the 3rd line, constituting of meropenem, piperacillin-tazobactam, and fluconazole. On the 11th day of life, upon request from the parents, despite the clinical condition being critical, all invasive supports were withdrawn, and the neonate was transferred to the nursery.

The patient NN was also a PT and LBW neonate who required an NICU admission at the 2nd hour of its life (13th August) because of the respiratory distress syndrome. The patient received the 1st line empirical therapy for three days. Later, the therapy was upgraded to include the 3rd line antimicrobials, constituting of meropenem, ofloxacin, colistin, and fluconazole. Later, after 12 days of NICU stay, upon request from the parents, the patient was transferred to the nursery with withdrawal of all supports despite the clinical condition being critical. After 3 days of transfer in nursery (28th August), the patient developed culture-positive sepsis producing *E. kobei* (ID 66), and was treated accordingly.

The patient 31 was a PT and LBW neonate. It was admitted to the NICU at 3 hours of life (4th September) because of respiratory distress syndrome, and neonatal meningitis. On the same day, a complete sepsis investigation was carried out and the 1st line antimicrobial treatment was started. On the 3rd day of life (7th September), the blood culture was positive for *E. kobei* (ID 72). The antimicrobial therapy was upgraded to the 2nd line. On the 6th day of life (10th September), *Enterobacter* spp. was also isolated from the urine culture. The bacterial isolation (ID 73) persisted in blood culture till 5 days. The treatment was further elevated to the 3rd line, including meropenem, colistin, and fluconazole. On the 11th day of life (15th September), *E. kobei* (ID 83) was re-isolated from blood culture. On the 12th day of life, the neonate died due to *Enterobacter* sepsis leading to metabolic acidosis, and septic shock.

The patient 30 was a PT and VLBW neonate who was admitted to the nursery on the 1st day of life (2nd August) because of the respiratory distress. On the 20th day of life (21st August), he was transferred to the NICU for an intensive management of congenital pneumonia. After a week of NICU transfer, on 27th August, the patient developed sepsis caused by *K. pneumoniae*, and received ofloxacin, and piperacillin-tazobactam for two weeks. On the 40th day of life (12th September), the patient was transferred to nursery with a sample sent for the blood culture, which was later traced back to be culture-positive for *E. kobei* (ID 80).

The patient 33 was a PT and LBW neonate who was admitted to the nursery after few hours of birth (12th September) for respiratory distress. On the 1st day of life (13th September), the patient was transferred to NICU for respiratory distress syndrome, and was treated with the 1st line empirical therapy. On the 3rd day of life (15th September), the blood culture result was positive for *E. kobei* (ID 85). The 2nd line antimicrobial was started, which was later upgraded to the 3rd line, constituting of meropenem, colistin, and fluoconazole. On the 8th day of life, the blood culture was

positive for *K. pneumoniae,* and subsequently on the 13th day of life, *Enterobacter* spp. was isolated from the umbilical catheter tips. The antimicrobial treatment was accordingly adjusted to include aztreonam, gentamycin, and clindamycin. After 26 days of stay in NICU, the neonate improved clinically, and was discharged to the nursery.

The patient ST was the 11th patient of the study who was a PT and normal birth weight neonate. It required an NICU admission after an hour of its birth (6th September) because of severe respiratory distress. The 1st line antimicrobial treatment was started. On the 9th day of life (15th September), *E. kobei* (ID 84) was isolated from the blood culture. As per the AST report, the neonate was treated with the 2nd line, and then the 3rd line (meropenem, and piperacillin-tazobactam) antimicrobials. Despite the use of 3rd line antimicrobials for an extended period, the clinical condition worsened, and on the 19th day of life, the neonate died because of an exacerbated sepsis.

5.4.3 Summary results of environmental culture

Table 5.3 summarizes the microbiological results of various environmental samples collected during the outbreak event to identify any potential environmental source of infection. Though non-pathogenic bacteria and *Acinetobacter* spp. were occasionally isolated during the outbreak period, *Enterobacter* spp. was not isolated from any environmental samples. Similarly, nasal swab and nail bed samples collected from the admitted neonates as patient surveillance culture, and the hand swabs of the NICU medical staffs also failed to isolate *Enterobacter* spp.

Table 5.3. Summary of microbiological results of various environmental samples collected from NICU during the given outbreak period

Environmental samples (swab)	**Microbiological results***
Drinking water tap	No growth after 48 hours of incubation
Ventilator	No growth after 48 hours of incubation
Feeding trolley	No growth after 48 hours of incubation
Big trolley	No growth after 48 hours of incubation
Door handle	No pathogen isolated after 48 hours of incubation
Infusion pump	No growth after 48 hours of incubation
Suction jar	No growth after 48 hours of incubation
Monitor	No growth after 48 hours of incubation
Floor-1	No pathogen isolated after 48 hours of incubation
Floor-2	No pathogen isolated after 48 hours of incubation
Floor-3	Light growth of *Acinetobacter* spp.
Syringe pump	No growth after 48 hours of incubation
Warmer	No growth after 48 hours of incubation
Ambu bag	No growth after 48 hours of incubation
Nursing station desk-1	Light growth of *Acinetobacter* spp.
Nursing station desk-2	No pathogen isolated after 48 hours of incubation
Weigh machine	No pathogen isolated after 48 hours of incubation
Laryngoscope blade	No growth after 48 hours of incubation
Overhead warmer	No growth after 48 hours of incubation
Ventilator baby log	No growth after 48 hours of incubation
Phillips ventilator	No growth after 48 hours of incubation
Formula milk feed	No growth after 48 hours of incubation
Green hand washing tap	Heavy growth of *Acinetobacter* spp.
Phototherapy	No growth after 48 hours of incubation
Medicine trolley	No pathogen isolated after 48 hours of incubation
Reusable SpO2 probe	No pathogen isolated after 48 hours of incubation
Nasal swab-1	Heavy growth of CoNS
Nail bed (nail tips)	No growth after 48 hours of incubation
Nasal swab-2	No pathogen isolated after 48 hours of incubation
Personnel hand swabs	No growth after 48 hours of incubation

NICU bed rail-3	No growth after 48 hours of incubation
Bucket/Jug	Heavy growth of *Acinetobacter* spp.
Fan	No growth after 48 hours of incubation
Outer door handle	No pathogen isolated after 48 hours of incubation
Air conditioner	No pathogen isolated after 48 hours of incubation

*'No pathogen isolated after 48 hours of incubation' referred to the isolation of clinically insignificant bacteria such as *Micrococcus* spp., *Bacillus* spp., and poly-microbial growth after 48 hours of incubation.

5.4.4 The phylogenetic analysis of *E. kobei* outbreak clones and the AMR gene contents

The phylogenetic analysis of 11 non-duplicate *E. kobei* isolates obtained from the blood samples of the septic neonates between July-September 2016 provided an evidence for an outbreak of neonatal sepsis (Figure 5.3A). The resulting phylogenetic tree showed a clustering of the 11 *E. kobei* isolates recovered from the neonates, when mostly admitted at the NICU, in a 3 months period. The 11 *E. kobei* isolates were separated 13 core SNPs apart. The isolate 56, which was recovered from an NICU admitted neonate when he was temporarily transferred to an out-borne PICU unit, was genetically distant by 5 SNPs from rest 10 *E. kobei* isolates which were obtained from the NICU (ID 57, 60, 62, 63, 73, 84, and 85), and nursery (ID 61, 66, and 80). The average pairwise distance among the 10 *E. kobei* isolates was 3 SNPs. Additionally, it was found that 4 isolates, namely, 66, 73, 84, and 85 differed from each other by only 2 SNPs. Similarly, isolates 62, and 63 were genetically related to each other with only 1 SNP difference apart.

The 10 outbreak-associated isolates had a limited array of an identical AMR genes profile constituting of bla_{AmpC}, *fosA2*, $bla_{ACT\text{-}28}$, and *mcr-10* genes conferring resistance to the β-lactam, fosfomycin, carbapenem, and colistin, respectively. In phenotypic AST, a high number of isolates (8/10, 80%) showed an intermediate or resistant response to the imipenem Similarly, 80% (8/10) of the isolates had colistin MIC value ranging ≥3 mg/L. Similarly, all 10 outbreak causing *E. kobei* isolates had a virulence gene cassette constituting of *mrkABCF* and *fliA* genes encoding for

flagellum-specific sigma factor, and adhesin respectively. Alternativey, *E. kobei* isolate 56, which was potentially acquired from an out-borne PICU unit, carried an extensive array of AMR genes against beta-lactams ($bla_{\text{ACT-28}}$, $bla_{\text{CTXM-1}}$, $bla_{\text{TEM-1D}}$), aminoglycosides (*Aac3-IIa, AacA-ad StrA/B*), phenicols (*CatA1/B4*), quinolones (*QnrB1*), sulpha drugs (*DfrA5),* fosfomycin (*fosA2*), tetracycline (*TetAB*), and colistin (*Mcr-10*). Additionally, the isolate 56 also carried the virulence genes *hcp/tssD* encoding for T6SS toxin secretory system, and the salmochelin siderophore encoding gene *iroN.*

The conjugation experiment confirmed that the *mcr10*-carrying IncFIBK plasmid was transferable between *E. kobei,* and *Escherichia coli* J53, and demonstrated an acquired resistance to multiple antimicrobials (cefotaxime, erythromycin, ofloxacin, and ampicillin). Further, the IncFIB plasmid in *E. kobei* isolates exhibited high synteny and similarity to a self-transferable *mcr10*-carrying IncFIB plasmid identified in an *Enterobacter roggenkampii* isolate reported in China (accession number: CP048651) (coverage 60%, identity 99%, Figure 5.3B).

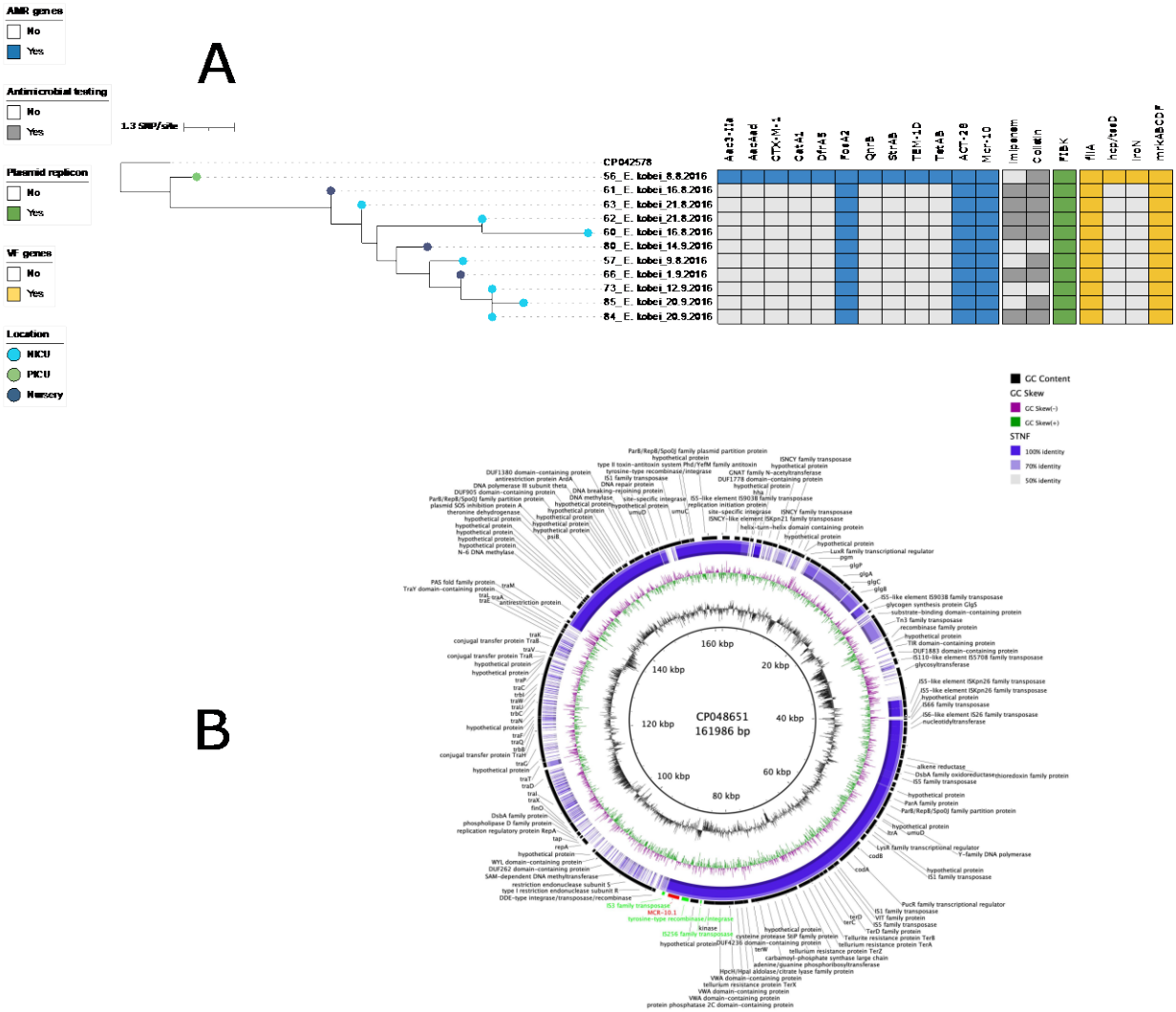


Figure 5.3. The phylogenetic tree, and an *mcr-10*-carrying plasmid structure of *E. kobei* causing neonatal sepsis at NICU and other neonatal departments at Patan hospital.

Figure A shows a phylogeny tree rooted with reference *E. kobei* strain C16 used as an outgroup. The node colours correspond to different neonatal departments of bacterial isolation. The tip labels correspond to the *E. kobei* isolates indicated the by ID numbers, also given with the dates of isolation. The scale bar estimates the number of SNPs per site. The heat map shows the presence (blue, dark grey, green and yellow color) or absence (light grey color) of the AMR genes, phenotypic antimicrobial resistance, plasmid replicon type, and virulence genes. Figure B shows the BLASTN comparison between IncFIBK plasmid identified from *E. kobei* isolate, and a reference plasmid from *E. roggenkampii* (Accession number CP048651). The outside ring indicates the gene annotation of reference sequence. *Mcr-10* gene has been highlighted in red, and the related insertion sequence elements are coloured in green.

5.5 Discussion

The study provided a high resolution genomic evidence of a clonal outbreak of neonatal sepsis caused by *E. kobei* in the NICU. The presence of AMR genes, particularly *mcr-10* conferring resistance to colistin, the last resort antimicrobial, could have offered an important adaptive antimicrobial mechanism for the *E. kobei* isolates to sustain in the NICU.

NICU remains a hotspot of infection outbreaks in the vulnerable neonates [40,226]. Similar to the NICU of other LMICs, NICU at Patan hospital had also suffered from several outbreaks of neonatal infections by GNB, such as *K. pneumoniae*, *Enterobacter* spp., and *Acinetobacter* spp. [73,74,124,140]. In this study, in a span of three-month period in mid-2016, a fatal outbreak was evidenced where 11/23 admitted neonates were infected of *E. kobei*, and 5/11 died of the resulting severe sepsis. The study showed that of 11 non-duplicate *E. kobei* isolates, 10 isolates were genetically close, with an average pairwise genetic distance of 3 SNPs, forming a single tight cluster. This confirmed for a monoclonal infection outbreak. Further, 3/11 *E. kobei* clones (61, 66 and 80) were isolated from the nursery after being discharged from the NICU, suggesting for confluent inter-unit transmission events. A successive carryover of the infecting clones for a continuum of 3 months suggested for a possibly compromised hospital IPC measures. A frequent turnover of NICU nursing staffs during this period, and a lack of a physically- and logistically-separated unit to provide an isolated ICU care to culture-positive septic neonates were the most prominent ambiguities in the existing IPC measures. The failure to isolate culture positive neonates potentially led to a series of inter-neonatal cross-transmission events resulting from the shared logistics among the neonates residing the same unit, resulting into a fatal outbreak. The outbreak could also have been originated from contaminated common environmental source/s in addition to a direct case to case transmission via contaminated hands as the average SNPs distance between the isolates was larger than zero.

In an attempt to identify a common source and mode of infection so as to prevent additional cases and future outbreaks, a routine route cause analysis was performed in association with the hospital IPC committee. As a part of routine protocol, an NICU audit was carried out summarizing the data of NICU admitted neonates on the demographic details including total number of admission, general outcome, distribution by gestational age and birth weight, department of admission from, NICU admission indication, tabulated master chart of indices including final diagnosis and final outcome for all admissions, the use of mechanical ventilator and ionotropes, the results of microbiological culture, and individual case presentation for mortalities. An environmental surveillance of frequently-touched NICU surfaces along with the culture of biological surveillance samples from the admitted patients and medical staffs was performed. In several similar studies, various environmental sources and modes of infection outbreaks have been identified that helped break the chain of further transmissions [40,228,235]. In this study, however, the surveillance failed to pinpoint the source of ongoing *Enterobacter* spp. infection. An earlier study from NICU of Patan hospital also reported of performing a rigorous environmental sampling, and surveillance culture of NICU HCWs during an NDM-1 positive *K. pneumoniae* linked neonatal sepsis outbreak in late 2011[73]. Though *K. pneumoniae* was isolated from several samples, such as laryngoscope blades, suction jar, tap water, hand, and rectal swab of few HCWs, none were ESBL producing, and therefore yielded an inconclusive result. Another genomic study undertaken on the outbreak of neonatal sepsis caused by *E. cloacae* in the NICU unit at Patan hospital between November 2012 and February 2013 identified a soap dispenser in one of the NICU sinks to be the infection source for a small cluster of infections, with zero SNPs distance[124]. However, the source could not be identified for rest of major clusters with large number of cases, suggesting for multiple transmission routes of infection. Several studies have identified *Enterobacter* spp., particularly *E. sakazakii* and *E. asburiae* as frequent contaminants of powdered infant formula milk and underscored their association in the causation of neonatal infections such as sepsis, meningitis, and

necrotizing enterocolitis [236–238]. As infant formula milk is a non-sterile product serving as an optimal bacterial growth media and specific *Enterobacter* spp. can thrive in dry environment, it has been recommended to take hygienic precaution in preparation and handling of such products, particularly in NICU where vulnerable neonates are heavily reliant on such feed [236]. Despite the established association between the infant formulae milk and neonatal sepsis due to *Enterobacter* spp., in this outbreak study, the culture of formulae milk sample failed to grow any *Enterobacter* spp.

Performing additional microbiological or genomic analyses on the environmental samples during the difficult times of outbreak in resource-strapped settings, such as LMICs could be, therefore challenging and even impossible. A limited laboratory capacity in hospitals of LMICs may already have been so overwhelmed by the increased number of clinical samples because of an outbreak, that a further processing of a large number of environmental samples can be almost unattainable. Therefore, though being ideal to pin-point the environmental source of infection, the environmental surveillance may be difficult to roll out in such low-resource scenario. Though an isolation unit could not be established at the study time, a multi-disciplinary approach was followed to limit the cross-transmission of infection. The preventive strategy included an extensive environmental decontamination, strict implementation of preventive measures, such as reinforcement of standard hand hygiene practice including that for the parents/guardians, three-monthly refresher training on hand washing technique for all NICU medical staffs including doctors by letting them watch the hand-washing video, followed by a semi-annual hand washing audit; allocation of all daily care essentials, such as thermometer, stethoscope, chlorhexidine swab container, vaseline for heel prick, tape for fixation, scissors, artery forceps, saturation probe, pen, calculator, and measuring tape for individual neonates by the cot side; avoiding keeping fomites such as X-ray films, pen etc. on the baby cot; the use of disposable ventilator circuit; a proper

decontamination of all equipment after discharge or transfer of the patient which included surface cleansing of the used equipment, such as ventilator, infusion pump, phototherapy unit, incubator, radiant warmer, stethoscope, glucometer, scissors, thermometers, and cot-side table with 1% Virkon or Klorkleen; autoclaving connecting tubes for oxygen and suction devices, and connectors after immersing in 2% glutaraldehyde for 20 minutes; immersing all re-usable tubes, suction devices, humidifier bottles, laryngoscope blades, ambu bags, masks in 2% glutaryaldehyde for 6 hours before autoclaving; every fortnightly high dusting and cleaning of filter mat for babylog; every semi-annual change of incubator air filter, babylog filter mat and cleaning of air conditioner; annual changing of dust filter and microfilter; and fumigation after any suspicious outbreak due to MDR organisms. The bundle for invasive procedures were also revised to include the training of healthcare personnel involved in inserting and maintaining catheters; using maximal aseptic barrier precautions during invasive procedure including hand washing, alcohol hand rub, followed by the use of sterile hand gloves (for venous blood collection and urinary catheter insertion), and additional use of sterile gown, cap and mask for other invasive procedures, such as insertion of central venous catheters (UVC, UAC, femoral, PICC, internal jugular, subclavian), pericardial-, pleural-, and ascitic- fluid tapping, chest tube insertion, endotracheal intubation, endotracheal tube suctioning, and lumbar puncture; using chlorhexidine (> 0.5%), iodine, followed by chlorhexidine (> 0.5%) for skin preparation, using tegaderm in pre-term neonates, and avoiding routine replacement of central venous catheters. Further, two nurses were dedicated to take care of the culture positive neonates only. As a result of adherence to stringent IPC measures, the rate of overall bacterial sepsis was dramatically reduced in subsequent months. No new cases of neonatal sepsis caused by *Enterobacter* spp. were detected for next nine months. These interventional approaches were integrated into the hospital IPC measures. In April 2017, the hospital was also able to establish a separate neonatal isolation unit (NISO) equipped with NICU service and with the provision of dedicated resources, including the nurses. After the establishment of NISO, the

infection outbreak events plummeted till now. There were sporadic culture-positive neonatal infections, which were immediately secluded and managed separately in NISO before they could become a source of cross-infection.

At Patan hospital, PICU provided an intensive care service, primarily to the out-borne neonates. Compared to the inborne neonates, who were borne and admitted at Patan hospital, the infection among out-borne neonates can be caused by distinct bacterial flora, possibly indicating community- or prior healthcare-acquired infections. Interestingly, I found that *E. kobei* isolate 56, which was potentially acquired from the PICU by the patient 25 upon a temporary transfer, was genomically more distant from rest of 10 outbreak associated clones. Further, unlike the outbreak cluster, the isolate 56 carried an extensive range of AMR, and virulence genes. This finding indicates the complexity of bacterial etiology, and the subsequent therapeutic difficulty for sepsis management in out-borne sepsis. Therefore, the findings suggest to provide a separate care for in-borne and out-borne neonates, which was usually practiced at Patan hospital.

Though several AMR genes were lacking in the outbreak causing *E. kobei* isolates, the finding that all isolates carried bla_{ACT28} gene was interesting and clinically relevant. In clinical isolates of *E. kobei*, the expression of chromosomal bla_{ACT28} gene has been shown to be associated with decreased outer membrane permeability leading to impaired carbapenem influx, and thus carbapenem resistance [239]. In this study as well, 80% (8/10) of *E. kobei* isolates showed non-susceptibility to imipenem despite the presence of any carbapenemase encoding genes. Further, the detection of *mcr-10* gene encoding for colistin resistance in all *E. kobei* isolates was also a clinically important finding. In phenotypic AST as well, 80% (8/10) of isolates had colistin MIC value ranging ≥3 mg/L. This finding may infer to the increased selective pressure imposed on the bacterial isolates of neonatal sepsis in our NICU resulting from an excessive clinical use of

colistin. Next, the presence of *mrk* gene cluster encoding for biofilm formation in all *E. kobei* isolates is of clinical significance. Studies have demonstrated the role of *mrk* gene cluster in the expression of type 3 fimbriae facilitating the formation of dense biofilms [240,241]. This finding raises the possibility of colonization and establishment of *E. kobei* isolates in the NICU environment thereby serving as a source of persistent infection to the admitted neonates. The environmental sampling carried out during the study period however failed to identify the environmental source of bacterial colonization. A more extensive environmental surveillance might have figured out the environmental source.

5.6 Conclusions

The findings of this study provided a genomic evidence for an outbreak of neonatal sepsis caused by *E. kobei* in NICU at Patan hospital, which also spanned across nurseries. The results showed that all *E. kobei* infections that peaked during mid-2016 among the in-borne neonates in the neonatal units were due to the expansion of a specific clone of *E. kobei*, sharing an identical but limited profile of plasmid replicons, virulence genes, and AMR genes including a novel colistin resistance *mcr-10*. My study findings highlighted the importance of rigorous IPC measures to contain, and prevent further hospital outbreaks. The results also emphasized on the need to segregate the out-borne neonates from the in-borne neonates as to prevent potential complexities of therapeutic management arising from the distinct corresponding bacterial aetiologies.

Chapter 6

The genomic epidemiology, antimicrobial resistance, and virulence factors of *Enterobacter cloacae* complex causing bloodstream infections in a tertiary care hospital in Nepal

6.1 Abstract

The BSIs caused by carbapenemase-positive ECC species are increasing internationally suggesting that ECC are the potential emerging pathogens. Aiming to understand the molecular epidemiology of ECC causing BSIs, I performed WGS on 80 *Enterobacter* spp. isolated from the patients with clinically significant BSIs, and admitted to the ER at a major tertiary hospital in Nepal between April 2016 and October 2017. I investigated the phylogenetic relationships in the ECC isolates, and the bacterial genomic determinants of virulence and AMR. I identified 5 ECC species, and 13 STs. *E. xiangfangnesis* ST171, one of the emerging carbapenem resistant international ECC clones with epidemic potential, was the most prevalent (42%, 33/80) ECC isolates. The phylogenetic analysis showed a large (>19,400 SNPs) core genome SNP distance between major STs, and relatively small genetic distance (<30 SNPs) between the isolates of each prevalent STs. The findings suggested for a potential recent importation of major STs of ECC followed by a rapid clonal expansion. Further, a genomic evidence for the resistance to all major antimicrobial classes except for colistin and macrolides was detected in the ECC isolates. A limited number of isolates also carried bla_{NDM-1} (n=3), and bla_{OXA-48} (n=1) carbapenemase genes. The virulence factors encoding for the siderophores (24%), T6SSD (25%), and fimbriae: (54%) were detected. My study highlighted that the MDR ECC isolates are important pathogens of BSIs among ER attending patients who could have been either referred from other hospital/s or came directly from the

community. Though of low prevalence, the carbapenem resistance observed in ECC isolates raised concern about further dissemination, thereby underscoring the need for a continued surveillance to identify the MDR ECC isolates with an epidemic potential.

6.2 Introduction

Bacteria belonging to ECC are the opportunistic GNB and comprise part of the healthy gut microbiota of humans, and animals. ECC are also one of the members of '*E. coli* and the ESKAPE' group of pathogens, and are associated with MDR nosocomial infections [63]. The ECC bacteria are intrinsically resistant to several narrow- and extended-spectrum β-lactam antimicrobials because of a constitutive expression of the chromosomally located AmpC gene [195,242]. Additionally, a rapid emergence, and spread of carbapenem-resistant *Enterobacter cloacae* complex (CREC) variants have been widely reported [243–245].

The MDR isolates of ECC often colonize the high-contact surfaces in ICUs, and therefore the bacterial isolates can be frequently exposed to the broad-spectrum antimicrobials that are routinely used in ICUs [79,124]. When hospital IPC measures are compromised, the contaminating bacteria can cause hospital infections in vulnerable patient populations [124,246]. In the community, the overuse of broad-spectrum antimicrobials may facilitate the selection, and colonization of MDR ECC bacterial isolates in the human gut [246]. The colonized MDR ECC isolates may serve as a reservoir for endogenous source of CO-BSIs, specifically in the individuals having debilitated health conditions, such as reduced immunity, malignancy, burns, diabetes, and severe illnesses requiring prolong hospitalization, and antimicrobial therapy [246].

The BSIs are prevalent in patients seeking for medical care in OPD and ER departments of the hospitals [247–249]. Further, the prevalence of BSIs caused by MDR non-*Salmonella*

Enterobacteriaceae isolates is high in LMICs [132]. In addition to *E. coli* and *K. pneumoniae,* the MDR ECC have been increasingly prevalent bacterial pathogens causing BSIs [250,251]. The growing prevalence of BSIs caused by MDR ECC bacteria threatens the current antimicrobial therapies. The genomic investigations aid in providing an improved clinical care through a precise identification of the *Enterobacter* species, the detection of the AMR profiles, and the understanding of bacterial population structure. Such data are also helpful in detecting an emergence, and spread of clinically, and epidemiologically relevant *Enterobacter* spp. In this study, aiming to characterize the species, AMR genes, and phylogenetic relations of ECC isolates associated with BSIs, I performed a WGS and comprehensive genomic analyses of ECC bacteria isolated from the patients admitted in the ER of a major tertiary hospital in Nepal.

6.3 Methods

6.3.1 The study setting and study design

This was a retrospective study conducted on ECC bacteria isolated from routine blood cultures of ER attending patients in the microbiology laboratory at Patan Hospital.

6.3.2 An ethical approval and a consent to participate

The isolates of *Enterobacter* spp. investigated in this chapter were a subset of *Enterobacter* spp. isolates studied in chapter 3, and therefore this study was conducted under the same approval of chapter 3. An ethical approval for conducting chapter 3 was obtained from NHRC under the approval registration number of 120/2012 for the research entitled “Prevalence of extended spectrum beta-lactamase (ESBL) pathogens and factors responsible for ESBL associate infections within Patan Hospital”. The information used in this retrospective study was derived from the microbiology database of Patan Hospital which was devoid of any patient identifying information.

As the data did not contain any identifying information on the patients, the informed consents were not required.

6.3.3 The ascertainment of case

In ER at Patan hospital, a blood culture investigation was requested if a patients had an undifferentiated fever for ≥48 hours at a time of presentation to the hospital. After a blood sample was collected for the microbiological culture, the patient was empirically treated with an intravenous third generation cephalosporins. If the patient showed a clinical improvement, the patient was discharged with a prescription of an oral azithromycin. Alternatively, if the patient failed to show expected clinical improvement or undergo further deterioration, the patient was transferred to an appropriate inpatient ward. In ER, the patients usually stayed for a maximum of 12-24 hours, before being discharged or transferred.

6.3.4 The microbiological procedure for blood culture investigation

As a part of routine clinical care, a blood sample was collected at the bedside of the patient by an attending nurse following an aseptic skin preparation using a standard protocol as described in the methods section 2.6. A routine microbiological processing for bacterial isolation, bacterial identification, and AST was performed as described in the methods section 2.6. The GNB that were lactose-fermenting, motile, indole-negative, urease-negative, citrate-positive, hydrogen sulfide non-producing, and giving acid/acid reaction with gas production in the triple sugar iron (TSI) test were presumptively identified as *Enterobacter* spp. The pure culture colonies of *Enterobacter* spp. were routinely stored at -80°C.

6.3.5 The ascertainment of ECC bacterial isolates

The microbiology database of Patan Hospital was searched for *Enterobacter* spp. originating from blood cultures of the patients attending to the ER between April 2016 and October 2017. The duplicate *Enterobacter* isolates with identical AST profiles originating from the same patient were excluded from the study.

6.3.6 The bacterial conjugation experiment

The conjugation experiment was performed between each of the donor ECC isolates: ST171, ST134, and ST1377 and the recipient *E. coli* J53 (sodium azide resistant). The overnight LB cultures of the donor and recipient bacterial cells were combined in equal volumes (5 mL). Bacteria were conjugated for 12 hours in LB broth at 37^{o}C. The *E. coli* trans-conjugants were selected on MacConkey agar containing sodium azide (100 mg/l), and ceftriaxone (6 mg/l). For conjugation experiments, the conjugation frequency was calculated as the number of trans-conjugants per recipient cell. Further, AST was performed on the *E. coli* J53 trans-conjugant to confirm for the transfer of the plasmid-mediated AMR genes.

6.3.7 The whole genome sequencing

The WGS of 80 *Enterobacter* spp. isolates was performed as described in the methods section 2.8.

6.3.8 The genomic analysis

The genomic analysis on WGS data was performed as per the bioinformatics pipeline as described in the methods section 2.8.4.

6.3.8.1 Pairwise SNP distance determination

The genetic distance between 3 predominant *Enterobacter* STs (ST171, ST134, and ST1377) in my collection were compared with each other, as described in the method section 2.8.4.2. Roary [161] was used to construct a pan genome for the 3 STs with a blastp percentage identity of 99%, and a core definition of 99%. The SNP-sites v2.1.3 [162] was used to extract the SNP sites from the core gene alignment (968 genes), resulting in an alignment of 44,409 SNPs. The pairwise genetic distances (the difference in the number of SNPs) within and between the 3 STs were estimated from the SNP alignment using ape (v4.1), and adegenet (v2.0.1) packages in R (v3.3.2) [163].

6.3.8.2 The phylogenetic analysis

A pairwise SNP distance analysis showed substantially large SNP differences between the 3 predominant STs (ST171, ST134, and ST1377), but limited SNP differences between the isolates of individual STs. To gain a further phylogenetic resolution for each of the STs, I reconstructed a pan genome for individual STs using Roary (blastp percentage identity of 99% and core definition of 99%). The core gene alignment of each ST was subjected to Gubbins (v2.3.2) [252] to remove regions of recombination, followed by extraction of SNP sites using SNP-sites v2.1.3 [162]. The number of core genes of ST171, ST134, and ST1377 were 3,833, 3,542, and 3,415, respectively, giving rise to corresponding core SNPs of 27, 6, and 6. The SNP alignment of each ST was used to construct a ML phylogenetic tree using IQ-TREE v1.4.4 [164], with the best-fit evolutionary model (K3Pu+ASC) identified based on the Bayesian Information Criterion in jModelTest implemented in the software. Support for the ML tree was assessed via 100 pseudo-replicates. As a limited genetic diversity was observed among the isolates of major STs, a genomic comparison was also carried out for ST171 by comparing the isolates of my study with 93 isolates of ST171 from the public databases (Merck Study for Monitoring Antimicrobial Resistance Trends

(SMART) (2008–2014) and the AstraZeneca global surveillance program (2012–2014), currently known as the INFORM Global Surveillance Study of Antimicrobial Resistance) [253].

6.4 Results

6.4.1 Demographic description

Between April 2016 and October 2017, 127 *Enterobacter* isolates were obtained from the blood samples across different departments at Patan hospital. Sixty three percent (80/127) of *Enterobacter* spp. were originated from ER attending patients with clinically-confirmed BSIs. Available demographic data including the geographical location of these patients were limited and fragmented, while pre-clinical data on prior hospital care were entirely missing. Based on the available data, the median age of ER patients positive with *Enterobacter* was 41 years (IQR: 30-59.5), with a nearly equal male to female ratio (0.92:1).

6.4.2 The species and Sequence Types (STs) of *Enterobacter* isolates

I found 5 species and 13 STs of *Enterobacter* isolates (Figure 6.1 A). *E. xiangfangensis* (73%, 58/80), and *E. hormaechei* (21%, 17/80) were the 2 most common species, followed by *E. hoffmannii* (4%, 3/80), *E. asburiae* (1%, 1/80), and *E. quasihormaechei* (1%, 1/80). Among the identified STs, ST171 was found to be the most predominant (42%, 33/80), followed by ST134 (26%, 18/80), ST1377 (16%, 13/80), STNF (sequence type not identified) (7%, 6/8), and several diverse singletons (n=8). The 3 dominant STs (ST171, ST134, and ST1377) collectively accounted for 80% of the total isolates. ST171 and ST134 isolates belonged to *E. xiangfangensis*, while ST1377 isolates were identified as *E. hormaechei.*

6.4.3 The phenotypic antimicrobial susceptibility

All 80 *Enterobacter* spp. isolates were resistant to cefotaxime (Figure 6.1 B). A high proportion of isolates were resistant to gentamicin (83%, 66/80), chloramphenicol (81%, 65/80), cotrimoxazole (91%, 73/80), and ciprofloxacin (60%, 48/80). The resistance to carbapenems was low instead, with resistance to meropenem and imipenem being found in 5% (4/80), and 8% (6/80) of isolates, respectively. At the species level, *E. xiangfangensis*, and *E. hormaechei* isolates exhibited dissimilar resistance patterns to gentamicin, chloramphenicol, and ciprofloxacin. In comparison to *E. hormaechei* isolates (n=17), *E. xiangfangensis* isolates (n=58) were more commonly resistant to gentamicin (93% vs 38%), and chloramphenicol (92% vs 38%), but more commonly susceptible to ciprofloxacin (53% vs 81%) (Figure 6.1 C, 6.1 D). However, the proportions of resistant isolates of *E. xiangfangensis*, and *E. hormaechei* were comparable against cefotaxime (100% vs 100%), meropenem (5.2% vs 5.8%), and cotrimoxazole (92% vs 94%).

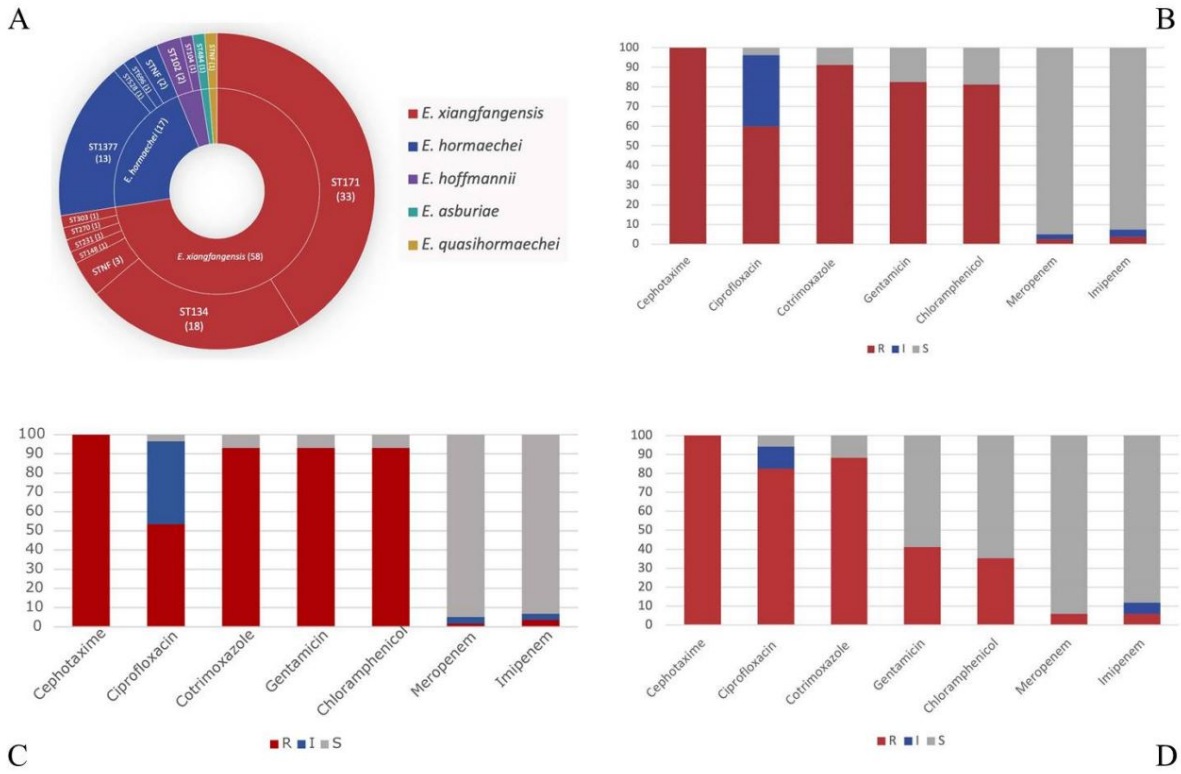


Figure 6.1. The species, ST, and phenotypic antimicrobial suscetptibility profiles of *Enterobacter* isolates.

Figure A shows the species distribution of *Enterobacter* isolates and the distribution of STs within each species. Figures B, C and D show the phenotypic AST profile of all isolates, AST results of *E. xiangfangensis*, and the AST profile of *E. hormaechei*, respectively.

6.4.4 The profile of antimicrobial resistance genes

A high proportion of *Enterobacter* isolates had several AMR genes conferring resistance to all classes of antimicrobial agents with the exception of carbapenems, macrolides, and colistin (Figure 6.2). Of note, >95% of *E. xiangfangensis* isolates harboured an extensive array of AMR genes, including *bla*$_{\text{CTX-M-1}}$- *bla*$_{\text{ACT}}$- *qnrB- dfrA5- strA/B- Aac3-IIa- AacAad- bla*$_{OXA-1}$- *bla*$_{TEM-1D}$- *catA- tetA- sulII- fosA2*. Three *E. xiangfangensis* isolates additionally carried the carbapenem resistance genes, *bla*$_{\text{OXA-48}}$ (ST171) and *bla*$_{\text{NDM-1}}$ (ST134 and ST231). *E. hormaechei* isolates had less extensive AMR gene profiles than the AMR profile of *E. xiangfangensis*. High proportion of *E. hormaechei* isolates had 4 AMR genes, namely *bla*$_{\text{ACT}}$ (100%), *qnrB* (100%), *bla*$_{\text{CTX-M-1}}$ (94%), and *dfrA5* (76%). The *bla*$_{\text{NDM-1}}$ carbapenemase gene was detected in one *E. hormaechei* ST528isolate.

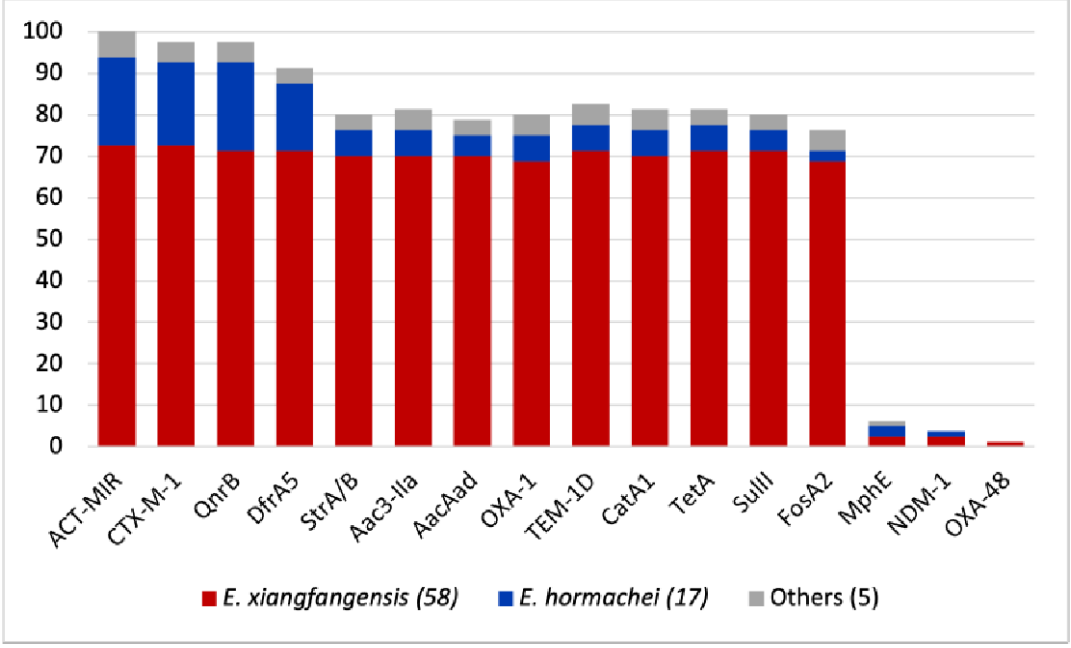


Figure 6.2 The distribution of AMR genes by *Enterobacter* species.

The results for minor species *E. asburiae* (n=1), *E. hoffmannii* (n=3), and *E. quasihormaechei* (n=1) were grouped as 'Others'

6.4.5 The phylogenetic analysis

I sought to investigate the phylogenetic relatedness of the three predominant STs (ST171, ST134, and ST1377) of *Enterobacter* isolates. The average pairwise SNP distance between ST171, and ST134 (*E. xiangfangensis*) was 19,429 SNPs (range: 19,429-19,431 SNPs), indicating that the two STs were genetically distantly related though being belonged to the same species. ST171, and ST134 isolates were more distantly related to ST1377 (*E. hormaechei*) than to each another, with average pairwise SNP differences of 36,197 SNPs (ST171 versus ST1377), and 34,201 SNPs (ST134 versus ST1377) respectively. Alternatively, the genetic variation between the *Enterobacter* isolates within each ST was limited despite the lack of geographical clustering; the ST171 isolates varied by only 27 SNPs, while isolates within ST134, and ST1377 each differed by only 6 SNPs. The *Enterobacter* isolates within each ST formed a single distinct cluster (Figure 6.3).

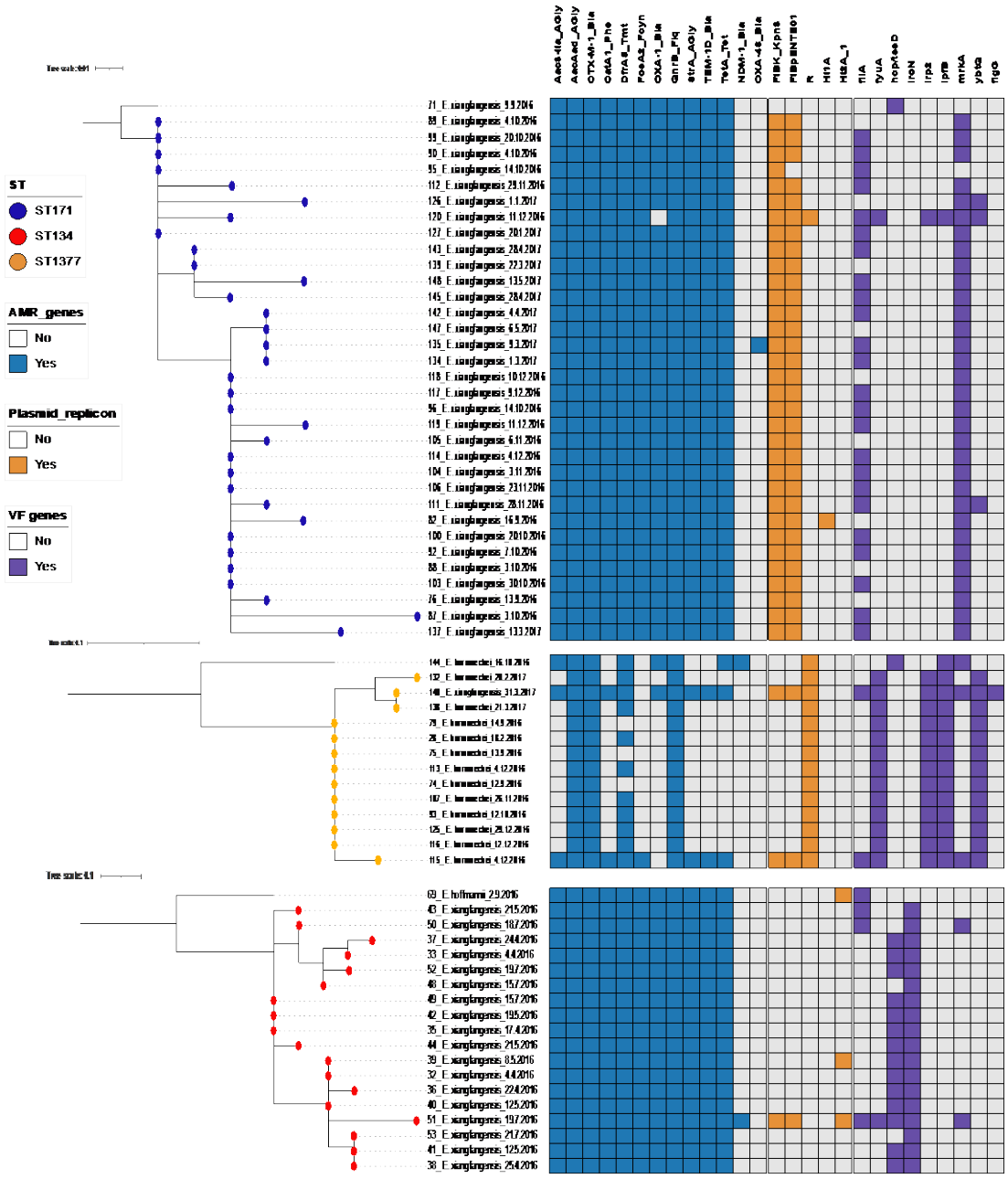
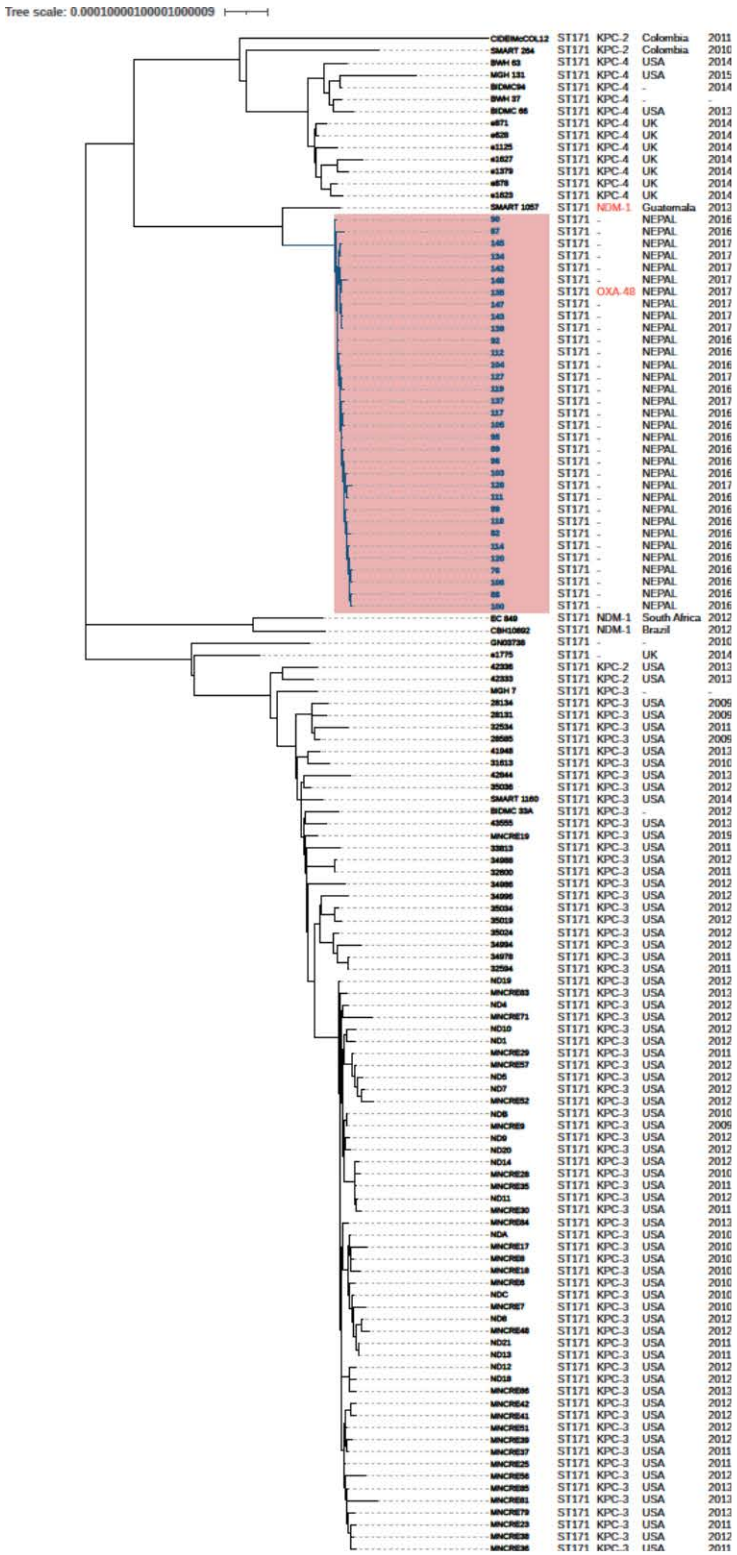


Figure 6.3. The recombination excluded core genome based maximum likelihood phylogenetic trees for isolates of ST171 (blue dots), ST1377 (brown dots), and ST134 (red dots) of *Enterobacter* species.

The presence of an array of AMR genes (blue), plasmid replicons (brown) and virulence genes (purple) for each isolate are also shown in colored square boxes to the right of the trees. Grey shading indicates the absence of the character state.

As a low genetic variability was observed among the ECC bacterial isolates within each major STs, the ST171 isolates, the most prevalent ST in this study, were subjected to a global comparison to obtain a tentative SNPs rate of ECC bacteria. A low core genome mutation rate was observed where ST171 isolates of my study differed from the global collection of 93 ST171 isolates over a period of 8 years by a total of 1,286 SNPs only (Figure 6.4).


Tree scale: 0.00010000100001000009
CIDEIMxCOL12 ST171 KPC-2 Colombia 2011
SMART 264 ST171 KPC-2 Colombia 2010
BWH 63 ST171 KPC-4 USA 2014
MGH 131 ST171 KPC-4 USA 2015
BIDMC94 ST171 KPC-4 - 2014
BWH 37 ST171 KPC-4 - -
BIDMC 66 ST171 KPC-4 USA 2013
e871 ST171 KPC-4 UK 2014
e628 ST171 KPC-4 UK 2014
e1125 ST171 KPC-4 UK 2014
e1627 ST171 KPC-4 UK 2014
e1379 ST171 KPC-4 UK 2014
e878 ST171 KPC-4 UK 2014
e1623 ST171 KPC-4 UK 2014
SMART 1057 ST171 NDM-1 Guatemala 2013
90 ST171 - NEPAL 2016
87 ST171 - NEPAL 2016
145 ST171 - NEPAL 2017
134 ST171 - NEPAL 2017
140 ST171 - NEPAL 2017
148 ST171 - NEPAL 2017
135 ST171 OXA-48 NEPAL 2017
147 ST171 - NEPAL 2017
143 ST171 - NEPAL 2017
138 ST171 - NEPAL 2017
92 ST171 - NEPAL 2016
112 ST171 - NEPAL 2016
104 ST171 - NEPAL 2016
127 ST171 - NEPAL 2017
119 ST171 - NEPAL 2016
137 ST171 - NEPAL 2017
117 ST171 - NEPAL 2016
105 ST171 - NEPAL 2016
98 ST171 - NEPAL 2016
89 ST171 - NEPAL 2016
96 ST171 - NEPAL 2016
103 ST171 - NEPAL 2016
126 ST171 - NEPAL 2017
111 ST171 - NEPAL 2016
99 ST171 - NEPAL 2016
118 ST171 - NEPAL 2016
82 ST171 - NEPAL 2016
114 ST171 - NEPAL 2016
120 ST171 - NEPAL 2016
76 ST171 - NEPAL 2016
106 ST171 - NEPAL 2016
86 ST171 - NEPAL 2016
100 ST171 - NEPAL 2016
BC 849 ST171 NDM-1 South Africa 2012
CIH10602 ST171 NDM-1 Brazil 2012
GN09736 ST171 - - 2010
e1775 ST171 - UK 2014
42336 ST171 KPC-2 USA 2013
42333 ST171 KPC-2 USA 2013
MGH 7 ST171 KPC-3 - -
28134 ST171 KPC-3 USA 2006
28131 ST171 KPC-3 USA 2009
32534 ST171 KPC-3 USA 2011
28585 ST171 KPC-3 USA 2009
41948 ST171 KPC-3 USA 2013
31613 ST171 KPC-3 USA 2010
42944 ST171 KPC-3 USA 2013
35036 ST171 KPC-3 USA 2012
SMART 1180 ST171 KPC-3 USA 2014
BIDMC 33A ST171 KPC-3 - 2012
43555 ST171 KPC-3 USA 2013
MNCRE19 ST171 KPC-3 USA 2015
33813 ST171 KPC-3 USA 2011
34088 ST171 KPC-3 USA 2012
32600 ST171 KPC-3 USA 2011
34095 ST171 KPC-3 USA 2012
34096 ST171 KPC-3 USA 2012
35034 ST171 KPC-3 USA 2012
35019 ST171 KPC-3 USA 2012
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34094 ST171 KPC-3 USA 2012
34978 ST171 KPC-3 USA 2011
32594 ST171 KPC-3 USA 2011
ND19 ST171 KPC-3 USA 2012
MNCRE83 ST171 KPC-3 USA 2013
ND4 ST171 KPC-3 USA 2012
MNCRE71 ST171 KPC-3 USA 2012
ND10 ST171 KPC-3 USA 2012
ND1 ST171 KPC-3 USA 2012
MNCRE29 ST171 KPC-3 USA 2011
MNCRE57 ST171 KPC-3 USA 2012
ND5 ST171 KPC-3 USA 2012
ND7 ST171 KPC-3 USA 2012
MNCRE52 ST171 KPC-3 USA 2012
ND8 ST171 KPC-3 USA 2010
MNCRE9 ST171 KPC-3 USA 2009
ND9 ST171 KPC-3 USA 2012
ND20 ST171 KPC-3 USA 2012
ND14 ST171 KPC-3 USA 2012
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MNCRE84 ST171 KPC-3 USA 2013
NDA ST171 KPC-3 USA 2010
MNCRE17 ST171 KPC-3 USA 2010
MNCRE8 ST171 KPC-3 USA 2010
MNCRE18 ST171 KPC-3 USA 2010
MNCRE6 ST171 KPC-3 USA 2010
NDC ST171 KPC-3 USA 2010
MNCRE7 ST171 KPC-3 USA 2010
ND6 ST171 KPC-3 USA 2012
MNCRE46 ST171 KPC-3 USA 2012
ND21 ST171 KPC-3 USA 2011
ND13 ST171 KPC-3 USA 2011
ND12 ST171 KPC-3 USA 2012
ND18 ST171 KPC-3 USA 2012
MNCRE86 ST171 KPC-3 USA 2013
MNCRE42 ST171 KPC-3 USA 2012
MNCRE41 ST171 KPC-3 USA 2012
MNCRE51 ST171 KPC-3 USA 2012
MNCRE39 ST171 KPC-3 USA 2012
MNCRE37 ST171 KPC-3 USA 2011
MNCRE25 ST171 KPC-3 USA 2011
MNCRE56 ST171 KPC-3 USA 2012
MNCRE85 ST171 KPC-3 USA 2013
MNCRE81 ST171 KPC-3 USA 2013
MNCRE79 ST171 KPC-3 USA 2013
MNCRE23 ST171 KPC-3 USA 2011
MNCRE38 ST171 KPC-3 USA 2012
MNCRE36 ST171 KPC-3 USA 2011

Figure 6.4. The recombination excluded core genome based maximum likelihood phylogenetic trees of *Enterobacter* spp. ST171 isolates of this study and carbapenem resistant ST171 global isolates of SMART and AstraZeneca global surveillance program.

The country of origin, year of isolation, and the presence of carbapenemase gene for each isolate were tabulated. The isolates of my study are highlighted in pink shading.

All ST171 and ST134 isolates carried a comparable group of AMR genes, namely *Aac3-IIa-AacAad-bla*$_{CTX\text{-}M\text{-}15}$*-bla*$_{OXA\text{-}1}$*-bla*$_{TEM\text{-}1D}$*-dfrA5-FosA2-qnrB-strAB-tetA,* conferring resistance to aminoglycosides, third-generation cephalosporins, fosfomycin, trimethoprim, quinolones, streptomycin, and tetracyclines. All ST1377 isolates carried fewer AMR genes, constituting of *AacAad-bla*$_{CTX\text{-}M\text{-}15}$*-bla*$_{OXA\text{-}1}$*-dfrA5-qnrB.* The distribution of virulence factors was distinct across the three STs. A majority of ST171 isolates had the flagella-encoding *fliA* gene, and type-3 fimbriae biosynthetic cluster (*mrkA-F*), whereas ST134 isolates predominantly carried a siderophore Salmochelin gene cluster, and an *hcp/tssD* gene associated with type VI secretion system (T6SS). ST1377 isolates had a siderophore, Yersiniabactin gene cluster, and *lpfB-lpfC* genes encoding long polar fimbriae (Figure 6.3).

All ST171 isolates carried a large IncFIB plasmid that displayed a substantial genetic similarity to a non-AMR IncFIB plasmid of a clinical *E. hormaechei* isolate detected in the USA (accession number: CP031569) (coverage: 90%, identity: 99%). The plasmid contained self-transfer modules, and multiple heavy metal resistance gene clusters against mercury, copper, iron, and arsenic (Figure 6.4). The conjugation experiments confirmed that the IncFIB plasmid was transferable conferring the resistance to multiple antimicrobials (ceftriaxone, erythromycin, streptomycin, chloramphenicol, ampicillin, gentamicin, and cotrimoxazole). Although ST134 and ST171 showed an identical AMR gene profile, I was unable to detect any specific plasmid replicon type among

ST134 isolates. However, the conjugation experiments determined that ST134 also carried a self-transferable plasmid conferring an MDR phenotype. This finding infers that the plasmid replicon carried by ST134 isolates might be a novel plasmid not found in the PlasmidFinder database. Further, I found that all ST1377 isolates harboured an IncR plasmid with high synteny and similarity to an IncR plasmid from an environmental *K. pneumoniae* isolate reported from Japan (accession number: AP021911.1) (coverage 73%, identity 99%, Figure 6-5). The plasmid was also transmissible, and mediated resistance to ceftriaxone, erythromycin, cotrimoxazole, and ofloxacin.

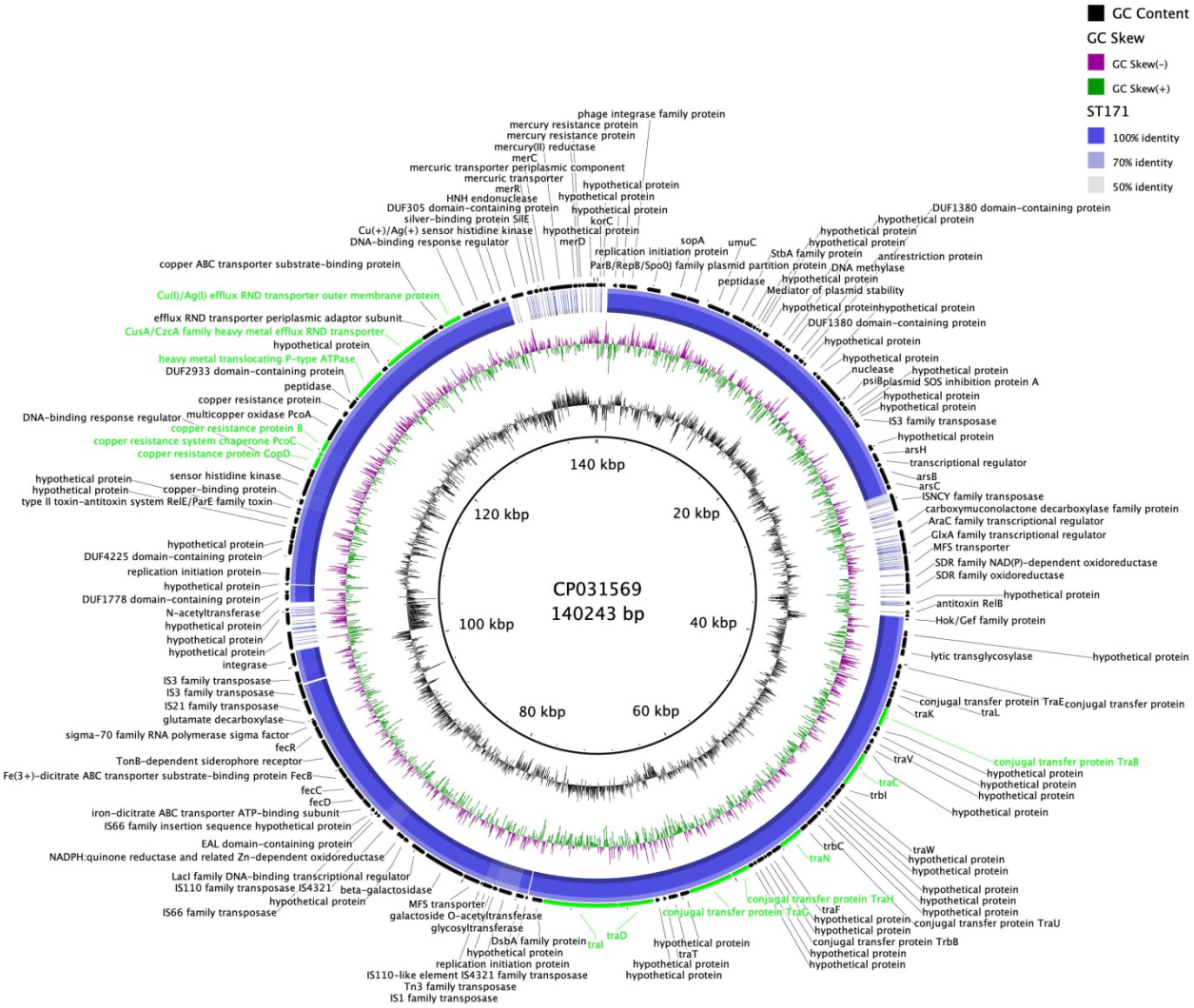

Figure 6.5. The circular map of IncFIB plasmid of *E. xiangfangensis* ST171

The innermost ring represents the IncFIB plasmid of a clinical *E. hormaechei* isolate detected in USA (accession number: CP031569) used as a reference and its coordinates. The second inner ring in black represents the GC content, followed by next concentric ring in purple/green representing GC skewness compared to the reference. The outer thicker blue ring indicates the BLASTn comparison of the IncFIB plasmid of *E. xiangfangensis* ST171 with the reference. The outermost black circle in arrow-headed lines depicts the coding sequences of *E. hormaechei* CP031569 in forward strand. The self-transfer modules of the plasmid and multiple heavy metal resistance gene clusters are labelled in green color.

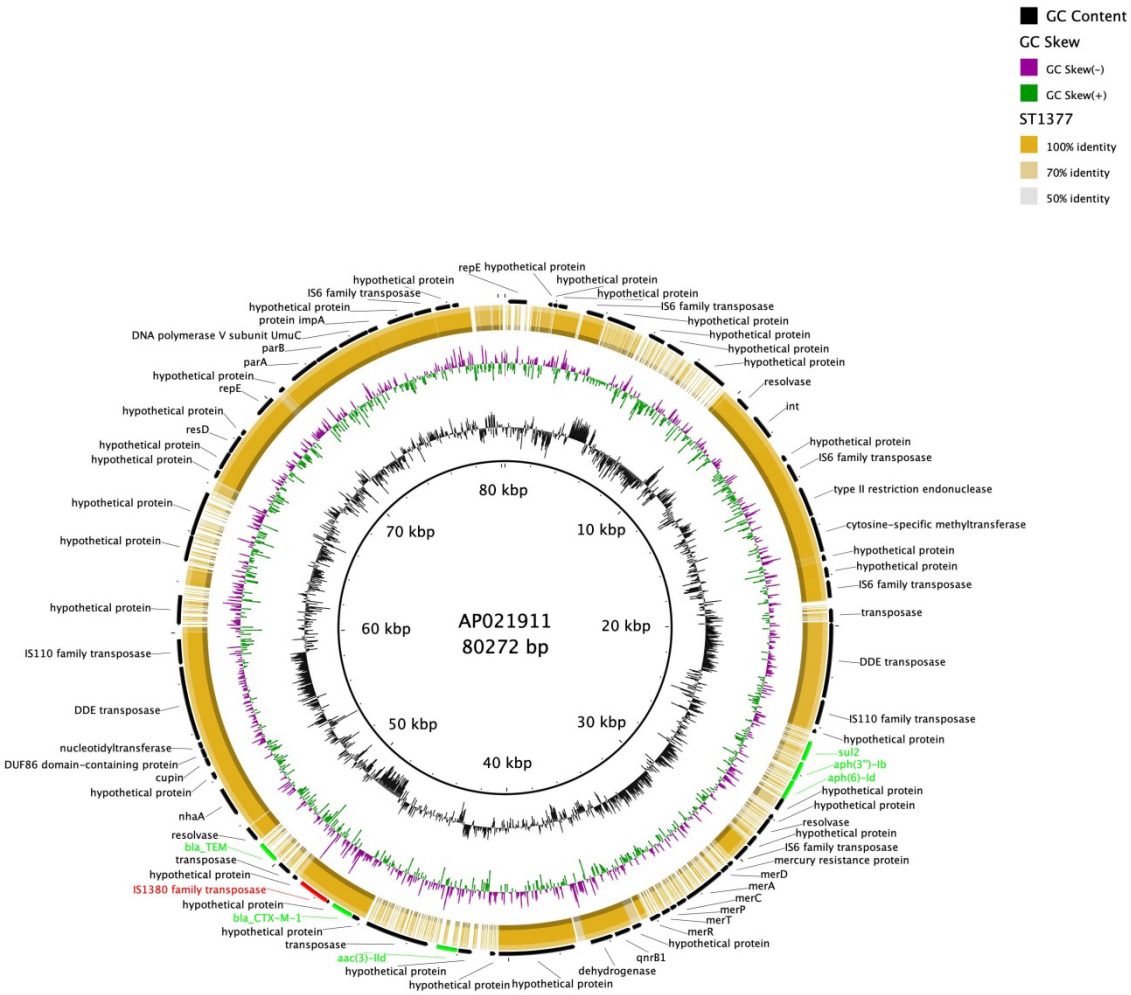

Figure 6.6. The circular map of IncR plasmid of *E. hormaechei* ST1377

The innermost ring represents an IncR plasmid from an environmental *Klebsiella pneumoniae* isolate reported from Japan (accession number: AP021911.1) used as a reference and its coordinates. The second inner ring in black represents the GC content, followed by next concentric ring in purple/green representing GC skewness compared to the reference. The outer thicker brown ring indicates the BLASTn comparison of the IncR plasmid of *E. hormaechei* ST1377 with the

reference. The outermost black circle in arrow-headed lines depicts the coding sequences of *Klebsiella pneumoniae* AP021911.1 in forward strand. The antimicrobial resistance gene clusters are labelled in green color.

6.5 Discussion

The identification of *Enterobacter* species/genotype associated with infection is important for understanding the epidemiological landscape, and AMR trends in ECC organisms. In this genomic epidemiological study, I found a diverse species and STs of *Enterobacter* isolates causing BSI. *E. xiangfangensis* (predominantly ST171, and ST134), followed by *E. hormaechei* (ST1377) were the most prevalent species, and STs circulating in this setting. The dominance of the two species of *Enterobacter* species in clinical samples was in concordance with a genomic study comprising a global collection of 170 CREC isolates between 2008 and 2014 [253]. Notably, I found that *E. xiangfangensis* isolates carried more AMR genes than *E. hormaechei*. The observation suggested that different *Enterobacter* species can exhibit different propensities to acquire the AMR genes, therefore, an accurate identification of species can be used to guide an initial treatment.

The prevalence of BSI caused by MDR ECC bacteria has increased over the last decade, thereby reducing the therapeutic options [132,250]. The fact that all ECC isolates from this study were ESBL-positive, while some were additionally resistant to carbapenems raised a serious concern. A high prevalence of BSIs caused by ESBL-producing ECC can induce an increased use of carbapenems in the clinical settings, subsequently leading to an increased risk of enrichment of the CREC variants. Earlier studies had suggested that widespread use of carbapenems had produced an antimicrobial pressure, that served as a key factor for a global emergence, and proliferation of ST171 as a dominant CREC clone [254]. A routine AMR surveillance with the integration of WGS would rapidly capture the emergence of and track the transmission of novel *Enterobacter* species/genotypes, including the carbapenem-resistant variants.

I found a limited genetic diversity among each population of 3 predominant ECC in Nepal. There are several potential explanations for this observation, including a recent strain importation followed by rapid clonal expansion, a recent population bottleneck, or community outbreaks. However, the possibility of community outbreak was less probable owing to the failure of a geospatial clustering. Additionally, Patan hospital being a referral hospital, a substantial proportion of ER attending patients may have originated from other surrounding hospitals where the infecting ECC isolates may have been acquired, leading to the observed limited genetic diversity. Further, comparison of ST171 isolates of this study with a global collection of carbapenem resistant ST171 ECC bacteria isolated from various parts of the world, primarily the United States and United Kingdom between 2009 and 2017 revealed smaller SNPs variations inferring that the core genome of ECC bacteria is highly conserved. Such small core genome mutation rate should be one of the reasons for low genetic diversity among the ECC isolates as observed in this study. The strain importation and subsequent rapid clonal spread could also potentially be the factor shaping for the observed population genetic structure in this setting. The factors that drove the transmission of such clones were largely unknown. Several studies have reported that asymptomatic carriage of ECC bacteria in body parts, such as gut, anterior nares, skin, and upper respiratory tract may act as an endogenous source of invasive infections in susceptible individuals [255]. The ability of ECC to colonize humans may play an important role in the transmission, and the development of drug-resistant phenotypes [221,242]. An improved understanding in the potential source and transmission chain of ECC organisms is required to improve the public health control measures.

6.6 Conclusions

I found that *E. xiangfangnesis* ST171, *E. xiangfangnesis* ST134, and *E. hormaechei* ST1377 were the predominant *Enterobacter* species/genotypes causing BSIs in a tertiary care hospital in

Kathmandu, Nepal. Further, I reported an extensive presence of AMR genes across several classes of antimicrobials and multiple virulence factors among ECC clones, potentially enabling the pathogens to thrive and disseminate. My study highlighted that MDR ECC clones were important pathogens of BSIs in the hospital settings, thereby underscoring the need for continued surveillance to better understand the transmission dynamics, and identify the MDR clones with epidemic potential.

Chapter 7

General Discussion

Patan hospital, being the third largest tertiary hospital in Kathmandu, Nepal, represents a tertiary healthcare facility of Nepal. With over 250,000 outpatients and over 21,000 inpatients taking healthcare services annually, the hospital statistics of Patan hospital mirrors the characteristics of community health and status of hospital care of urban-suburban Nepal, respectively. A 23 years retrospective study undertaken between 1992 and 2014 on the bacterial pathogens of BSIs provided an evidence of an increasing trend in the proportion of MDR and AMR among non-*Salmonella* GNB of the Enterobacteriaceae family in the hospital [132]. Abundant studies on typhoidal *Salmonella enterica* had taken place at Patan hospital, but not on other GNB. Therefore, it called for a further investigation to meticulously elucidate the characteristics of bacteraemia caused by non-*Salmonella* GNB and associated AMR in a higher resolution, justifying an entire series of this study.

AMR is a continuously emerging global public health problem, threatening the currently available antimicrobial therapy. LMICs including Nepal are major contributors to the growing burden of global crisis of AMR [256]. Several studies reported from various hospitals in Nepal provided an evidence that AMR was an amplifying problem in bacterial pathogens in the country [129,136,256–258]. At Patan hospital, a 23 years retrospective study had revealed an MDR proportion of 51% (n=5,319) among non-*Salmonella* bacterial pathogens causing BSIs [132], while in this study I detected the proportion of MDR to be 90% (n=1,955). This temporal increase in the proportion of MDR suggested that an increasing AMR among GNB continued to be a major problem at Patan hospital. Additionally, the AMR genes conferring resistance to extended spectrum beta-lactam and carbapenem antimicrobials were detected in a considerable proportion of

GNB pathogens in the tertiary hospital. These findings emphasized the need for a continued AMR surveillance in healthcare settings ensuring the monitoring of an ever changing evolution and spread of AMR among key bacterial pathogens. The integrated data of AMR surveillance also aids in the development of an updated antibiogram. Such data provide crucial evidence-based information for updating the existing protocol of empirical antimicrobial therapy making it more specific. Additionally, a continued AMR surveillance also plays an integral role in improving institutional IPC measures by identifying the specific hospital departments having high prevalence of resistant infections.

A high AMR towards multiple classes of antimicrobials was evidenced among the bacterial isolates in this study. In a prospective study in NICU, higher proportions of GNB were resistant to ampicillin (100%, 38/38), cefotaxime (74%, 28/38) and ampicillin-sulbactam (55%, 21/38). Earlier study conducted in NICU of Patan hospital also reported higher resistance among blood isolates of *Klebsiella* species towards cefotaxime (90%, 19/21), ampicillin-sulbactam (66%, 14/21), and chloramphenicol (65%, 13/20) [259]. Such findings are important in considering and revisiting the efficacy of the currently used antimicrobial protocol. Based on the results of my study, the resistance of GNB to aminoglycosides was relatively low (34%, 13/38 to amikacin, and 39%, 15/38), suggesting that the use of ampicillin and amikacin as the first line empirical therapy is still efficient. Despite high proportion of bacterial isolates being resistant against ampicillin, the latter is still included in the first line empirical therapy for neonatal sepsis at Patan hospital. It is because of the reason that though GBS is rarely isolated from blood culture, the paediatricians still believe GBS to be an important cause of EOS. Further in my study, <50% (39%, 15/38) of GNB were resistant to cotrimoxazole. However the latter is not considered for the treatment of neonatal sepsis at Patan hospital. In the setting where resistance to beta-lactam antimicrobials is high, my result

highlights the potential applicability of old antimicrobials like cotrimoxazole for the therapeutic management of difficult-to-treat infections.

Another important finding of my study is that all isolates of *Enterobacter* spp. originating from the ER attending patients were resistant to cefotaxime. However, all febrile patients suspected of BSIs attending to ER at Patan hospital are empirically treated with intravenous third generation cephalosporin. At Patan hospital, the most prevalent diagnosis for acute bacterial BSIs is typhoid fever, and an empirical therapy has been developed to target typhoidal *Salmonella*. My study findings provided evidence that the use of third generation cephalosporin for all suspected bacterial BSIs at ER is not optimal at Patan hospital. As my study was a retrospective study, the outcome of patients in terms of clinical improvement, the need for transfer, and upgrade of antimicrobials remained unknown. Based on my research findings, it was possible that the patients producing positive blood culture results for *Enterobacter* spp. and empirically treated with intravenous third generation cephalosporin did not improve clinically, requiring a transfer to an inpatient department and upgrade of the antimicrobial therapy. This calls for conducting a prospective study on ER attending patients with suspected bacterial BSIs with an aim of collecting the data on microbiological, therapeutic, and outcome details. With an increasing prevalence of non-Salmonella enteric GNB in causation of acute BSIs, and increasing resistance to extended spectrum beta-lactam antimicrobials, it may pose a diagnostic and therapeutic challenge to the attending physician to distinguish the underlying aetiology, and empirically treat based on the clinical signs at ER admission. As typhoid is the most prevalent cause of bacterial BSIs in Nepal, it may mask the other aetiologies. The use of various diagnostic tests such as rapid antigen test for *Salmonella enterica*, CRP, complete blood cell counts etc. may help in accurate diagnosis and subsequent treatment of BSIs at ER.

In this study, it was identified that PICU-NICU was the first inpatient department with the highest prevalence of blood culture positivity during the study period constituting of 14% (295/2,153) of all isolates. Similar finding had also been observed in the earlier 23 year retrospective study, where 21% (2,217/10,496) of blood culture isolates originated from the paediatric ICU population at Patan hospital. Depending on the local epidemiology, NICU admitted neonates may acquire infection either vertically or horizontally. In my prospective NICU study (chapter 4), it was observed that 80% of sepsis episodes were of LOS type, implying the horizontal transmission being a major mode of infection in the unit. With various invasive modalities of neonatal care being associated with increased odds of neonatal sepsis in the risk factor study, it further pointed out that the hospital acquisition of infections was prominent in this unit during the study period. Further, the mono-clonal transmission dynamics of *Enterobacter* sepsis episodes confirmed for the horizontal acquisition, and direct/indirect cross-transmission events in the NICU. The history of recurrent infection outbreak events in the NICU unit also indicated the same, inferring to suboptimal hospital IPC measures and/or persistent source of environmental colonization.

Reducing the prevalence of nosocomial infections in hospitals is the fundamental objective of IPC practices which also plays a crucial role in reducing the overall use of antimicrobials, and subsequent the reductions of AMR. Because of limited resources, the nosocomial infection control is often the least prioritized area in hospitals of LMICs including Nepal. There is no formal national guideline in place for IPC measures in Nepal [260]. Limited IPC practices, poor hospital waste management, poor knowledge of HCWs, and inadequate resources of universal precautions are great challenges in Nepal. One hospital-based survey from Nepal reported that 47% of 17 different sized hospitals in Nepal lacked hospital IPC manual, 59% didn't have any functional infection control committee, and 65% of hospitals didn't have any provision of providing IPC trainings to their staffs [261]. Another survey based study on 324 HCWs from five hospitals in

Kathmandu reported that only 56% of medical doctors complied to hand hygiene, only 33% used protective aprons while handling biological samples, and 91% recapped the needles on a regular basis [260]. Patan hospital is one of the few tertiary hospitals with a formal IPC manual and an IPC committee. Despite the formulation of IPC manual and committee, available resources in the hospital are limited to enable the manual to be equally operational. The lack of isolation room in high risk departments to seclude septic patients, limited access to the logistics of universal precaution, high patient to doctor ratio, and insufficient and poor infrastructural designs are still the challenges of the hospital to ensure an optimal functionality of the IPC practices. Departments, such as NICU, where the load of immune-compromised patients is high, are specifically at increased risk of infections particularly during outbreaks. Higher infections loads are specifically true during the times of high bed occupancy, understaffing, and lack of staff training and audit. During the study period, there was a frequent turnover of the nursing staffs in the NICU, which potentially resulted into a breach in the existing IPC measures contributing to the infection outbreak events. A substantial reduction observed in the number of infectious cases after a reinforcement of stringent aseptic measures such as extensive environmental decontamination with fumigation, standard hand hygiene practice, aseptic protocol for all invasive procedures, proper decontamination of equipment before sharing, seclusion of culture-proven cases, and allocation of dedicated nursing staffs for clinical care of the culture-positive cases in my research highlighted the importance of continued IPC intervention practices in the prevention of HAIs.

In this study, of a total 98 *Enterobacter* spp., (18 being derived from NICU, and 80 from ER patients) subjected to WGS analysis, *E. kobei* was isolated only from the NICU. The emergence of *E. kobei* as a cause of neonatal sepsis at Patan hospital NICU was intriguing, and may infer to the predilection of *E. kobei* towards neonatal infections. *E. kobei* has been seldom reported in various clinical specimens, such as bronchoalveolar lavage, blood, and abscess [262,263]. Till date, there

are no explicit reports on *E. kobei* as a cause of neonatal sepsis. This may be because of two main reasons. Firstly, the taxonomic assignment of *Enterobacter* spp. is highly complex, and is being consecutively updated [79]. Further, species differentiation of the ECC bacteria, and precise identification of *E. kobei* is challenging based on routinely used conventional identification methods [79]. This may have resulted into the under reporting of *E. kobei* in clinical specimens. Secondly, the detection of *E. kobei* from the NICU admitted neonates only in my study may just be of a local significance, rather than the result of an explicit association of *E. kobei* with neonatal infections. Like other ECC bacteria, *E. kobei* may be associated with opportunistic nosocomial infections among immunocompromised individuals in high dependency hospital units, such as neonates admitted to NICU as evidenced in my study.

The detection of *E. kobei* as a cause of outbreak of neonatal sepsis in my study instead infers to the possibility of establishment of *E. kobei* in our NICU environment, though the possibility of direct case-to-case transmission can't be excluded. All isolates of *E. kobei* isolated from the neonatal samples were devoid of all tested virulence genes except for the *mrkABCDF* gene cluster encoding for type 3 fimbriae [240]. The type 3 fimbriae has been demonstrated to facilitate binding to the host cells, and formation of dense biofilms [240,241]. The detection of *mrkABCDF* gene cluster in all neonatal isolates of *E. kobei* therefore suggests for the possibility of environmental establishment of *E. kobei* in NICU by the formation of biofilms. This may explain for the history of recurrent persistence of infection outbreaks in our unit with an apparent cessation after an extensive environmental fumigation only.

The possibility of an environmental colonization as a source of infection is further corroborated by the fact that during the outbreak, not all neonates were infected simultaneously, but were infected intermittently in a span of 3 months. Further, the *E. kobei* isolates from the infected neonates were

not identical with zero SNPs. Except for 2 isolates which were just 1 SNP apart from each other inferring direct cross transmission, the average SNPs distance for rest of the outbreak causing *E. kobei* isolates was 3 SNPs. This observation infers that majority of infections might have been acquired from one or more common environmental or biological source/s of colonization. The environmental surveillance carried out by the hospital during the study period however could not identify the environmental source of *E. kobei* colonization. Further, it was intriguing that all neonatal *E. kobei* isolated from NICU lacked all AMR genes except for bla_{ACT28}, *fosA2*, and *mcr-10*. This was in contrary to *Enterobacter* spp. isolated from the ER attending patients and *E. kobei* isolated from PICU, in which an extensive array of AMR genes against several classes of antimicrobials were detected. The isolation of AMR genes lacking *E. kobei* isolates from the neonates despite the clinical administration of several classes of antimicrobials suggests for the protective effect of biofilm produced in vivo by *E. kobei*. The ability of *E. kobei* to inflict a fatal infection outbreak for 3 consecutive months in an NICU despite the absence of an extensive array of AMR and virulence genes strengthens the speculation on the importance of biofilm formation in the pathogenicity, virulence, and persistence of *E. kobei* in the observed NICU outbreak. Biofilms have been observed to protect bacterial pathogens against host defense mechanisms, and prevent the therapeutic concentration of antimicrobials from reaching the bacterial cells [240]. Additional experimental studies are required to establish the speculation on biofilm formation by *E. kobei* isolates in our unit.

In addition to the possibility of an environmental colonization as a potential source of infection, another possibility was the colonized neonate and/or mother as a source of infection. In this study, nasal and nail bed swabs from few admitted neonates were tested, but *Enterobacter* spp. was not isolated. Studies have pointed out into potential role of bacterial gut colonization in the development of sepsis, particularly in premature neonates [221,222,264,265]. Immature gut

barrier, native immunity, decreased gastric acid secretion, low levels of protective mucous, and decreased intestinal motility in premature and LBW neonates have been identified to be the factors making the neonatal gut prone to bacterial colonization [266]. Further, an increased empirical use of broad-spectrum antimicrobials in NICU may hinder the gut colonization by commensal bacterial flora and facilitate the colonization by antimicrobial resistant nosocomial bacterial pathogens instead, increasing the risk of sepsis [266]. The stool or rectal swab samples of the neonates were not tested in routine surveillance by the hospital during the study period. This calls for the need to conduct an epidemiological surveillance culture of stool samples of NICU admitted neonates to monitor and characterize neonatal gut colonization in NICU. Further a high resolution genomic analysis will be required to discern the genotype of colonizing isolate from the sepsis causing isolate. During the study, I performed WGS on bacterial culture of stool samples collected from the mothers once postnatally, and that collected from the neonates on the NICU admission day and weekly thereafter. However, detailed analyses to investigate the association between colonization and infection could not be performed because of limitation of time during the thesis.

Antibiotic stewardship is one of the crucial steps to curtail the AMR among the bacterial pathogens by facilitating a rational use of antimicrobial agents. Though ministry of health and population of Nepal has formulated a national antibiotic treatment guidelines in 2014, and a national antimicrobial resistance containment action plan in 2016, most of the hospitals in Nepal lack a functional antibiotic stewardship policy [129,267]. The improper use of antimicrobials is also common in hospitals where over- and irrational- prescription of antimicrobials by the medical doctors is common [256]. At Patan hospital, only the NICU department has a formal antimicrobial stewardship policy where an empirical antimicrobial treatment protocol has been formulated which is annually reviewed based on an updated antibiogram that is reviewed every year. The policy is strictly followed by all paediatricians in the units. The other departments of the hospital however

lack a stewardship program. A high prevalence of AMR observed among the bacterial pathogens of BSIs isolated from other inpatient hospital departments and ER may suggest for the overuse of antimicrobials. This observation underpins the need for the implementation of an antibiotic stewardship throughout all hospital departments.

In chapter 6 of my study, because of the lack of prior clinical and demographic information, it was not possible to accurately distinguish if the observed BSI was acquired in community or as a result of recent exposure to health care institution prior to current admission. Because patients with acute febrile illnesses mostly seek urgent healthcare in ER department, the infections present among ER attending patients at the time of admission may be considered as community-onset type. Few studies have generally considered BSIs observed among ER patients at admission as community acquired [247]. Though this may be true in a subset of ER visiting febrile patients, the rest of patients may have hospital- or health care- acquired infection as a result of recent health care exposure. A detailed clinical data on variables, such as prior hospitalization in last 3 months, transfer from other hospital or health care facility, the need for recurrent health care procedures such as haemodialysis, chemotherapy etc., history of surgery in past 3 months etc. would be required to accurately distinguish CA-BSIs from HA-BSIs. Compared to HA-BSIs, the studies on epidemiology of CO-BSIs are less reported. With properly collected epidemiological data, the genomic analyses of bacterial pathogens isolated from ER patients can, therefore provide a unique opportunity to explore the epidemiology of CO-BSIs, including transmission dynamics of the pathogens and their genomic determinants of AMR and virulence and identification of bacterial clones of concern with epidemic potentials and carrying transmissible cassettes for multiple AMR and virulence genes.

In this study, the blood culture positivity due to non-*Salmonella* GNB was found to be the highest (24%, 511/2,153) among ER attending patients. ER of Patan hospital was reported to have the

highest prevalence of blood culture positivity (36%, 3,808/10,496) since past ≥23 years. Also, noteworthy was the fact that like in my study, *Enterobacter* spp. had consistently been the most prevalent bacterial pathogen among BSI suspected ER patients at Patan hospital since past ≥23 years [132]. In contrary to my results, compared to the other bacterial genus, the contribution of *Enterobacter* spp. as a causative agent of BSIs is much less pronounced in other studies reported from several parts of the world [268–272]. Further, compared to other Enterobacteriaceae bacterial pathogens such as *K. pneumoniae*, there were less reported genomic analyses on *Enterobacter* spp. ECC being identified as an emerging member of '*E. coli* and the ESKAPE' pathogen of BSIs, and its predominance, particularly among ER admitted patients provided a premise to carry out a genomic study on this bacterial pathogen. The resulting findings were interesting and perplexing.

The detection of diverse species and STs of *Enterobacter*, several of which had never been reported before from Nepal were interesting. More interesting and concerning was the detection of an extensive array of AMR and virulence gene cassettes in BSI causing *Enterobacter* spp., isolated from ER attending patients. Further, *E. kobei* isolated from PICU differed from that isolated from NICU by just 5 core genome SNPs, but carried a repertoire of several accessory genes encoding AMR and virulence that were entirely lacking in NICU isolates. Such research findings suggested that AMR was a concern among outpatient infections, which may have been derived from other hospitals or community, demanding for a further research. A metagenomics approach on the gut colonization of people and associated AMR together with a carefully collected epidemiological data can provide invaluable information to understand an ongoing epidemiology of infections among ER and OPD attending outpatients.

The detection of low genetic diversity among *Enterobacter* spp. isolates of major STs causing BSIs among ER attending patients was intriguing. Small core SNPs based genetic distance in the

isolates indicated that the ECC isolates within each STs were genetically closely related, although available limited demographic data failed to show any geographic clustering. The most likely explanation for this observation is that the BSIs were acquired by a majority of patients from common hospitals or health institutions where they received healthcare service before visiting to the ER at Patan hospital. This is a likely scenario as Patan hospital is a referral tertiary care hospital. Alternatively, if the infections were assumed to have been acquired from community, such finding was difficult to explain because of lack of complete and accurate demographic, clinical, and pre-clinical information on the patients. I suspected that the location of residence provided by most of the patients was incorrect. The patients might have provided the permanent address, which refers to the parental homes or places of birth, rather than their current residential locations in Kathmandu. My speculations are supported by the fact that if the patients residing out of Kathmandu valley had acute febrile illness requiring an emergency medical care, they would have sought the hospitals nearby their residence, instead of visiting at Patan hospital in Kathmandu, which would take several hours or days of commute. If majority of patients were from within the Kathmandu valley, it may indicate for a common source of infection. It may be that the patients yielding clonal *Enterobacter* spp. have potentially shared a close occupancy in the community. The observed high degree of clonality may also be explained based on the hypothesis that the isolates of each major STs might had been recently introduced in Nepal, which were then selected, and expanded resulting in a rapid clonal expansion. The observed ambiguity warrants for a further well-planned genomic research on *Enterobacter* spp. isolates derived from outpatients with an aim of collecting a true representation of demographic data.

My thesis research has produced important and interesting findings. It highlighted into a high burden of antimicrobial resistance among four predominant and clinically important members of '*E. coli* and the ESKAPE' group of GNB at Patan hospital. My research identified ER and NICU

as two specific hospital departments where burden of antimicrobial resistant BSIs was the highest, and thus informed the hospital to direct preventive measures in these specific outpatient and inpatient departments respectively. My research identified that during the study period, a majority of BSIs among inborne neonates in NICU were acquired horizontally rather than vertically from the mother, and were significantly associated with an increased use of various invasive procedures, thereby underscoring the need to revise and strengthen the IPC measures at NICU. Compared to other bacterial pathogens, the contributory role of *Enterobacter* spp. as a causative agent of BSIs has been less pronounced in other parts of the world. In contrast, I found that *Enterobacter* spp. was one of the predominant non-*Salmonella* bacterial pathogens of BSIs among ER attending outpatients as well as NICU admitted neonatal population at Patan hospital. The finding on the aetiological role of *mcr-10* and biofilm encoding *mrk* gene cluster positive *E. kobei* of novel ST as a sole driver of a fatal infection outbreak at an NICU for a continuum of 3 months despite the presence of limited AMR and virulence genes was interesting. Finally, the detection of several species and STs of BSI causing ECC bacteria from ER carrying a multitude of AMR and virulence genes, but relatively limited genetic diversity for each major STs was equally important and intriguing.

Compared to other Enterobacteriaceae members, the epidemiological and genomic studies on *Enterobacter* spp. are less reported. My research findings on important aetiological role of ECC bacteria as a causative agent of invasive BSIs among the patients of diverse age groups (neonates in NICU and adults in ER) and clinical background demands for further epidemiological and genomic studies on ECC bacteria to establish the clinical significance. With limited existing information available on *E. kobei*, my research findings ask new questions on the pathogenesis, clinical significance, and epidemiology including the source and mode of transmission of *E. kobei*.

The failure to identify colonized environmental source/s of infection, and an inability to conduct a continued surveillance culture of the NICU admitted neonates and medical staffs to identify potential role of body colonization on subsequent infection were the limitations of the study. This calls for undertaking a more detailed surveillance culture on scientifically and thoughtfully selected specimens. In my research chapter on *Enterobacter* spp. isolated from ER patients, I could not distinguish if the BSI was acquired in community or from other health care institutions. Patan hospital being a major referral tertiary care hospital, though a majority of BSIs could have been hospital acquired among referred patients, a subset of BSIs might have been acquired from the community among the patients with no recent history of hospital exposure. If the latter was true, my research findings that ECC bacteria of major STs lacked core genetic diversity, and carried a vast array of AMR genes across multitude of antimicrobial classes imposes an important clinical significance. This demands to conduct a well-planned longitudinal study on blood isolates of *Enterobacter* spp. among the outpatients with carefully collected data on pathogen genomics and patients' demographic, therapeutic, laboratory, and outcome details.

Chapter 8

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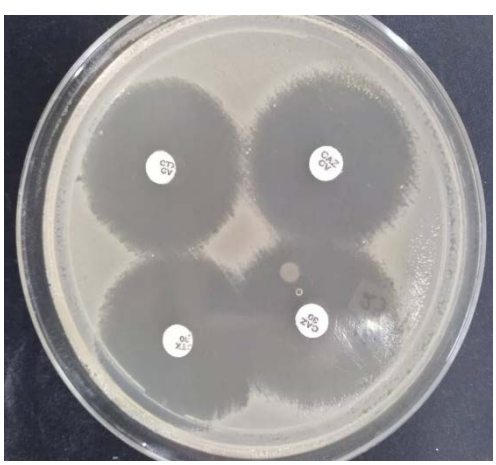
Chapter 9

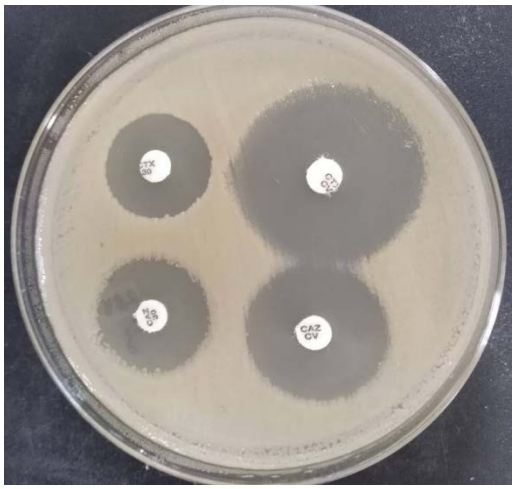
Appendices

9.1 The representative images of phenotypic confirmatory tests for ESBL positivity by combination disc diffusion method

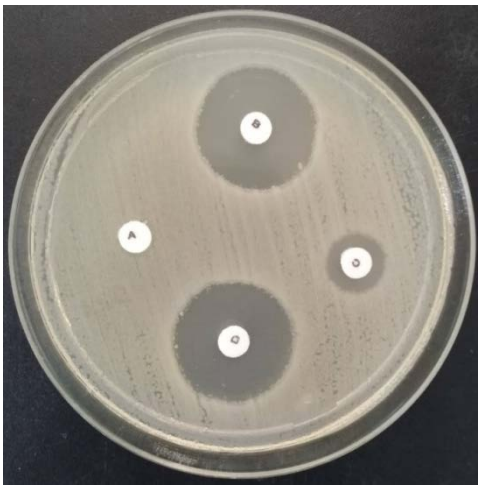


a.

b.



c.

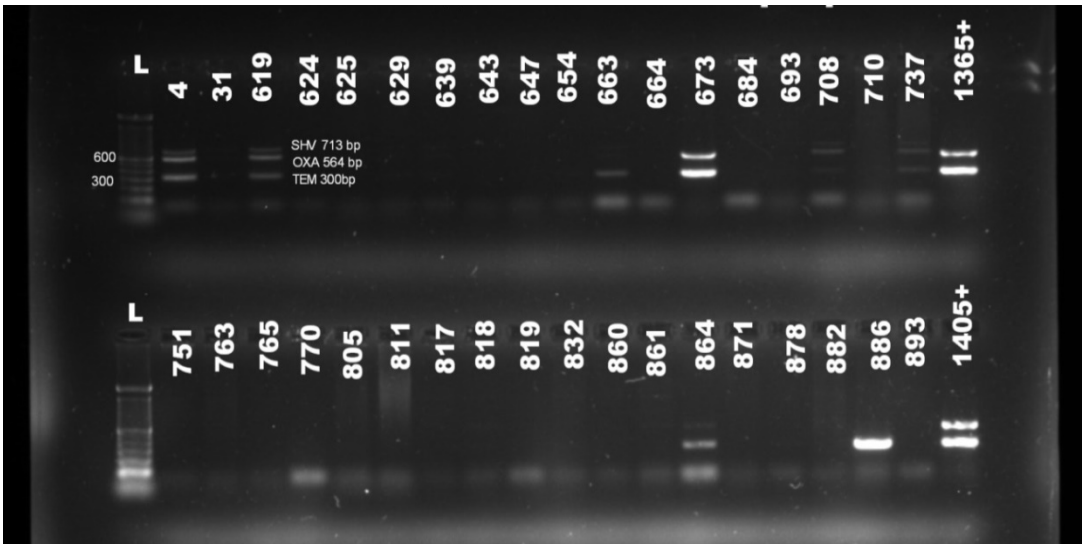


d.

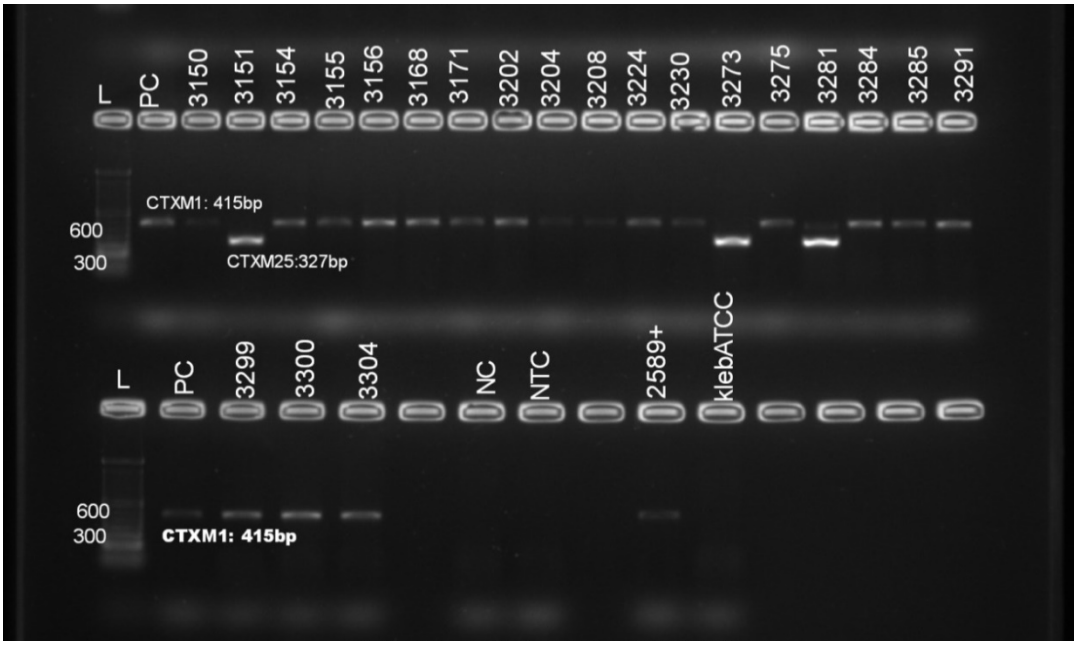
Figure 9.1 The results of the phenotypic ESBL confirmatory test.

Figures a, b show results for *E. coli* ATCC strain 25922 while figures c and d show results for *K. pneumoniae* ATCC strain 700603. *E. coli* showed negative test result for both ESBL and AmpC, while *K. pneumoniae* showed positive test results for ESBL but negative for AmpC production in the tests carried out using D62C (a, c) and D68C (b, d) antimicrobial disc sets.

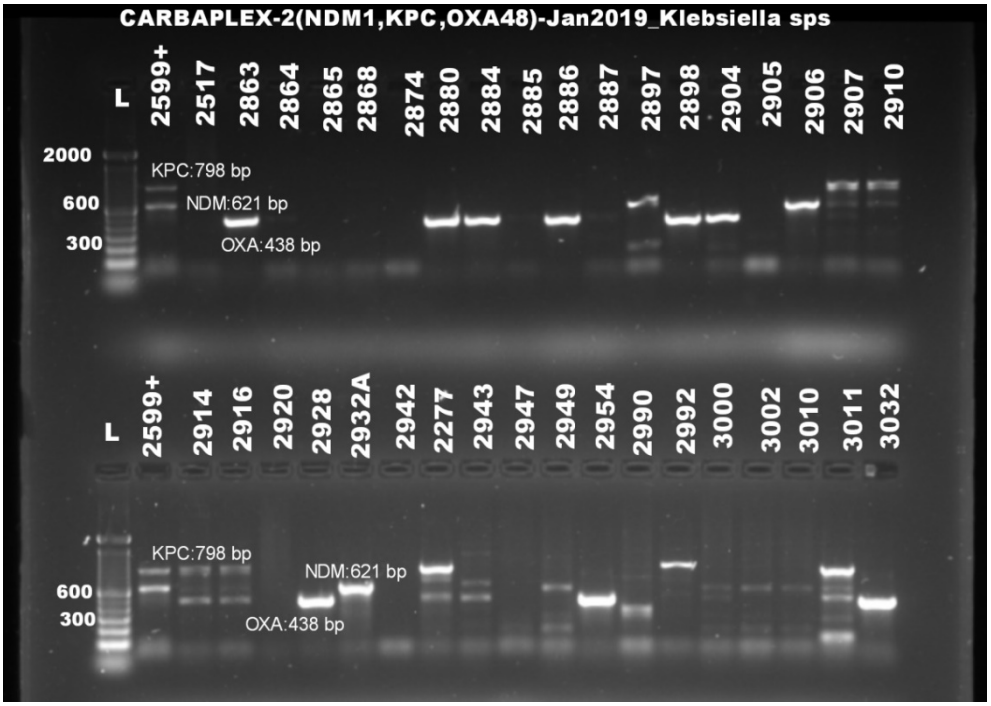
9.2 Representative gel electrophoresis images of multiplex PCR for AMR genes



a.



b.


CARBAPLEX-2(NDM1,KPC,OXA48)-Jan2019_Klebsiella sps
L
2599+
2517
2863
2864
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2868
2874
2880
2884
2885
2886
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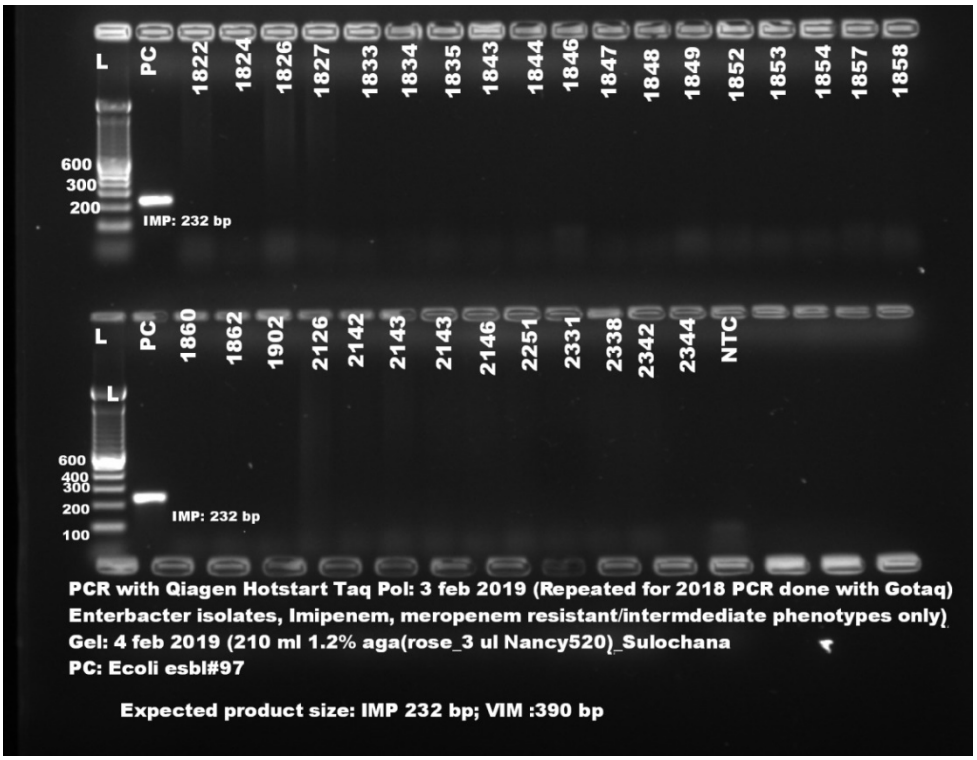
2000
600
300
KPC:798 bp
NDM:621 bp
OXA:438 bp
L
2599+
2914
2916
2920
2928
2932A
2942
2277
2943
2947
2949
2954
2990
2992
3000
3002
3010
3011
3032
KPC:798 bp
NDM:621 bp
OXA:438 bp
600
300

c.


L
PC
1822
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IMP: 232 bp
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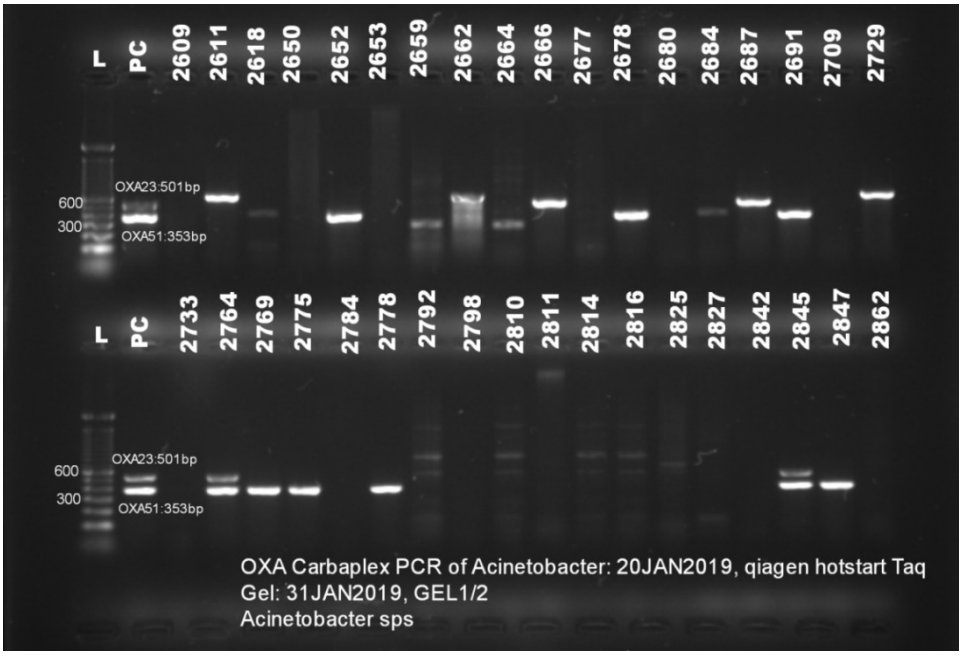
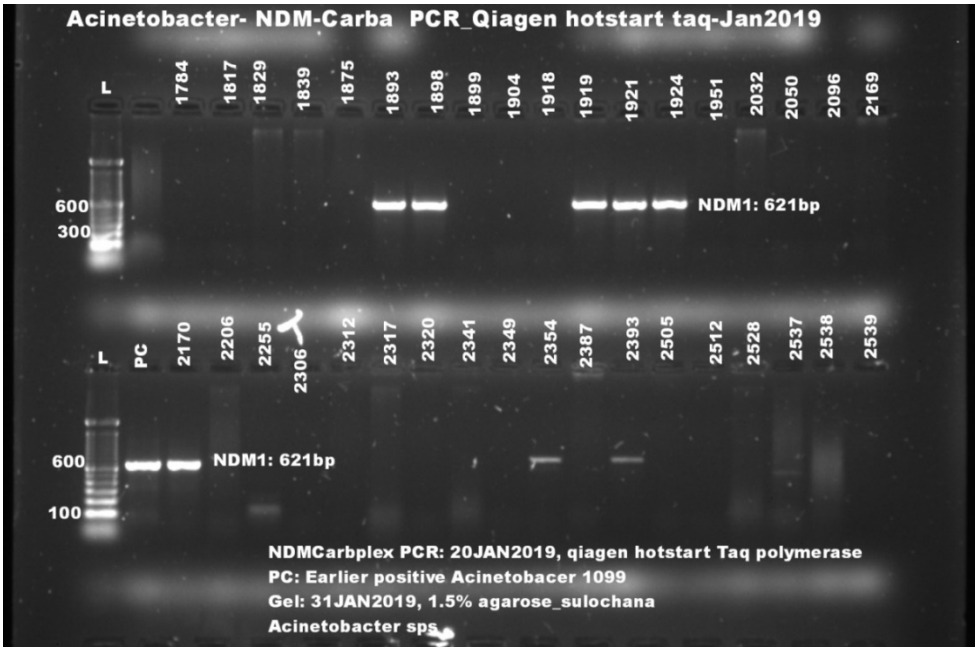
PC
1860
1862
1902
2126
2142
2143
2143
2146
2251
2331
2338
2342
2344
NTC
L
600
400
300
200
100
IMP: 232 bp
PCR with Qiagen Hotstart Taq Pol: 3 feb 2019 (Repeated for 2018 PCR done with Gotaq)
Enterbacter isolates, Imipenem, meropenem resistant/intermdediate phenotypes only)
Gel: 4 feb 2019 (210 ml 1.2% aga(rose_3 ul Nancy520)_Sulochana
PC: Ecoli esbl#97
Expected product size: IMP 232 bp; VIM :390 bp

d.



e.

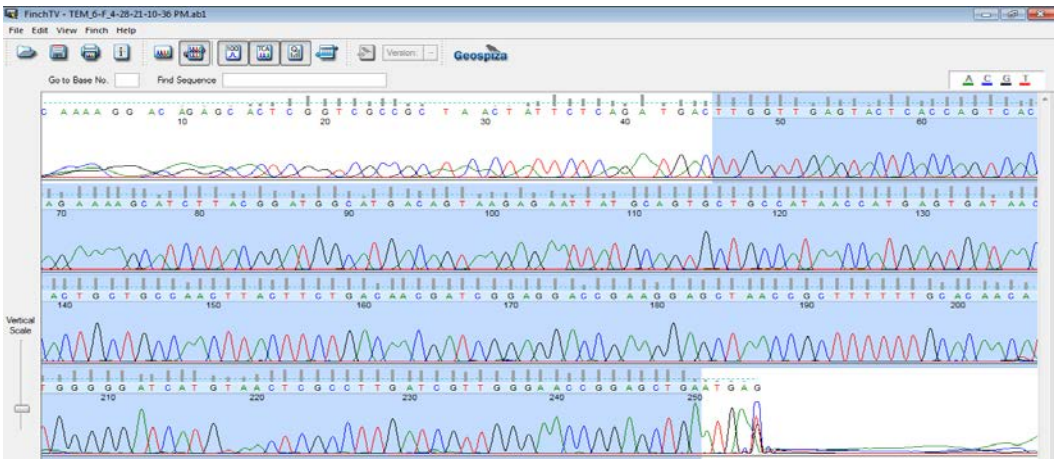


f.

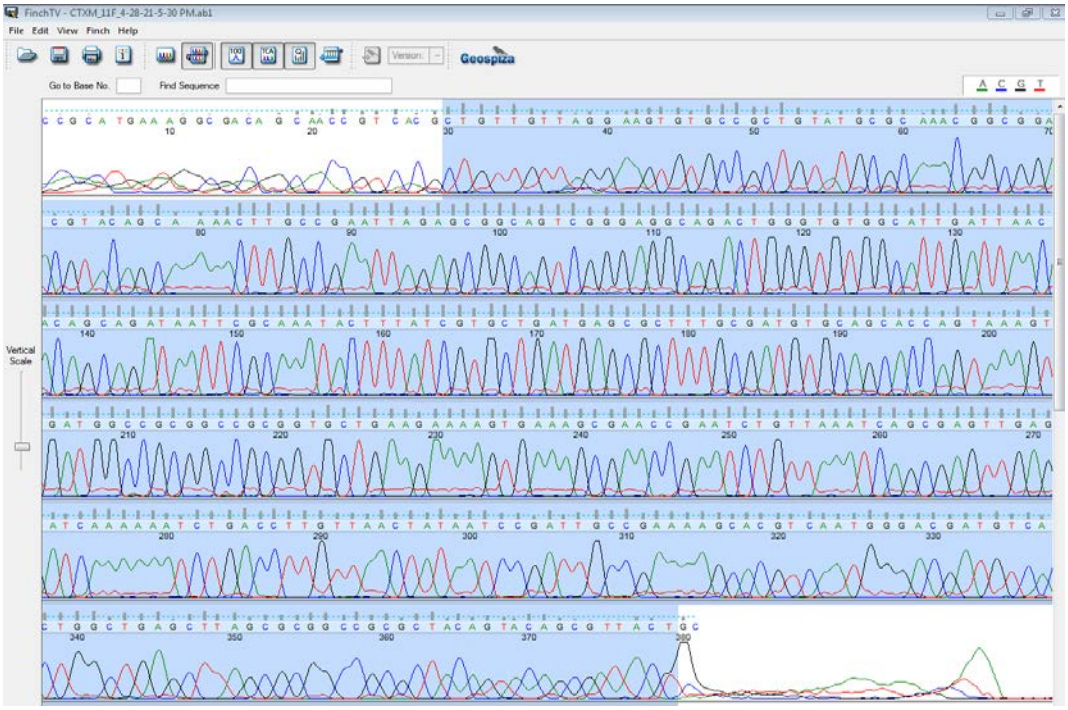
Figure 9.2 The results of multiplex PCR test for screening the AMR genes.

a. TSO, b. CTX-M, c. Carba-1, d. Carba-2, e. A-Carba 1 and f. A-Carba 2 multiplex PCR tests. PC, KlebATCC or +: positive control; NC: negative control; NTC: no template control

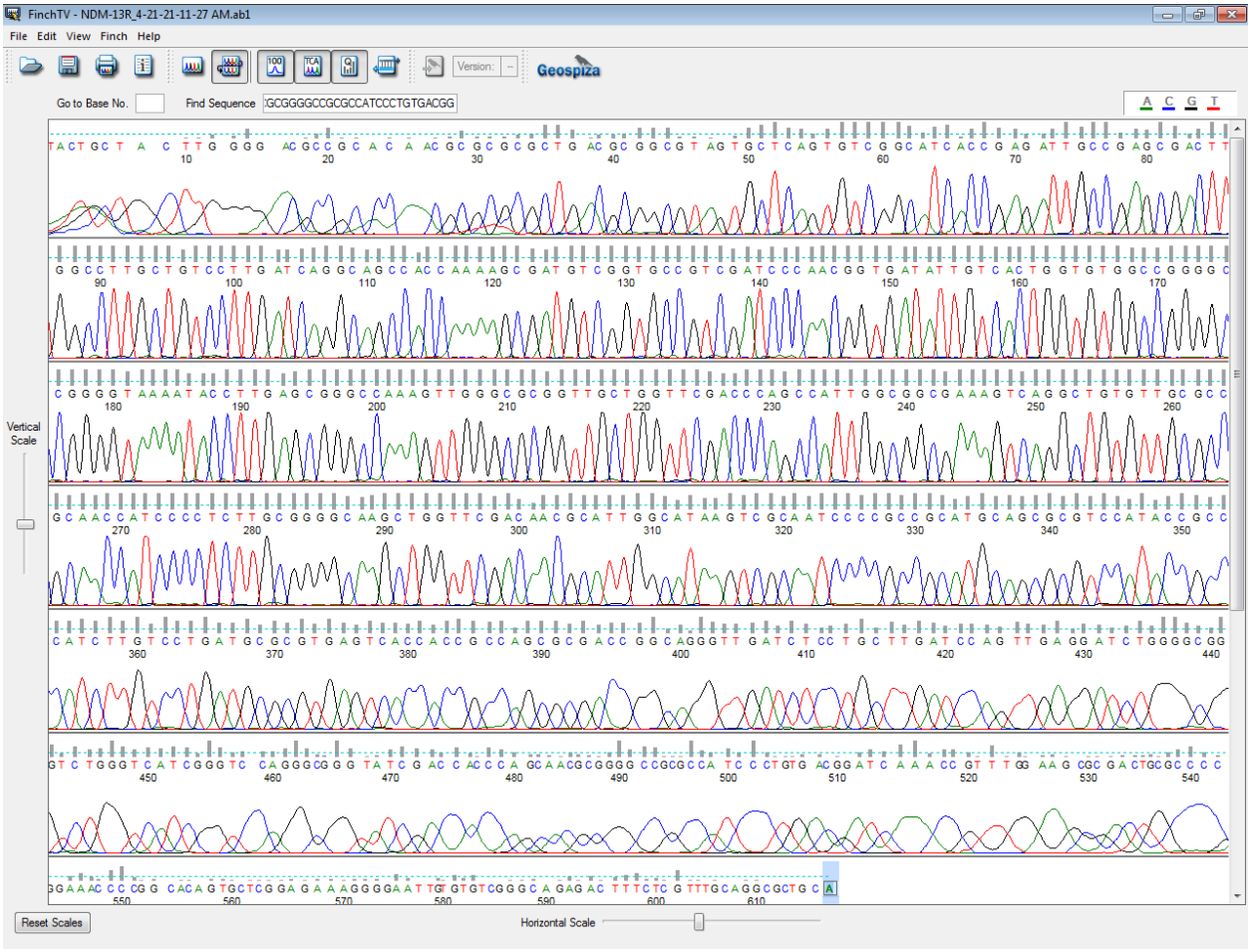
9.3 Representative electropherogram traces of Sanger DNA sequencing for AMR gene amplicons



a.



b.



c.

Figure 9.3 The electropherogram trace results of Sanger DNA sequencing for the PCR products of various AMR genes.

a. TEM, b. CTX-M1, c. NDM-1 PCR products electrophemogram

9.4 An informed consent form used for Chapter 4

Consent form

Consent to participate in the neonatal intensive care unit (NICU) study

- I have been informed and fully understood the benefits and drawbacks of this study. I agree to be a part of this study and also agree for my child's data to be collected.
- I know who to contact if I need more information. I understand that confidentiality will be preserved. I understand that I am free to withdraw from the study at any time without affecting the care I or my baby will receive. I agree that my child' samples will be used for research as mentioned in the information sheet,
- I agree for the study team to access my and my child's medical records

Participant Number: 17NP - ___ ___ - ___ ___ ___

Child's name: [_____]

Mother's name: [_____] Signature: [_____]

Doctor's name: [_____] Signature: [_____]

Date: [___/___] [___/___] [___/___/___] [Day] [Month] [Year]

If the mother of the participating neonate is illiterate, ask another person but her husband who is independent to the study, to explain to the mother what is written in the informed consent form and get their name and signature in the space below.

The mother has been informed and fully understands the benefits and drawbacks of this study. She agrees to be a part of this study and also agrees for her child's data to be collected.

Intermediary's name: [_____] Signature: [_____]

Doctor's name: [_____] Signature: [_____]

Date: [___/___] [___/___] [___/___/___] [Day] [Month] [Year]

There will be no cost to you for participating in this study. The study will not pay for any of your routine clinical care as requested by your doctor.
The samples we collect from your child are not extra samples, it is collected from routine samples for clinical use. Being in the study will be of no direct benefit to you or your baby, but will help us gain a better understanding of risk factors for neonatal blood infections and the associated micro-organisms and hopefully help us prevent this in the future.

Confidentiality.

You identification will not be disclosed at any point in time. All data will be stored securely at all times with only the specific study investigation team having access. Everyone will be provided Personal Identification Numbers (PIN) and all databases will be stored with only the PIC. Your identification will be kept separately from the main database.

Voluntary participation.

The decision to take part in this study or not is solely yours. You are under no compulsion to take part in this part in this study. If you choose to change your mind at a later date once you have enrolled in this study today, you have the right to withdraw from this study at any point in time without it affecting any of your rights. The treatment and management for you and your child will remain the same irrespective of your decision to remain or leave the study.

If you have any further questions now or later you can either ask your treating physician or one of the doctors listed below:

Dr Shrijana Shrestha

Dr Suchita Joshi

Dr Abhilasha Karkey

Patan Hospital

Phone number: 5521024, 5522278, 5522266

Consent form

Consent to participate in the neonatal intensive care unit (NICU) study

- I have been informed and fully understood the benefits and drawbacks of this study. I agree to be a part of this study and also agree for my child's data to be collected.
- I know who to contact if I need more information. I understand that confidentiality will be preserved. I understand that I am free to withdraw from the study at any time without affecting the care I or my baby will receive. I agree that my child' samples will be used for research as mentioned in the information sheet,
- I agree for the study team to access my and my child's medical records

Participant Number: 17NP - ___ ___ - ___ ___ ___

Child's name: [_____]

Mother's name: [_____] Signature: [_____]

Doctor's name: [_____] Signature: [_____]

Date: [___/___] [___/___] [___/___/___] [Day] [Month] [Year]

If the mother of the participating neonate is illiterate, ask another person but her husband who is independent to the study, to explain to the mother what is written in the informed consent form and get their name and signature in the space below.

The mother has been informed and fully understands the benefits and drawbacks of this study. She agrees to be a part of this study and also agrees for her child's data to be collected.

Intermediary's name: [_____] Signature: [_____]

Doctor's name: [_____] Signature: [_____]

Date: [___/___] [___/___] [___/___/___] [Day] [Month] [Year]

9.5 A case report form (CRF) used for recording data for Chapter 4

Study number: [_____]

Date: [___/___] [___/___] [___/___/___] [Day] [Month] [Year]

Demographic data

Mother's hospital number: [_____]

Name: [_____] Age: [_____] years Occupation: [_____]

Address: [_____]

Contact No: [_____]

Date of admission of mother: [___/___] [___/___] [___/___/___/___] [Day] [Month] [Year]

Date of study enrollment: [___/___] [___/___][___/___/___/___] [Day] [Month] [Year]

Admission from: ☐ Home

☐ Hospital

If from hospital: ☐ Private Hospital

☐ Government Hospital

☐ Lower Health Facilities

Duration of stay: [_____] days [_____] weeks

Obstetric History

Parity of mother: [_____] Abortion: ☐ Yes ☐ No

Reason for abortion: [_____]

Anti natal care check : ☐ Done ☐ Not done

TT Injection: ☐ Taken
☐ Not taken [If taken, no of doses: _____]

Were there any problems during pregnancy?

☐ Yes [**if yes**, fill the table below]
☐ No

S. no	During first trimester	During second trimester	During third trimester
1			
2			
3			
4			

Microbiological investigations of mother:

S. no.	Date sample sent [Day] [Month] [Year]	Sample	Isolate
1			
2			
3			
4			
5			

High Vaginal Swab:

Date: [___/___] [___/___][___/___/___/___] [Day] [Month] [Year]

Findings:

Ultrasound findings:

Date: [___/___] [___/___][___/___/___/___] [Day] [Month] [Year]

Number of PV examinations:

S.No.	Date of Examination	Frequency

Ante-partum antibiotics (in the previous 4 months):

☐ Taken ☐ Not Taken ☐ Not known

Perinatal period

Duration of labor pain: [_____] hours

PPROM/ PROM: ☐ Yes ☐ No

If yes, use of antibiotics:

S.N	Antibiotics	Days	Comments
1			
2			
3			

Foetal distress: ☐ Yes ☐ No

If yes, Heart Rate: ☐ Bradycardia ☐ Tachycardia

If non-stress testing was done, findings:

Date: [___/___] [___/___][___/___/___/___] [Day] [Month] [Year]

Induction of labor ☐ Done ☐ Not Done

Mode of delivery: ☐ Normal ☐ Vaginal/Assisted ☐ Cesarean

Amniotic fluid colour: ☐ Clear ☐ Meconium mixed ☐ Foul smelling

Gender: ☐ Male ☐ Female

Gestational age: [____] weeks

Weight of child: [____] grams

APGAR score: [ ] at 1 min & [ ] at 5 min

Suctioning: ☐ Done ☐ Not done

Resuscitation: ☐ Done ☐ Not done

Intubation: ☐ Done ☐ Not done

Post natal period

Ward: [_____] Bed No: [_____]

Date: [___/___] [___/___][___/___/___/___] [Day] [Month] [Year]

Post Partum Fever: ☐ Yes ☐ No

Microbiological testing

S.No	Date sample sent [Day] [Month] [Year]	Sample	Isolate
1			
2			
3			
4			
5			

Infants ID: [_____]

Date of Admission: [___/___] [___/___][___/___/___/___] [Day] [Month] [Year]

Indication for admission: [_____]

Date of Examination: [___/___] [___/___][___/___/___/___] [Day] [Month] [Year]

Head circumference: [_____] centimeters

Length: [_____] centimeters

Differential Diagnosis:

Invasive devices: ☐ Yes ☐ No

[**If yes**, fill the table below]

S. no	Lines/Tubes/Drains:	Date of insertion	Date of removal	Total Days

Antibiotic treatment received by neonate in the NICU:

S. no	Medicine	Start date	End date	Total days
		/ /	/ /	
		/ /	/ /	
		/ /	/ /	
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		/ /	/ /	
		/ /	/ /	
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Outcome

Length of stay in NICU: [_____] days

Length of stay in Hospital: [_____] days

Final Diagnosis: [_____]

Final Outcome: ☐ Survived ☐ Dead ☐ Need to follow up
☐ DNR ☐ LAMA

Readmission into the NICU

Date of Admission: [___/___] [___/___][___/___/___/___]

Readmission from: ☐ Home

☐ Other Hospital

☐ Other ward of Patan Hospital

If from other wards of Patan Hospital, which ward was it? [_____]

Duration of stay in the other ward: [_____] days

Final Outcome (after readmission): ☐ Survived

☐ Dead

☐ Need to follow up

☐ LAMA

Microbiology investigations:

S. no	Date sample sent [Day] [Month] [Year]	Sample	Isolate
1	/ /		
2	/ /		
3	/ /		
4	/ /		
5	/ /		
6	/ /		
7	/ /		
8	/ /		
9	/ /		
10	/ /		

For cerebrospinal fluid (CSF) cultures:

Isolate No.				
Date	/ /	/ /	/ /	/ /
RBC				
WBC				
Neutrophils				
Lymphocytes				
Protein				
Sugar				

Laboratory investigations:

S.N	Date [dd] [mm] [yyyy]	/ /	/ /	/ /	/ /	/ /	/ /	/ /
	Day							
	WBC							
	Neutrophil							
	Lymphocytes							
	Eosinophil							
	Monocytes							
	Hct/Hb							
	Platelets							
	MCV/MCHC							
	ESR							
	CRP							
	PT/INR							
	APTT							
	Total Bilirubin							
	Direct Bilirubin							
	Alkphosp							
	SGPT							
	SGOT							
	Creatinine							
	Glucose							
	Sodium							
	Potassium							
	Urea							
	Calcium							
	Others:							
1.								
2.								

Ultrasound Reports:

Date [dd] [mm] [yyyy]	Day	Results
/ /		
/ /		
/ /		

Study number: [_____] ☐ **Obstetric History (Mother)** ☐ **Post Natal (Mother)** ☐ **Neonate**

Antibiotic Susceptibility Testing:

Date of isolation [mm/dd/yyyy]	/ /		/ /		/ /		/ /		/ /	
	ISOLATE 1		ISOLATE 2		ISOLATE 3		ISOLATE 4		ISOLATE 5	
	Specimen-		Specimen-		Specimen-		Specimen-		Specimen-	
ANTIBIOTIC	Sensitivity	MIC	Sensitivity	MIC	Sensitivity	MIC	Sensitivity	MIC	Sensitivity	MIC
Amikacin										
Amoxicillin										
Amoxicillin-clavulanic										
Ampicillin										
Ampicillin Sulbactam										
Azithromycin										
Aztreonam										
Cefotaxime										
Cefoxitin										
Ceftriaxone										
Chloramphenicol										
Ciprofloxacin										
Colistin										
Gentamycin										

Date of isolation [mm/dd/yyyy]	/ /		/ /		/ /		/ /		/ /	
ANTIBIOTIC	ISOLATE 1		ISOLATE 2		ISOLATE 3		ISOLATE 4		ISOLATE 5	
	Sensitivity	MIC	Sensitivity	MIC	Sensitivity	MIC	Sensitivity	MIC	Sensitivity	MIC
Imipenem										
Meropnem										
Nalidixic Acid										
Nitrifurantoin										
Ofloxacin										
Penicillin										
Piperacillin Tazobactam										
Teicoplanin										
Tetracycline										
Tigecycline										
Trimethoprim-sulphamethoxazole										

Follow up sheet

Study number: [_____] **Day:** [_____]

Date of examination: [___/___] [___/___] [___/___/___] [Day] [Month] [Year]

Weight: [_____] grams

Respiratory Support:

☐ Spontaneous ☐ CPAP ☐ Ventilation

☐ Nasal Prong

CNS Status:	Seizure:	☐ Yes	☐ No
	Lethargy:	☐ Yes	☐ No
CVS Status:	HR: [_____]	BP: [_____]	CRT: [_____]
	Ionotropes:	☐ Yes	☐ No
	Fluid Bolus:	☐ Yes	☐ No
	If yes, number of bolus: [_____]		
GI Status:	Distension:	☐ Yes	☐ No
	GI bleed:	☐ Yes	☐ No
	Breast Feeding:	☐ Yes	☐ No
	Spoon Feeding:	☐ Yes	☐ No
If yes,	Enteral feeding:	☐ Yes	☐ No
Skin Status:	Ulcer/ Wound	☐ Yes	☐ No
Blood transfusion:		☐ Yes	☐ No
Canula insertion:		☐ Yes	☐ No
Foley's catheter		☐ Yes	☐ No
UAC/UVC		☐ Yes	☐ No
ET tube		☐ Yes	☐ No

1

Follow up sheet

Study number: [_____] **Day:** [_____]

Date of examination: [___/___] [___/___] [___/___/___] [Day] [Month] [Year]

Weight: [_____] grams

Respiratory Support:

☐ Spontaneous ☐ CPAP ☐ Ventilation

☐ Nasal Prong

CNS Status:	Seizure:	☐ Yes	☐ No
	Lethargy:	☐ Yes	☐ No
CVS Status:	HR: [_____]	BP: [_____]	CRT: [_____]
	Ionotropes:	☐ Yes	☐ No
	Fluid Bolus:	☐ Yes	☐ No
	If yes, number of bolus: [_____]		
GI Status:	Distension:	☐ Yes	☐ No
	GI bleed:	☐ Yes	☐ No
	Breast Feeding:	☐ Yes	☐ No
	Spoon Feeding:	☐ Yes	☐ No
If yes,	Enteral feeding:	☐ Yes	☐ No
Skin Status:	Ulcer/ Wound	☐ Yes	☐ No
Blood transfusion:		☐ Yes	☐ No
Canula insertion:		☐ Yes	☐ No
Foley's catheter		☐ Yes	☐ No
UAC/UVC		☐ Yes	☐ No
ET tube		☐ Yes	☐ No

9.6 The details on submission of whole genome DNA sequences of *Enterobacter* spp.

Table 9.1. The accession numbers of WGS data of *Enterobacter* spp. submitted to the European nucleotide archive.

Sample alias	Accession number	forward_file_name	reverse_file_name
98Q0100	ERS6485182	98Q0100_1.fastq.gz	98Q0100_2.fastq.gz
98Q0103	ERS6485183	98Q0103_1.fastq.gz	98Q0103_2.fastq.gz
98Q0104	ERS6485184	98Q0104_1.fastq.gz	98Q0104_2.fastq.gz
98Q0105	ERS6485185	98Q0105_1.fastq.gz	98Q0105_2.fastq.gz
98Q0106	ERS6485186	98Q0106_1.fastq.gz	98Q0106_2.fastq.gz
98Q0107	ERS6485164	98Q0107_1.fastq.gz	98Q0107_2.fastq.gz
98Q0109	ERS6485187	98Q0109_1.fastq.gz	98Q0109_2.fastq.gz
98Q0111	ERS6485188	98Q0111_1.fastq.gz	98Q0111_2.fastq.gz
98Q0112	ERS6485189	98Q0112_1.fastq.gz	98Q0112_2.fastq.gz
98Q0113	ERS6485165	98Q0113_1.fastq.gz	98Q0113_2.fastq.gz
98Q0114	ERS6485190	98Q0114_1.fastq.gz	98Q0114_2.fastq.gz
98Q0115	ERS6485166	98Q0115_1.fastq.gz	98Q0115_2.fastq.gz
98Q0116	ERS6485167	98Q0116_1.fastq.gz	98Q0116_2.fastq.gz
98Q0117	ERS6485191	98Q0117_1.fastq.gz	98Q0117_2.fastq.gz
98Q0118	ERS6485192	98Q0118_1.fastq.gz	98Q0118_2.fastq.gz
98Q0119	ERS6485193	98Q0119_1.fastq.gz	98Q0119_2.fastq.gz
98Q0120	ERS6485194	98Q0120_1.fastq.gz	98Q0120_2.fastq.gz
98Q0123	ERS6485195	98Q0123_1.fastq.gz	98Q0123_2.fastq.gz
98Q0125	ERS6485168	98Q0125_1.fastq.gz	98Q0125_2.fastq.gz
98Q0126	ERS6485196	98Q0126_1.fastq.gz	98Q0126_2.fastq.gz
98Q0127	ERS6485197	98Q0127_1.fastq.gz	98Q0127_2.fastq.gz
98Q0128	ERS6485198	98Q0128_1.fastq.gz	98Q0128_2.fastq.gz
98Q0132	ERS6485169	98Q0132_1.fastq.gz	98Q0132_2.fastq.gz
98Q0133	ERS6485181	98Q0133_1.fastq.gz	98Q0133_2.fastq.gz
98Q0134	ERS6485199	98Q0134_1.fastq.gz	98Q0134_2.fastq.gz
98Q0135	ERS6485200	98Q0135_1.fastq.gz	98Q0135_2.fastq.gz
98Q0137	ERS6485201	98Q0137_1.fastq.gz	98Q0137_2.fastq.gz
98Q0138	ERS6485170	98Q0138_1.fastq.gz	98Q0138_2.fastq.gz

98Q0139	ERS6485202	98Q0139_1.fastq.gz	98Q0139_2.fastq.gz
98Q0140	ERS6485171	98Q0140_1.fastq.gz	98Q0140_2.fastq.gz
98Q0142	ERS6485203	98Q0142_1.fastq.gz	98Q0142_2.fastq.gz
98Q0143	ERS6485204	98Q0143_1.fastq.gz	98Q0143_2.fastq.gz
98Q0144	ERS6485172	98Q0144_1.fastq.gz	98Q0144_2.fastq.gz
98Q0145	ERS6485205	98Q0145_1.fastq.gz	98Q0145_2.fastq.gz
98Q0147	ERS6485206	98Q0147_1.fastq.gz	98Q0147_2.fastq.gz
98Q0148	ERS6485207	98Q0148_1.fastq.gz	98Q0148_2.fastq.gz
98Q028	ERS6485173	98Q028_1.fastq.gz	98Q028_2.fastq.gz
98Q029	ERS6485160	98Q029_1.fastq.gz	98Q029_2.fastq.gz
98Q032	ERS6485208	98Q032_1.fastq.gz	98Q032_2.fastq.gz
98Q033	ERS6485209	98Q033_1.fastq.gz	98Q033_2.fastq.gz
98Q035	ERS6485210	98Q035_1.fastq.gz	98Q035_2.fastq.gz
98Q036	ERS6485211	98Q036_1.fastq.gz	98Q036_2.fastq.gz
98Q037	ERS6485212	98Q037_1.fastq.gz	98Q037_2.fastq.gz
98Q038	ERS6485213	98Q038_1.fastq.gz	98Q038_2.fastq.gz
98Q039	ERS6485214	98Q039_1.fastq.gz	98Q039_2.fastq.gz
98Q040	ERS6485215	98Q040_1.fastq.gz	98Q040_2.fastq.gz
98Q041	ERS6485216	98Q041_1.fastq.gz	98Q041_2.fastq.gz
98Q042	ERS6485217	98Q042_1.fastq.gz	98Q042_2.fastq.gz
98Q043	ERS6485218	98Q043_1.fastq.gz	98Q043_2.fastq.gz
98Q044	ERS6485219	98Q044_1.fastq.gz	98Q044_2.fastq.gz
98Q046	ERS6485220	98Q046_1.fastq.gz	98Q046_2.fastq.gz
98Q048	ERS6485221	98Q048_1.fastq.gz	98Q048_2.fastq.gz
98Q049	ERS6485222	98Q049_1.fastq.gz	98Q049_2.fastq.gz
98Q050	ERS6485223	98Q050_1.fastq.gz	98Q050_2.fastq.gz
98Q051	ERS6485224	98Q051_1.fastq.gz	98Q051_2.fastq.gz
98Q052	ERS6485225	98Q052_1.fastq.gz	98Q052_2.fastq.gz
98Q053	ERS6485226	98Q053_1.fastq.gz	98Q053_2.fastq.gz
98Q058	ERS6485227	98Q058_1.fastq.gz	98Q058_2.fastq.gz
98Q067	ERS6485161	98Q067_1.fastq.gz	98Q067_2.fastq.gz
98Q068	ERS6485162	98Q068_1.fastq.gz	98Q068_2.fastq.gz
98Q069	ERS6485163	98Q069_1.fastq.gz	98Q069_2.fastq.gz
98Q070	ERS6485174	98Q070_1.fastq.gz	98Q070_2.fastq.gz
98Q071	ERS6485228	98Q071_1.fastq.gz	98Q071_2.fastq.gz

98Q074	ERS6485175	98Q074_1.fastq.gz	98Q074_2.fastq.gz
98Q075	ERS6485176	98Q075_1.fastq.gz	98Q075_2.fastq.gz
98Q076	ERS6485229	98Q076_1.fastq.gz	98Q076_2.fastq.gz
98Q077	ERS6485177	98Q077_1.fastq.gz	98Q077_2.fastq.gz
98Q079	ERS6485178	98Q079_1.fastq.gz	98Q079_2.fastq.gz
98Q081	ERS6485179	98Q081_1.fastq.gz	98Q081_2.fastq.gz
98Q082	ERS6485230	98Q082_1.fastq.gz	98Q082_2.fastq.gz
98Q087	ERS6485231	98Q087_1.fastq.gz	98Q087_2.fastq.gz
98Q088	ERS6485232	98Q088_1.fastq.gz	98Q088_2.fastq.gz
98Q089	ERS6485233	98Q089_1.fastq.gz	98Q089_2.fastq.gz
98Q090	ERS6485234	98Q090_1.fastq.gz	98Q090_2.fastq.gz
98Q091	ERS6485235	98Q091_1.fastq.gz	98Q091_2.fastq.gz
98Q092	ERS6485236	98Q092_1.fastq.gz	98Q092_2.fastq.gz
98Q093	ERS6485180	98Q093_1.fastq.gz	98Q093_2.fastq.gz
98Q095	ERS6485237	98Q095_1.fastq.gz	98Q095_2.fastq.gz
98Q096	ERS6485238	98Q096_1.fastq.gz	98Q096_2.fastq.gz
98Q099	ERS6485239	98Q099_1.fastq.gz	98Q099_2.fastq.gz

The WGS was performed on the Illumina Hiseq X Ten platform using the Nextera XT library construction protocol that generated the insert size of 150 bp