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Onyekwuo, Anthony

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The Burden of Nosocomial Neonatal Sepsis at the Chris Hani Baragwanath Academic Hospital using Conventional Culture Methods and Minimal Invasive Tissue Sampling

by

Anthony Onyekwuo

(801139)

A dissertation submitted to the School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine in the field of Microbiology

Declaration

I Anthony Uchenna Onyekwuo, hereby declare that this dissertation titled " The Burden of Nosocomial Neonatal Sepsis at the Chris Hani Baragwanath Academic Hospital using Conventional Culture Methods and Minimal Invasive Tissue Sampling" is my original work and has not been submitted in part or in whole for any other degree at this or any other institution. All sources used or referred to in the preparation of this dissertation have been duly acknowledged in the text and in the bibliography.

I understand the academic principles and guidelines set forth by the University of the Witwatersrand regarding plagiarism and the proper citation of sources. Any contribution from other individuals or sources has been properly credited, and I have obtained any necessary permissions to use copyrighted material.

I take full responsibility for the content, structure, and arguments presented in this dissertation. Any errors or omissions are entirely my own. This dissertation represents the culmination of my independent research and academic efforts during the specified period of study, under the guidance of my supervisors, Prof. Ziyaad Dangor, Prof. Shabir A. Madhi, and Dr. Jeannette Wadula.

I also declare that the data and findings presented in this dissertation are genuine and have not been manipulated or falsified in any way. The methodologies employed in this research are accurately described, and any deviations from standard procedures have been duly explained.

Sign 09/09/2024



Abstract

Background: Neonatal sepsis is a clinical syndrome characterised by systemic signs of infection occurring within the first 28 days after birth, accompanied by the isolation of microorganisms in blood or cerebrospinal fluid (CSF) cultures. It can be categorised into early-onset sepsis (EOS) and late-onset sepsis. EOS occurs within the first 72 hours of life and usually results from vertical transmission of organisms from the mother, often occurring in utero. Late-onset sepsis occurs between 3 and 28 days and may be horizontally transmitted by the mother, other close contacts, or the community. Nosocomial neonatal sepsis, a subset of late-onset sepsis acquired within healthcare facilities, is associated with multiple factors and is often caused by pathogens such as *Staphylococcus aureus, Klebsiella pneumoniae,* and *Acinetobacter baumannii*. This type of sepsis is defined as occurring at least 48 hours after admission or birth. In Sub-Saharan Africa in 2017, a significant proportion of neonatal deaths (75%) occurred within the first seven days of life, with 50% occurring within the first 24 hours. This study aims to investigate the burden of nosocomial sepsis in neonates at Chris Hani Baragwanath Academic Hospital from 2016 to 2019, specifically focusing on cases caused by Methicillin-resistant *Staphylococcus aureus, Klebsiella pneumoniae,* and *Acinetobacter baumannii*.

Methods: This was a retrospective descriptive study of the incidence, and laboratory and clinical characteristics of *Acinetobacter baumannii, Klebsiella pneumoniae* and Methicillin-resistant *Staphylococcus aureus* on blood and/or cerebrospinal fluid in neonates hospitalised (at least 48 hours after admission or birth) at the CHBAH from January 2016 to December 2019. We identified cases through the National Health Laboratory Services- Cooperate Data Warehouse database and cross-referenced that with the neonatal discharge database, and the extracted demographics, laboratory and clinical data.

Results: Over the study period, we identified 859 culture-confirmed nosocomial neonatal sepsis caused by *Acinetobacter baumannii* (462; 53.8%), *Klebsiella pneumoniae* (323; 37.6%) and Methicillin Resistant *Staphylococcus aureus (*MRSA) (74; 8.6%). The incidence (per 1000 live births) of *Acinetobacter baumanni and Klebsiella pneumoniae* remained consistent over the study period, and was highest at 4.03 (95%CI: 3.36-4.80) and 2.91 (95%CI: 2.34-3.57) in 2017, respectively. The incidence of MRSA declined from 1.59 (95%CI: 1.16-2.13) in 2016 to 0.21 (95%CI: 0.08-0.43; p=<0.001). The case fatality rate was 54.5%, 40.6% and 31.1% for

Acinetobacter baumannii, *Klebsiella pneumoniae* and MRSA respectively. Most (68.4%) neonates with *Acinetobacter baumannii* had very low birth weight and 162 (35.1%) were HIV-exposed. Similarly, a large proportion (52.0%) of neonates with *Klebsiella pneumoniae* were very low birth weight and 34.7% were HIV-exposed. *Acinetobacter baumannii* had an overall resistance of 71.9% to amikacin, 66% to cefepime, and 66.5% to ceftazidime.

Conclusion: The incidence and case fatality rates of nosocomial *Acinetobacter baumannii* and *Klebsiella pneumoniae* were high in this setting, particularly amongst neonates with low birth weight. The study emphasises the need for improved infection prevention and control measures, antibiotic stewardship, implementation of enhanced therapeutic approaches, and efforts to minimise prolonged hospital stays.

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I would like to express my sincere gratitude to all those who have contributed to the realisation of this endeavor. My heartfelt thanks go to the National Health Laboratory Service, the Cooperate Data Warehouse of the NHLS -, and the Respiratory and Meningococcal Pathogen Research Unit RMPRU now Wits-Vaccine and Infectious Diseases Analytics, whose specific contribution significantly enriched the quality of this work.

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List of Abbreviations

AME	Aminoglycoside modifying enzyme
API	Analytical Profile Index
CA	Community-associated
CDA	Complete diagnostic autopsy
CDC	Centre for Disease Control
CDW	Cooperate Data Warehouse
CLSI	Clinical Laboratory Standards Institute
CoD	Cause of death
CPE	Carbapenemase-Producing Enterobacterales
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
HA	Healthcare-associated
HAI	Healthcare-associated infection
HIV	Human Immunodeficiency Virus
HvKp	Hypervirulent *Klebsiella pneumoniae*
Ig	Immunoglobulin
Lab	Laboratory
LMIC	Low-middle-income countries
LPS	Lipopolysaccharide
MBLs	Metallo-β-lactamases
MDR	Multidrug resistance
MIC	Minimum Inhibitory Concentrations
MITS	Minimally Invasive Tissue Sampling
MRSA	Methicillin-resistant *Staphylococcus aureus*
OMPs	Outer membrane proteins
PBP	Penicillin Binding Protein
PDR	Pan drug resistance
PMTCT	Prevention from Mother to child Transmission
RIT	Repeat Infection Time
RMPRU	Respiratory and Meningococcal Pathogen Research Unit
RNA	Ribonucleic acid
RVD	Retroviral Disease
SANAS	South African National Accreditation System
TSS	Toxic Shock Syndrome
TSST	Toxic Shock Syndrome Toxin
WHO	World Health Organisation
XDR	Extensive drug-resistance

Chapter 1: Introduction and Literature Review

1.1 Introduction

An estimated 3.6 million neonatal deaths were reported worldwide in 2015, with the majority occurring in low-and middle-income countries (LMICs) [1]. All-cause neonatal mortality is estimated at 34 per 1000 live births in LMICs with most of these deaths happening within the first week of life [2]. South Africa has a neonatal mortality rate of 22 per 1000 live births, primarily attributed to complications of prematurity or intrapartum events, congenital malformations, and infections [3]. There has been significant progress in reducing global neonatal mortality, with a 51% reduction observed from 1990 to 2017, decreasing from 37 deaths per 1000 live births to 18 deaths per 1000 live births [4].

Neonatal sepsis is a clinical syndrome characterised by systemic signs of infection within the first 28 days after birth and isolating micro-organisms on blood or cerebrospinal fluid (CSF) cultures [5]. Sepsis occurring within the first 72 hours of life is defined as early-onset sepsis (EOS), usually resulting from vertical transmission of organisms from the mother, with infection often occurs in utero [6]. Late-onset sepsis (LOS) occurs between 3 and 28 days and may be horizontally transmitted by the mother, other close contacts or the community [6]. In Sub-Saharan Africa in 2017, a significant proportion of neonatal deaths, 75% occurred within the first seven days of life, with 50% occurring within the first 24 h [4, 7, 8].

On the other hand, nosocomial sepsis, defined as a healthcare-associated infection (HAI), is an infection acquired in a hospital or other healthcare facility, usually occurring within 48-72 hours of admission [9]. The mortality rate from nosocomial sepsis in LMICs is uncertain but is estimated to be 3-20 times higher than in high-income settings [1]. The incidence of nosocomial neonatal sepsis in high-income settings ranges from 6 to 9 cases per 1000 births, whereas in LMICs, the incidence is higher, ranging from 15 to 62 cases per 1000 births [9]. Risk factors for nosocomial infections in neonates include prematurity, low birth weight, prolonged hospital stay and indwelling devices with human immunodeficiency virus (HIV) exposure posing an additional risk factor in LMICs [10]. Amongst several bacterial causes, Gram-positive bacteria, particularly *Staphylococcus*

aureus, and Gram-negatives such as *Klebsiella pneumoniae* and *Acinetobacter baumannii* are the leading etiologic agents of nosocomial sepsis in neonates [1].

1.2 *Acinetobacter baumannii*

Acinetobacter baumannii has emerged as a significant concern in clinical settings, now as a leading cause of Healthcare-Associated Infections (HAIs) [11, 12]. It has the ability to resist multiple classes of antibiotics due to chromosome-mediated genetic elements and can persist for prolonged periods on inanimate surfaces and medical devices [13]. *Acinetobacter baumannii* accounted for 13% of all culture-confirmed sepsis, with an incidence of 4.3/1000 live births and a prevalence of 22.8/1000 admissions [13, 14]. The World Health Organisation (WHO) has declared *Acinetobacter baumannii* as a Group-1 priority pathogen that urgently requires new antimicrobials [15]. *Acinetobacter baumannii* isolates exhibit broad resistance to all classes of antibiotics, including 100% resistance to carbapenems and piperacillin/tazobactam, but are often susceptible to colistin [3].

1.2.1 Morphology

Acinetobacter baumannii is an opportunistic Gram-negative coccobacillus exhibiting pleomorphism [11]. It is strictly aerobic, catalase-positive, oxidase-negative, non-fermenting, and non-motile [11]. At 37°C, *Acinetobacter baumannii* forms greyish-white smooth but slightly mucoid colonies when cultured on sheep blood agar media [13]. *Acinetobacter baumannii* often produces a capsule, which is a polysaccharide layer surrounding the cell wall. This capsule provides protection against desiccation, phagocytosis, and antibiotics, contributing to the bacterium's virulence and ability to survive in various environments. The size of *Acinetobacter baumannii* cells ranges from 0.5 to 1.5 micrometers in diameter and 1.0 to 3.0 micrometers in length [16].

1.2.2 Pathogenesis and virulence factors of *Acinetobacter baumannii*

The lipid polysaccharides content of the outer membrane of *Acinetobacter baumannii* often acts in form of adhesins to facilitate the attachment of the bacterium to numerous surfaces as well as epithelial cells and medical devices also providing it with defense against desiccation, harsh environmental conditions, antibiotics and routinely used disinfectants, [16-18]. Figure 1.1 shows

the cell wall structure of *Acinetobacter baumannii* revealing the presence of capsular lipopolysaccharides in the outer membrane.

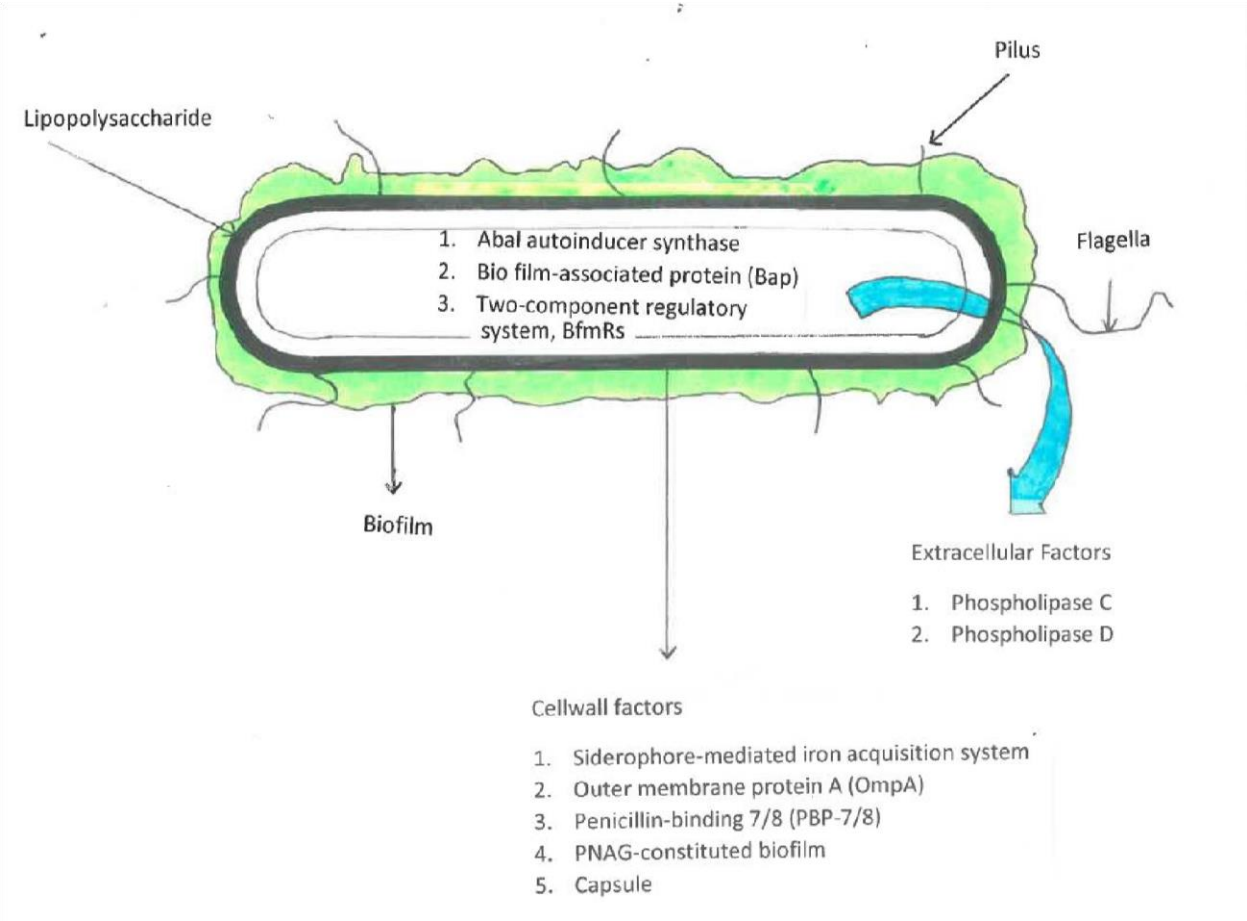


Figure 1.1 Cell wall structure of *Acinetobacter baumannii* [20].

The infections caused by *Acinetobacter baumannii* result from multiple complex virulence factors including capsules, porins, cell wall lipopolysaccharides, various enzymes, biofilm formation, and iron-acquisition systems, amongst others. These factors collectively enable the pathogen to survive adverse environmental conditions leading to severe health conditions or infections such as pneumonia, septicaemia, meningitis and urinary tract infections contributing to increased morbidity and mortality rates [17].

1.2.3 Membrane proteins of *Acinetobacter baumannii*

Outer membrane proteins (OMPs) are major immunogenic proteins located in the outer membrane of bacteria. They facilitate the transport of antibiotics and amino acids, provide advantages in host fitness including stress tolerance, virulence, and resistance to antimicrobials, and enable immune evasion. OMPs are beta barrel-shaped monomeric or trimeric porins that regulate the movement of micro-molecules into and out of the periplasmic space across all Gram-negative bacteria. In *Acinetobacter baumannii*, various types of OMPs include OmpA, CarO, and OprD-like porins, as well as Omp proteins ranging from 33- to 36 kDa, AbuO, TolB, DcaP, Oma87/BamA, NmRmpM, CadF, and OprF, among others [21-23]. OmpA in *Acinetobacter baumannii* mediates apoptosis and participates in cell invasion [24]. It also functions as a transporter and plays a considerable role in biofilm formation, interaction with epithelial cells using fibronectin, and subsequent dissemination into the bloodstream [25]. OmpA is among the most abundant OMPs in *Acinetobacter baumannii* and interacts with efflux pumps to expel antimicrobial compounds from the periplasmic space [26-28]. Figure 1.2 reveals the three-dimensional structure and pore sizes of OmpA (A), CarO (B), and OprD (C).

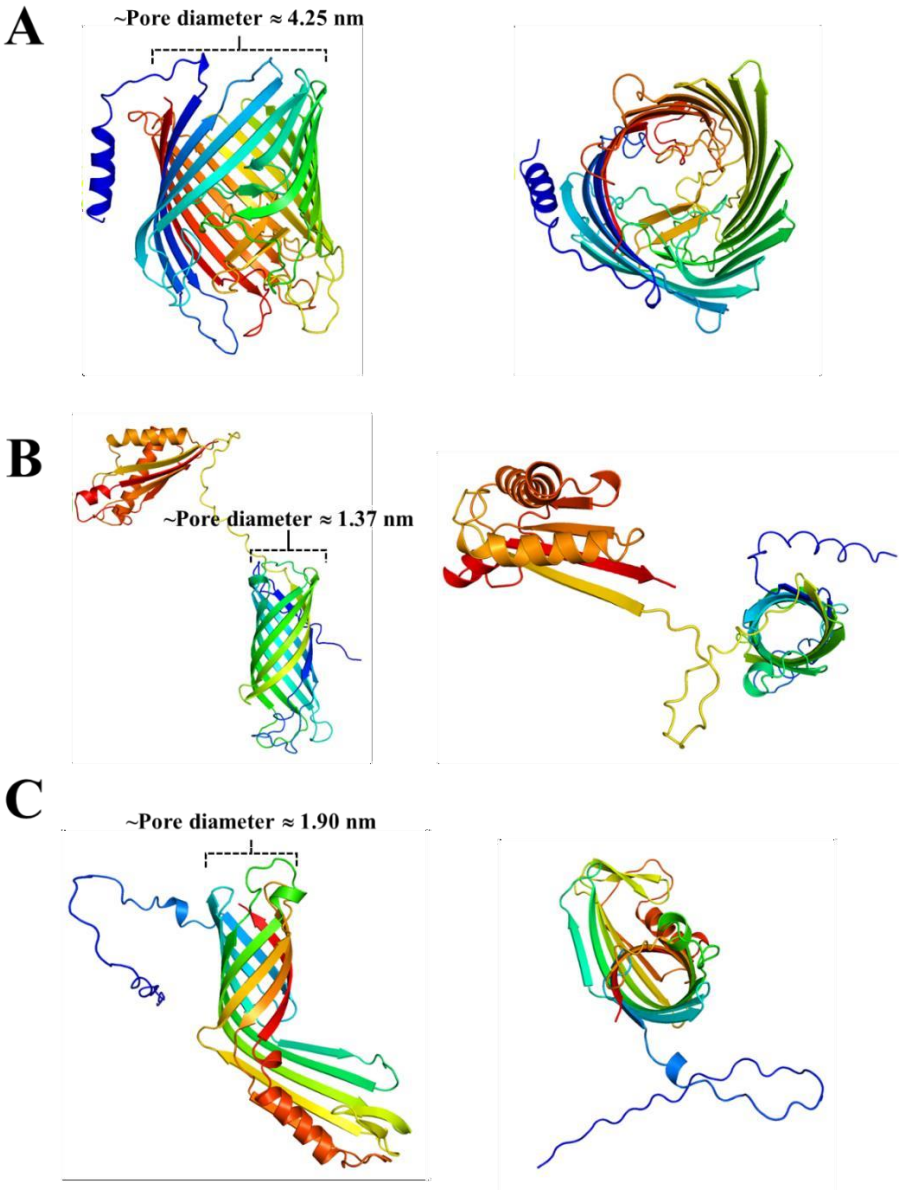


Figure 1.2. The three-dimensional crystal structure and estimated pore sizes of *Acinetobacter baumannii* OmpA, CarO and OprD. The predicted three-dimensional folds of (A), (B) and (C) and data files corresponding to each structure were extracted from the UniProtKB database (https://www.uniprot.org/) and submitted to PyMol molecular modelling software, which was used to render the cartoons showing the secondary structural elements of each porin. The images on the left-hand side depict the side view of the beta-barrel, and the images on the right-hand side depict the top view of the beta-barrel structures. The images were coloured according to the polarity of the polypeptide chain, with the N-terminus and the C-terminus represented by blue and red, respectively. The pore sizes were measured using the same PyMol molecular modelling software.

Table 1.1: **Structure and function of major outer membrane proteins of *Acinetobacter baumannii*.** Summary of the role of the OMPs of *Acinetobacter baumannii* in antibiotic resistance and virulence [19].

OMP's	Weight (kDa)	Structure	Function
OmpW	Not studied	Eight-stranded-beta-barrel shaped.	Colistin binding site. Facilitates iron uptake into the cell
Omp33-36	33-36	Yet to be studied	Implicated in imipenem resistance. Also induces apoptosis in host cells by activating caspases 3 and 9.
OmpA	28-36	Eight-stranded-beta barrel shaped.	Mediates attachment to host cells via fibronectin
OprD/OccAB1	43	Eighteen-stranded-beta barrel shaped	Allows diffusion of basic amino acids and beta-lactam class of antibiotics into the cell. -
CarO	25-29	Eight-stranded Beta barrel-shaped.	Uptake of glycine and ornithine. Also implicated in carbapenem resistance.
DcaP	47-50	Sixteen-stranded-beta barrel shaped	An Omp with preference for anionic compounds. Involved in transport of phthalates into the cell.
AbuO	50.2 (Theoretical)	Three-domains-four-stranded beta barrel, αhelical barrel and α-β mixed barrel	Homolog of *E. coli* TolC protein. Involved in pH and bile salt tolerance.

Both the presence of lipopolysaccharide and a capsule have been found to play active roles in all Gram-negative bacteria virulence as they often evade phagocytosis [29]. Figure 1.3 reveals the capsular polysaccharide of *Acinetobacter baumannii* as a virulence factor as it illustrates the various virulence determinants in *Acinetobacter baumannii*. Enzymes such as phospholipase C and D endow the bacterium with toxicity to epithelial cells and persistence in human serum, respectively. [30] These highly hydrolytic enzymes possess lipolytic activity against phospholipids of human cell walls [30]. Biofilm formation has emerged as a significant pathogenic feature in *Acinetobacter baumannii* as it confers antibiotic resistance evading immune system clearance and promoting interactions between the pathogen and its host. This process involves the formation of pilli and facilitates cell attachment, thereby enhancing the pathogen's ability to persist and cause infection [18, 20, 21].

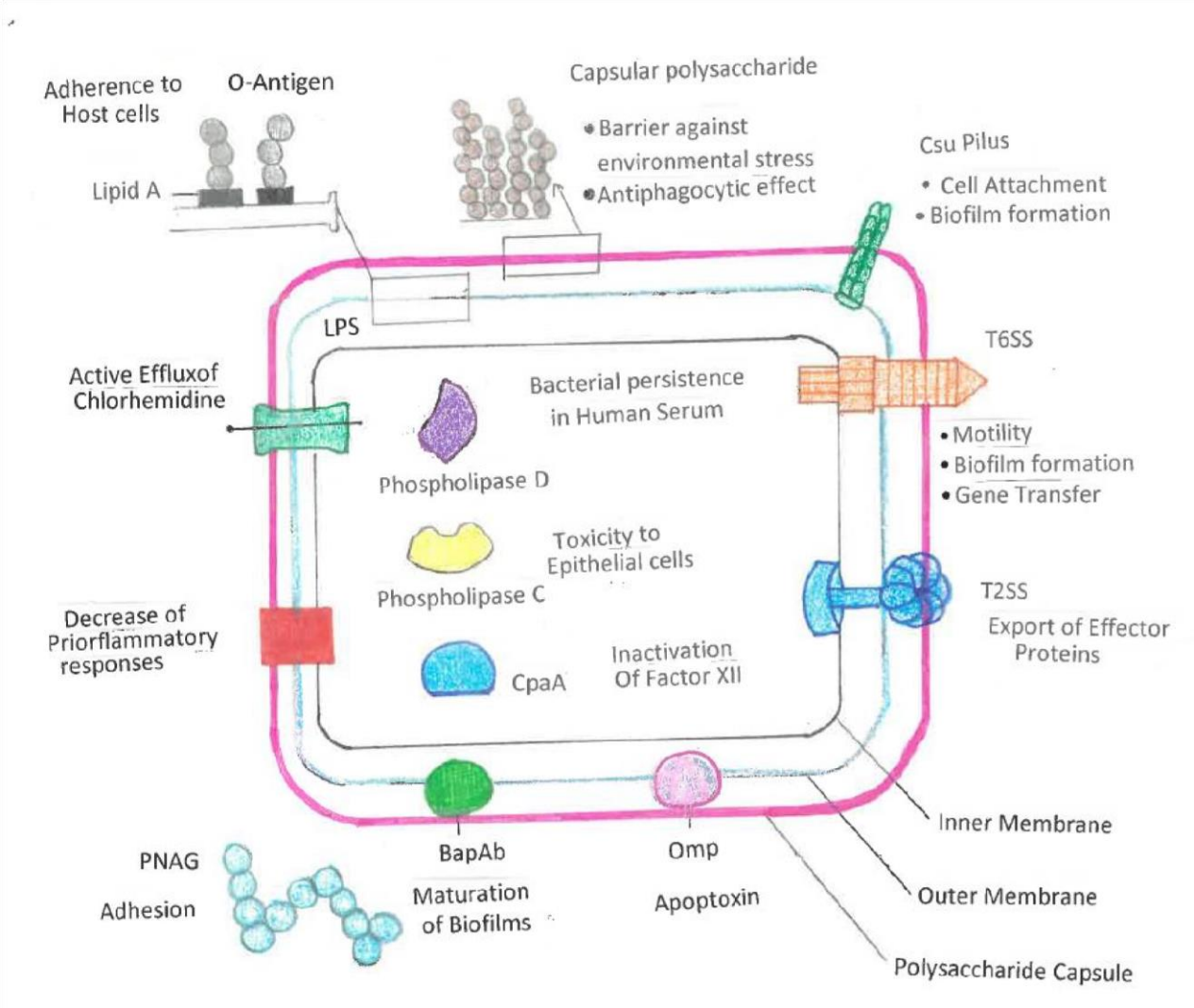


Figure 1.3 Illustration of virulence determinants possessed by *Acinetobacter baumannii* [29]..

Bacteremia caused by *Acinetobacter baumannii* has become a significant contributor to bloodstream infections (BSI) in healthcare settings, often arising from sources like the respiratory tract or intravenous catheters. This condition carries a mortality rate of up to 40%, underscoring its severity and impact on patient outcomes [22]. Frequently, *Acinetobacter baumannii* has been isolated from skin and soft tissues in patients with severe burns and wound infections following traumatic injuries [23, 24]. Though research on *Acinetobacter baumannii* infections has solely been concentrated on blood stream, it has been reported that it can occasionally cause urinary tract infections (UTI), especially with in-dwelling urinary catheters [25].

1.2.4 Immunology of *Acinetobacter baumannii*

Neonates are highly vulnerable to infections due to their inadequate developmental and functional mechanisms of innate and adaptive immunity. Recent research has shown that the vulnerability of neonates to infections is influenced by factors such as the mode of exposure, precursor frequency of lymphocytes and antigenic dose. The low frequency and quantity of precursor cells along with the quality of innate immunity present challenges in developing robust adaptive immune responses in neonates [26].

The role of neutrophils, T lymphocytes (Th1, Th2. Tregs), B cells and antigen presenting cells cannot be overemphasised in understanding neonatal immunity. This understanding is crucial for the development of therapeutic vaccines against infectious agents aimed at combating infectious agents. Also, the proliferation of T lymphocytes, their assistance in B-cell immunity and the subsequent production of antibodies by B-cell are known to influence adaptive immunity and can enhance vaccine efficacy.

Several other factors could affect neonatal immune development and response to infection, including maternal antibodies, maternal nutrition, maternal HIV, malaria, TB status, plasma factors, and allergens. Hence, maternal vaccinations should be considered to generate neonatal protective immunity, even as neonates may receive multiple priming doses of vaccines and booster vaccines to achieve immunity [26].

The outer membrane lipid polysaccharides (LPS) of *Acinetobacter baumannii* facilitate the modulation of immune response, phagocytosis, and complement activation leading to increased virulence and immune evasion of antibody-mediated destruction, resulting in persistent infection in the host [27].

1.2.5 Laboratory diagnosis of *Acinetobacter baumannii*

Laboratory techniques for isolating *Acinetobacter baumannii* from blood and CSF cultures as outlined in the laboratory method section in Chapter 2 involves the microscopic examinations of Gram-stained smears of both blood and CSF specimens revealing a Gram-negative coccobacilli. Colonies of cultures displaying a greyish-white appearance on blood agar and non-lactose fermentation on MacConckey

agar, along with Gram-negative coccobacilli, are used to prepare a 0.5 McFarland standard solution. *Acinetobacter baumannii* isolates exhibiting intrinsic resistance to certain antibiotics, including aminoglycosides, are subjected to confirmation by polymerase chain reaction (PCR) to determine the presence of specific antibiotic resistance genes. The results are interpreted based on the Clinical and Laboratory Standards Institute (CLSI) guidelines [28].

In addition to conventional culture methods, molecular diagnostics such as real-time PCR, multiplex PCR, and next-generation sequencing (NGS) have been increasingly used to improve the speed and accuracy of diagnosing *Acinetobacter baumannii* infections. Automated identification systems such as Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS and VITEK 2 have been evaluated in the literature and compared to manual identification methods, showing significant improvements in diagnostic efficiency and accuracy.

1.2.6 Antimicrobial susceptibility of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and Methicillin-resistant *Staphylococcus aureus*

In addition to *Acinetobacter baumannii*, *Klebsiella pneumoniae* and Methicillin-resistant *Staphylococcus aureus* (MRSA) causing an increased burden of nosocomial neonatal infections, many of these pathogens have developed increased resistance to commonly used antibiotics. This resistance poses significant therapeutic challenges in the management of neonatal infections [29]. Multidrug resistance (MDR) is defined as resistance to at least one agent in three or more antimicrobial classes [30]. Extensive drug-resistance (XDR) is characterised by resistance to at least one agent in all but two or fewer antimicrobial categories indicating that bacterial isolates remain susceptible to only one or two antimicrobial classes. In contrast, pan drug resistance (PDR) refers to resistance to all agents in all antimicrobial classes [30]. Antibiograms are valuable tools used to determine the antimicrobial susceptibility of microbial isolates [31]. This information is often used to aid clinicians in selecting empiric antibiotic therapy and monitoring resistance trends within and across healthcare institutions [32]. Susceptibility testing is conducted by using various laboratory techniques, including broth micro dilution, agar dilution, and disk diffusion testing methods, amongst other commercial testing methods, to determine the antimicrobial resistance of

clinical isolates. Kirby-Bauer disk diffusion antimicrobial susceptibility testing is discussed below in section 1.2.7. Minimum Inhibitory Concentrations (MICs) can also be performed by broth micro dilution method. This allows for the detection of strains exhibiting MDR, XDR and PDR characteristics. The Clinical and Laboratory Standards Institute (CLSI) usually provides guidelines regarding the selection of antimicrobial categories for antimicrobial susceptibility testing as well as corresponding breakpoints for interpreting test results. Table 1.2 shows the list of recommended antibiotic agents for susceptibility testing of *Acinetobacter baumannii* by the World Health Organisation (WHO) [28].

1.2.7 Susceptibility Testing Methods

Determining antimicrobial susceptibility is crucial in guiding effective treatment for infections caused by *Acinetobacter baumannii* and *Klebsiella pneumoniae*. The following are the primary methods used for susceptibility testing:

1. **Kirby-Bauer Disk Diffusion Method** involves applying antibiotic-impregnated disks onto an agar plate inoculated with the test organism. The diameter of the inhibition zone around the disk is measured after incubation and interpreted using CLSI guidelines. This method is widely used due to its simplicity and cost-effectiveness [33].

2. **Broth Microdilution Method**: This quantitative method determines antibiotics' minimum inhibitory concentration (MIC). Serial dilutions of antibiotics are prepared in a broth medium and inoculated with the test organism. The MIC is the lowest concentration of the antibiotic that prevents visible growth. This method is considered the gold standard for susceptibility testing [34].

3. **Automated Systems**: VITEK 2 and MicroScan WalkAway provide rapid and accurate susceptibility results. These systems automate the process of broth microdilution, and their results are interpreted based on CLSI guidelines. They also can identify resistance mechanisms and patterns [35].

4. **E-test (Gradient Diffusion Method)**: The E-test combines aspects of the disk diffusion and broth microdilution methods. An antibiotic gradient is used on a plastic strip placed on an agar plate inoculated with the test organism. The MIC is determined by the point at which the inhibition ellipse intersects the strip [36].

5. **Molecular Methods**: Polymerase chain reaction (PCR) and next-generation sequencing (NGS) are used to detect specific resistance genes. These methods provide rapid and precise identification of genetic determinants of resistance, aiding in the understanding of AMR mechanisms [37].

1.2.8 Mechanisms of resistance

Antibiotic resistance in *Acinetobacter baumannii* in clinical settings is increasingly alarming with the enzymatic hydrolysis mediated by beta-lactamases being the most common resistance mechanism in *Acinetobacter baumannii* to beta-lactams **(**β-lactams), and all penicillins and cephalosporins are easy targets except cephamycins [38, 39]. These enzymes exist in classes, e.g. Ambler class A, B, C and D. While there are zinc-dependent metallo-β-lactamases (MBLs) that strongly hydrolyse both beta-lactams and carbapenems (except aztreonam, but are inhibited by metal chelators such as ethylene-di-amine-tetra-acetic acid), there has been reported also other enzymes that were detected in *Acinetobacter baumannii* to hydrolyse carbapenem [40-42].

Both *Acinetobacter baumannii* and *Klebsiella pneumoniae* exhibit complex and diverse mechanisms of antibiotic resistance, leading to multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pan drug-resistant (PDR) profiles. These resistance profiles pose significant challenges for treatment and management, making it crucial to understand the underlying mechanisms. *Acinetobacter baumannii* is naturally resistant to several antibiotics, including aminoglycosides and β-lactams. The bacteria's ability to acquire resistance genes through horizontal gene transfer and mutations has further contributed to resistance against carbapenems, cephalosporins, and fluoroquinolones. Fundamental resistance mechanisms in *A. baumannii* include the production of carbapenemases, such as OXA-type enzymes, the action of efflux pumps that expel antibiotics from the bacterial cell, and modifications to antibiotic target sites, making the drugs less effective.

Klebsiella pneumoniae exhibits resistance through various mechanisms, most notably the production of extended-spectrum beta-lactamases (ESBLs) and carbapenemases like KPC, NDM, and OXA-48, which hydrolyse β-lactam antibiotics and render them ineffective. Increasing reports of resistance to colistin, a last-resort antibiotic, are concerning, with mechanisms involving structural modifications of lipopolysaccharides (LPS) mediated by the *mgrB* gene. Additionally, *K. pneumoniae* employs efflux pumps to remove antibiotics from the bacterial cell and exhibits loss of porins, which reduces antibiotic

uptake. Understanding these resistance mechanisms and accurately interpreting antimicrobial susceptibility testing (AST) results are essential for managing infections caused by MDR organisms like *A. baumannii* and *K. pneumoniae*. This knowledge is critical in tailoring appropriate therapeutic strategies to combat these resistant pathogens and improve patient outcomes.

Non-enzymatic mechanism of β-lactams and other antibiotics resistance

Besides the secretion of proteins such as enzymes to facilitate antimicrobial resistance, non-enzymatic mediated responses such as multi-drug efflux systems by efflux pumps help the bacterium to resist several antimicrobial drugs and chemicals [43]. Resistance to ribosome-targeting antibiotics such as tetracyclines is achieved by the secretion of proteins that protect bacteria ribosomes [44].

Mutation of the antimicrobial target site or enzyme

Though efflux pumps may be effective on different antibiotics, mutation of antibiotics target enzymes is another mechanism of resistance detected in *Acinetobacter baumannii* as in the case of mutation of flouroquinolones target enzyme – DNA gyrase and DNA topoisomerase IV [45]. On the other hand, polymyxins, such as colistin, which was rarely used due to its effect on the kidneys, were recently reconsidered following the increase in antibiotic resistance in *Acinetobacter baumannii.* Still, recent resistance to colistin has been reported, and this is due to *Acinetobacter baumannii* chromosomal encoding of phosphoethanolamine (PetN) and added to the lipid A of the outer membrane protein to alter its structure as well as mutate the gene needed for lipid A synthesis thereby leading to a low expression of LPS synthesis cofactors [46, 47].

Enzyme modification of aminoglycosides

Enzyme modification of aminoglycosides is a significant mechanism by which bacteria develop resistance to this class of antibiotics. Aminoglycosides, such as gentamicin, amikacin, and tobramycin, function by binding to the bacterial 30S ribosomal subunit, leading to the inhibition of protein synthesis and ultimately causing bacterial cell death [48, 49]. However, bacteria can evade the lethal effects of these antibiotics through the production of specific modifying enzymes that chemically alter the aminoglycosides [50]. Three primary types of enzymes are involved in this modification: aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs), and aminoglycoside nucleotidyltransferases (ANTs) [51]. These enzymes act by attaching chemical groups

acetyl, phosphate, or adenyl groups, respectively to the aminoglycoside molecule. This modification reduces the drug's affinity for the bacterial ribosome, rendering it ineffective at inhibiting protein synthesis. The genes encoding these modifying enzymes are often carried on plasmids, which can be easily transferred between bacteria through horizontal gene transfer. This contributes to the rapid spread of resistance within bacterial populations [52]. The presence of these enzymes in clinical isolates of pathogenic bacteria is a major challenge in treating infections, as it limits the effectiveness of aminoglycosides, which are often used as a last resort for severe infections. Addressing this form of resistance requires the development of novel aminoglycoside derivatives that can evade enzymatic modification or the use of enzyme inhibitors in combination with aminoglycoside therapy. The resistance of aminoglycosides by *Acinetobacter baumannii* has been reported to result from the production of aminoglycoside modifying enzyme AME [53].

1.2.9 Therapeutics

Treatment and Management of Acinetobacter baumannii

Managing infections caused by *Acinetobacter baumannii* involves a multifaceted approach, including appropriate antibiotic selection based on susceptibility profiles, infection control measures, and supportive care. Based on the results of susceptibility testing or a combination regimen, the treatment regimen may involve the use of carbapenems, colistin, or other antibiotics to which the isolate is susceptible.

Implications for Treatment

Due to the high levels of antibiotic resistance encountered in clinical settings, combination therapy has become a critical strategy in managing severe infections. This approach involves using two or more antibiotics together to enhance their efficacy, broaden the spectrum of activity, and prevent the emergence of further resistance [54]. The rationale behind combination therapy is that it targets multiple bacterial pathways or functions simultaneously, reducing the likelihood that bacteria will develop resistance to all the drugs used [55]. For example, the combination of colistin, a last-resort antibiotic, with a carbapenem is often employed to combat infections caused by carbapenem-resistant strains, particularly those caused by multidrug-resistant Gram-negative bacteria like *Klebsiella pneumoniae* or Pseudomonas aeruginosa [56]. Colistin disrupts the bacterial cell membrane, increasing

permeability, while carbapenems inhibit cell wall synthesis [57]. When used together, these antibiotics can achieve a synergistic effect, enhancing bacterial killing even in strains that have developed resistance to carbapenems alone [58]. This strategy not only increases the likelihood of successfully treating the infection but also helps prevent the emergence of resistance to either antibiotic. By attacking the bacteria from multiple angles, combination therapy can limit the ability of bacteria to survive and adapt, thereby curbing the spread of resistant strains. Additionally, combination therapy may reduce the dosage needed for each drug, potentially decreasing the risk of toxicity and side effects associated with higher doses of single-drug treatments. However, the use of combination therapy must be carefully managed to avoid potential drug interactions and to ensure that the selected antibiotics are truly synergistic, as inappropriate combinations could lead to antagonism or increased toxicity. [59].

Novel antibiotics and adjuvants have emerged as crucial tools in the fight against multidrug-resistant (MDR) organisms. Among these, antibiotics like ceftazidime-avibactam and meropenem-vaborbactam represent significant advancements [60]. These drugs are specifically designed to overcome resistance mechanisms that render traditional antibiotics ineffective. Ceftazidime-avibactam combines a third-generation cephalosporin with a non-β-lactam β-lactamase inhibitor, which protects the antibiotic from degradation by β-lactamase enzymes produced by resistant bacteria [61]. Similarly, meropenem vaborbactam pairs a carbapenem with a novel β-lactamase inhibitor, enhancing its activity against carbapenem-resistant *Enterobacteriaceae* [62]. In addition to these new antibiotics, adjuvants play a critical role in combination therapies. These substances do not possess significant antibacterial activity on their own but work by inhibiting bacterial resistance mechanisms, thereby restoring the efficacy of antibiotics. β-lactamase inhibitors are a prime example, as they prevent the enzymatic breakdown of β-lactam antibiotics, allowing these drugs to effectively target and kill resistant bacteria. Together, these innovations offer hope in addressing the growing threat of antibiotic resistance [63].

Optimising dosing regimens based on pharmacokinetics and pharmacodynamics (PK/PD) principles is essential for maximising therapeutic efficacy against resistant pathogens. PK refers to how the body absorbs, distributes, metabolises, and excretes a drug, while PD relates to the drug's effects on the organism, particularly its ability to kill or inhibit bacterial growth. By understanding and applying these principles, clinicians can tailor dosing strategies to achieve optimal drug concentrations at the infection site. For instance, prolonged infusions of antibiotics can maintain drug levels above the

minimum inhibitory concentration (MIC) for extended periods, enhancing their effectiveness. Similarly, higher doses may be necessary to overcome resistance mechanisms, ensuring that the drug exerts sufficient pressure to eradicate the pathogen without promoting further resistance. This PK/PD-guided approach is crucial in managing infections caused by multidrug-resistant bacteria [64].

Certain other principles which may apply when selecting antimicrobial therapy in drug-resistant Gram-negative bacilli GNBs infections are as follows; when isolating a strain of XDR-GNB, such as *Acinetobacter baumannii*, it is crucial to differentiate between infection and colonisation. Susceptibility testing results should guide the choice of appropriate antibiotics. Suppose the strain is resistant to all tested antibiotics. In that case, those showing intermediate susceptibility or a zone of inhibition or MIC value close to the breakpoints may be selected for combination therapy at higher doses. Combination therapy is often used to manage XDR-GNB infections. Adjustments in antibiotic doses should be considered for patients with hepatic or renal impairment and elderly patients. Controlling possible sources of infection and eliminating risk factors is essential for effectively addressing the primary disease. A few antibiotics are available for XDR-GNB infections, and Table 1.3 highlights them [65].

Table 1.2 Suggested antimicrobials for susceptibility testing by WHO.

Antimicrobial category	**Antimicrobial agent**	***Acinetobacter baumannii***
Penicillins	Ampicillin	No
β-Lactam/β-lactamase inhibitor combinations	Amoxicillin–clavulanate	No
	Ampicillin–sulbactam	Yes
	Cefoperazone–sulbactam	Yes
	Ticarcillin–clavulanate	Yes
	Piperacillin–tazobactam	Yes
Third/fourth-generation cephalosporin	Cefotaxime or ceftriaxone	Yes
	Ceftazidime	Yes
	Cefepime	Yes
Monobactams	Aztreonam	No
Cephamycins	Cefoxitin	No
	Cefmetazole	No
Carbapenems	Ertapenem	No
	Imipenem	Yes
	Meropenem	Yes
Aminoglycosides	Gentamicin	Yes
	Amikacin	Yes
Fluoroquinolones	Ciprofloxacin	Yes
	Levofloxacin	Yes
Sulphonamides	Trimethoprim–sulphamethoxazole	Yes
Chloramphenicol	Chloramphenicol	No
Tetracyclines	Doxycycline	Yes
	Minocycline	Yes
Glycylcyclines	Tigecycline	Yes
Polymyxins	Polymyxin E	Yes
	Polymyxin B	Yes
Others	Fosfomycin	No

Yes: Antimicrobial suggested for susceptibility testing; No: Antimicrobial not suggested for susceptibility testing.

Table 1.3 Available antibiotics for XDR-GNB infections

Infection	Two-drug combination	Three-drug combination
XDR *Acinetobacter baumannii*	**Sulbactam-based combinations**	
	(Cefoperazone-sulbactam or Ampicillin-sulbactam) + Tigecycline	Cefoperazone-sulbactam + Tigecycline + Carbapenems
	(Cefoperazone-sulbactam or Ampicillin-sulbactam) + Doxycycline	Cefoperazone-sulbactam + Doxycycline + Carbapenems
	Sulbactam + Carbapenems	
Tigecycline-based combinations		
	Tigecycline + (Cefoperazone-sulbactam or Ampicillin-sulbactam)	Tigecycline + Carbapenems
	Tigecycline + Polymyxin	Tigecycline + Polymyxin + Carbapenems
Polymyxin-based combinations		
	Polymyxin + Carbapenems	Imipenem + Rifampicin + (Polymyxin or Tobramycin)
	Polymyxin + Tigecycline	Polymyxin + Tigecycline + Carbapenems

1.2.10 Prevention

One most important risk factor for drug resistance in GNB is long-term exposure to antibiotics, especially extended-spectrum antibiotics [66]. The rapid spread of resistant clones and the pressure arising from the choice of antibiotics in combination has led to the rise in XDR-GNB infections, thereby necessitating the integration of appropriate infection control measures as well as effective antibiotic stewardship [67-69].

Basic and cost-effective infection prevention and control measures include hand hygiene to limit the spread of resistant microorganisms via healthcare workers' hands. Timely notification to appropriate authorities or infection control units upon identifying resistant strains is also crucial. Additional precautionary measures may involve partial separation of beds, single rooms, and minimising the sharing of devices such as stethoscopes, thermometers, sphygmomanometers, and infusion pumps [67, 70].

Prompt screening of patients using perianal and rectal swabs for CRE, nasopharyngeal samples, and wound secretions for XDR non-fermenters is essential. This can be performed using

conventional or rapid diagnostic methods to quickly identify resistant bacteria in high-prevalence wards such as Intensive Care Units (ICUs). Patients in such wards should be appropriately isolated [68, 71]. Regular disinfection of environments and surfaces frequently contacted by health workers and patients is also necessary [68, 72]. Strict adherence to clinical indications for antibiotic use and developing a rational formulary is highly recommended [73].

1.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, lactose-fermenting, facultative anaerobic bacillus with humans being a reservoir [74]. *Klebsiella pneumoniae* isolates are often resistant to most classes of antibiotics, including 73.9% to cefotaxime, but susceptible to carbapenems and amikacin but generally resistant to other aminoglycosides. It is responsible for a significant proportion of healthcare-associated infections, including septicemia, urinary tract infections, pneumoniae, and soft tissue infections, especially in immunocompromised hosts such as neonates. The gastrointestinal tract of hospitalised infants and the hands of health care workers serve as reservoirs for the transmission of the organism, accounting for multiple hospital outbreaks with an overall mortality rate of 30% [75, 76].

Treatment of infections that caused significant mortality and morbidity during the pre-antibiotic era was made possible by the emergence of antibiotics. However, the extensive use of these antibiotics in humans as medication and in agriculture and veterinary medicine has shown a possible association with an increase in the emergence of antibiotic-resistant bacteria [77]. These antibiotic-resistant bacteria have not only increased the burden of nosocomial neonatal infections, they have caused therapeutic challenges [78]. *Klebsiella pneumoniae*, which are resistant to first and second-line WHO-recommended treatment options, require carbapenems, but the emergence of Carbapenemase-Producing Enterobacterales (CPE), involves the use of colistin, the last-line antibiotic and yet has widely reported resistance patterns [79].

1.3.1 Clinical epidemiology of the *Klebsiella pneumoniae*

Klebsiella species are generally ubiquitous and thus commonly found in soil, water, and other surfaces. *Klebsiella pneumoniae* usually colonise the upper respiratory tract and gut of humans,

including several mucosal surfaces [80-82]. Recent studies revealed that the prevalence of nasopharyngeal colonisation rates in hospitalised patients rose to 19%, but can be as high as 77% in the gastrointestinal tract [83]. *Klebsiella pneumoniae* colonises the mucosal surfaces in humans as well as the gastrointestinal tract and nasopharynx once acquired from environmental reservoir [83]. Rates of colonisation may differ by body sites and sources of acquisition with nosocomial nasopharyngeal colonisation rates slightly higher, up to 19% and gastrointestinal colonisation increase among hospitalised patients as high as 77% in a study by Davis and Matsen, 1974. Several underlying diseases have also been identified as risk factors for infection, since they weaken host defenses and therefore increase susceptibility to infection.

The clinical presentations of neonatal sepsis caused by *Klebsiella pneumoniae* are usually nonspecific, including fever or hypothermia, feeding intolerance, tachypnea and apnea [84] with a remarkable increase in C-reactive protein (CRP) level however an active infection is established when *Klebsiella* is found in the bloodstream [85]. Figure 1.4 shows colonial morphology and characteristics of *Klebsiella pneumoniae* on Mac Conckey agar.



Figure 1.4; Colonial Morphology of *Klebsiella pneumoniae* on Mac Conkey agar [86].

1.3.2 Colonial morphology of *Klebsiella pneumoniae* on blood agar

The lactose fermenting, mucoid strain of *Klebsiella pneumoniae* that is capable of causing infection manifested in multiple organs in healthy humans are referred to as hypervirulent *Klebsiella pneumoniae* (HvKp) and it was initially reported in 1986 in Taiwan where it was responsible for causing *pneumoniae* complicated with endolphthalmitis, meningitis and liver abscess. Due to the hypermucoviscosity of some strains of HvKp they were then referred to as hypermucoviscous *Klebsiella pneumoniae* [87]. North America had the first case of HvKp infection with a 38 year old American black man who was diagnosed with fever and headache complicated with liver abscess, meningitis and endolphthalmitis [88]. Reports of *Klebsiella pneumoniae* infections involving multiple organs such as in liver abscess, endolphthalmitis, necrotising fasciitis, meningitis in bacteremia patients are currently on the rise and this is indicative of a high prevalence of HvKp globally thus requiring immediate attention in patients with a positive culture, and due to high

lethality, early diagnosis and intervention play a key role in limiting the disease severity and death due to hvKp [88].

1.3.3 Pathogenesis of *Klebsiella pneumoniae*

Pathogenicity of *Klebsiella pneumoniae* is driven by the bacterium's ability to produce several virulence factors following the presence of specific virulence genes that are encoded for them. These virulence factors include antibiotic resistance enzymes, siderophores, capsules and fimbrial adhesins, formation of biofilm, iron uptake, hypermucoviscosity and synthesis of lipopolysaccharides as all these proffer its ability to invade, adhere and develop infection in humans [89, 90]. Several clinical features of *Klebsiella pneumoniae* are due its profound ability to resist several antibiotics especially the third generation cephalosporin such as ceftriaxone, ceftazidime, and cefotaxime as well as plasmid mediated encoding of the SHV, TEM, and C-TXM genes that contain the extended beta lactamase enzyme leading to MDR [91-93]. A study in Iraq found that 62.5% of *Klebsiella pneumoniae* isolates exhibited hypermucoviscosity, while 100% showed siderophores, biofilm formation, and capsule production. MDR isolates accounted for 84.375%, XDR 12.5%, and PDR 3.12%. The rate of extended-spectrum beta-lactamase production was 62.5%. The study concluded a relationship between the presence of virulence genes and antibiotic resistance ability [92]. In research by Esra Deniz Candan and Nilüfer Aksöz in Turkey, carbapenem resistance was identified in 50 *Klebsiella pneumoniae* isolates from various sources, including the rectum, bile, trachea, blood, bronchial, and wound cultures. Resistance genes such as blaKPC, blaIMP, blaVIM, blaNDM, and blaOXA were detected in 33 of the 50 isolates using multiplex PCR [94].

1.3.4 Immunological response to *Klebsiella pneumoniae* infection

Following infection with *Klebsiella pneumoniae*, the innate immune response is first activated upon recognition of the bacterium by pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). These PRRs recognise bacterial patterns like outer membrane proteins and lipopolysaccharides, triggering the release of pro-inflammatory cytokines, such as tumor necrosis

factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), and the recruitment of immune cells to the infection site [95]. This response involves macrophages, neutrophils, and natural killer (NK) cells [96]. The adaptive immune response follows, with B cells producing antibodies against *Klebsiella pneumoniae* antigens, leading to bacterial eradication through opsonisation. B cells produce antibodies like IgM and IgG that neutralise bacteria and enhance phagocytosis, while T cells facilitate bacterial clearance through antibody production [95, 96]. The infection also elicits cell-mediated adaptive immune responses and humoral immune responses. Antigen-presenting cells (APCs) present bacterial antigens to activate specific T and B cells [95, 96]. Certain mechanisms, mediated by receptors like FcRn, enable the active transport of immunoglobulin G (IgG) across the placenta from mother to fetus, providing temporary immunity. This process begins early in fetal development and increases towards the end of gestation, resulting in preterm and extreme preterm newborns having insufficient levels of protective antibodies, as most are transferred during full-term pregnancy [97, 98].

1.3.5 Lab diagnosis of *Klebsiella pneumoniae*

Laboratory diagnosis of *Klebsiella pneumoniae* involves Gram staining from specimens such as cerebrospinal fluid, blood, sputum, urine, pleural effusion, pericardial effusion, synovial fluid, and abscess materials for presumptive identification. Direct culture on media like blood, chocolate, and MacConkey agars provides colonial morphology and characteristics for further identification. On Gram staining, short, plump, encapsulated Gram-negative bacilli are observed, while blood and chocolate agar cultures reveal round, raised colonies. On MacConkey agar, lactose-fermenting colonies are pink, raised, and mucoid [99]. In addition to traditional culture methods, automated identification systems such as MALDI-TOF MS and VITEK 2 are currently used for rapid and accurate identification of *Klebsiella pneumoniae*. Molecular diagnostics, including PCR and NGS, offer advanced options for detecting specific resistance genes and providing detailed microbial profiles. See the Laboratory Methods section in Chapter 2.

1.3.6 Antimicrobial susceptibility of *Klebsiella pneumoniae*

Antibiotic susceptibility testing is performed using the Kirby-Bauer disk diffusion method or the automated MicroScan NM37 minimum inhibitory concentration (MIC) panel (MicroScan WalkAway 96 SI Siemens System). Additionally, the micro broth dilution technique is employed to further assess antimicrobial susceptibility. The results, including zone diameters from disk diffusion and MIC readings from the broth dilution, are evaluated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [56].

1.3.7 Mechanisms of resistance in *Klebsiella pneumoniae*

The global incidence of MDR bacteria has become a serious global challenge in public health as this has led to increased mortality and elevated healthcare cost from a prolonged hospital stay and has, on the other hand, created limited therapeutic options for the treatment of severe infections of which *Klebsiella pneumoniae* is one of the MDR organisms implicated [100-103]. Microorganisms use several mechanisms to develop resistance to antimicrobial agents. These include the production of the enzyme beta-lactamase, loss of target OMP, biofilm formation, capsule production, structural alteration of target sites, integron and efflux pump [104]. The thick polysaccharide capsule facilitates the resistance to phagocytosis [104].

Carbapenem-Resistant in Klebsiella pneumoniae

Several mechanisms are used by microorganisms to develop resistance to antimicrobial agents. These include the production of the enzyme beta-lactamase, loss of target OMP, biofilm formation, and structural alteration of target sites, integron and efflux pump as well as the production of a thick polysaccharide capsule which facilitates resistance to phagocytosis [104]. The emergence of carbapenem resistance in *Klebsiella pneumoniae* can be dated back to the 1990s and has become a global menace with significantly high mortality, and this is due to the production of carbapenemases, which is classifiable into Amber classes A, B, C and D as mentioned earlier where class A is the *Klebsiella pneumoniae* carbapenemase (KPC), class B-(the metalloenzymes NDM, VIM, and IMP) and class D is (oxacillin enzyme, OXA) [105, 106]. Besides the emergence of beta-lactamase, carbapenemase, biofilm-producing *Klebsiella pneumoniae* and MDR *Klebsiella pneumoniae*, reports of colistin (which is now used as the last treatment option for KPC-producing *Klebsiella pneumoniae*)

resistance in *Klebsiella pneumoniae* is also on the rise and this mediated by the mutations and subsequent loss of function of the *mgrB* gene a negative regulator of the PhoPQ signaling system [69, 107-109].

1.3.8 Treatment and prevention

Due to disease complexity and therapeutic approaches often resulting in multiple organ dysfunction syndrome (MODS), reduced antimicrobial susceptibility of nosocomial pathogens and sometimes acute kidney injuries, treating very ill patients with sepsis, particularly in ICUs, has been very difficult [110-112]. At the establishment of infection on diagnosis, the results of antibiotic susceptibility testing determine the regimen [113, 114].

1.4 *Staphylococcus aureus*

Staphylococcus aureus is widely distributed across the globe and commonly found in both healthcare and community settings. The first strain of MRSA was observed in 1961 with subsequent MRSA strains and vancomycin-resistant strains reported in the USA and Japan in 1982 and 2002, respectively [115, 116]. MRSA has emerged as a significant global antimicrobial pathogen posing a threat to public health [117]. Given the prevalence of MRSA in hospital and communities, there have been numerous published reports on the trend in various regions worldwide [3, 117-119].

1.4.1 Morphology of *Staphylococcus aureus*

The name *Staphylococcus aureus* accounts for the morphology of the bacterium, derived from the Greek words "staphyle" (bunch of grapes) and "coccus" (spherical bacteria). Its colonial appearance on blood agar is yellow to yellowish-white, reflecting the Latin word "aureus," meaning gold [120]. *Staphylococcus aureus* is a disease-causing bacterium of the genus *Staphylococci*. It typically measures about 1 µm in size and is characterised as a facultative anaerobe. Gram staining reveals its Gram-positive nature, and it is non-motile and non-spore forming. It often exhibits hemolysis on blood agar, with a Gram stain appearance of bluish-grape-like structures. Figure 1.5 shows the morphological appearance of *Staphylococcus aureus* as creamy-white colonies on blood agar.



Figure 1.5; Colonial morphology of *Staphylococcus aureus* on blood agar media

1.4.2 Cell wall of *Staphylococcus aureus*

The cell wall of *Staphylococcus aureus* is composed of an extremely complex multicomponent structure that maintains the bacteria's integrity, shape, size, pressure-regulating potential, and chemical properties. These are the potential target for antibiotics [121-123]. The major component of the cell wall is the peptidoglycan, a macromolecule composed of glycan strands connected via peptide bridges forming a mesh-like structure surrounding the cell, providing it with mechanical strength and regulating the internal osmotic pressure. Therefore, inhibition of its synthesis or disruption of its integrity would result in cessation of cell growth; hence this is a target for antibiotics [124-127]. Since Penicillin-binding proteins (PBPs) are required for cell wall synthesis, they are, therefore, the main target of beta-lactam antibiotics [128].

1.4.3 Clinical representation of *Staphylococcus aureus* Infection and Disease

Staphylococcus aureus is a Gram-positive opportunistic pathogen causing a range of diseases, including pneumonia, septic arthritis, bacteremia, and skin infections such as blisters, styes, furunculosis, mastitis, phlebitis, meningitis, necrotising fasciitis, and deep-seated infections like osteomyelitis and endocarditis [129, 130]. It colonises the skin and mucous membranes of humans, including nasal passages, as part of the normal flora. It can also cause pus-forming infections and toxin-mediated illnesses such as food poisoning and toxic shock syndrome by releasing enterotoxins and superantigens into the bloodstream [130, 131].

1.4.4 Immunology of *Staphylococcus aureus*.

The innate immune response is crucial against MRSA infections, involving the recruitment of neutrophils to the infection site and the release of granulocyte chemotactic protein 2 (GCP2, CXCL6), complement component C5a, and interleukin-8 (IL-8, CXCL8) [132, 133]. In humoral immunity, B cells produce Immunoglobulin G (IgG) against Staphylococcus aureus antigens, though the bacterium can evade this through mechanisms such as protein A-mediated interference with antibody function [134]. Cellular immunity involves effector T cells like Th1 and Th17 cells, which produce cytokines such as interferon-gamma (IFN-γ) and interleukin-17 (IL-17), promoting phagocytosis and inflammation [135].

1.4.5 Laboratory Diagnosis of *Staphylococcus aureus*

Laboratory diagnosis of *Staphylococcus aureus* involves identifying catalase-positive, creamy to white colonies on blood agar media and a blue to purple appearance on Gram staining. MRSA is identified through its coagulase production ability and resistance to methicillin, cloxacillin, oxacillin, and cefoxitin [114, 136]. These steps include initial culturing, biochemical tests, and molecular techniques as discussed below.

The laboratory diagnosis of *Staphylococcus aureus* involves a combination of culturing, biochemical tests, antibiotic susceptibility testing, and molecular methods to ensure accurate identification. Culturing on blood agar typically reveals creamy to white colonies with possible golden-yellow pigmentation and zones of hemolysis. Gram staining shows Gram-positive cocci in clusters. Key biochemical tests include the catalase test, which differentiates *Staphylococcus* species from

Streptococcus by detecting oxygen bubbles when exposed to hydrogen peroxide, and the coagulase test, which confirms S. aureus by detecting its ability to clot plasma. Methicillin-Resistant *S. aureus* (MRSA) is identified through antibiotic susceptibility testing, such as the Kirby-Bauer disk diffusion method and Minimum Inhibitory Concentration (MIC) testing. Molecular methods, like PCR, detect the mecA gene, responsible for methicillin resistance. Additional biochemical tests, such as mannitol fermentation and DNase activity, further characterise *S. aureus*. Automated systems like VITEK and MALDI-TOF MS offer rapid identification and susceptibility testing, enabling prompt diagnosis and treatment. This is elaborately discussed the journal article [137].

1.4.6 Antimicrobial Susceptibility Testing of Methicillin-Resistant *Staphylococcus aureus*

The use of penicillin to treat *Staphylococcus aureus* infections was effective until resistance was identified in 1942, initially in hospitals and subsequently in communities [138]. This resistance led to a significant decrease in the therapeutic efficacy of penicillin by the 1950s [138]. Modern clinical practice involves antimicrobial susceptibility testing using the Kirby-Bauer disk diffusion method, with results interpreted according to CLSI guidelines [139].

1.4.7 Mechanism of Resistance in *Staphylococcus aureus*

Methicillin resistance in *Staphylococcus aureus* is determined by the acquisition of the mecA gene, which encodes the low-affinity PBP2A through the *staphylococcal* cassette chromosome mec (SCCmec). A new methicillin resistance gene, mecC, has also been discovered in humans, animals, and food products [140, 141]. Among 1914 cases of *Staphylococcus aureus*, 557 (29.1%) were MRSA infections, with 44 cases (2.3%) as Community-associated (CA)-MRSA infections and 513 (26.8%) as Healthcare-associated (HA)-MRSA infections [142]. CA-MRSA strains are found in hospitals, while HA-MRSA strains are identified in communities [143]. MRSA rates are notably high in South Africa, with isolates classified as SCCmec type III (41%) and type IV (31%), associated with HA and CA infections, respectively [144].

Pathogenesis and Virulence Factors of Staphylococcus aureus

Adherence: The production of fibronectin and fibrinogen-binding proteins, along with the expression of specific surface proteins, facilitates binding and promotes attachment to laminin and fibronectin within the body. Clumping factor and coagulase promote attachment to blood clots and traumatised tissue. In infections leading to osteomyelitis, adhesins binding to collagen play a significant role. The adherence of bacteria to collagen enables biofilm secretion, which makes eradication more challenging [114].

Invasion: A virulence factor of *Staphylococcus aureus* is its ability to invade the host and spread within body tissues through the secretion of invasins such as alpha and beta-toxins. These toxins create pores in the membrane of susceptible cells, leading to leakage of cellular contents and damage to sphingomyelin-rich lipid membranes by beta-toxin (sphingomyelinase toxin), which is not often seen in *Staphylococcus aureus* strains isolated from humans [114].

Evasion: The production of a polysaccharide-rich microcapsule, visible only by electron microscopy, helps Staphylococcus aureus evade phagocytosis. The binding of Protein A to IgG in the incorrect orientation interferes with phagocytosis and opsonisation. Additionally, the production of leucocidin, a toxin targeting polymorphonuclear leukocytes (phagocytic cells), enhances the bacterium's ability to evade the immune system and promotes its survival and spread [114].

Toxin: Besides alpha and beta toxins, *Staphylococcus aureus* releases other toxins such as enterotoxins and exotoxins with superantigen activity, causing Toxic Shock Syndrome Toxin (TSST-1) and food poisoning. Superantigens bind directly to major histocompatibility complex class II receptors, while enterotoxins and TSS toxins cause nonspecific T cell stimulation, leading to the release of large amounts of cytokines and symptoms of toxic shock syndrome [114]. Resistance to multiple antibiotics has led to the emergence of MRSA, posing significant therapeutic challenges [145].

1.4.9 Treatment and Prevention of *Staphylococcus aureus* Infection

Antimicrobials for treating *Staphylococcus aureus* infections target various bacterial components, including the cell wall, cell membrane, DNA/RNA synthesis, ribosomes (protein synthesis), and folic acid biosynthesis [146]. Vancomycin remains the standard treatment for severe MRSA infections, though its efficacy is compromised by nephrotoxicity, persistent bacteremia, and non-susceptible strains. Alternatives include linezolid, daptomycin, teicoplanin, and arbekacin. Drainage of abscesses caused by *Staphylococcus aureus* infections is also recommended [147, 148].

1.5 Minimally Invasive Tissue Sampling

The identification of microbial ecosystems is challenging using conventional culture techniques of micro-organisms. Non cultivation techniques (also known as cultivation-independent techniques) such as molecular techniques serve as a valuable tool for the investigation of microbial ecosystems ensuring the detection of non-viable bacteria using the nucleic acid sequence [149, 150]. Also, post-mortem minimally invasive tissue sampling is a potential alternative to the gold standard (blood culture technique) and compared to a complete autopsy for identifying causes of death in children

Minimally Invasive Tissue Sampling (MITS) techniques have been explored globally and locally to address diagnostic challenges and improve patient outcomes. Studies have shown that MITS can effectively diagnose various infections and diseases with less risk and discomfort than traditional methods. A review of recent literature indicates several studies from different regions that have employed MITS with success in clinical settings [3]. In a recent study using MITS, it was reported that 74.4% of 90 infection-related deaths among children were healthcare associated, mainly due to multidrug-resistant *Acinetobacter baumannii* (52.2%), *Klebsiella pneumoniae* (22.4%), and *Staphylococcus aureus* (20.9%) [3]. In the same study, it was concluded that MITS has the potential to address the knowledge gap on specific causes of neonatal mortality by mitigating against the lower sensitivity of routine blood culture, sample contamination, and/or translocation of enteric bacteria into the bloodstream [3].

1.5.2 Antemortem versus Postmortem Diagnosis of Sepsis

Antemortem diagnosis of sepsis involves identifying and confirming sepsis in a living patient through clinical evaluation, laboratory tests, and occasionally imaging studies to detect infection, inflammation, and organ dysfunction. This process is challenging due to the nonspecific nature of sepsis symptoms, such as fever, tachycardia, and hypotension, which can overlap with other conditions. Diagnostic delays are common, as the early signs of sepsis can be subtle, making timely diagnosis and treatment difficult. Laboratory tests like blood cultures are critical but can be time-consuming and yield false negatives. Biomarkers such as procalcitonin and C-reactive protein (CRP) can assist in diagnosis but are not definitive on their own, necessitating a combination of clinical judgment and laboratory data [151-153].

Postmortem diagnosis of sepsis is made after death, often through an autopsy or MITS, to identify signs of infection and inflammation. This process is complicated by the need to interpret whether pathogens found postmortem were significant antemortem infections or merely contaminants introduced during or after death. Decomposition and postmortem changes can obscure sepsis indicators, making distinguishing true infections from artefacts difficult. Histopathological examination of tissues can reveal infection, but the interpretation is complex due to these postmortem changes. The significance of pathogens found in the antemortem or postmortem must be carefully interpreted, considering clinical correlation, treatment response, and the potential for contamination during postmortem procedures. Understanding the temporal relationship between pathogen presence and clinical symptoms, supported by histological evidence, is crucial in determining the role of these pathogens in sepsis [154, 155].

1.6 Research justification

A significant number of neonatal deaths occur in LMICs where nosocomial infections are associated with high mortality rates, especially among premature neonates. Limited data exists regarding the incidence, antimicrobial resistance and mortality rates of nosocomial sepsis in South Africa [156].

1.7 Aim and objectives

This study aimed to characterise the incidence and case fatality rates of nosocomial sepsis within the neonatal unit at the Chris Hani Baragwanath Academic Hospital (CHBAH) from 1st January 2016 to 31st December 2019. Additionally, we aimed to analyse the distribution patterns of the antibiograms of prevalent nosocomial pathogens including *Acinetobacter baumannii, Klebsiella pneumoniae* and *Staphylococcus aureus*. Furthermore, we described the pathogens responsible for neonatal nosocomial sepsis in neonates that demised through minimally invasive tissue sampling (MITS) methods. The complete diagnostic autopsy (CDA) is the gold standard for determining the cause of death (CoD). However, its widespread implementation in LMICs may face challenges due to multiple factors such as resource constraints, cultural and religious beliefs and limitations in pathology capacity [156].

The objectives of the study are as follows:

- To determine the incidence measured per 1000 live births and case fatality rates of nosocomial neonatal sepsis attributed to *Acinetobacter baumannii, Klebsiella pneumoniae* and MRSA at the CHBAH from 1st January 2016 to 30th December 2019.
- To describe the clinical and laboratory characteristics of nosocomial sepsis caused by *Acinetobacter baumannii, Klebsiella pneumoniae and* MRSA
- To determine the antibiotic susceptibility profiles of *Acinetobacter baumannii, Klebsiella pneumoniae and* MRSA.
- To describe the burden of nosocomial pathogens (*Acinetobacter baumannii, Klebsiella pneumoniae and* MRSA detected by the minimal invasive tissue sampling method versus pathogens detected by conventional culture methods.
- To determine risk factors associated with increased mortality in neonates hospitalised with nosocomial sepsis caused by *Acinetobacter baumannii, Klebsiella pneumoniae and* MRSA.

Chapter 2: Materials and Methods

2.1 Study design

This retrospective descriptive study investigated neonates hospitalised at the Chris Hani Baragwanath Academic Hospital (CHBAH) who were diagnosed with microbiologically confirmed nosocomial sepsis caused by *Acinetobacter baumannii, Klebsiella pneumoniae and* MRSA from 1st January 2016 to 31stth December 2019.

2.2 Study Population

The study was conducted at the CHBAH, a secondary-tertiary hospital located in Soweto, where approximately 75% of the 30 000 annual births takes place [3]. Public healthcare services are provided is free of charge to all pregnant women and children under 6 years of age. [3]. Clinicians at CHBAH maintain a low threshold for investigating neonates suspected of nosocomial sepsis or meningitis, adhering to standardised guidelines. Routine blood and CSF cultures are performed as part of the diagnostic protocol. Clinical manifestations of nosocomial sepsis may vary and can be non-specific [157]; features of sepsis may include:

- bradycardia and or tachycardia,
- lethargy, refusal to suck and poor cry,
- prolonged capillary refill time or poor perfusion,
- hypotonia or absence of neonatal reflexes,
- hypothermia or fever (hypothermia appears to be more common in preterm low birth weight infants),
- respiratory distress or apnea,
- hypoglycemia or hyperglycemia and
- metabolic acidosis [3].

Blood and CSF culture remains the gold standard for the diagnosis of sepsis and meningitis, and can take 48-72 hours to become available [157]. The choice of antibiotics would depend on the antimicrobial susceptibility profiles and the prevailing flora in the specific clinical unit [158].

Initiation of antibiotic therapy is guided by clinical features and a positive septic screen [158]. At the CHBAH, common antibiotics for nosocomial infections include piperacillin-tazobactam and amikacin. Alternatives such as meropenem or vancomycin may be considered if there is suspicion of extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae* or MRSA infections.

2.3 Sample size

Since this is a retrospective study, a formal sample size calculation was not conducted.

2.4 Study method

Nosocomial sepsis was defined as the presence of a positive blood and/or CSF culture obtained at least 48 hours after admission or birth (for newborns admitted from birth) [159]. The NHLS microbiology laboratory database from the CDW was used to identify positive blood and/or CSF cultures for *Acinetobacter baumannii, Klebsiella pneumoniae and* MRSA, with antibiotic susceptibility profiles (permission was obtained from the Academic Affairs and Research Management System- AARMS of NHLS). The NHLS is an accredited South African National Accreditation System (SANAS) laboratory. The extracted information from the NHLS CDW included patient demographics, test date (date of specimen collection and test performance), the organisms isolated, their antibiotics susceptibility profile and the patient outcome. This database was then cross-referenced with the neonatal discharge database (permission from Prof. Shabir A. Madhi), and the extracted information contained demographics and clinical data. The following variables were extracted from both databases:

- patient age,
- gender,
- gestational age,
- birth weight,
- maternal pre-existing health condition,
- maternal HIV (including PMTCT), Rh and RPR status,
- newborn HIV status,
- mode of delivery,
- APGAR score,

- duration of hospitalisation,
- presence of congenital anomalies,
- ante-mortem cultures,
- post-mortem cultures,
- intensive care and mechanical ventilation,
- central line and total parental nutrition,
- discharge diagnoses/outcome
- feeding mode
- birth canal
- aetiology
- antibiotics (over 51 in number)

To ensure data integrity and consistency, initially we merged the NHLS CDW and the neonatal discharge datasets using hospital number as a common identifier. This yielded a total of 1,019 observations. Following this merge, duplicate cases was identified and subsequently excluded from the analysis. A duplicate case was based on two conditions; having the same hospital number in the database with admission records captured within 14 days. This was to conform with the Centre for Disease Control and Prevention (CDC) criteria for which a 14-day time frame for repeat infection was considered (RIT) [160].

2.5 Laboratory methods

Blood culture specimens are obtained through peripheral blood draws or a central or arterial line for neonates in the neonatal unit. These blood samples are aseptically inoculated into pediatric blood culture BacT/ALERT PF bottles containing specialised media designed for small volume samples and charcoal for antibiotic neutralisation following local guidelines. The bottles are then loaded into a blood culture processing machine such as the BacT/ALERT system - BioMerieux [159]. Positive blood cultures and CSF samples are then processed according to the standard methodology. The specimens are initially gram-stained and then cultured onto 5% blood agar, chocolate agar medium and brain heart infusion (BHI) broth for CSF samples only.

Incubation is carried out at 37^0C for 18-24 hours under 5%-10% CO_2 atmospheric conditions to facilitate optimal growth [159]. Colonies cultured are used for antibiotic susceptibility testing using the Kirby Bauer disc diffusion method or automated MicroScan NM37 minimum inhibitory concentration (MIC) panel (MicroScan WalkAway 96 SI Siemens) System and supported with the micro broth dilution technique where disk contents of antimicrobial agents and their corresponding zone diameters and MIC readings are taken in accordance to the Clinical and Laboratory Standards Institute (CLSI) recommendations for *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Staphylococcus aureus* [139].

The MITS procedures were performed by trained personnel. Prior to sample collection for molecular testing and microscopy, culture and susceptibility testing, the body surface from which the procedure was to be performed was washed with water and then decontaminated with 70% alcohol. Blood and CSF samples were collected, along with brain, liver and lung tissue samples for various analysis [149]. Laboratory testing included microscopy, culture and susceptibility testing, as well as molecular testing using methods using Multiplex Polymerase Chain Reaction assay (e.g., Fast-Track Diagnostics^{TM}) [3].

2.6 Statistical analysis

Incidence and case fatality rates

We calculated the in-facility annual incidence rate of nosocomial neonatal sepsis using the formula:

$$Incidence = \frac{Number\ of\ live\ birth\ with\ infection\ in\ a\ specified\ time\ period}{Total\ number\ of\ live\ birth\ in\ the\ same\ period} \times 1000$$

For each organism, we estimated the annual case fatality rate of nosocomial neonatal sepsis using the formula below:

$$Case\ fatality\ rate = \frac{Number\ of\ infection\ \ deaths\ in\ a\ specified\ time\ period}{number\ of all\ cases\ in\ the\ same\ time\ period} \times 100$$

Clinical characteristics

A descriptive analysis of the clinical characteristics of nosocomial sepsis during the study period was conducted, followed by a multinomial logistic regression (MLR) to investigate the effect of demographic and clinical characteristics on nosocomial sepsis infection. *Acinetobacter baumannii* was chosen as the reference category, being the most frequently observed organism. The estimated odds ratios, confidence intervals, and p-values associated with each predictor variable were reported for *Klebsiella pneumoniae* and MRSA. Statistical significance was set at a p-value < 0.05.

Risk factors associated with mortality

The logistic regression models were fitted to assess the association between the demographic and clinical variables and outcome variables. First, we employed the binary logistic regression to assess the impact of each potential predictor on the outcome variable with two measurement levels (death or alive), and this is denoted by:

$$Log(P) = \left(\frac{P_i}{1 - P_i}\right) = \beta_0 + \beta_1 x_i, \qquad (3)$$

Where P_i is the probability of dying from nosocomial neonatal sepsis infection and the x_i is for instance, the weight category of the neonates. β is the regression coefficient, and exp ($\beta_0 + \beta_1 x_i$) the odds. The application of this model is detailed elsewhere [161, 162].

Antibiotic susceptibility profiles

We used a frequency table and bar plot to characterise the antibiotic susceptibility profiles of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and MRSA. Only antibiotics with no single record of observation were excluded from the analysis.

Nosocomial pathogens detected by MITS

The MITS data source was imported and merged with the working dataset and identify any additional cases that were not identified in the NHLS data. These were presented as frequencies.

2.7 Ethical clearance

Before the commencement of this study, ethical clearance was obtained from the Human Research

Ethics Committee (HREC) of the University of the Witwatersrand. Ethics clearance certificate no: M201050.

Chapter 3: Results

Over the study period, 859 culture confirmed nosocomial neonatal sepsis cases were caused by *Acinetobacter baumannii, Klebsiella pneumoniae* and MRSA *were* identified. There were 462 (53.8%) cases of *Acinetobacter baumannii*, 323 (37.6%) cases of *Klebsiella pneumoniae* and 74 (8.6%) cases of MRSA.

3.1 Incidence (per 1000 live births) and case fatality rates

The incidence (per 1000 live births) and case fatality rates of *Acinetobacter baumannii, Klebsiella pneumoniae and Staphylococcus aureus* are presented in Figure 3.1 and Figure 3.2. The incidence of *Acinetobacter baumanni and Klebsiella pneumoniae* was highest at 4.03 (95%CI: 3.36-4.80) and 2.91 (95%CI: 2.34-3.57) in 2017, respectively. While the incidence of *Acinetobacter baumanni and Klebsiella pneumoniae* remained consistent over the study period, the incidence of MRSA declined from 1.59 (95%CI: 1.16-2.13) in 2016 to 0.21 (95%CI: 0.08-0.43; p=<0.001) by December 2019. The overall case fatality rate was 47.3% (95%CI: 43.9-50.7); the case fatality rate was 54.5%, 40.6% and 31.1% for *Acinetobacter baumanni, Klebsiella pneumoniae and MRSA* respectively. Case fatality rates fluctuated over the study period and were lowest in 2017 for *Acinetobacter baumanni and MRSA*.

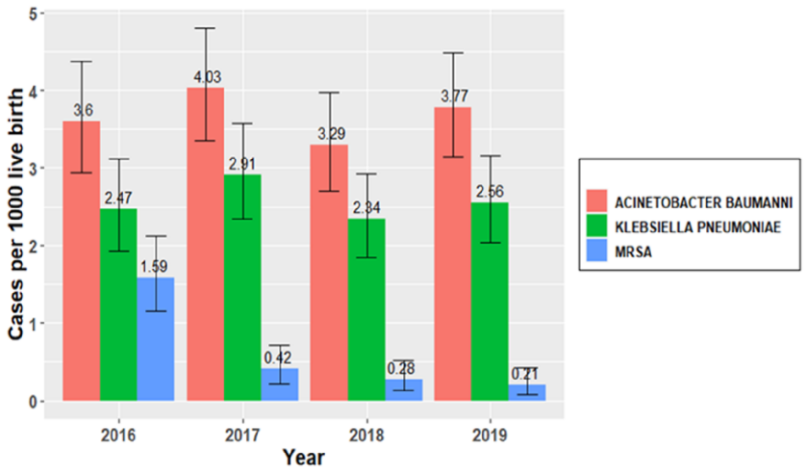


Figure 3.1: Incidence (per 1000 live births) of *Acinetobacter baumannii, Klebsiella pneumoniae* and MRSA

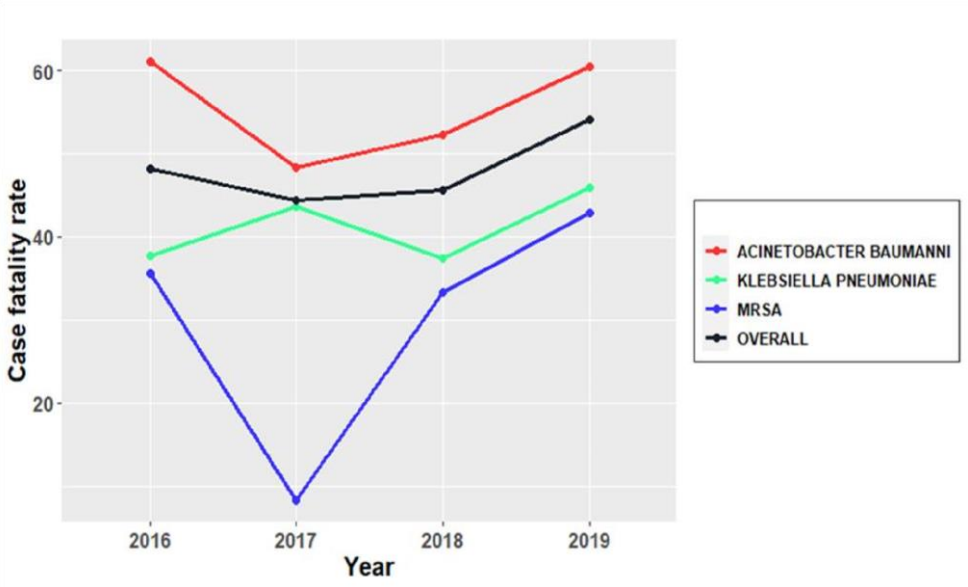


Figure 3.2: Case fatality rates of *Acinetobacter baumannii, Klebsiella pneumoniae* and MRSA

3.2 Clinical and Laboratory characteristics

Neonatal clinical characteristics for the three organisms are shown in Table 3.1. Of the 462, culture confirmed *Acinetobacter baumannii* cases, 235 (50.9%) were female. Most (n=394, 85.3%) were born preterm, 316 (68.4%) were very low birth weight (<1500g), 244 (52.8%) were born via caesarian section and 162 (35.1%) were HIV-exposed. Of the 323, culture confirmed *Klebsiella pneumoniae* cases, 182 (56.3%) were female, 264 (81.7%) were born preterm, 168 (52.0%) were very low birth weight, 182 (56.3%) were born via caesarian section and 112 (34.7%) were HIV-exposed. Of the 74 MRSA cases, 34 (45.9%) were females, 66 (89.2%) were born preterm, 54 (73.0%) were very low birth weight, 47 (63.5%) were born via caesarian section and 18 (24.3%) were HIV-exposed.

In the multinomial logistic regression (MLR), *Acinetobacter baumannii* was the reference category (Table 3.2). Neonates with low birth weight, compared to those with very low birth weight, had twice the odds of being infected with *Klebsiella pneumoniae* than with *Acinetobacter baumannii* (OR = 2.0, 95% CI: 1.4-2.8, p<0.001). Additionally, neonates with normal birth weight compared with very low birth weight were approximately twice as likely to be infected with *Klebsiella pneumoniae* as with *Acinetobacter baumannii* (OR = 2.0, 95% CI: 1.4-2.8, p = 0.005). However, there is no statistically significant association between birth weight and MRSA infection compared to *Acinetobacter*

baumannii. Furthermore, infants who demised had significantly lower odds of being infected with *Klebsiella pneumoniae* (OR = 0.4, 95% CI: 0.2-0.6, $p < 0.001$) and MRSA. (OR = 0.6, 95% CI: 0.4-0.8, $p < 0.001$) compared to those who were discharged home, with *Acinetobacter baumannii* as the reference category.

Table 3.1: Clinical characteristics of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and MRSA neonatal sepsis

Variable	**Category**	***Acinetobacter baumannii* (N=462)**	***Klebsiella pneumoniae* (N=323)**	**MRSA (N=74)**
Gender	Female	235 (50.9%)	182 (56.3%)	34 (45.9%)
	Male	227 (49.1%)	141 (43.7%)	40 (54.1%)
Gestational age	<37 weeks	394 (85.3%)	264 (81.7%)	66 (89.2%)
	≥37 weeks	55 (11.9%)	51 (15.8%)	8 (10.8%)
	Missing	13 (2.8%)	8 (2.5%)	0 (0%)
Apgar score	0-3	62 (13.4%)	32 (9.9%)	8 (10.8%)
	4-6	125 (27.1%)	84 (26.0%)	15 (20.3%)
	7-10	215 (46.5%)	174 (53.9%)	45 (60.8%)
	Missing	60 (13.0%)	33 (10.2%)	6 (8.1%)
Birth Weight	<1500g	316 (68.4%)	168 (52.0%)	54 (73.0%)
	1500-2499g	98 (21.2%)	104 (32.2%)	13 (17.6%)
	≥2500g	48 (10.4%)	48 (14.9%)	7 (9.5%)
Delivery mode	Caesarian	244 (52.8%)	182 (56.3%)	47 (63.5%)
	Vaginal	199 (43.1%)	126 (39.0%)	26 (35.1%)
	Missing	19 (4.1%)	15 (4.6%)	1 (1.4%)
Rhesus factor	Negative	32 (6.9%)	18 (5.6%)	1 (1.4%)
	Positive	367 (79.4%)	268 (83.0%)	65 (87.8%)
	Missing	63 (13.6%)	37 (11.5%)	8 (10.8%)
RVD	Negative	283 (61.3%)	199 (61.6%)	52 (70.3%)
	Positive	162 (35.1%)	112 (34.7%)	18 (24.3%)
	Missing	17 (3.7%)	12 (3.7%)	4 (5.4%)
Feeding discharge	Breast	69 (14.9%)	49 (15.2%)	16 (21.6%)
	Formula	62 (13.4%)	41 (12.7%)	13 (17.6%)
	Missing	331 (71.6%)	233 (72.1%)	45 (60.8%)
Outcome	Discharged Home	203 (43.9%)	185 (57.3%)	50 (67.6%)
	Demised	252 (54.5%)	131 (40.6%)	23 (31.1%)

Table 3.2: Multinomial logistic regression comparing clinical characteristics in neonates with *Klebsiella pneumoniae* and MRSA to *Acinetobacter baumannii*

Variable	***Klebsiella pneumoniae*** **OR (95% CI)**	**MRSA OR (95% CI)**
Gender (female)	0.7 (0.5-1.1)	0.8 (0.5-1.4)
Gestational age (≥37 weeks)	1.5 (1.0-2.4)	2.0 (1.0-4.0)
Birth Weight (1500-2499g)	2.0 (1.4-2.8)*	1.2 (0.6-2.3)
Birth Weight (≥2500g)	2.0 (1.4-2.8)*	1.5 (0.7-3.2)
RVD (positive)	0.8 (0.6-1.2)	0.6 (0.4-1.0)
Outcome (demised)	0.4 (0.2-0.6)*	0.6 (0.4-0.8)*

*$p<0.05$

3.3 Antibiotic Susceptibility Patterns

Figures 3.3 A, B and C show detailed antibiotic susceptibility profiles of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and MRSA, respectively, from 2016 to 2019 in CHBAH. *Acinetobacter baumannii* had an overall resistance of 71.9% (332 of 462 isolates) to amikacin for 2016 (75 of 102 isolates (73.5%), 2017 (83 of 126 isolates = 65.9%), 2018 (70 of 104 isolates (65.4%) and 2019 (104 of 127 isolates (81.9%) were largely similar. Resistance of *Acinetobacter baumannii* to cephalosporins over the years 2016, 2017, 2018 and 2019 were as follows: cefepime: 74.5%, 67.5%, 58.9% and 63.8% respectively with an overall resistance of 66.0%; and ceftazidime: 76.5%, 69.0%, 62.6% and 59.1% respectively with an overall resistance of 66.5%. There was also an observable increase in resistance to carbapenems at the end of the study period with a frequency of resistance to imipenem of 89% and meropenem of 88.4% in 2019.

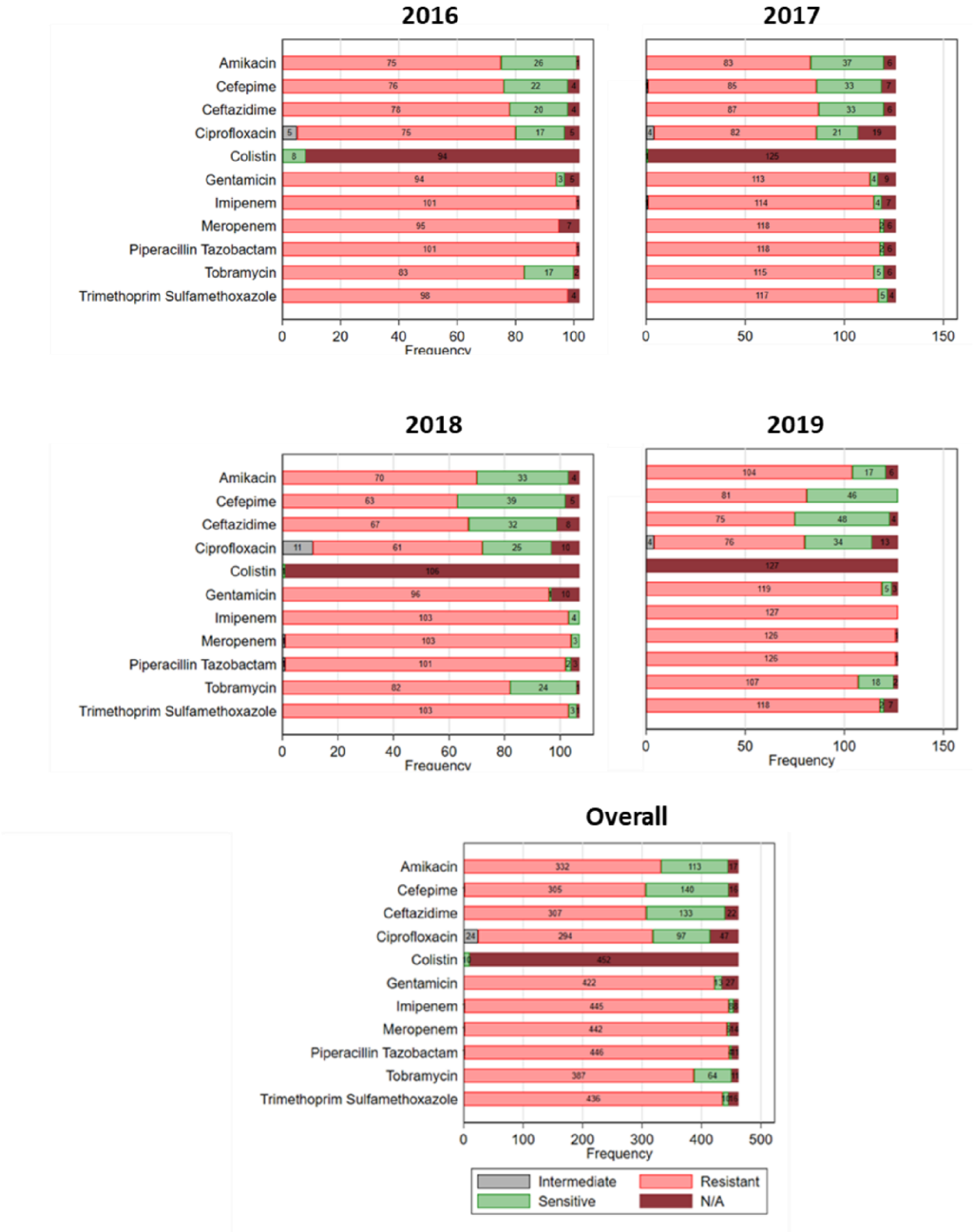


Figure 3.3A: Antibiotic susceptibility profile of *Acinetobacter baumannii*

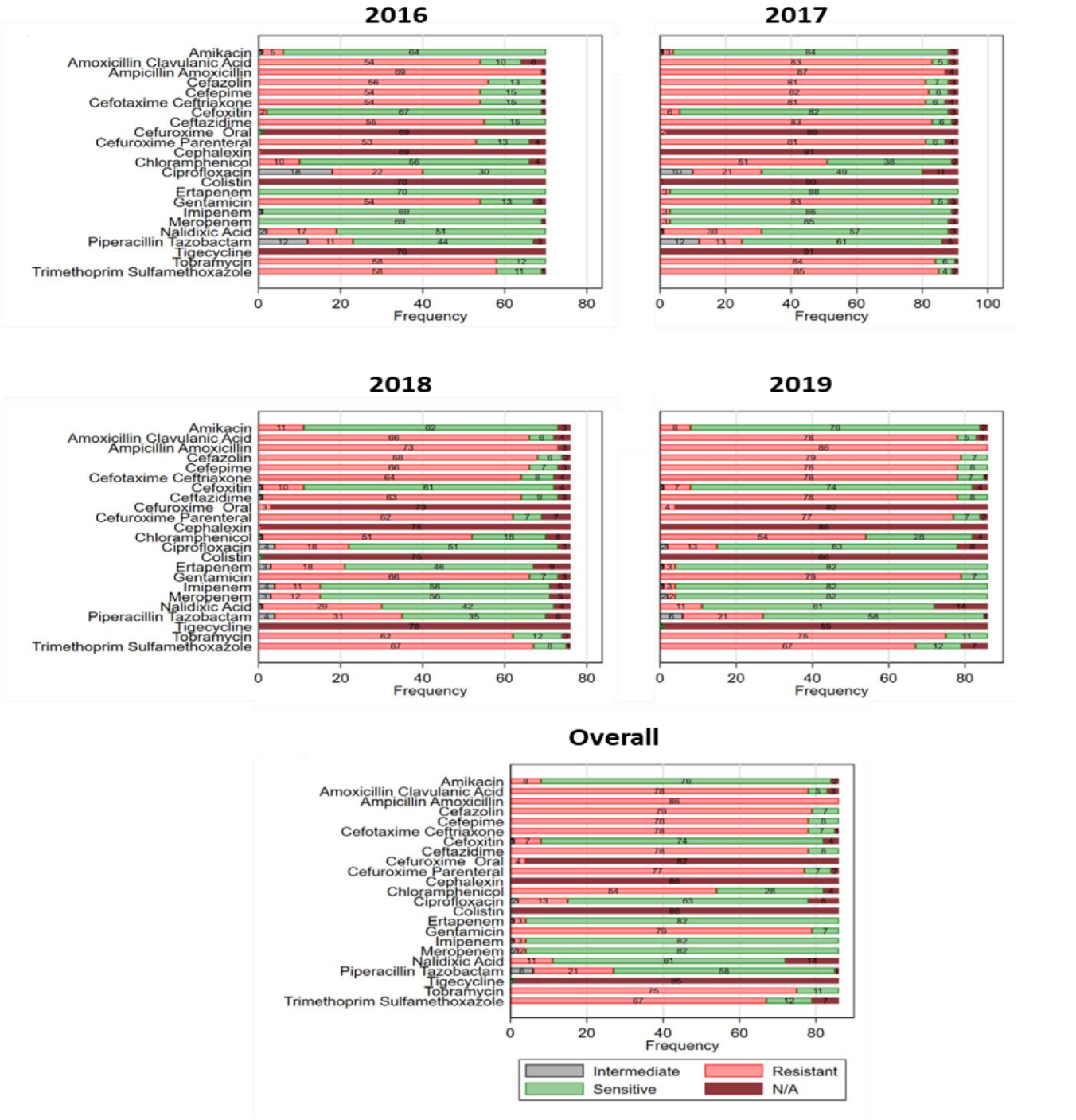


Figure 3.3 B: Antibiotic susceptibility profile of *Klebsiella pneumoniae*



Figure 3.3C: Antibiotic susceptibility profile of MRSA

*NA represents not tested.

The predominant multidrug-resistant (MDR) profiles observed for the organisms in the study were as follows:

Acinetobacter baumannii: This pathogen exhibited extensive drug resistance, characterised by high resistance to multiple classes of antibiotics, as demonstrated in Figure 3.3 A. The resistance was notably high for amikacin (71.9%), cefepime (66.0%), ceftazidime (66.5%), and carbapenems (89% for imipenem and 88.4% for meropenem in 2019).

Klebsiella pneumoniae predominantly displayed extended-spectrum beta-lactamase production, which contributes to its resistance to several antibiotics, including cephalosporins. For resistance patterns for ceftriaxone, piperacillin/tazobactam, and carbapenems (Figure 3.3 B). Ceftriaxone: Resistance to ceftriaxone was prevalent, with an overall resistance rate observed to be significant across the study period. Specific resistance rates were not detailed in the extracted section but were noted to be high. Piperacillin/tazobactam: Resistance to piperacillin/tazobactam was also observed but specific percentages were not detailed in the extracted section.

Carbapenems: Although susceptibility to carbapenems remained high during the study period, there was a concern about the increasing prevalence of carbapenemase-producing *Klebsiella pneumoniae*. The study did not provide specific resistance percentages for carbapenems but indicated an emerging resistance trend.

MRSA showed high resistance to multiple antibiotics but remained generally susceptible to vancomycin and linezolid (Figure 3.3 C).

3.4 The burden of the pathogens detected by the MITS versus NHLS culture methods

MITS was only undertaken during the 2018 and 2019 study period. During this period, on the MITS database, there were 88 deaths from *Acinetobacter baumannii* (n=39), *Klebsiella pneumoniae* (n=44) and MRSA (n=5). During the same 2018/2019 period, there were 287 deaths from *Acinetobacter baumannii* (n=131), *Klebsiella pneumoniae* (n=67) and MRSA (n=6) on the NHLS database. Only seven cases of *Acinetobacter baumannii* and *Klebsiella pneumoniae* were common to both databases (Table 3.3). Notably, MITS was not performed on all NHLS deaths. Using the MITS procedure, which includes culture and molecular testing, four of 16 (25%) deaths had both *Acinetobacter baumannii* and *Klebsiella pneumoniae* identified in the same participant (Table 3.4). Most of the deaths for

Acinetobacter baumannii (36/39; 92.3%) and *Klebsiella pneumoniae* (36/44; 81.8%) detected through MITS were the immediate cause of death in the causal chain (Figure 3.4).

Table 3.3 Deaths identified through NHLS and MITS databases for 2018 and 2019

Organism	**NHLS database**	**MITS database**	**Common to both databases**
Acinetobacter baumannii	131	39	7
Klebsiella pneumoniae	67	44	7
MRSA	6	5	0

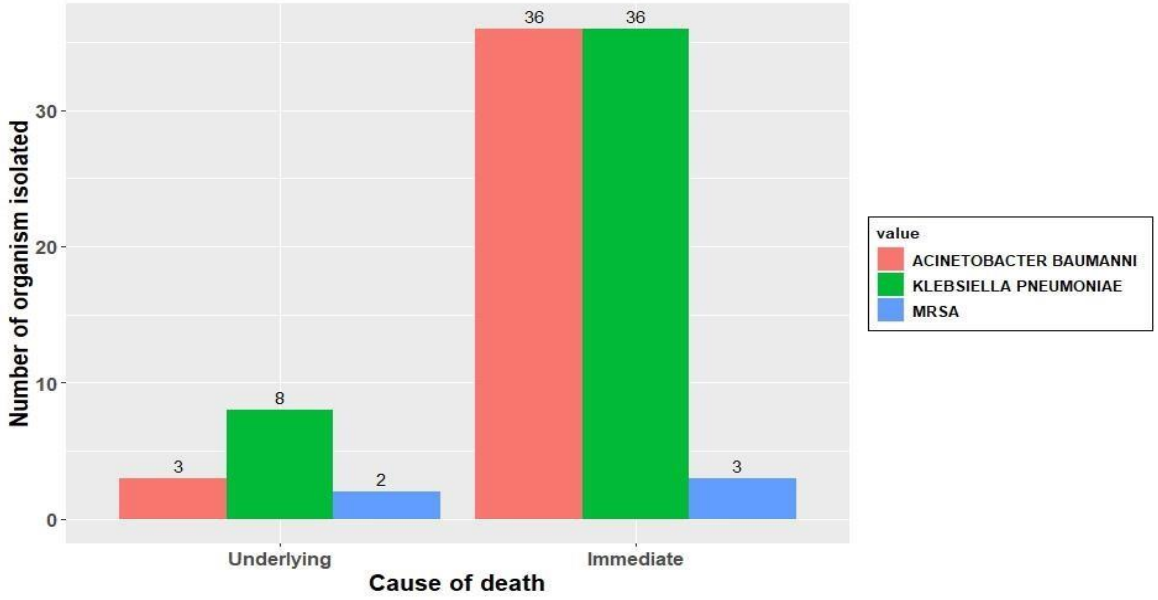


Figure 3.4: Frequency of organism in the causal chain of death

Table 3.4: *Acinetobacter baumannii* and *Klebsiella pneumoniae* common to both methods

Year	Pathogen 1 (MITS)	Pathogen 2 (MITS)	NHLS-detected pathogen
2018	*Acinetobacter baumannii*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
2018	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	No pathogen detected
2018	*Acinetobacter baumannii*	*Acinetobacter baumannii*	No pathogen detected
2018	*Klebsiella pneumoniae*	*Acinetobacter baumannii*	*Klebsiella pneumoniae*
2019	*Enterococcus faecium*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
2019	*Acinetobacter baumannii*	*Klebsiella spp*	*Klebsiella pneumoniae*
2019	*Acinetobacter baumannii*	*Acinetobacter baumannii*	No pathogen detected
2019	*Acinetobacter baumannii*	*Streptococcus agalactiae*	*Acinetobacter baumannii*
2019	*Acinetobacter baumannii*	*Acinetobacter baumannii*	No pathogen detected
2019	*Acinetobacter baumannii*	*Acinetobacter baumannii*	No pathogen detected
2019	*Streptococcus agalactiae*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
2019	*Acinetobacter baumannii*	*Klebsiella pneumoniae*	*Acinetobacter baumannii*
2019	*Acinetobacter baumannii*	*Acinetobacter baumannii*	No pathogen detected
2019	*Acinetobacter baumannii*	*Klebsiella pneumoniae*	No pathogen detected
2019	*Klebsiella pneumoniae*	*Enterococcus faecium*	*Klebsiella pneumoniae*

3.5 Risk factors associated with increased mortality

Table 3.5 presents the risk factors associated with increased mortality. Neonates admitted to the ICU had a 90.2 (95%CI: 55.8-151.0) increased odds of mortality than neonates not admitted to the ICU. Neonates born at term compared to preterm (OR: 0.5; 95%CI: 0.3-0.8) had fewer odds of mortality. Similarly, neonates born with a normal birth weight compared to very low birth weight had fewer odds of mortality (OR: 0.4; 95%CI: 0.2-0.6).

Table 3.5: Factors associated with mortality in neonates with *Acinetobacter baumannii*, *Klebsiella pneumoniae* and MRSA sepsis.

Variable	Category	Demised (N=406)	Alive (N=438)	OR (95% CI)	p-value
Gender	Female	219 (53.9%)	224 (51.1%)	0.9 (0.7-1.2)	0.416
	Male	187 (46.1%)	214 (48.9%)		
Gestational age	<37 weeks	352 (86.7%)	361 (82.4%)		
	≥37 weeks	38 (9.4%)	73 (16.7%)	0.5 (0.3-0.8)	**0.003**
	Missing	16 (3.9%)	4 (0.9%)		
Apgar score	0-3	51 (12.6%)	51 (11.6%)		
	4-6	110 (27.1%)	107 (24.4%)	1.0 (0.6-1.6)	0.908
	7-10	190 (46.8%)	239 (54.6%)	0.8 (0.5-1.2)	0.298
	Missing	55 (13.5%)	41 (9.4%)		
Birth weight	<1500g	293 (72.2%)	235 (53.7%)		
	1500-2499g	78 (19.2%)	134 (30.6%)	0.5 (0.3-0.6)	**<0.001**
	≥2500g	33 (8.1%)	69 (15.8%)	0.4 (0.2-0.6)	**<0.001**
	Missing	2 (0.5%)	0 (0%)		
Delivery mode	Caesarian	208 (51.2%)	257 (58.7%)	1	
	Vaginal	173 (42.6%)	173 (39.5%)	1.2 (0.9-1.6)	0.137
	Missing	25 (6.2%)	8 (1.8%)		
Rhesus factor	Negative	18 (4.4%)	33 (7.5%)	1	
	Positive	308 (75.9%)	380 (86.8%)	1.5 (0.8-2.7)	0.191
	Missing	80 (19.7%)	25 (5.7%)		
RVD	Negative	257 (63.3%)	269 (61.4%)	1	
	Positive	133 (32.8%)	152 (34.7%)	0.9 (0.7-1.2)	0.551
	Missing	16 (3.9%)	17 (3.9%)		
Feeding discharge	Breast	16 (3.9%)	115 (26.3%)	1	
	Formula	8 (2.0%)	106 (24.2%)	0.5 (0.2-1.3)	0.177
	Missing	382 (94.1%)	217 (49.5%)		
Wards	Non-ICU	29 (7.1%)	352 (80.4%)	1	
	ICU	312 (76.8%)	42 (9.6%)	90.2 (55.8-151.0)	**<0.001**
	Missing	65 (16.0%)	44 (10.0%)		
Organism name	*Acinetobacter baumannii*	252 (62.1%)	203 (46.3%)	1	
	Klebsiella pneumoniae	131 (32.3%)	185 (42.2%)	0.6 (0.4-0.8)	<0.001
	MRSA	23 (5.7%)	50 (11.4%)	0.4 (0.2-0.6)	<0.001

Chapter 4: Discussion

4.1 Discussion

The study examined the incidence, clinical characteristics, antibiotic susceptibility patterns, associated risk factors and case fatality rates of nosocomial neonatal sepsis caused by *Acinetobacter baumannii*, *Klebsiella pneumoniae* and Methicillin-resistant *Staphylococcus aureus* at the CHBAH from January 1, 2016, to December 30, 2019.

Incidence and case fatality rates

The incidence of *Acinetobacter baumannii* and *Klebsiella pneumoniae* remained high throughout the study period, and compared similarly to the incidence of the national late-onset sepsis estimates of 4.9 per 1000 livebirths where *Acinetobacter baumannii* and *Klebsiella pneumoniae* were the most prevalent pathogens [163]. This also compares similarly to other LMICs but is three-fold higher than estimates reported in high-income settings [6].

Alarmingly, half (54.5%) of the neonates with nosocomial *Acinetobacter baumannii* and 40.6% with *Klebsiella pneumoniae* demised during the study period. All three pathogens showed an increasing trend in case fatality rates which may indicate evolving resistance patterns and challenges in treating infections caused by these pathogens. The observed trends in case fatality rates also underscore the significance of antimicrobial resistance and risk of mortality associated with these pathogens [164, 165]. Furthermore, comprehensive antimicrobial stewardship programs and infection prevention and control measures need to be ongoing to mitigate against these adverse neonatal health outcomes [166-169].

Clinical management and treatment of multidrug resistant nosocomial neonatal sepsis is challenging in LMICs [170, 171]. Healthcare providers face complexities in optimising therapeutic strategies, including timely diagnosis, appropriate antimicrobial selection, and supportive care, to effectively manage infections and reduce mortality rates. Most neonates were predominantly born preterm and had very low birth weight, indicating vulnerability among preterm neonates [172, 173]. These findings emphasise the need for continuous improvement in clinical practices and evidence-based guidelines to enhance neonatal sepsis care, particularly amongst preterm neonates [174, 175].

Importantly, we identified risk factors associated with mortality in neonates hospitalised with nosocomial sepsis caused by *Acinetobacter baumannii*, *Klebsiella pneumoniae* and MRSA. Admission to ICU showed a 90-fold (95%CI: 55.8-151.0) increased odds of mortality. Furthermore, neonates born at term compared with a normal birth weight had less odds of mortality (OR: 0.4; 95%CI: 0.2-0.6). These findings are comparable to studies undertaken in LMICs [176]. However, we did not find an increased odd of mortality in children born to HIV-infected mothers, this may be explained by an improved access to antiretroviral therapy in women during pregnancy.

Antibiotic Susceptibility Patterns

There was high resistance in *Acinetobacter baumannii* to antibiotics such as amikacin, cefepime, and ceftazidime over the study period. In addition, there was a notable rise in resistance to carbapenems towards the latter part of the study. The high levels of antibiotic resistance observed in *Acinetobacter baumannii* are consistent with previous studies documenting the emergence and spread of multidrug-resistant strains of *Acinetobacter baumannii* worldwide [177]. This highlights the urgent need for effective antimicrobial stewardship and infection control measures and underscores the challenges in managing infections caused by this pathogen in LMICs. Strict adherence to hand hygiene protocols, isolation precautions, and environmental cleaning are essential for preventing the transmission of multidrug-resistant *Acinetobacter baumannii* strains and reducing the incidence of nosocomial infections [178]. Further research is warranted to develop novel antimicrobial agents and alternative treatment strategies for infections caused by multi-drug-resistant *Acinetobacter baumannii* strains. Additionally, efforts to better understand the mechanisms of antibiotic resistance and transmission dynamics of *Acinetobacter baumannii* are essential for informing targeted interventions and improving patient outcomes. For *Klebsiella pneumoniae,* antibiotic susceptibility patterns reveal concerning trends with resistance to multiple antibiotics, including cephalosporins. Fluctuation levels of resistance to piperacillin/tazobactam was observed, underscoring the importance of monitoring this resistance as piperacillin/tazobactam is a crucial empiric treatment choice for nosocomial sepsis. Specific resistance percentages were not provided in the extracted section, but the need for its inclusion in the study's susceptibility patterns was emphasised.

This observed increase in resistance over the study period suggests a rise in multidrug-resistant strains, posing challenges for treatment. Although susceptibility to carbapenems remained high during the study period, recent studies have shown an increase in the prevalence of carbapenemase-producing

Klebsiella pneumoniae among neonates across various settings [169]. Overall, susceptibility to vancomycin and linezolid was high among cases of MRSA. We, however, did observe resistance to rifampicin in some cases.

Minimal invasive tissue sampling

Among deceased cases evaluated using MITS, *Acinetobacter baumannii* and *Klebsiella pneumoniae* were often the immediate cause of death in the causal chain. Previous studies have demonstrated the utility of MITS for post-mortem diagnosis of infectious diseases, particularly in resource-limited settings where access to full diagnostic autopsy facilities may be limited. The ability of MITS to detect a polymicrobial infection compared to conventional culture methods is also noted in our study with a quarter of deaths having both organisms. By identifying a higher number of pathogens, MITS can provide more comprehensive insights into the burden of infectious diseases and facilitate targeted interventions to mitigate transmission and improve patient outcomes. The findings underscore the importance of continued research and development in post-mortem diagnostic techniques, including MITS, to improve our understanding of infectious diseases and enhance diagnostic capabilities in both clinical and public health settings.

4.2 Limitations of the study

Our study had several limitations. One of the major limitations of this retrospective study was the reliance on an existing database and missing data for certain variables. Furthermore, we did not have data on the proportion of neonates that were HIV-infected. We were also unable to provide an estimate of the burden of all-cause culture confirmed sepsis and therefore unable to estimate the prevalence of these pathogens at CHBAH. Lastly, MITS was only performed on a proportion of neonates from the CHAMPS identified catchment areas. We identified cases of *Acinetobacter baumannii, Klebsiella pneumoniae* and MRSA sepsis on MITS that were not in the NHLS database and therefore possibly missed antemortem.

Chapter 5: Conclusion and Recommendation

5.1 Conclusion

Findings regarding the significant burden of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and MRSA *sepsis* reveal persistent multidrug resistance, thereby highlighting the importance of infection control measures in healthcare settings, including strict adherence to hygiene practices, antimicrobial stewardship, and surveillance protocols to prevent transmission and reduce the incidence of nosocomial infections in neonatal populations. The study poses a significant burden of nosocomial neonatal sepsis attributed to *Acinetobacter baumannii* at CHBAH. This research provides valuable insights into the clinical and microbiological aspects of neonatal sepsis, facilitating more informed decision-making and targeted interventions aimed at improving patient outcomes. Furthermore, the findings highlight the urgent need for multifaceted interventions to address neonatal sepsis effectively. Enhanced surveillance, antimicrobial stewardship, and diagnostic capabilities are crucial for tackling the escalating threat of antimicrobial resistance and reducing mortality rates associated with nosocomial neonatal sepsis.

5.2 Recommendations

Healthcare facilities must prioritise stringent infection control measures to prevent the nosocomial transmission of pathogens. This includes strict adherence to hand hygiene protocols, thoroughly disinfecting equipment and surfaces, ensuring appropriate use of personal protective equipment, and implementing isolation precautions as needed. Regular education and training sessions for staff on infection control practices are essential to ensure compliance and efficacy. Antimicrobial stewardship programs play a crucial role in optimising antibiotic use and mitigating antimicrobial resistance. Healthcare facilities should enhance these programs by promoting appropriate antibiotic prescribing practices, conducting regular antimicrobial susceptibility testing, and monitoring antibiotic consumption patterns. Multidisciplinary antimicrobial stewardship teams can help develop guidelines for antibiotic use and educate healthcare providers on the importance of judicious antibiotic use.

Robust data collection systems are essential for accurate epidemiological surveillance of nosocomial infections. Healthcare facilities should invest in improving data collection systems to ensure completeness and accuracy of data. This includes implementing electronic medical records systems, standardising data collection processes, and training staff on data entry protocols. Comprehensive and reliable data are crucial for monitoring trends, identifying outbreaks, and informing evidence-based interventions.

Further research is necessary to explore additional risk factors and interventions for reducing mortality in neonatal sepsis. This may involve conducting prospective studies to identify novel risk factors, evaluating the effectiveness of interventions such as immunomodulatory therapies or probiotics, and exploring the impact of socio-economic factors on neonatal outcomes. Collaborative research efforts involving multidisciplinary teams can help advance our understanding of neonatal sepsis and inform the development of targeted interventions.

Improving prenatal care services is essential for reducing the incidence of low birth weight and preterm births, which can increase the vulnerability of neonates to nosocomial infections. Healthcare facilities should focus on providing comprehensive prenatal care, including regular antenatal visits, screening for maternal infections, nutritional support, and education on healthy lifestyle behaviours. Addressing underlying maternal risk factors can help reduce the occurrence of adverse neonatal outcomes and prevent the need for prolonged hospital stays.

Consideration should be given to integrating MITS into routine diagnostic protocols for enhanced pathogen detection and mortality attribution. Minimally Invasive Tissue Sampling offers several advantages over conventional culture methods by providing rapid and accurate identification of causative pathogens, particularly in cases of neonatal sepsis. Healthcare facilities should explore the feasibility of incorporating MITS into existing diagnostic protocols and invest in staff training for proficient utilisation of MITS techniques, thereby optimising its potential advantages.

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Appendices

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

MEDICAL ADVISORY COMMITTEE

CHRIS HANI BARAGWANATH ACADEMIC HOSPITAL

PERMISSION TO CONDUCT RESEARCH

Date: 28th September 2021

TITLE OF PROJECT:

The Burden of Nosocomial Neonatal Sepsis at the Chris Hani Baragwanath Academic Hospital using Conventional Culture Methods and Minimal Invasive Tissue Sampling.

UNIVERSITY: Witwatersrand

Principal Investigator: Mr A Onyekwuo

Department: NHLS

Supervisor : Prof S Madhi / Prof Z Dangor / Dr J Wadula

Permission Head Department (where research conducted): Yes

NHRD No. 202109_045

The Medical Advisory Committee recommends that the said research be conducted at Chris Hani Baragwanath Academic Hospital. The CEO / management of Chris Hani Baragwanath Academic Hospital is accordingly informed and the study is subject to:-

- **Permission having been granted by the Committee for Research on Human Subjects of the University of Witwatersrand.**
- The Hospital will not incur extra costs as a result of the research being conducted on its patients within the hospital
- The MAC will be informed of any serious adverse events as soon as they occur
- Permission is granted for the duration of the Ethics Committee Approval.

.....
Recommended
(On behalf of the MAC)
Date: 28/09/2021

.....
Approved/Not Approved
Hospital Management
Date: 29/09/2021

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

12 July 2021
Person No: 801139
PAG

Mr AU Onyekwuo
Unit 64
Sunset Gardens
Hefer Street
Naturena
2095
South Africa

Dear Mr Anthony Onyekwuo

Master of Science in Medicine: Approval of Title

We have pleasure in advising that your proposal entitled *The burden of nosocomial neonatal sepsis at the Chris Hani Baragwanath Academic Hospital using conventional culture methods and minimal invasive tissue sampling.* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Mr A Onyekwuo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M201050

NAME: (Principal Investigator)	Mr A Onyekwuo
DEPARTMENT:	School of Pathology Department of Clinical Microbiology and Infectious Diseases Medical School University
PROJECT TITLE:	*The burden of nosocomial neonatal sepsis at the Chris Hani Baragwanth Academic Hospital, using conventional culture methods and minimally invasive tissue sampling*
DATE CONSIDERED:	2020/10/30
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Professors Z Dangor & SA Madhi; Dr J Wadula
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/11/11

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in October and therefore reports and re-certification will be due in the month of **October** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

15/11/2021
Date

Academic Affairs and Research
Modderfontein Road, Sandringham, 2031
Tel: +27 (0)11 386 6142
Fax: +27 (0)11 386 6296
Email: babatyi.kgokong@nhls.ac.za
Web: www.nhls.ac.za

06 November 2020

Applicant: Anthony Onyekwuo
Institution: National Health Laboratory Service
Department: Medical Microbiology
Email: anthony.onyekwuo@nhls.ac.za
Tel: 011 489 8730 **Cell:** 061 372 3620

CC: Jeanette Wadula
Senior Pathologist

Re: Provisional Approval to access National Health Laboratory Service (NHLS) Data

Your application to undertake a research project **"THE BURDEN OF NOSOCOMIAL NEONATAL SEPSIS AT THE CHRIS HANI BARAGWANATH ACADEMIC HOSPITAL USING CONVENTIONAL CULTURE METHODS AND MINIMAL INVASIVE TISSUE SAMPLING"** using data from the NHLS database has been reviewed. This letter serves to advise that the application has been provisionally approved. For full approval to be granted and for you to be given access to the data you have requested, you need to satisfy the following conditions:

- Ethics approval is obtained from a recognised SA Health Research Ethics Committee.
- Processes are discussed with the relevant NHLS departments (i.e. Information Management Unit and Operations Office) and are agreed upon.
- Confidentiality is maintained at participant and institutional level and there is no disclosure of personal information or confidential information as described by the NHLS policy.
- A final report of the research study and any published paper resulting from this study are submitted and addressed to the NHLS Academic Affairs and Research office and the NHLS has been acknowledged appropriately.
- NHLS Data cannot be used to track patients as no pre-approval/consent is obtained from Patients.

Please send any requested documents through to academic.research@nhls.ac.za. Once your application has received full final approval, we will confirm with you in writing. Should you wish to speak with us, please contact us on 011 555 0367.

Yours sincerely,

P.P. Refilwe Mochochoko

Dr Babatyi Malope-Kgokong
National Manager: AAR

Chairperson: Prof Eric Buch CEO: Dr Karmani Chetty