Assessment of antibiotic resistance in pathogens causing neonatal sepsis, associated mortality and recommended treatment options in low- and middle- income countries.

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Summary

Sepsis is a leading cause of neonatal mortality, particularly in low- and middle-income countries (LMICs). Current studies evidence alarming rates of antimicrobial resistance (AMR) in bacterial pathogens causing neonatal sepsis, however, these are often single site studies, limiting the extrapolation of data to inform policy change.

BARNARDS was undertaken in 12 sites across Africa and South Asia. Mothers attending clinical sites in labour or presenting neonates with suspected sepsis were enrolled and data regarding mother socio-demographics and birth of the neonate was collected. Associated antibiotic use was collated retrospectively, and neonates followed up for 60 days. Isolates grown from positive blood cultures were sent to the UK for further analyses, including minimum inhibition concentrations, whole genome sequencing, frequency of resistance and pathogenicity indexing.

Over 30 bacterial species were identified from blood cultures across BARNARDS sites with *Klebsiella* spp., *E. coli* and *Staphylococcus aureus* most identified discounting outbreaks, which were prominently caused by *Klebsiella* spp. and *Serratia marcescens*. High rates of AMR were seen against multiple antibiotics, with 71.5% of Gram-negative bacteria resistant to ampicillin and/or gentamicin, recommended first-line treatment. Furthermore, 83% resistance was found against recommended second-line treatments cefotaxime and 80% against ceftriaxone, slightly reduced for ceftazidime (60%). Sites often veered from ampicillin and gentamicin due to known resistance and varied antibiotics were prescribed by sites, including piperacillin-tazobactam and amikacin, amoxicillin-clavulanate and amikacin, ceftazidime and amikacin in addition to ampicillin and gentamicin. Amikacin and ceftazidime could act as potential empirical therapy alternatives for neonatal sepsis due to lower prevalence of resistance, reported survival, current usage, cost, and availability. However, antibiotic usage was uneven between countries, which could not be accounted for regarding

reported survival with usage of a specific antibiotic. Availability, price and whether costs were deferred to patients varied between sites and needs to be considered when deliberating alternative treatments. Empirical therapy for neonatal sepsis urgently needs to be re-evaluated in LMICs.

Contents

Table of Contents

List of abbreviations

AMR	Antimicrobial resistance
ARCCA	Advanced research computing at Cardiff University
ATCC	American Type Culture Collection
BARNARDS	Burden of antibiotic resistance in neonates from developing societies project
BC	Child Health Research Foundation, Chittagong, Bangladesh
BK	Kumudini Women's Medical College, Dhaka, Bangladesh
bla	Beta-lactamase gene
CDCP	Centres for disease control and prevention
CCS	Culture confirmed sepsis
CDS	Clinically diagnosed sepsis
CFU	Colony forming units
CI	Confidence interval
CoNS	Coagulase negative staphylococci
CU	Cardiff University
DeNIS	Investigators of the Delhi Neonatal Infection study
DNA	Deoxyribonucleic acid
EOS	Early-onset sepsis
ESBL	Extended spectrum Beta-lactamases
ESS	St Paul's Millenium Medical College in Addis Ababa, Ethiopia in affiliation with Children's Hospital Boston
EUCAST	European committee on antimicrobial susceptibility testing
FDA	Food and drug administration
FICI	Fractional inhibitory concentration index
g	Gram
GBS	Group-B streptococcus
GLASS	Global Antimicrobial Resistance and Use Surveillance Survey
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
HC	Healthcare
ID	Identification
IN	Sukhlal Karnani Memorial Hospital, in affiliation with the National Institute of Cholera and Enteric Diseases and the Institute of Postgraduate Medical Education and Research, Kolkata, India
IPC	Infection prevention and control
LMICs	Low- and middle- income countries
LOS	Late-onset sepsis
MALDI-ToF MS	Matrix Assisted Laser Desorption Ionization-Time of Flight Mass spectrometry
MBL	Metallo-beta-lactamase
mcr	mobile colistin resistance

MDR	Multi-drug resistant
mg/mL	Milligram per millilitre
MH	Mueller Hinton
MIC	Minimum inhibition concentration
MIC_{50}	Minimum Inhibitory Concentration for 50% of isolates tested
MIC_{90}	Minimum Inhibitory Concentration for 90% of isolates tested
MLST	Multi locus sequence type
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin susceptible *Staphylococcus aureus*
NDM	New-Delhi metallo-beta-lactamase
NK	Murtala Muhammad Specialist Hospital, Kano, Nigeria
NN	National Hospital Abuja, Abuja, Nigeria
NW	WUSE General Hospital, Abuja, Nigeria
OR	Odds ratio
PC	Healthcare centres in Malpur village and Maira Bhagwal, Islamabad, Pakistan
PCR	Polymerase chain reaction
PDR	Pan-drug resistant
PP	Pakistan Institute of Medical Sciences (PP) in affiliation with Quaid-i-Azam University, Islamabad, Pakistan
RK	Centre Hospitalier Universitaire de Kigali hospital, Kigali, Rwanda
RNA	Ribonucleic acid
RPM	Revolutions per minute
RU	Kabgayi hospital, Kigali, Rwanda
SCC	staphylococcal cassette chromosome
ST	Sequence type
UK	United Kingdom
UN	United Nations
UN IGME	United Nations Inter-agency Group for Child Mortality Estimation
UNICEF	United Nations Children's Fund
UTI	Urinary tract infection
VRE	Vancomycin resistant Enterococcus
WGS	Whole genome sequencing
WHO	World Health Organisation
XDR	Extensively drug resistant
ZAT	Tygerberg hospital, Cape Town, South Africa
β	Beta
β-lactam	Beta-lactam
µg/mL	Microgram per millilitre
°C	Degrees Celcius

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Publications

Publications arising from work carried out during this thesis include the following:

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  The work I contributed towards this paper is based on chapter 4.0
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  This work is composed of chapter 5

Currently under review at PLOS Medicine:

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  Proposed title: “Associations in admission cases of neonatal sepsis for outcome, culture confirmed sepsis, common pathogens and antibiotic resistance in low- and middle-income countries”.

  This work is the basis for chapter 6

1.0 Introduction

1.1 Antimicrobial Resistance

A pathogen is categorised as resistant to an antimicrobial when a treatment used against a certain organism is no longer effective. Antimicrobial resistance (AMR) refers to resistance in bacteria, fungi and viruses, with antibiotic resistance specifically referring to resistance in bacteria. The World Health Organisation (WHO) and the Centres for Disease Control and Prevention (CDCP) state that AMR is currently one of the leading threats to global human health, carrying the risk of returning to a pre-antibiotic era. The antibiotic resistance collaborators (Murray *et al*., 2022) estimated that 1.27 million deaths were caused by AMR in 2019. In addition to making infections harder to treat, routine operations and procedures will be accompanied with significantly higher risks as use of antibiotics as prophylactics will have diminished efficacy to prevent infections. Increased AMR will also be associated with extensive costs, with the World bank group (2017) estimating that AMR could cost more than $1 trillion annually worldwide by 2050.

1.1.1 Overuse and misuse as a driver for AMR

AMR is rising globally, accelerated by the overuse and misuse of antibiotics. Many patients seeking medical care for common colds, coughs and sore throats are prescribed antibiotics in the UK (Hawker *et al.*, 2014), with patterns of antibiotic prescriptions following that expected with cold and flu season in Europe and higher use of broad-spectrum antibiotics (Goossens *et al*., 2005). Goossens *et al.* (2005) also correlated countries with higher use of antibiotics with higher rates of resistance. Studies by Dempsey *et al.* (2014) and Fletcher-Lartey *et al.* (2016) found that GPs prescribed antibiotics for upper respiratory tract infections due to uncertainty of the cause, pressure from patients and a belief that their prescriptions will not make a difference to rates of antibiotic resistance. Alternatively, some clinicians are

incentivised, particularly in private healthcare, for use of antibiotics from pharmaceutical companies and receive monetary rewards for prescribing these (Tang *et al.*, 2016).

Antibiotic resistance is high in low- and middle- income countries (LMICs), partly due to increased overuse and misuse of antibiotics. Many antibiotics are available over the counter without a prescription with no limit on purchases, leading to self-medication, unnecessary use of antibiotics, or inadequate dosing of antibiotics (Kotwani *et al*., 2021; Morgan *et al*., 2011; Awad *et al*., 2005; Thamlikitkul, 1988). Furthermore, there is a higher prevalence of counterfeit drugs in LMICs, with evidence of lower quality products or dosage than stated on labels (Newton *et al*., 2006; Obodozie *et al.*, 2006). These factors contribute to the rise in antibiotic resistance, as patients may take antibiotics too regularly, or may be taken at sup-optimal doses. Prolonged application at sub-optimal doses below therapeutic quantities are key drivers for antimicrobial resistance in bacteria (Roberts *et al*., 2008). This leads to a survival advantage for resistant mutants, which will replicate and readily pass these genes onto the surviving susceptible bacteria, which have not been killed due to insufficient dosing of antibiotic, through multiplication and horizontal gene transfer creating a larger population of resistant bacteria (Viswanathan, 2014). Therapeutic doses will kill more of the target bacteria quickly, preventing spread of such genes and eliminating more bacteria.

1.1.2 Antibiotic use in agriculture as a driver for AMR

Issues from overuse causing AMR are exacerbated by the use of numerous medically important antibiotics in agriculture, with 80% of antibiotics sold in the United States for use in agriculture (FDA, 2016), 70% of which are medically important for human infections (Food and Drug Administration, 2014). These are mainly used as preventatives for infections and growth promoters, having been found to increase growth by 4-8% (Ewing and Cole, 1994). FDA data from 2015 estimated that 95% of antibiotics used in farms in America were administered in feed (74%) and drinking water (21%) (FDA, 2016), leading to the

assumption only 5% of antibiotics administered in farm animals were used to directly treat infections. Antibiotic use in agriculture is often unregulated, adding additional causes for rises in AMR, particularly in intensive farming practices. Bacteria can spread quickly in intensively farmed animals, due to close proximity, poor living conditions and poor nutrition received by the animals, which causes the farmers to apply prophylactic treatment through feed, along with uses for augmented growth (Oliver *et al*., 2011; Sibergeld *et al.,* 2008). Inadequate sanitation, poor infection control practices and improper food handling practices enhance the contamination of meat, leading to potential human colonisation.

1.1.3 One Health

The One Health approach considers the environment, animals, and human populations in terms of diseases, water contamination and AMR and how these may interact with one another and colonisation or infections in human populations. Through the one-health approach, antibiotic use in agriculture is a strong focus as a potential source of AMR genes due to the high associated antibiotic use, in addition to run off from farms into rivers.

There has been debate over the link of resistance between animals and humans. Mather *et al*. (2013) found that antimicrobial use in humans alone mainly dictates the AMR prevalence in infections. Cox and Ricci (2008) highlight that despite bans or restrictions placed on antibiotics for use as feed additives in animals, rises in resistance rates in human populations were not prevented.

On the other hand, some research has found a correlation between use of antibiotics in livestock and AMR in humans (O'Neill, 2015, Lazarus *et al*., 2015). Liu *et al.* (2016) found strong links between animals and humans for mobile colistin resistance (mcr) mechanism mcr-1. Hummel *et al*. (1986), found plasmid mediated resistance to streptothricin in farmed pigs, following its use as a growth promoter. They also found this plasmid mediated

resistance in the farm employees, their families and a man with no direct contact to the farms. Mather *et al.* (2013) found a multidrug resistant strain of *Salmonella typhimurium* (DT104) was found in both humans and animals, though concluded that this resistance was contained separately within humans and within animals, with a few transmissions across the groups. They also found that human isolates had greater diversity, suggesting that sources of resistance came from additional sources.

1.1.4 The spread of AMR bacteria

Horizontal gene transfer is a rapid means of spreading resistance genes, mainly achieved via conjugation, whereby one bacterium will provide a copy of a resistance gene on a plasmid or mobile element to another bacterium. Other means of horizontal spread include transformation, when a bacterium dies and any associated resistance genes are released into the environment ready to be picked up by other bacteria, and transduction via bacteriophages. Bacteriophages are bacterial viruses which enter a bacterium and use their resources to replicate. Following attachment to the bacterial cell, bacteriophages introduce the viral genome into the host cytoplasm before entering either a lytic or lysogenic replication strategy. In lytic replication cycles, the bacteriophage manufactures proteins via utilisation of the host ribosomes to create multiple copies of the original phage before lysing the bacterial cell, releasing the newly copied bacteriophages. During lysogenic replication cycles, the bacteriophage genome is integrated into the host cell chromosome, known as prophage DNA, which is replicated within the bacterial DNA and passed onto daughter bacterial cells. These are able to convert back to lytic replication cycles if required, such as when the bacterial cell undergoes stress, known as phage induction (Kasman and Porter, 2022; Ptashne, 2006). They may pick up segments of the host DNA during the replication process which they may pass onto other bacteria cells when entering a new host for replication (Hyder and Streitfield, 1978; Oliver *et al*., 2009). In addition to horizontal gene transfer, bacteria can develop

mutations in genes that will alter the efficacy of the antibiotic and can pass on chromosomally encoded resistance genes vertically. These mutations often carry a fitness cost and so will usually only have an advantage to the cell during selective pressure in the presence of antibiotic application (Munita and Arias, 2016).

1.1.5 Mechanisms of action common antibiotics

Antibiotics have varied mechanisms of action, all targeting elements of the bacterial cell which differ from mammalian cells, to prevent action against mammalian cells. This includes inhibition of: the cell wall, protein synthesis, nucleic acid synthesis, folate synthesis and cell membrane disruption (Table 1.1) (Kapoor *et al.*, 2017; Reygaert, 2018).

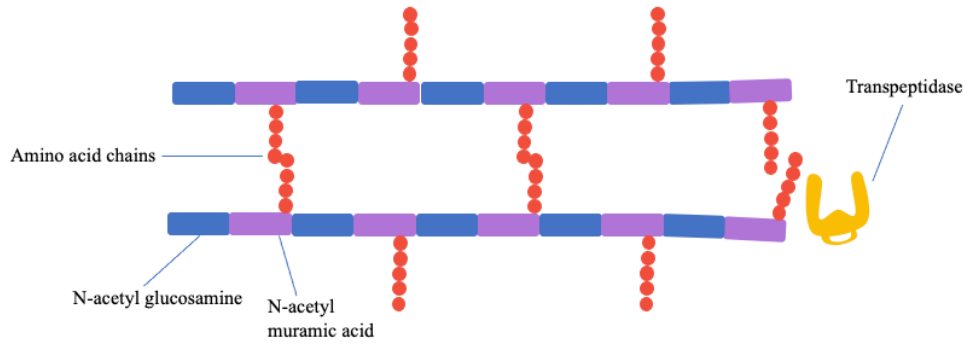
Table 1.1 Main antibiotic mechanisms of action and associated antibiotic classes that utilise each mechanism. Adapted from Reygaert, 2018.

Mechanism of action		Antibiotics
Inhibit cell wall synthesis		ß-lactams (carbapenems, cephalosporins, monobactams. Penicillins) Glycopeptides
Depolarize cell membrane		Lipopeptides Polymyxins
Inhibit protein synthesis	Bind to 30S ribosomal subunit	Aminoglycosides Tetracyclines
	Bind to 50S ribosomal subunit	Chloramphenicol Lincosamides Macrolides Oxazolidinones Streptogramins
Inhibit nucleic acid synthesis		Quinolones (Fluoroquinolones)
Inhibit metabolic pathways (folate synthesis)		Sulfonamides Trimethoprim

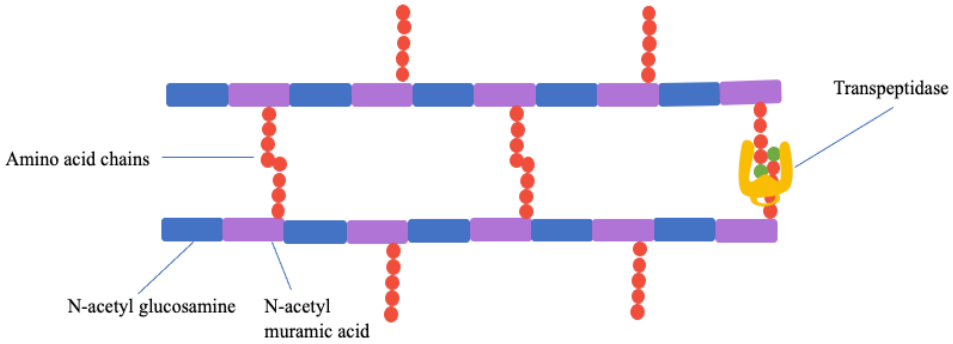
1.1.5.1 Beta-lactam mechanism of action

β-lactams are a large class of antibiotics containing a β-lactam ring and cover important medical applications that account for 65% of all injectable prescriptions in the United States (Bush and Bradford, 2016) and make up a wide range of important antibiotics including penicillins, cephalosporins, monobactams and carbapenems.

β-lactams antibiotics work by binding to transpeptidase which is responsible for cross linking of amino acids in the peptidoglycan layer of the cell wall (Figure 1.1). This prevents cell wall building affecting the bacterial structure and the high internal osmotic pressure causes to the cell to lyse. β-lactamases are enzymes that interact with the β-lactam antibiotics that hydrolyse the β-lactam ring, making it incapable of binding to transpeptidase to disrupt cell structure. This can occur in both Gram-positive and Gram-negative bacteria, however Gram-positive bacteria intrinsically release β-lactamases out of the cell, whereas these are trapped in the peptidoglycan layer in Gram-negative cells, therefore they are more effective against β-lactam antibiotics in the cell (Toth *et al*., 2015).



A



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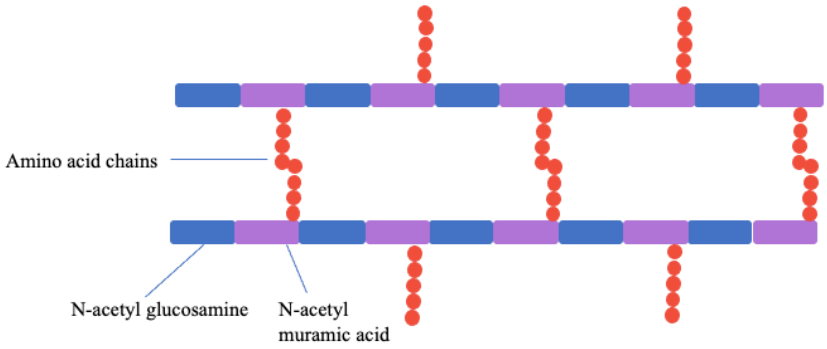

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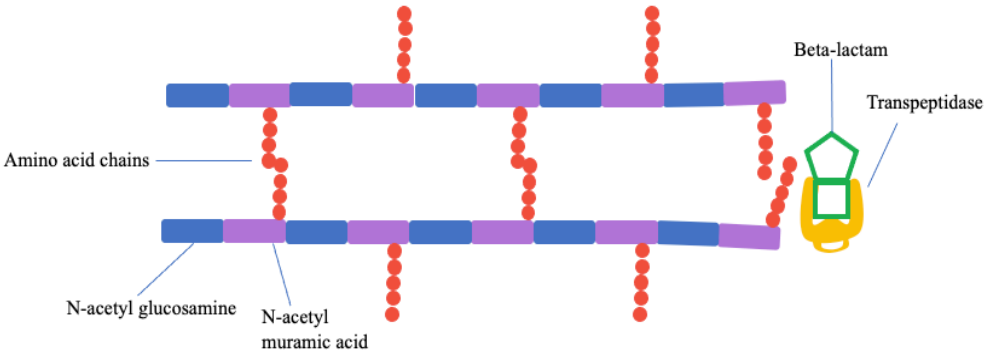


D

Figure 1.1. ***A.*** *Peptidoglycan later of a bacterial cell wall made up of N-acetyl glucosamine and N-acetyl muramic acid. These are joined together by chains of amino acids, which are cross-linked together via transpeptidase.* ***B.*** *Transpeptidase carries out cross-linking in addition to alanyl-carboxy-transpeptidase, also known as the penicillin binding proteins.* ***C.*** *Stable peptidoglycan section with all amino acids joined.* ***D.*** *Beta-lactam antibiotic bind to the transpeptidase (penicillin binding protein) to prevent them from carrying out cross-linking of the cell wall. Image modified from video 'β-lactamases: Mechanisms of action and resistance'. https://www.youtube.com/watch?v=qBdYnRhdWcQ&t=245s. Last accessed on 24.09.2022.*

1.1.6 Mechanisms of resistance

Bacteria have developed various mechanisms to overcome the action of antibiotics, encoded for by diverse resistance genes. Efflux pumps are a common mechanism of resistance, particularly in Gram-negative bacteria, whereby antibiotics are pumped out of the cell, preventing toxic concentrations of antibiotic within the cell (Pearson *et al*., 1999), as seen in many Gram-negative species, such as *P. aeruginosa* for varied antibiotics (Aeschlimann, 2003). Alternatively, some bacteria are able to reduce permeability, preventing the antibiotic from entering the cell (CDC, 2018), as seen in *E. coli* with

norfloxacin (Cohen *et al*., 1989). Others achieve resistance by alteration of the antibiotic binding site, preventing the antibiotic from taking effect, such as with Tet(M) in Streptococcus spp. (Connell *et al.*, 2003). However, unless the modification is small these can accompanied with a high fitness cost, although this has been shown to vary on the mutation and the species in addition to the cost-benefit of the presence of antibiotics (Melnyk *et al*., 2015).

Another technique developed by both Gram-positive and Gram-negative bacteria is the use of enzymes to change the structure of the antibiotic, via acetylation, phosphorylation or adenylation, resulting in added bulk of the antibiotic, steric hindrance, and reduced affinity of the antibiotic to the target (Munita and Arias, 2016). Alternatively, there are bacterial enzymes that actively break down the antibiotic, as seen with Beta-lactamases, which hydrolyse the amide bond of the Beta-lactam ring for certain Beta-lactams (penicillins and narrow-spectrum cephalosporins) rendering them ineffective (Bush, 2018).

1.1.6.1 Mechanism of action Beta-lactamases

There are four main classes of β-lactamases under the Ambler classification system (Ambler, 1980), termed A, B, C and D based on similarity of their sequence motifs. Classes A, C and D use serine for enzyme activity, while class B consists of metallo-β-lactamases (MBLs) which use zinc for this enzymatic reaction. The increasing global prevalence of MBLs is concerning, as these enzymes have the ability to hydrolyse all β-lactams, including carbapenems, aside from monobactams and emerged on mobile genetic elements in pathogenic bacteria in the late 1990s (Laraki *et al*., 1999), which has allowed them to rapidly disseminate globally. β-lactamases can be inhibited by β-lactamase inhibitors, such as tazobactam and sulbactam (Drawz and Bonomo, 2010), which can be administered alongside the by the β-lactam antibiotic. These β-lactamase inhibitors bind to the β-lactamases, with high affinity, either permanently, as seen in clavulanic acid, or

temporarily, as demonstrated by avibactam (Drawz and Bonobo, 2010) However, these are only effective against class A β-lactamases and not effective against the other classes (Steward *et al*., 2001).

Different β-lactamases are effective against varied classes of β-lactams (Table 1.2). Within each class of β-lactamases, there are multiple enzyme families and variations within each family, which may have diverse activity. These are frequently located on plasmids with the ability to transfer horizontally and it is possible that some bacteria will have more than one β-lactamase (Tenover *et al*., 2020). All four classes contain carbapenemases, with the ability to break down carbapenems, the last resort group of antibiotics. The most clinically significant carbapenemases include *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$ and *bla*$_{\text{NDM-1}}$ (Kumarasamy *et al*., 2010). The gene *bla*$_{\text{CTX-M-15}}$ is a dominant extended spectrum Beta-lactamase (ESBL) with widespread global prevalence (Canton and Coque, 2006).

Table 1.2. Key enzyme families and main associated enzyme families per class of Beta-lactamase.ESBL=Extended spectrum Beta-lactamase. Adapted from Nagshetty et al., 2021)

Class of Beta-lactamase	Key enzyme families	Key resistance
A	TEM	Penicillins, cephalosporins (some ESBLs)
	SHV	Penicillins, cephalosporins
	CTX-M	ESBLs
	KPC	Carbapenems
B	NDM	Carbapenems
	VIM	Carbapenems
C	CMY	Carbapenems
	AMPC	Penicillins and cephalosporins
D	OXA	ESBLs and carbapenems

There is abundant variation in β-lactamase enzymes, and there are currently 7,166 enzymes in the β-lactamase database (www.bldb.eu; Naas *et al.*, 2017). This has risen from

4,300 in 2019 (Tooke *et al.*, 2019), conveying how rapidly they could be changing, or more likely an increased WGS capacity and knowledge to identify this variety. β-lactamase enzymes are currently the most common and worrying form of resistance as provide vast resistance to range of antibiotics, including ESBLS and last resort treatments (Livermore, 2012; Kumarasamy *et al.*, 2010). Furthermore, they are usually found on plasmids and therefore have a high potential to spread rapidly amongst bacterial populations and have spread globally (Nordmann *et al.*, 2011).

1.1.7 Multidrug resistance

Antibiotic resistance has been worsened by the increase in Multidrug resistant (MDR) bacteria, that demonstrate non-susceptibility to at least one treatment in three or more classes of antibiotics (Magiorakos *et al*., 2012). An infection with a MDR bacteria can increase mortality rates, due to reduced treatment options. Furthermore, development of extensively drug resistant (XDR) and pandrug-resistant (PDR) bacteria, that are defined by Magiorakos *et al*. (2012) as organisms resistant to at least one agent in all but two or fewer antibiotic classes and those resistant to all available antibiotic classes, respectively have made some infections nearly impossible to treat. Pan-drug resistance has been found in *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Zhi-Wen *et al.*, 2015), easily adopting varied resistance mechanisms, including reduced permeability, mutations to antibiotic targets, efflux pumps and B-lactamase production (Karageorgopoulos and Falagas, 2008). Betrosian *et al*. (2008) have found some success treating MDR *Acinetobacter baumannii* with colistin monotherapy achieving resolution of symptoms in 60% (9/15) of patients with standard dosing and 61.5% (9/13) patients when treated with a high dose (9g every 8hours) of ampicillin/ sulbactam. Similar results (60.3% resolution) were found by Ye *et al.* (2011) using tigecycline for treatment of MDR *A. baumannii.*

A group of organisms that have rapidly growing MDR and cause a great deal of nosocomial infections are known as the ESKAPE pathogens. This group of pathogens is a major health threat and composes of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (Rice, 2008). These pathogens have a wide range of AMR mechanisms, that are frequently plasmid mediated, able to transfer across species. Further to this, these species are able to form biofilms, aiding their potential to cause nosocomial infections as well as providing further protection from antibiotics and host immune responses (Bales *et al.*, 2013).

1.1.8 WHO priority list pathogens

In 2017, the WHO published a list of priority pathogens for research and development of new antibiotics (World Health Organisation, 2017), listed as critical, high, or medium priority (detailed in Table 1.3). This was based on collated data from multiple countries, although there were no pre-defined criteria for prioritising infectious diseases and varied per country (Krause, 2008). Multi-criteria decision analysis best practices were utilised to overcome this, and prioritisation of pathogens were based on the impact of infections: associated mortality rates, need of hospitalisation, length of hospital stay following infection, incidence in community settings, transmissibility. Additionally, taken into consideration was the frequency of AMR, potential future AMR, ease of prevention through hygiene and vaccinations, remaining effective treatment options and whether new antibiotics are currently being developed. Within this report, MDR *Mycobacterium tuberculosis* is listed as a priority pathogen which will not be detailed further, as is outside the remit of this thesis, followed by the second section with an overview of other priority AMR bacteria

Table 1.3. Bacteria with associated AMR listed in the WHO priority pathogen lists published in 2017 (WHO, 2017).

Priority level	**Species**	**Antibiotic resistance**
Critical	*Acinetobacter baumannii*	Carbapenem resistant
	Pseudomonas aeruginosa	Carbapenem resistant
	Enterobacteriaceae	Carbapenem resistant Third generation cephalosporin resistant
High	*Enterococcus faecium*	Vancomycin resistant
	Helicobacter pylori	Clarithromycin resistant
	Salmonella spp.	Fluoroquinolone resistant
	Staphylococcus aureus	Vancomycin resistant Methicillin resistant
	Campylobacter spp.	Fluoroquinolone resistant
	Neisseria gonorrhoeae	Third generation cephalosporin resistant Fluoroquinolone resistant
Medium	*Streptococcus pneumoniae*	Penicillin non-susceptible
	Haemophilus influenzae	Ampicillin resistant
	Shigella spp.	Fluoroquinolone resistant

All the pathogens of critical importance according to the WHO (World Health organisation, 2017) are Gram-negative. However, Gram-positive pathogens are included in the high priority, which included vancomycin resistant *Enterococcus faecium* (VRE), vancomycin resistant *Staphylococcus aureus* and methicillin resistant *S. aureus* (MRSA) and in the medium priority (penicillin non-susceptible *Streptococcus pneumoniae*) (Table 1.2).

1.1.9 Nosocomial infections

High proportions of resistance for preferable treatments are reported from various regions, particularly for hospital acquired infections (WHO, 2014) and nosocomial infections are associated with increased mortality and morbidity in addition to prolonged hospital stays. The WHO collated data from various surveillance systems and studies from around the world reporting rates of nosocomial infection rates. From the data the WHO approximated that of

between 5.7-19.1% of hospitalised patients acquire a nosocomial infection in LMICs and a rate of 3.5-12% HICs (WHO, 2011), although Suetens *et al*. (2018) report rates of up to 19.2% in intensive care units across Europe. However, the true global rate of nosocomial infections remains unknown due to lack of reliable surveillance systems. Hospital acquired infections can occur through contact with healthcare workers, contamination of nearby surfaces or medical equipment. Common nosocomial infections include central venous line blood stream infections, catheter associated UTI, post-surgery infection of skin or soft tissue, pneumonia following use of a ventilator (Sikora and Zahra, 2022). As previously mentioned, ESKAPE pathogens commonly cause nosocomial infections, a great concern due to their ability to remain in the environment due to the formation of biofilms and associated MDR.

1.1.10 Burden of AMR

A study was carried out by the antimicrobial resistance collaborators (2022) to investigate the impact of AMR. From available data, they estimated that globally 1.27 million deaths were attributable to AMR in 2019. Wide variation in estimates were seen in different countries. Europe and central Asia had the lowest estimated rate of deaths associated with AMR (283,000) and highest numbers in South Asia (1.39 million). Rates per 100,000 people were highest in Sub-Saharan Africa (98.9 per 100,000) followed by South Asia (76.8 per 100,000). The Review on Antimicrobial Resistance (2014) estimated mortality due to AMR bacteria to rise to 10 million by 2050, with 4.73 million deaths in Asia and 4.15 million in Africa, if actions are not taken to diminish this (Laxminarayan *et al.,* 2013).

1.1.10.1. Burden of AMR in HICs vs LMICs

The CDC (2019) estimates that in the USA over 2.8 million antibiotic resistant infections occur per annum, responsible for approximately 35,900 deaths, updated from an estimation of 23,000 deaths in 2013 (CDC, 2013). Thorpe *et al.* (2018) estimated that AMR

added $1,383 to treatment of a patient with a bacterial infection, a 92% increase to non-AMR infections, increasing healthcare cost in the US by $2.2 billion annually. Within the EU, AMR is estimated to be responsible for 33,000 deaths, costing 1.5 billion euros annually (European commission, 2022).

The first case of carbapenem resistant Enterobacteriaceae isolated from a clinical specimen arose in 2001 in North Carolina, United Stated (Yigit *et al*., 2001). Throughout 2006-2015, rates of carbapenem resistant *K. pneumoniae* and *Enterobacter cloacae* spread from Northeast states across to most of the US (Wilson *et al*., 2017). A review carried out by Annavajhala *et al*. (2019) demonstrated that *bla*-$_{\text{KPC}}$ was the most common carbapenemase allele found in *Enterobacter cloacae* in the US and Canada and were commonly found in Europe, in addition to VIM variants. On the other hand, *bla*-$_{\text{KPC}}$ and *bla*-$_{\text{VIM}}$ were not found in Africa or Southeast Asia (Annavajhala *et al*., 2019; Carvalho *et al*., 2022), but *bla*-$_{\text{NDM}}$ was more commonly found in in Asia and Africa and throughout Eastern China (Jin *et al*., 2018), in addition to IMP-encoding genes. *Bla*-$_{\text{OXA-48}}$ had as spread around Europe, Africa and the Middle East and Southeast Asia (Annavajhala *et al*., 2019; Carvalho *et al.,* 2022).

LMICs have additional risk factors, including limited access to flushing toilets, clean water, or soap, increasing risk of infections. Floods disproportionately affect LMICs, which can bring communities into contact with contaminated water sources. Rivers are often contaminated with faecal matter, and pit latrines are common in many LMIC regions, which can be flooded, exposing communities to contaminated flood water and increased amounts of bacteria, particularly Enterobacteriaceae and their associated AMR genes.

1.1.11 Current AMR surveillance

Data availability varies widely between continents, with well-structured AMR surveillance systems in place through most of Europe and good data availability through the Americas. However, there is insufficient data available within Africa, with only a few

countries surveying AMR, with no formal framework for collaboration for the countries that do perform AMR surveillance (WHO, 2017). The Eastern Mediterranean regions also have limited AMR surveillance data and AMR governance is lacking in addition to overburdened healthcare centre and limited technology for reporting (Talaat *et al.*, 2022). Southeast Asia had few national surveillance systems in place but has recently embraced the need for structured surveillance with regional database, although still has high variation per country (Zellweger *et al*., 2017). The Western Pacific region has a varied amount of data available depending on the area, with established systems in the higher-income countries. Good collaborative links were set up in the 1980s to share AMR data, although this crumbled after 20 years but there are still national surveillance schemes (WHO, 2014).

Global surveillance of AMR in is currently improving, with initiatives such as Neonatal AMR research network established in 2017 across 12 countries (Li, 2020). The burden of antibiotic resistance in neonates from developing societies (BARNARDS) project was set up in 2015 to monitor AMR prevalence in neonatal sepsis in 12 sites across seven LMIC countries. In 2015, the WHO set up the Global Antimicrobial Resistance and Use Surveillance Survey (GLASS), aiming to standardise AMR surveillance globally, collaborating with AMR regional networks to monitor AMR, antibiotic consumption data as well as additional focused surveys including burden, one-health (WHO, accessed 2022). Furthermore, in in 2016, a £265 million UK aid program, The Fleming Fund, was set up to support AMR surveillance across 24 LMICs that importantly help some countries to establish national surveillance systems and aid in refurbishing laboratories (The Fleming Fund, accessed 2022).

However, data from developing countries is still sparse. Some hospitals do not have access to microbiology or blood culture facilities and so cannot confirm biological sepsis and will not be able to gather information on pathogens commonly causing infections, or

resistance profiles (Jacobs *et al.*, 2019). This shortage of facilities will increase propensity to prescribe broad spectrum antibiotics, as clinicians will not have any information on the type of pathogen they are treating, which will further drive AMR in commonly used broad-spectrum antibiotics. Other hospitals in LMICs have automated blood cultures but lack the ability to accurately identify the organism or, importantly sufficient facilities to obtain reliable information on the bacterial antibiotic susceptibility profile. Furthermore, hospitals with these facilities in LMICs often face issues of inadequate training or depletion of stocks of consumables, or lengthy shipping or customs delays (Mukadi *et al*., 2015; Fitzgibbon and Wallis, 2014). Many hospitals in LMICs do not have access to information technology or linked electronic health records, a valuable tool for surveillance (Birkhead *et al*., 2015). Additionally, hospitals often undertake manual data entry and information is input in a way that does not allow timely analyses. Healthcare systems in LMICs are overburdened and nurses often do not have the time to complete additional forms for surveillance. Furthermore, there is often inadequate training to complete forms correctly (Mahomed *et al*., 2017).

1.1.12 Lack of new antibiotics

Throughout the history of antibiotics, AMR has developed within a few years following the release of a new antibiotic (Figure 1.2), and this situation has occurred for nearly all antibiotics. Therefore, AMR has always posed an issue to modern medicine, with rises of AMR exacerbated in recent years, presumably due to the extensive overuse of antibiotics. Development of new antibiotics has also slowed in the past two decades (Boucher *et al.*, 2013), with 15 of 18 major pharmaceutical companies pulling out of antibiotic development completely, mainly due to lack economic appeal as antibiotic courses are short and relatively low priced compared to treatments for cancer or chronic illnesses (Bartlett *et al*., 2013). Gould and Bal (2013) suggest that pharmaceutical companies may be dubious about investing in production of new antimicrobials, as antimicrobial stewardship programs

are advising restraint against use of antimicrobials and that new antibiotics may be reserved for last line treatment in cases of MDR organisms. Development of AMR may also cause their investment to have an unexpectedly short shelf life, a problem inflated for treatment of Gram-negative organisms, which are more likely to rapidly acquire resistance.

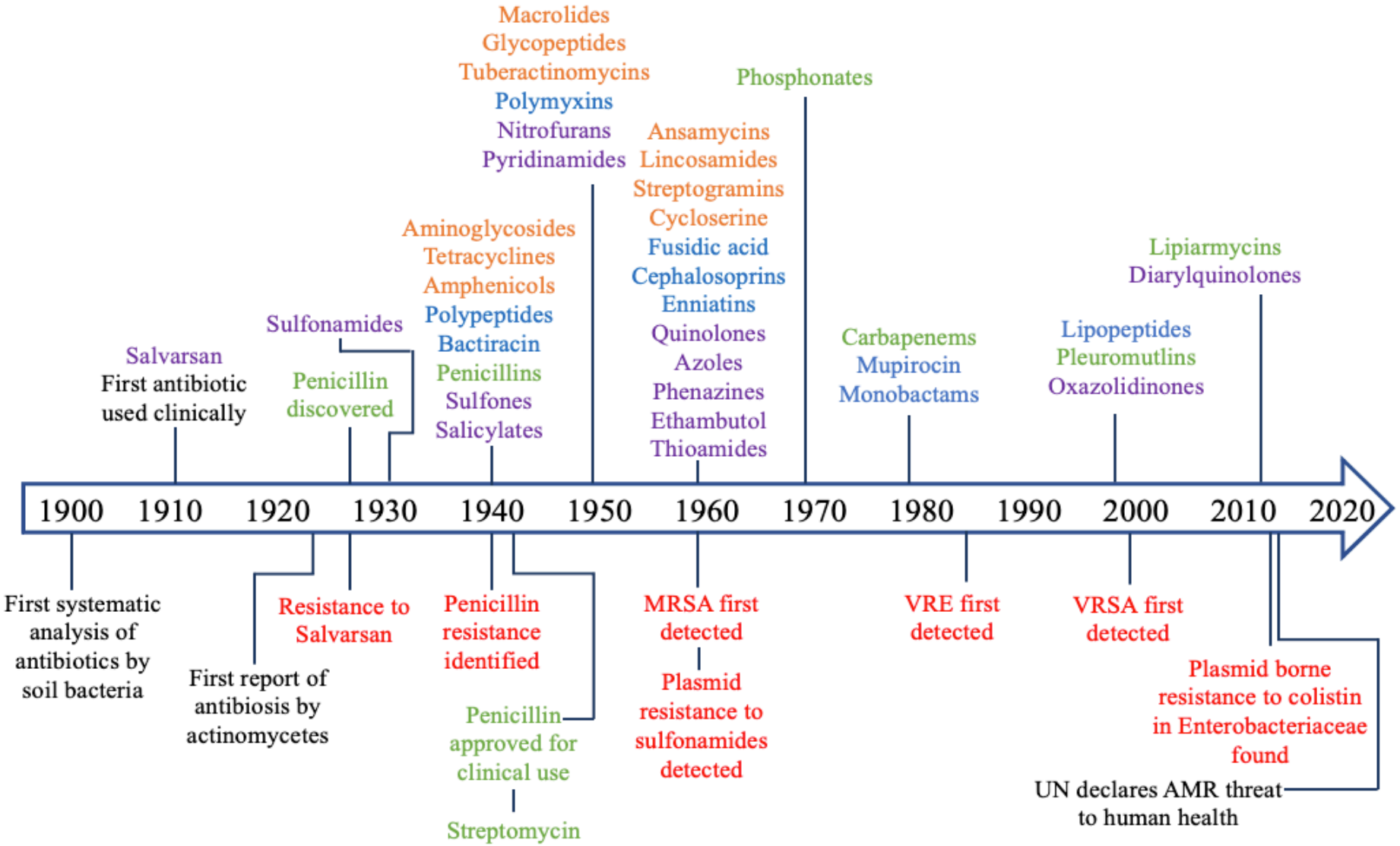


Figure 1.2. Timeline of significant discovery of new antibiotic classes over the past 120 years. The top shows discovery of new clinically used. Orange text displays antibiotics that are natural products produced from actinomycete; Blue text display antibiotics that are produced naturally from other bacteria; Green text show antibiotics that originate from fungal natural products; and purple text shows synthetic antibiotics. Other significant dates and detection of key resistance are noted underneath the timeline. Adapted from Hutchings et al., 2019.

1.2 Child and neonatal mortality

An estimated five million children under the age of five years died in 2020 (UNICEF, 2021). According to the WHO (2018), deaths in children under five years of age were most often caused by pneumonia, diarrhoea, injury, malaria, or congenital causes. Within the neonatal period, defined as the first 28 days of life, pre-term birth, intrapartum-related complications (causing birth asphyxia) and infections are reported as the leading causes for death (Figure 1.3) (WHO, 2022).

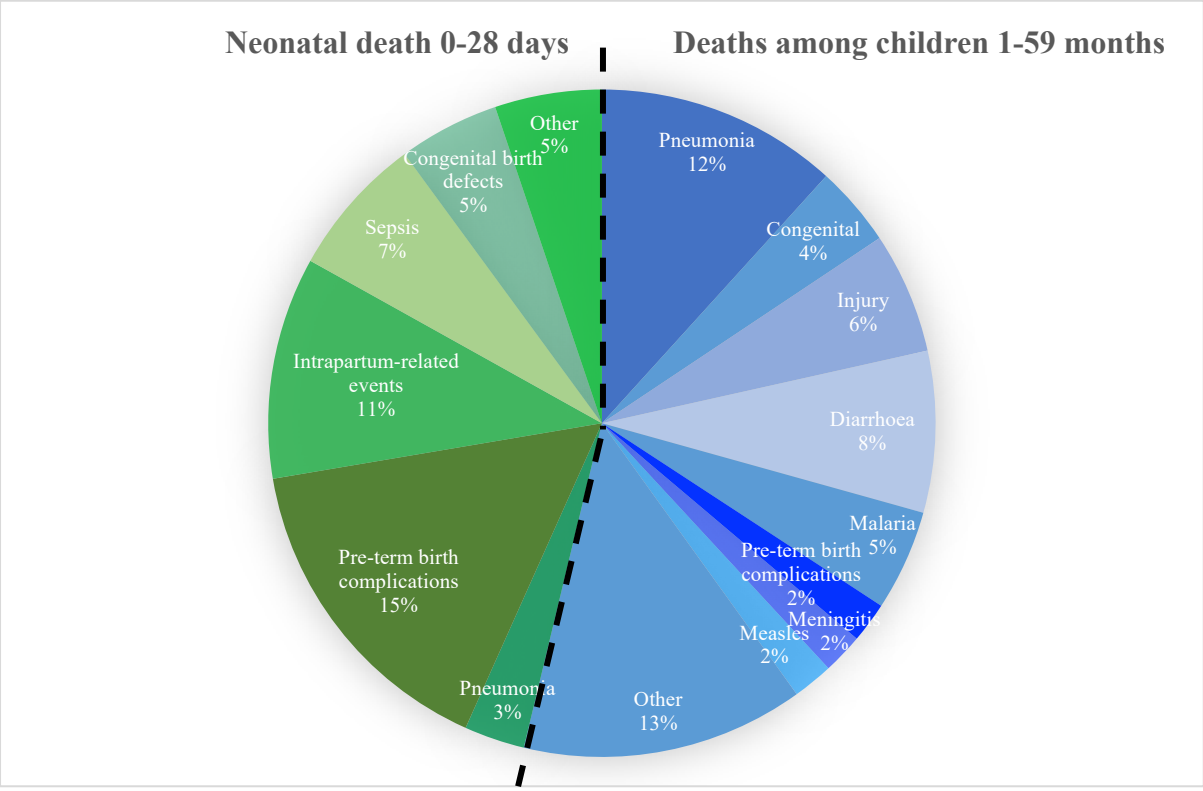


Figure 1.3. Common causes of death for children under five and for neonates, globally in 2018. Adapted from Source: WHO and maternal and child epidemiology estimation group interim estimates produced in September 2019.

Mortality in children under five has reduced by 59.6% since 1990, from 93.23 per 1,000 live births in 1990 to 37.7 per 1,000 live births in 2019 globally (WHO, 2022). Reductions in neonatal mortality has decreased in recent years, recorded as 17.4 per 1,000 live births in 2019, reduced by 52.4% since 1990 (36.7 per 1,000 live births) (WHO, 2022).

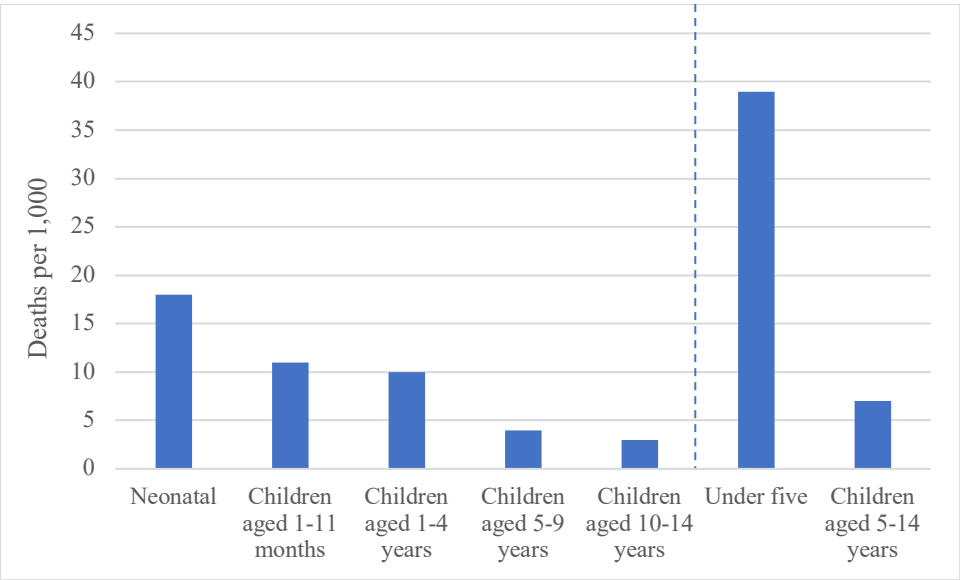


Figure 1.4. Global mortality rates and number of reported deaths by age, 2018. NB. All figures were based on unrounded numbers, Chart adapted from UNICEF, 2019.

Neonatal mortality is an imperative health concern, with 47% of all child deaths under five years of age occurring within the neonatal period in (UNICEF, 2018) (Figure 1.4). Globally in 2018, 2.5 million children died within the first 28 days of life, and it has been reported that 75% of neonatal deaths occur within the first week of life, with a high proportion dying within the first 24 hours (UNICEF, 2019). Developing countries bear the burden of 98% of neonatal mortality worldwide (Bryce *et al.*, 2005), with the highest rates occurring in Sub-Saharan Africa (43% of global deaths, average of 28 deaths per 1,000 live births) followed by Central and Southern Asia (36% of global deaths, average of 25 deaths per 1,000 live births) (WHO, 2019). These rates are ten times higher than for high-income countries (WHO, 2019) and as Figure 1.5 displays, these rates reach over 40 deaths per 1,000 live births in certain parts of Africa and Asia (You *et al.*, 2015).

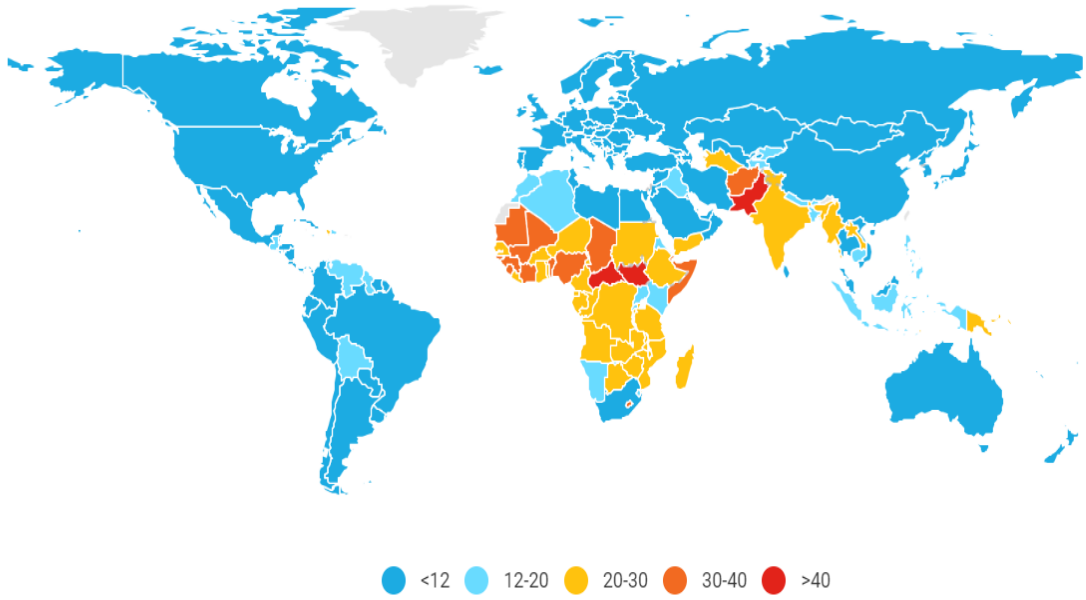


Figure 1.5. Neonatal mortality rate (deaths per 1,000 live births) in 2018 by country. Source: United Nations Inter-agency Group for Child Mortality Estimation (UN IGME) 2019. You et al, 2015.

Furthermore, a lack of reliable data primarily in LMICs is likely contributing to an underestimation of neonatal mortality, so rates are likely higher in these settings (Fleischmann-Stuzek *et al*., 2018; Antimicrobial resistance collaborators, 2022; Fleischmann *et al*., 2021). Similarly, the causes of death within LMICs may not be accurately accounted for and poor data exists for many LMICs, with less than 10% of complete data from countries in Africa, or those with data lacking in specificity (Mathers *et al*., 2005). This is due to similar issues in AMR surveillance, including a lack of infrastructure in many LMIC settings, insufficient funding, overburdened healthcare systems with many staff working for both the public and private sector due to inadequate wages in the public sector, lacking regulations and reporting processes and an absence of electronic record keeping or laboratory information management systems (Naz *et al*., 2012; Rattanaumpawan *et al*., 2018; Turner *et al*., 2021). Cause of death may be unknown in many cases, as hospitals in these regions also often face inadequate microbiological support with insufficient diagnostic facilities (Yadav *et al*. 2021). Additionally, many births in LMICs do not take place in hospital settings and

capturing inclusive data on this cohort born outside of tertiary hospitals is challenging (Montagu *et al*., 2011). Therefore, mortality is often underestimated, and causes of death may be inaccurate, with an underrepresentation of some common causes, including sepsis which necessitates a positive blood culture, with a bacterial pathogen found in the blood for confirmation.

1.3 Neonatal sepsis

Sepsis is defined as an immunological reaction of the body to a bloodstream infection that can be life threatening and requires treatment. Upon recognition of a bloodstream infection by innate immune cells the pathogen-associated molecular patterns on the invading pathogen activate the immune cell pattern recognition receptors. Cascades of pro-inflammatory molecules (e.g. leukotrienes, prostaglandins) and anti-inflammatory cytokines, trigger the dilation of blood vessels reducing vascular resistance leading to a systemic inflammatory response. The release of these chemicals activate the complement system, and stimulate permeability of endothelial cells, increasing vascular permeability, allowing fluid to leak into surrounding tissues and also stimulate disseminated intravascular coagulation. White blood cells are also signalled which release tumour necrosis factor-alpha, interleukins interferon-gamma, further stimulating sustained macrophage T cell activation sustaining the inflammatory response (Greer, 2015; Rittirsch *et al*., 2008). 'Severe sepsis' includes cellular and organ dysfunction due to the decreased blood flow and hypercoagulation causing perturbations in microcirculation (Abraham, 2000), which is then followed by septic shock, accompanied by extreme hypotension due to the decreased systemic vascular resistance and can lead to death (Delano and Ward, 2016; Lever and Mackenzie, 2007). Neonatal sepsis refers to sepsis in neonates (new-borns up to 28 days of age). The immune response to a bloodstream infection in neonates will vary from that in adults due to the immature immune system (Basha *et al*., 2015). The adaptive immune system is limited in neonates, with

immature T cells and only previous exposure to maternal alloantigens. Neonates rely mainly upon the innate immune system, which is muted in newborns, particularly premature newborns, thought to enable tolerance to maternal antibodies (Simon *et al*., 2015). As with adult sepsis, immune cell pattern recognition receptors (toll-like receptors) are activated upon recognition of pathogen associated molecular patterns on pathogens, although this stimulates reduced pro-inflammatory cytokines compared to adults (Levy *et al*., 2004). Neonates also undergo decreased leukocyte recruitment, further reduced in pre-term neonates, potentially contributing to the risk of infection, with reduced phagocyte activity in the first three days of life (Fillias *et al*., 2011; Nussbaum *et al*., 2013). Neonates have lower activation of the complement system, which is again lower in pre-term neonates (Wynn and Levy, 2011).

Within neonates, sepsis is defined as early onset sepsis (EOS) or late onset sepsis (LOS). There is divergence within the literature regarding the terminology of EOS, either as sepsis developing within the first 72 hours of life, or the first seven days of life (Fleischman *et al*., 2021), with most studies using the first 72 hours to define EOS. Some literature states that EOS refers to sepsis occurring within 72 hours for preterm infants and seven days in full-term infants (Mukhopadhyay and Puopolo, 2012). EOS is thought to be due to vertical transmission from the mother during the intrapartum period or during birth. LOS refers to neonatal sepsis developed after the first 72 hours (or seven days) of life and is thought to be from infections acquired from the surrounding environment, often associated with nosocomial or community acquired infections (Hornik *et al.*, 2012).

Symptoms for neonatal sepsis include but are not limited to lethargy, irritability, high or low temperature, poor feeding, jaundice, bradycardia, tachycardia, respiratory distress (Gerdes, 1991; European Medicines Agency, 2010). These symptoms are vague, overlap with multiple other diseases and the definition of symptoms vary in different healthcare centres. Therefore, sepsis can be difficult to diagnose and may be mistaken for other illnesses and

vice versa. To confirm infection in the blood following clinical diagnosis, patients need to have a blood sample taken and a positive blood culture, where a pathogen is cultured from the blood, confirms sepsis, known as culture confirmed or biologically confirmed sepsis. There is discrepancy between studies reporting rates of neonatal sepsis, with some reporting rates as those clinically diagnosed, whereas others only report numbers of culturally confirmed sepsis. It is possible a patient may still have biological sepsis following a negative blood culture. Extracting sufficient volumes of blood in neonates, particularly those of low birthweight is challenging, and one of the reasons why rates of neonatal sepsis and associated deaths may be underestimated.

1.3.1 Burden of neonatal sepsis globally

The Global Burden of Disease Study (James *et al*., 2018) estimated an incidence of just over 1.3 million (95% confidence interval [CI] 792,700 to 2,284,900) cases of neonatal sepsis per annum worldwide, estimated to cause approximately 203,000 (95% CI 178,700 to 267,100) deaths per annum (Roth *et al.*, 2018). However, Ranjeva *et al*. (2017) reported higher rates, estimating neonatal sepsis to cause 177,500 to 302,870 deaths annually in sub-Saharan Africa alone. Data collated by the WHO and the maternal and child epidemiology estimation group model projected a higher number, with 375,000 neonatal deaths caused by sepsis globally in 2018 (UN IGME, 2019) and higher estimations again have been reported by Liu *et al*. (2015) claiming 421,000 (95% CI 269,000 to 688,000) neonatal sepsis related deaths per annum in 2013 globally. Fleischman *et al*. (2018) combined data from 26 studies, including four from high-income countries, 20 from middle-income studies and only two from low-income studies, mostly based on single sites. They found an overall rate of 17.6% mortality in neonates with sepsis, with some studies reporting clinically diagnosed sepsis, others culture confirmed sepsis and a high heterogeneity between studies.

The variation in data is due to a lack of sufficient surveillance alongside a lack of standardised diagnosis and reporting methods between hospitals/ studies in addition to variation between hospital sites regarding population, level of care and available facilities. Rates of neonatal sepsis and associated mortality in LMICs is often based on small studies at limited or single healthcare facilities (Fleischmann *et al*., 2021). Due to a lack of standardised methodologies between clinical sites, vague symptoms of neonatal sepsis, among many other factors rates of reported sepsis can vary greatly between sites and so it is difficult to generalise these findings across continents. Furthermore, confirmation of neonatal sepsis is challenging in LMIC environments. Many healthcare facilities do not have automated blood culture facilities, and some have no microbiology facilities and cannot confirm cases of sepsis. In addition to an overall deficiency of data, there are very few community-based studies, which Fleischmann-Struzek *et al*. (2018) found to be associated with higher incidence rates 8,549/100,000) compared to hospital-based studies (1,986/100,000). The sparsity of data, particularly in LMICs leads to a likely underestimation of the true burden of neonatal sepsis.

1.3.1.1 LMICs

A study by Bangi & Devi (2014) found an incidence of 6% sepsis in paediatric wards from a tertiary care hospital in India, with an high mortality rate of 46.7% in neonates that were clinically diagnosed with sepsis in the years 2013-2014, similar to 2003-2004 figures of 48.0%. The DeNIS study (2016) reported cases of diagnosed sepsis associated with 26% mortality, which rose to 48% mortality for those with culture confirmed sepsis. Similarly high rates of mortality were reported in a study in a neonatal intensive care unit (NICU) in Egypt, reporting an overall mortality rate of 45.7% (51% in EOS and 42.9% in LOS) (Shehab El-Din *et al.*). Comparable mortality rates were found in a study based in Iraq, which reported 44.2% mortality in neonates with sepsis (Jumah and Hassan, 2007). Lower rates were found

in a study in Iran, a middle-income country, with an overall mortality rate of 19.8%, although this rate included coagulase negative Staphylococci (CoNS), a potential contaminant, which was associated no mortality (Movahedian *et al*., 2006). Further detail on the burden of neonatal sepsis is provided in chapter 3.

1.3.1.2 HICs

The burden of neonatal sepsis is much lower in HICs, and the associated mortality rate is also much lower. Shin *et al.* (2009) reported clinical neonatal sepsis incidence in South Korea as 30.5 per 1,000 live births with 4.7% mortality rate and confirmed sepsis as 6.1 per 1,000 live births with a lower mortality rate of 2.2%.

Gianonni *et al*. (2018) onset of sepsis for 429 infant cases in Switzerland. They found the majority of cases of sepsis to be hospital acquired LOS (62%), followed by 20% EOS and community acquired LOS (18% of cases). The mortality has been combined to dovetail with other results and reported an overall mortality rate of sepsis of approximately 10.95%, highest for EOS. Similar rates of 9.7% mortality were found in hospital acquired cases of sepsis in Belgium (Verstrate *et al*., 2014).

1.3.2 Aetiology

Sepsis can be caused by parasites, viruses, bacteria or fungi, but is most commonly bacterial. A study carried out in Japan demonstrated 1% of sepsis cases were caused by fungus (Umemura *et al*., 2021). Marchetti *et al*. (2004), found *Candida* spp. caused 2.9% of sepsis cases in a Swiss tertiary care hospital 1991-2000, although it has been reported more commonly in America, causing up to 5% of sepsis cases, more common as a nosocomial infection (Delaloye and Calandra, 2014).

In HICs, a large proportion of neonatal sepsis cases are caused by Gram-positive bacteria (Testoni *et al*., 2014), with Group B streptococcus (GBS) (*Streptococcus agalactiae*) fond to be a leading cause in a study by Hamada *et al.* (2008). However, rates of GBS

reportedly declined following this study period due to prenatal screening and use of intrapartum antibiotics (Centers for Disease Control and Prevention, 2004) but is still a major cause of neonatal sepsis in HICs, alongside *E. coli* (Doenhardt *et al*., 2020), particularly in EOS (Schrag *et al*., 2016; Stoll *et al*., 2020). In LMICs, Gram-negative pathogens cause approximatively half or more of all cases of neonatal sepsis, with low reports of GBS (DeNIS, 2016; Zaidi *et al*., 2005) with more cases caused by *S. aureus* in neonates from LMICs (Yadav *et al*., 2018). *S. aureus* was found to be the most common sepsis causing agent in South Korea in a study carried out by Shin *et al*. (2009). This was also found to be the second highest cause of LOS in a study in Toronto, Canada with *E. coli* as the highest (Testoni *et al*., 2009).

CoNS is commonly isolated from blood samples of sepsis patients (Stoll *et al*., 1996). However, as previously stated, there is variation between studies as to whether this is reported as a sepsis causing pathogen (Testoni *et al*., 2014; Movahedian *et al*., 2006) or as a contaminant as it is often present on the skin (Grice and Segre, 2013), so may be transferred to blood upon extraction if the area was not sterilised sufficiently, or from healthcare workers, but alternately could potentially infect patients due to high colonisation. Souvenir *et al.* (1998) approximated 72.8% of CoNS found in blood cultures to be contamination, with 24.7% determined as infections and 12% inconclusive, similar to approximations from Jean-Baptiste *et al.* (2011) and Mirrett *et al.* (2001), respectively reporting 73% and 67% of isolates as potential contaminants. A repeated blood culture is often required to determine if CoNS is sepsis causing rather than a contaminate but this will underestimate cases as the antibiotic treatment will in some cases clear the infection before a second blood draw is carried out.

1.3.3 Gram-negative bacteria in neonatal sepsis

A greater proportion of sepsis cases are found to be caused by Gram-negative pathogens in LMICs compared to HICs (Zaidi *et al*., 2005). This is concerning as Gram-negative bacteria (GNB) are associated with increased incidence of AMR, as the outer membrane provides an additional barrier against antibiotics. This prevents antibiotics, such as vancomycin from entering, along with providing additional resistance mechanisms including changes to porins, used by hydrophilic compounds, such as β-lactam antibiotics, or to the membrane properties to prevent hydrophobic antibiotics from entering (Miller, 2016). Furthermore, as previously mentioned, β-lactamases that break down β-lactam antibiotics are more effective in GNB, as the outer membrane retains the enzymes, increasing their concentration, improving activity against incoming β-lactam antibiotics (Toth *et al*., 2015).

Resistance genes are common in GNB, commonly found on plasmids or mobile elements that can be readily transferred between GNB via horizontal gene transfer (Holloway *et al*., 1969; Stokes and Gillings, 2011), providing the ability to transfer between species. This includes genes encoding carbapenemase resistance, such as NDM, KPC, VIM, OXA-48 (Pitout *et al*., 2015), that signify one of the biggest threats against antibiotics. Moreover, lipopolysaccharide on the outer member of Gram-negative bacteria, due to the lipid A component, is known to contribute to endotoxic shock, initiating dysregulated inflammatory pathways (Opal, 2010) due to over stimulation of the Toll-like receptor-4 gene (Poltorak e*t al*., 1998), associated with increased risk of septic shock and mortality.

1.3.4 AMR in neonatal sepsis

Although data on AMR in neonatal sepsis is lacking, particularly in LMICs, recent studies have shown high rates of AMR in pathogens associated with neonatal sepsis. A study by the Neonatal AMR research network (Li *et al.,* 2019) collated data from 39 hospitals in 12 LMICs serving a combined total of 228,500 live births per year. This study demonstrates high

incidence of AMR in Gram-negative isolates from neonatal units against a range of important antibiotics, such as third generations (3G) cephalosporins and carbapenems, particularly in Bangladesh, as shown in Table 1.4 below.

Table 1.4. Antimicrobial resistance patterns in the NeoAMR network. (Reproduced from NeoAMR: Neonatal AMR research network. Source: Li, G. et al., 2019.)

Country	**Gram-negative cultures resistant ≥1 third-generation cephalosporin, n (%)**	**Gram-negative cultures resistant to a carbapenem, n (%)**	**Gram-positive cultures resistant to a glycopeptide, n (%)**
Bangladesh	49/58 (84)	47/58 (81)	0/1 (0)
Brazil	17/57 (30)	5/57 (9)	0/12 (0)
Cambodia	6/9 (67)	0/9 (0)	0/2 (0)
China	78/185 (42)	13/185 (7)	0/84 (0)
Colombia	25/42 (60)	1/42 (2)	0/50 (0)
Greece	8/13 (62)	0/13 (0)	0/1 (0)
India	286/562 (51)	154/562 (27)	35/265 (13)
Nigeria	26/36 (72)	7/36 (19)	5/11 (45)
South Africa	427/627 (68)	245/627 (39)	0/394 (0)
Thailand	12/46 (26)	10/46 (22)	0/11 (0)

A literature review by Zaidi *et al.* (2005) assessing studies across multiple LMICs reported high levels of ampicillin resistance across studies in *E. coli* (82%) and *Klebsiella* spp., averaging 86%. Overall, 50% of *E. coli* and 71% of *Klebsiella* spp. isolates demonstrated resistance against gentamicin. Thaver *et al*. (2009) performed a literature review investigating resistance in three common pathogens causing neonatal sepsis (*S. aureus*, *Klebsiella* spp. and *E. coli*) from data collated 1991-1995 and 1996-2007 and found increased prevalence of resistance in all three species for all antibiotics tested, highest in *Klebsiella* spp. Resistance from the later time period showed 97% *Klebsiella* spp. isolates and 76% *E. coli* isolates resistant to ampicillin, 60% *Klebsiella* spp. and 14% *E. coli* resistant to gentamicin, 66% *Klebsiella* spp. vs 8% *E. coli* against third generation cephalosporins and 43% *Klebsiella* spp. vs 5% *E. coli* against amikacin.

The 2020 GLASS report found high levels of resistance in *Acinetobacter* spp. isolates in blood stream infections against aminoglycoside and carbapenems, ranging across minimal levels to above 90% for some countries. Within *E. coli* isolates, lower levels of carbapenem resistance were found, ranging from 0 to around 30%. Fluoroquinolone resistance was greater, ranging from approximately 10-85% and resistance against third generation cephalosporins reached 100% for one country, with a median range of around 15-65%. Higher resistance against third generation cephalosporins was also seen in *K. pneumoniae* isolates, again reaching 100%, with as low as 5% resistant isolates reported in other countries. Fluoroquinolone resistance range from approximately 8-88% and carbapenem resistance from low levels to up to 65% reported in one country. However, AST results are not reported for all species from all countries within the GLASS network, with only one country (Republic of Korea) providing data for all pathogens (WHO, 2020). There is a lack of sampling strategy, preventing incidence rates from being assessed within catchment areas.

A high number of the deaths caused by neonatal sepsis are considered to be caused by pathogens resistant to treatment, particularly in LMICs. A study carried out by Laxminarayan *et al*. (2016) estimated that just under one third of neonatal deaths may be caused by AMR and approximated that 214,000 of the 690,000 reported neonatal deaths due to sepsis worldwide each year could be attributable to resistant pathogens. However, this figure is based on limited studies from developing countries as data from LMICs is severely lacking and may overrepresent deaths due to AMR.

1.4 Research focus

This research is based on the 'burden of antibiotic resistance in neonates from developing societies' (BARNARDS) study. BARNARDS was set up to assess the burden neonatal sepsis across 12 sites in seven LMIC countries, including Bangladesh, Ethiopia, India, Nigeria, Pakistan, Rwanda, and South Africa. This study investigated incidence of

neonatal sepsis from enrolled neonates born at clinical sites or presented at clinical sites with signs of sepsis. Reported neonatal mortality was tracked for all neonates enrolled for 60 days following enrolment. The study identified common sepsis causing pathogens at clinical sites and associated antibiotic resistance profiles. Potential risk factors for the development of sepsis and mortality in both inborn and outborn neonates were also explored through questionnaire-based data which focused on demographics of the mother and birth related factors. This thesis focuses on the phenotypic resistance found in sepsis causing pathogens, addressing levels of resistance to treatment as a potential cause for mortality and assessing potential alternative treatments. Further to this, this thesis delves further into community acquired sepsis, investigating potential associated risk factors for mortality.

1.5 Added value

BARNARDS is the first multi-country study on neonatal sepsis that incorporated standardised and unified protocols between sites to combine clinical, microbiology, epidemiology, and genomic data. There is a lack of standardised protocols within literature in different studies, many of which are single site studies, with varying diagnostic definitions and varied microbiology skills and equipment. Wider analyses in the literature composes of reviews combining these varied single site studies, achieving important but flawed results.

As seen in previous literature, high resistance is found in multiple studies against a range of antibiotics commonly used in the treatment of neonatal sepsis. It is vital to investigate this further to determine how AMR may affect treatment. Of note, literature showed high resistance against ampicillin and gentamicin, the WHO recommended first-line treatment in LMICs, requiring further exploration.

Furthermore, most literature is based on sepsis acquired in the hospital setting, either as EOS or some studies focussed on hospital acquired LOS. Part of this research will explore

community acquired neonatal sepsis data and associated risk factors to begin to address this gap in research.

1.6 Aims and objectives

1. A main aim of this research was to assess standardised data across seven LMICs to determine rates of resistance against a range of medically important antibiotics.
2. Assess common antibiotic usage and target attainment
    a. Rate of resistance against WHO recommended empirical therapy
    b. Investigation of treatment appropriateness for organism and resistance profile.
    c. Did resistance to antibiotic therapy cause mortality?
3. Demographic and birth related risk factors for mortality following community acquired neonatal sepsis
    a. Additional challenges faced by LMICs regarding increased infection and mortality.

2.0 Methods

2.1 BARNARDS Study design and ethics

BARNARDS was an observational study analysing sepsis rates in neonates and infants between 0-60 days of life in LMICs between 01/11/2015 - 31/03/2018. For results within this thesis, the term neonates, which usually refers to 0-28 days of life will refer to neonates and infants 0-60 days of life. Twelve clinical sites across seven countries were included in the study (Figure 2.1), consisting mainly tertiary care hospitals and one community site in Pakistan. Countries with participating sites included Bangladesh (Kumudini Women's Medical College; Chittagong Maa-O-Shishu Hospital); Ethiopia (St. Paul's Hospital Millennium Medical College, Addis Ababa); India (Seth Sukhlal Karnani Memorial Hospital in collaboration with National Institute of Choleric and Enteric Diseases, Kolkata); Nigeria (National Hospital Abuja; WUSE District Hospital, Abuja; and Murtala Mohammad Specialist Hospital, Kano); Pakistan (Pakistan Institute of Medical Sciences; Bharu Kahu Rural Health Centre); Rwanda (Kabgayi Hospital; University Central Hospital Kigali); and South Africa (Tygerberg hospital, Cape Town) (Figure 2.1; Table 2.1).

Table 2.5. Sites included in BARNARDS per country and associated site abbreviations.

Country	Site	Site abbreviation
Bangladesh	Chittagong Maa-O-Shishu Hospital	BC
	Kumudini Women's Medical College	BK
Ethiopia	St. Paul's Hospital Millennium Medical College	ESS
India	Seth Sukhlal Karnani Memorial Hospital in collaboration with National Institute of Choleric and Enteric Diseases, Kolkata	IN
Nigeria	National Hospital Abuja	NN
	WUSE District Hospital	NW
	Murtala Mohammad Specialist Hospital	NK
Pakistan	Pakistan Institute of Medical Sciences	PP
	Bharu Kahu Rural Health Centre	PC
Rwanda	Kabgayi Hospital	RU
	University Central Hospital	RK
South Africa	Tygerberg hospital	ZAT

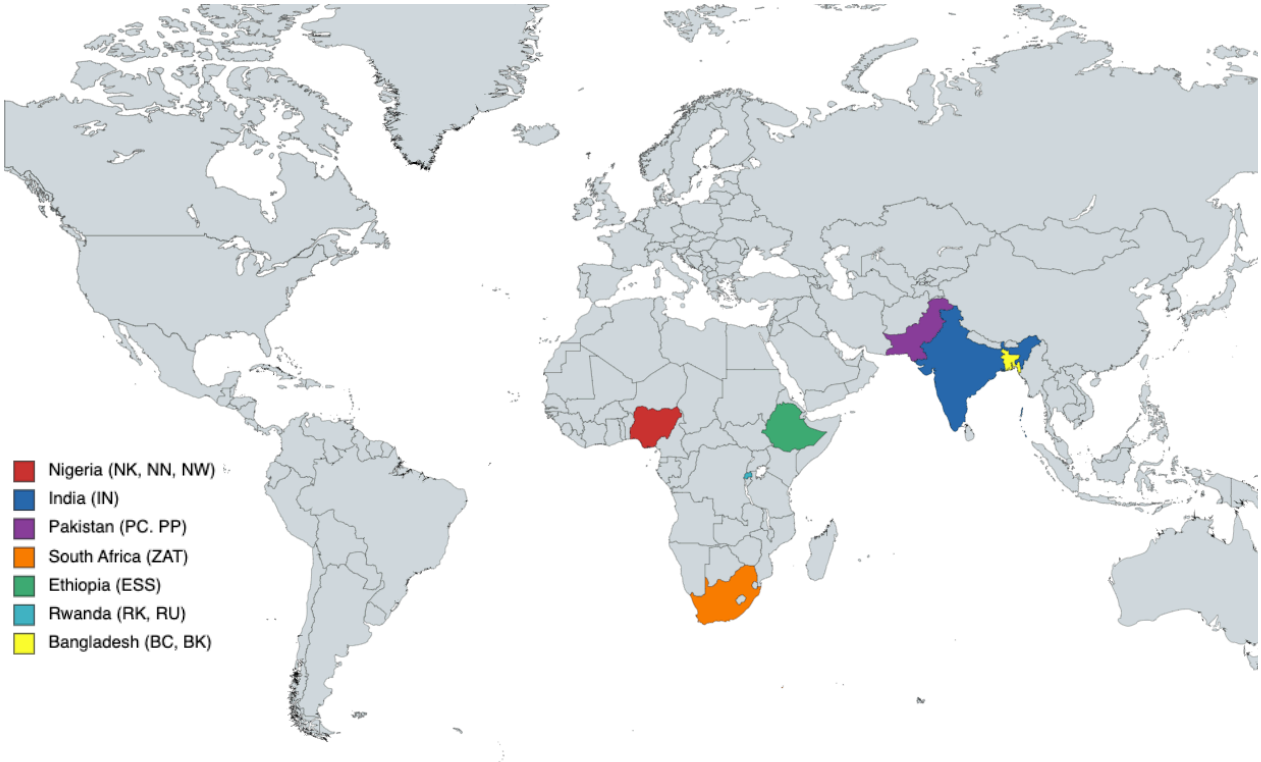


Figure 2.6. Global map with countries where BARNARDS sites located are highlighted. Made using Map Chart (https://www.mapchart.net/world.html)

Ethical approval was granted via local ethics committees. Mothers that arrived at one of the clinical sites in labour were informed about the study (forms written in local languages). All mothers that provided informed consent were enrolled at the clinical site together with their new-born, which made up an inborn cohort. In addition to these inborn mother-neonate dyads, outborn neonates that were born outside of the clinical site or were born at a site and later returned, with signs of sepsis were also enrolled alongside presenting carer. All participants could withdraw from the study at any time. Stillborns birthed at clinical sites were excluded from the study.

2.2 Questionnaire

Upon enrolment, mothers were asked 53 questions relating to their lifestyle, recent medical history and demographic information (Appendix, page 1-4). Questionnaires were posed to the mother/ guardian for the admissions neonates following admittance to the

neonatal intensive care unit (NICU). Data was input into the Bristol Online Survey (BOS) system for all sites except for the site in Ethiopia which used REDCap due staff familiarity with the software. Depending on logistics at various sites, research nurses either input data directly onto Bristol online survey (BOS) when collecting information from the mother, or input information onto hard copied of the questionnaire and later uploaded onto BOS. Data was pseudonymised and patient names were not entered into the system. All uploaded data was available to project staff at Cardiff University. If any patients decided they did not want to take part in the study following upload of their data, the relevant dataset was deleted from Cardiff University and removed from any downstream analyses. Data was downloaded monthly and potential mistakes queried with site contacts throughout the study. Any necessary amendments were made, and downloads saved as different versions.

Participant involvement lasted up to 60 days, inclusive of enrolment of the mother and neonate when admitted to hospital before/after labour or upon admittance of the neonate with suspected sepsis and a 60-day follow-up period by phone. This study did not require any additional hospital visits in addition to those required for their standard clinical care.

Information on antibiotic treatments for those with clinically diagnosed sepsis was obtained on site and retrospectively added to neonate clinical data. Research nurses followed up outcomes of enrolled neonates for a 60-day period following enrolment via house visits and phone calls at days 3, 7, 14, 28 and 60. Neonates of parents that could not be contacted were considered as alive until their age at last observation.

2.3 Sample collection

Neonates diagnosed with clinical sepsis had a blood sample taken at clinical sites, following standard clinical care practices. Signs of sepsis, phlebotomy checklists and microbiology protocols carried out at sites are linked through the following website https://www.ineosoxford.ox.ac.uk/research/barnards. Blood samples were incubated in a BD

BACTEC™ machine for up to four days. Following incubation, samples with positive blood culture were streaked onto Columbia blood agar and incubated overnight at 37°C. Purity of cultures was checked visually before a Gram-stain was performed. Antimicrobial susceptibility testing was carried out on site using the Kirby-Bauer method and resistance against antibiotics determined according to EUCAST v5.0 (EUCAST, 2015). Species identification was also carried out on site using an Enterosystem 18R kit (Liofilchem®) for Enterobacteriaceae. Bacterial isolates from positive blood cultures were stored on charcoal swabs before being sent to the UK under UN3373 transport regulations. It is possible that resistance plasmids may have been lost during transit or the initial incubation on Colombia blood agar but data on ESBL and MBL prevalence were collected by the sites to enable quality control at CU.

2.4 Sample processing, Cardiff University

All bacterial isolates obtained from positive blood cultures, again on charcoal swabs sent under UN3733 regulations. Rectal and environmental swabs were processed to obtain prevalence of resistance genes and isolates from positive blood cultures were processed for sequencing and antibiotic susceptibility profiling (Figure 2.2).

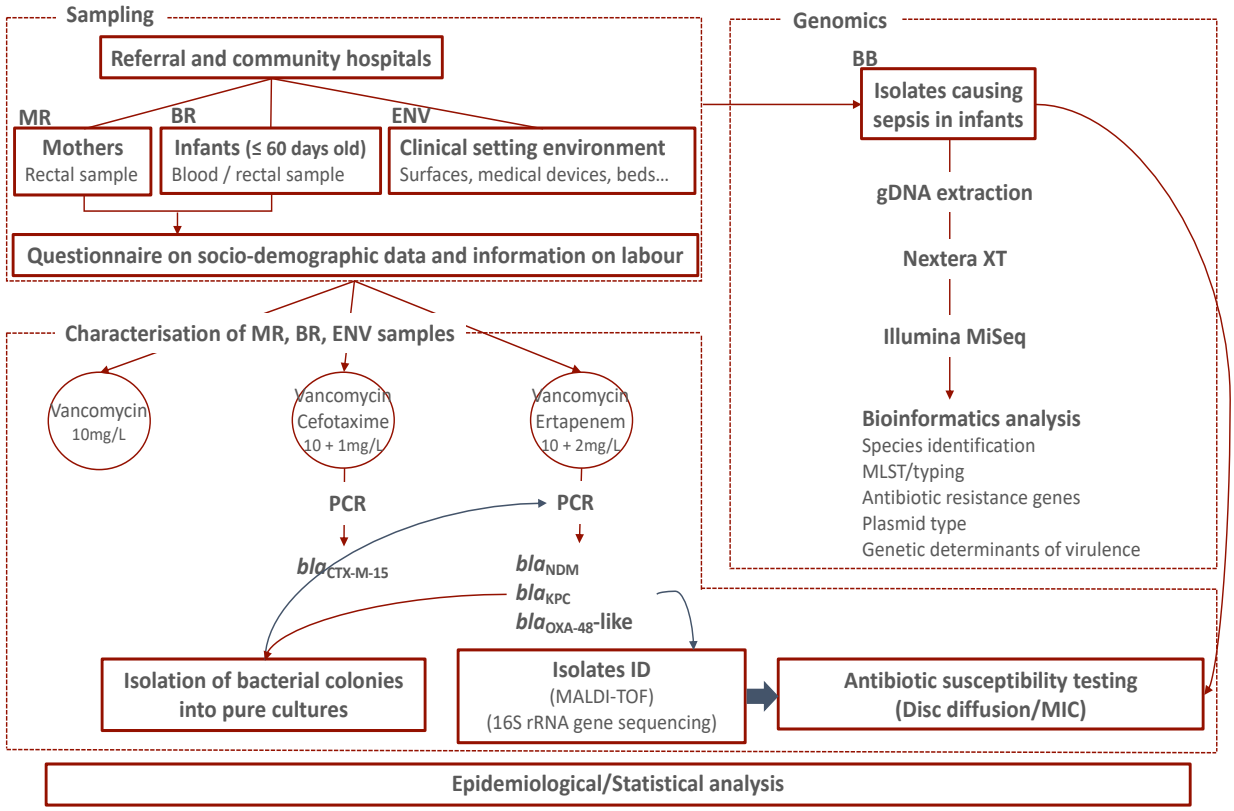


Figure 2. 2. Microbiology protocol for all samples received at Cardiff University, UK. Details for processing mother rectal (MR), baby rectal (BR), environmental (ENV) swabs and associated polymerase chain reaction (PCR) results for presence of Beta-lactamase genes $bla_{CTX\text{-}M\text{-}15}$, bla_{NDM}, bla_{KPC} and $bla_{OXA\text{-}48\text{-}like}$ are not detailed herein, as this was outside the remit of this thesis, which focuses on sepsis (BB) isolates.

2.4.1 Sepsis isolates whole genome sequencing preparation

Sepsis causing isolates sent to Cardiff University were streaked onto chromogenic urinary tract infection (UTI) agar (Oxoid Ltd., Brilliance^{TM} UTI Clarity^{TM} agar) and incubated overnight at 37°C. Gram-negative isolates were also grown on Chromatic ESBL agar (Liofilchem®) that contained vancomycin, alongside cefotaxime and ertapenem to prevent loss of cephalosporin or carbapenem resistance genes respectively prior to sequencing. The following day, these were checked visually for purity. Laboratory results from the original clinical site were checked for any isolates that demonstrated more than one colour on the agar and relevant isolate selected and purified. Pure isolates were processed for DNA extraction and stored at -80°C. For DNA extractions, a colony from each isolate was

transferred to 1.5 mL of autoclaved LB broth (Sigma-Aldrich®) in 2mL autoclaved Eppendorf tubes and incubated overnight in a shaking incubator at 120 revolutions per minute (rpm) and 37°C. The following day, isolates were spun down in a centrifuge for ten minutes at 13,000 rpm (relative centrifugal force = 17,950) and supernatant poured off into a bleach solution, leaving the pellet in the tube. These were placed in the shaker rack of the automated QIAGEN QIAcube® extractor (Figure 2.3). Proteinase K (Sigma-Aldrich®) and RNAse (Sigma-Aldrich®) were added to the microcentrifuge tube slots, pipette tips added to the racks (Figure 2.3) and reagent bottles were topped up with relevant reagents prior to a run. QIAGEN DNA spin columns and 1.5mL Eppendorf Tubes® were placed in QIAGEN rotor adapter cartridges and loaded into the centrifuge section of the QIAcube® and the machine was set to a bacterial pellet extraction program including RNAse treatment protocol. When this was completed, the DNA of each isolate was contained in individual 1.5mL Eppendorf Tubes®. These were labelled and the DNA was quantified using the Invitrogen Qubit 4 fluorometer (by Thermo Fisher Scientific) that utilises a fluorescent dye that increases its fluorescence upon binding with DNA. The Qubit was calibrated using Invitrogen by Thermo Fisher Scientific dsDNA HS standard #1 and #2. 2mL of each sample was added to 198mL of Invitrogen by Thermo Fisher Scientific dsDNA HS Assay kit and vortexed and DNA quantified measured using the Qubit, which provides DNA quantification in ng/mL. Isolates were then stored at -20ºC.

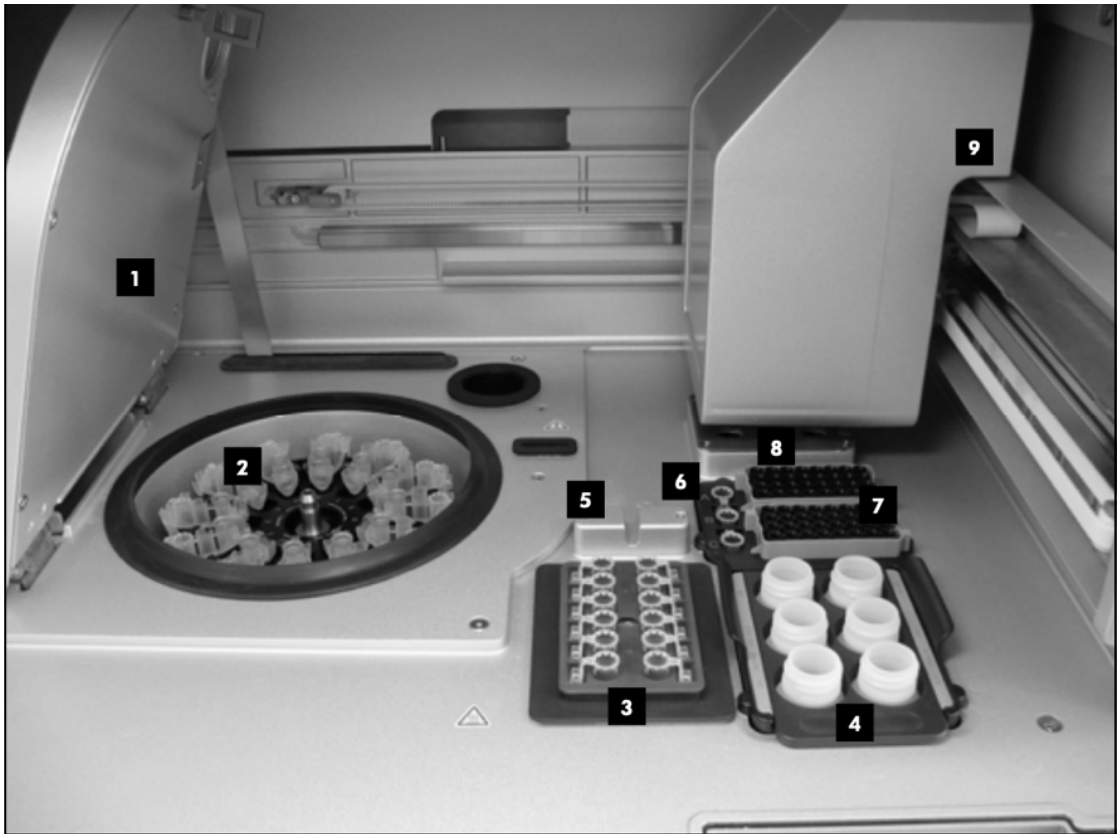


Figure 7.3 Internal features of the QIAcube® machine. 1. Centrifuge lid; 2. Centrifuge; 3. Shaker; 4. Reagent bottle rack; 5. Tip sensor; 6. Microcentrifuge tube slots; 7. Tip racks; 8. Disposal slots for tips and columns; 9. Robotic arm. Source: QIAcube® User manual version 1.3 March 2018.

Prior to sequencing, all samples containing over 10ng/µl of DNA were normalised to 10ng/µl via relevant addition of sterile MGW, before normalising all samples to a concentration of 0.2ng/µl. Library preparation (Figure 2.4) is then initialised via tagmentation whereby unfragmented DNA is cleaved via enrichment bead-linked transposome (eBLT) and washed with a tagment wash buffer. Unique adapter sequences are added to the tagmented DNA samples (Figure 2.4) prior to carrying out polymerase chain reaction (PCR) amplification under the conditions specified in Table 2.2.

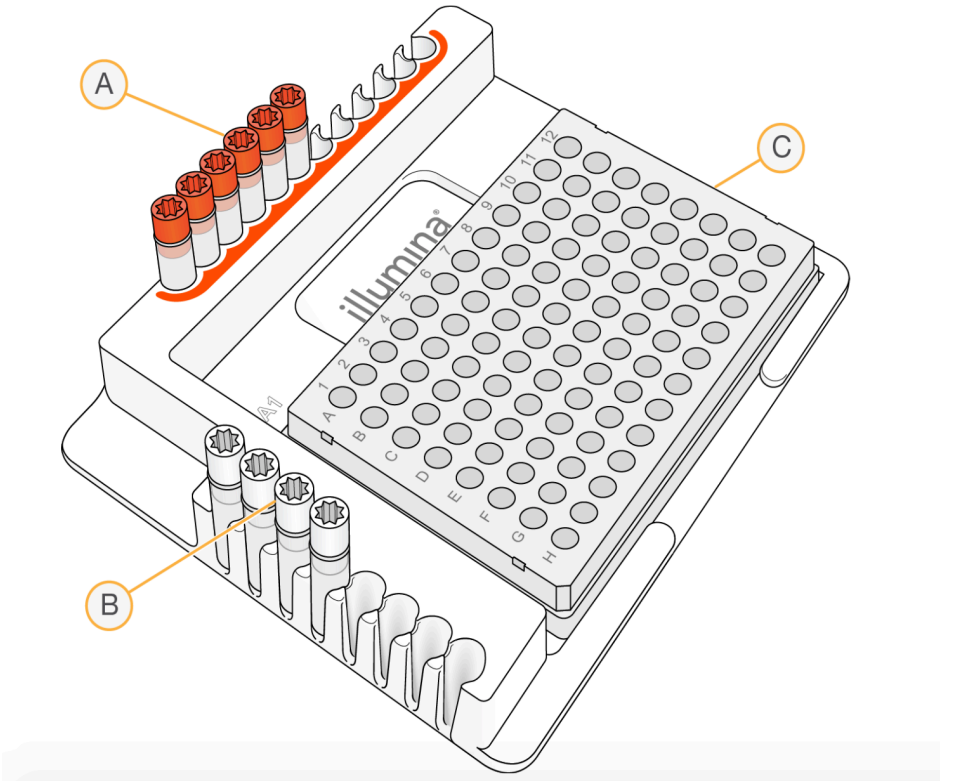


Figure 2.4. TruSeq Index plate arrangement to prepare 24 libraries using the the i7 primers (labelled A), i5 primers (labelled B) and PCR plate (labelled C).These primers allow binding of the DNA sections to the flow cell and and this setup allows each isolate to have a unique combination of the primers attached.

Table 2. 1. Polymerase chain reaction amplification conditions.

Temperature (°C)	Time (minutes)	Number of cycles
72.0	03:00	1
95.0	00:30	1
95.0 **55.0** **72.0**	00:10 00:30 00:30	12
72.0	05:00	1
10.0	∞	1

AMPure XP beads are used to select correct size of fragmented genomic DNA (gDNA). DNA is bound to the beads and unwanted contents separated and an ethanol wash followed by elution. A shaking step is then carried out, whereby NexteraXT beads (LNB1) are added to all samples and placed in a shaker for 30 minutes. Samples were washed and eluted back into buffer. Following this, 5µl of 96 samples were pooled into two Eppendorf

tubes (48 separate isolate DNA each). Pooled samples were diluted and heat-denatured at 96°C to achieve single stranded DNA and loaded onto MiSeqs for sequencing. Isolate preparation was carried out by myself and other colleagues working on the BARNARDS project. Whole genome sequencing was carried out mainly by colleagues Edward Portal and Kirsty Sands.

2.4.2 Whole genome sequencing bioinformatics analysis

Analyses of whole genome sequencing data was mainly analysed by colleagues Kirsty Sands and Maria Carvalho, with guidance from Robert Andrews, Cardiff University. This was carried out via the Advanced Research Computing at Cardiff (ARCCA). Following paired end sequencing (300bp sequences), Trim galore (v0.4.3) was used to trim adapters from reads and also trim any lower quality bases. FastQC files were used to assess read quality of each isolate and ensure there was no contamination. Once quality of reads was established, Flash (v1.2.11) was used to overlap the two reads generated before assembling reads into contigs suing SPAdes (v3.9.0). Contigs were then aligned using BWA followed by Pilon to detect any errors. Once any necessary corrections were made, Quality Assessment Tool (QUAST) reports were generated, whereby reports, summary tables and plots are produced (Gurevich *et al*., 2013). PROKKA was used for genome annotation (Seemann, 2014). Kmer finder was used for species identification and SRST2 for multi-locus sequence typing and identifying resistance genes and virulence genes (Inouye, 2014) (Sands *et al*., 2020).

2.4.3 Antibiotic susceptibility profiling

Panels of 14 antibiotics were used to determine antibiotic susceptibility profiles of Gram-positive and 20 antibiotics were tested against Gram-negative sepsis isolates. Agar dilution was performed to determine minimum inhibitory concentrations (MICs) and were

undertaken in batches of 80 isolates including quality control strains to ensure antibiotic concentrations in the plates were correct.

Four concentrations were tested for antibiotics around the breakpoint range, including two concentrations below, the breakpoint concentration and two concentrations above, in addition to a control plate with no antibiotics. Different concentrations were tested for different antibiotics, depending on their respective breakpoints (listed in Table 2.3; Table 2.4). Additional concentrations were tested for antibiotics that had no quality control strains that had a target MIC value within the range to be tested, with an additional concentration added for ampicillin for GPB and GNB in addition to a lower concentration added for tigecycline in GNB to incorporate control strain MICs.

Table 2. 2. Antibiotics used and associated resistance breakpoints and dilutions tested for Gram-positive bacteria.

Antibiotic	Resistance breakpoint (µg/mL)	Dilutions tested (µg/mL)
Ampicillin	>8	0,2, 4,8,16,32
Oxacillin	>2	0, 1, 2, 4, 8
Flucloxacin	Oxacillin breakpoint used >2	0, 1, 2, 4, 8
Levofloxacin	>1	0, 0.5, 1, 2, 4
Ciprofloxacin	>1	0, 0.5, 1, 2, 4
Gentamicin	>1	0, 0.5, 1, 2, 4
Amikacin	>16	0, 4, 8, 16, 32
Tobramycin	>1	0, 0.5, 1, 2, 4
Tigecycline	>0.5	0, 0.25, 0.5, 1, 2
Minocycline	>1	0, 0.25, 0.5, 1, 2
Rifampicin	>0.5	0, 0.03, 0.06, 0.12, 0.25
Vancomycin	>2	0, 1, 2, 4, 8
Azithromycin	>2	0, 1, 2, 4, 8
Linezolide	>4	0, 2, 4, 8, 16

Table 2. 3. Antibiotics used, associated resistance breakpoints and dilutions tested for Gram-negative bacteria.

Antibiotic	Resistance breakpoint (µg/mL)	Dilutions tested (µg/mL)
Ampicillin	>8	0, 2, 4, 8, 16, 32
Amoxicillin-clavulanate	>8	0, 4, 8, 16, 32
Piperacillin-tazobactam	>16	0, 4, 8,16, 32
Ceftriaxone	>2	0, 0.5, 1, 2, 4
Cefotaxime	>2	0, 0.5, 1, 2, 4
Ceftazidime	>4	0, 0.5, 1, 2, 4
Cefepime	>4	0, 0.5, 1, 2, 4
Imipenem	>8	0, 1, 2, 4, 8
Meropenem	>8	0, 1, 2, 4, 8
Ertapenem	>1	0, 0.25, 0.5, 1, 2
Aztreonam	>4	0, 0.5, 1, 2, 4
Gentamicin	>4	0, 1, 2, 4, 8
Amikacin	>16	0, 4, 8, 16, 32
Tobramycin	>4	0, 1, 2, 4, 8
Tigecycline	>2	0, 0.25 0.5, 1, 2, 4
Minocycline	NA	0, 0.5, 1, 2, 4
Fosfomycin	>32	0, 16, 32, 64, 128
Levofloxacin	>2	0, 0.5, 1, 2, 4
Ciprofloxacin	>0.5	0, 0.125, 0.25, 0.5, 1, 2
Colistin	>2	0, 1, 2, 4, 8

Firstly, 50mL falcon tubes were labelled with the concentrations to be tested for each antibiotic corresponding to four 35mL plates with an additional plate with 0µg/mL per antibiotic (the 0 µg/mL antibiotic plate will be used as antibiotic free growth control; and to make sure there is no carry over for each of the different antibiotics). According to the manufacturer's instructions (BD™, Fisher Scientific™), the appropriate amount of cation-adjusted Mueller-Hinton (MH) II was prepared and autoclaved the day before plate inoculation and kept at 55°C overnight. On the same day, bacterial isolates that were to be tested were streaked onto Oxoid Ltd. Brillance™ UTI Clarity™ agar and incubated overnight at 37°C. The following day, antibiotics were weighed out according to their individual potency and solutions made up using appropriate solvents according to the European

Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST v9.0, 2021). Stock solutions were made according to the following formula:

$$\frac{1000}{potency\ x\ 10}\ x\ Volume\ x\ Concentration = Weight$$

P = potency given by the manufacturer (µg/mg)

V = volume required (mL)

C = final concentration of solution (multiples of 1000) (mg/L)

W = weight of antibiotic in mg to be dissolved in volume V (mL).

Three stocks were made up, including the original stock (Stock A) with a concentration of 2560mL/L, Stock B that was made up from 500µl of stock A with 15.5mL of autoclaved de-ionised (DI) water (80mg/L) and a stock C from 500µl of stock B + 15.5mL of autoclaved DI water (2.5mg/L). Antibiotic dilutions were made by adding the amount of relevant antibiotic stock detailed in Table 2.5 into the appropriate labelled falcon tube.

Table 2. 4. Amount of each antibiotic stock to be added to 35mL of agar to create a range of concentrations (µg/mL)

Stock	Amount in 35mL agar	Concentration
Stock A	1.75 mL	128 µg/mL
	875 µL	64 µg/mL
	437.5 µL	32 µg/mL
	218.7 µL	16 µg/mL
	109.4 µL	8 µg/mL
Stock B	1.75 mL	4 µg/mL
	875 µL	2 µg/mL
	437.5 µL	1 µg/mL
	218.7 µL	0.5 µg/mL
	109.4 µL	0.25 µg/mL
Stock C	1.75 mL	0.125 µg/mL
	875 µL	0.06 µg/mL
	437.5 µL	0.03 µg/mL
	218.7 µL	0.015 µg/mL
	109.4 µL	0.008 µg/mL

Once the appropriate amount antibiotic solutions were added to each falcon tube, a tube was topped up to 35mL with molten MHII agar at approximately 50°C, lid placed on, gently inverted to mix and poured into the relevant square plate and left to set from approximately 30 minutes. Following this, plates were placed into the drying cabinet for approximately 10 minutes until dry and ready for inoculation.

Control strains were included in every batch. Control strains for Gram-negative batches included: *E. coli* American type culture collection (ATCC) 25922; *Pseudomonas aeruginosa* ATCC 27853; and VIM-positive *Pseudomonas aeruginosa* A70; NDM-1-positive *E. coli* IR60; NDM-1-positive *K. pneumoniae* IR35. Control strains for Gram-positive batches included: *E. coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853; NDM-1-positive *E. coli* IR60; *Staphylococcus aureus* 29213*;* and *Staphylococcus aureus* 25923. Control strains not labelled as ATCC strains were obtained from the National Health Service (NHS) Public Health Wales as characterised control strains.

Gram-negative bacterial cultures were checked phenotypically (Table 2.6) for purity via checking growth on the chromogenic agar was of a single colour before use: any mixed cultures were reisolated, stored and tested at a later date when pure. With a sterile cotton swab, several bacterial colonies were taken from a plate and transferred to a vial of Oxoid Ltd. sterile saline (0.85% NaCl w/v in water). These were made to the density of a McFarland 0.5 turbidity standard (approximately corresponding to 1–2 x 10^8 CFU/mL for *Escherichia coli*) for each bacterial plate.

Table 2. 5. Main colour distinctions of common Gram-negative pathogens on chromatic ESBL vancomycin plates (Liofilchem ®).

Colour	**Species**
Purple	*E. coli*
Blue	*Klebsiella* spp.
Green/blue	*Enterobacter* spp.
Translucent cream	*Pseudomonas aeruginosa*
Cream/beige	*Acinetobacter* spp.*; Burkholderia* spp.*; Ralstonia* spp.
White	*Proteus* spp.
White, changing to red, sometimes green	*Serratia* spp.

For preparation of the inoculation plate, 180µl of sterile saline was added to each required well of the Multi-point Inoculator (MPI) well mould, each of which corresponded to a single test isolate. To each well, 20µl of the 0.5 McFarland bacterial suspension for each isolate was added (1:10 dilution). The MPI was then used to inoculate plates, starting with the control 0 µg/mL antibiotic control growth plate and inoculating the plates with the lowest to the highest concentration for each antibiotic. Plates were left facing upwards on the bench for approximately 30 minutes or until inoculation spots have dried, before inverting and incubating overnight at 37°C. The following day, control plates were checked for growth, any with missing growth needed to be repeated. Plates were then read and the lowest concentration of bacterial inhibition for each isolate was recorded for all antibiotics. Control strains were checked against EUCAST QC breakpoints (EUCAST v9.0, 2021). Any antibiotics that had an incorrect QC strain breakpoint that were not within the target range were repeated.

MICs were assessed via MIC_{50} and MIC_{90} results, which demonstrate the MIC for 50% and 90% of the isolates analysed, respectively. MICs for isolates included in this study were also grouped into those that are deemed susceptible (S), required increased exposure (I) and resistant (R), interpreted through EUCAST breakpoints provided (v9.0, 2019). All breakpoints used are listed in Tables 2.7 and 2.8. Coverage for main antibiotic therapies was

determined as the percentage of isolates susceptible to at least one of the antibiotics in a combination. Isolates with MIC values determined as requiring 'increased exposure' were combined with resistant isolates. MIC values were plotted with outcome for neonates treated with each of the top four antibiotic combinations with no treatment change following initial therapy.

Table 2. 6. Breakpoints used to determine susceptibility profiles of Gram-positive bacteria analyses using EUCAST v9.0 breakpoints. ≤ S displays the concentration (µg/mL) (at or lower than) at which isolates were determined as susceptible and >R shows the concentration (µg/mL) (higher than) for isolates to be deemed resistant to each antibiotic. Isolates with MICs between these breakpoints were considered as requiring increased exposure. No breakpoint is provided for ampicillin for Staphylococcus aureus, as most are penicillinase producers and considered resistant.

Antibiotic	***Staphylococcus* sp.**	
	≤S (µg/mL)	**>R (**µg/mL)
Ampicillin	-	-
Oxacillin	2	2
Flucloxacin	2	2
Levofloxacin	1	1
Ciprofloxacin	1	1
Gentamicin	1	1
Amikacin	8	16
Tobramycin	1	1
Tigecycline	0.5	0.5
Minocycline	0.5	1
Rifampicin	0.06	0.5
Vancomycin	2	2
Azithromycin	1	2
Linezolide	4	4

Table 2. 7. Breakpoints used to determine susceptibility profiles from Gram-negative bacteria analysed (EUCAST v9.0[1]). ≤ S displays the concentration (µg/mL) (at or lower than) at which isolates were determined as susceptible and >R shows the concentration (µg/mL) (higher than) for isolates to be deemed resistant to each antibiotic. MICs between these breakpoints were considered as requiring increased exposure. Enterobacteriaceae breakpoints were available for all antibiotics tested, except for Minocycline. PK-PD breakpoints were also not available; therefore SIR was not analysed for minocycline. Where available, breakpoints for Pseudomonas sp. as determined by EUCAST. However, where these were not available, Enterobacteriaceae breakpoints were used. Similarly, Pseudomonas sp. (green) or Enterobacteriaceae (blue) breakpoints were used to determine Acinetobacter sp. isolate resistance. Where breakpoints were not available for Stenotrophomonas sp., Acinetobacter sp. breakpoints were used were available (orange), followed by Pseudomonas sp. (green) or Enterobacteriaceae (blue). Enterobacteriaceae breakpoints were used for Aeromonas sp. as no confirmed breakpoints were available.

Antibiotic	**Enterobacteriaceae**		**Pseudomonas***		**Acinetobacter**		**Stenotrophomonas**		**Aeromonas**		**PK-PD**	
	≤S	**>R**	**≤S**	**>R**	**≤S**	**>R**	**≤S**	**>R**	**≤S**	**>R**	**≤S**	**>R**
Ampicillin	8	8	8	8	8	8	8	8	8	8	2	8
Amoxicillin-clavulanate	8	8	8	8	8	8	8	8	8	8	2	8
Piperacillin-tazobactam	8	16	16	16	16	16	16	16	8	16	4	16
Ceftriaxone	1	2	1	2	1	2	1	2	1	2	1	2
Cefotaxime	1	2	1	2	1	2	1	2	1	2	1	2
Ceftazidime	1	4	8	8	8	8	8	8	1	4	4	8
Cefepime	1	4	8	8	8	8	8	8	1	4	4	8
Imipenem	2	4	4	4	2	4	2	4	2	4	2	4
Meropenem	2	8	2	8	2	8	2	8	2	8	2	8
Ertapenem	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Aztreonam	1	4	16	16	16	16	16	16	1	4	4	8
Gentamicin	2	4	4	4	4	4	4	4	2	4	-	-
Amikacin	8	16	8	16	8	16	8	16	8	16	-	-
Tobramycin	2	4	4	4	4	4	4	4	2	4	-	-
Tigecycline	ECOFF values for respective species used*[2]										0.5	0.5
Minocycline	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-	-
Fosfomycin	32	32	32	32	32	32	32	32	32	32	-	-
Levofloxacin	0.5	1	1	1	0.5	1	0.5	1	0.5	1	0.5	1
Ciprofloxacin	0.25	0.5	0.5	0.5	0.25	1	0.25	0.5	0.25	0.5	0.25	0.5
Colistin	2	2	2	2	2	2	2	2	2	2	-	-

**Burkholderia* sp. and *Ralstonia* sp. isolates were analysed with the same breakpoints as *Pseudomonas* sp., as these species are closely related and not in EUCAST v9.0.
**2*ECOFF values can be found for Tigecycline through link provided in the reference list.[2]

2.5 Isolate selection for evaluating empirical treatment given

A subset of neonates with culture confirmed sepsis were selected for inclusion in the evaluation of current WHO empirical treatment guidelines. Antibiotic consumption data was collated from all clinical sites and assessed. The four most common antibiotic combinations were selected for further analysis and only neonates that had antibiotic data, WGS data and were treated with one (or more) of these four combinations were included. The study size was based on the maximum number of cases treated with common antibiotic combinations found following retrospective clinical note review, with available WGS data.

Antibiotic susceptibility profiles were assessed for these neonates in addition to pharmacokinetic/pharmacodynamic (PK/PD) modelling to gauge whether dosing of antibiotics was appropriate for the pathogen considering the MIC result. Experiments investigating the frequency of resistance (FoR) were also carried out to compare the potential for resistance to arise in susceptible bacteria to a range of antibiotics. In addition, pathogenicity indexing and virulence factors were also taken into account to establish this effect on the reported outcome of the neonate in addition to resistance to antibiotic therapy. Finally costs of current and alternative antibiotics were assessed across BARNARDS sites to assess the practicality of using different antibiotics to treat neonatal sepsis. Methodologies for these individually are detailed in the following paragraphs.

2.5.1 PK/PD modelling

PK/PD analysis was performed by collaborators from Leiden Academic Centre for Drug Research, Leiden University, Netherlands. PK/PD modelling was carried out on the four most common antibiotic combinations used at clinical sites, which included six antibiotics (ampicillin and gentamicin, amoxicillin-clavulanate and amikacin, piperacillin-tazobactam and amikacin and ceftazidime and amikacin). Characteristics of neonates were taken into consideration (e.g. premature) and were utilised to derive reference values for body weight, serum creatinine and albumin (Blencowe *et al.*, 2019) for PTA simulations (Hartman *et al.*, 2020). Only neonates that were prescribed an antibiotic combination were assessed in each relevant antibiotic simulation. The MIC of the specific pathogen to that antibiotic was input in addition to the dosing schedule provided by the relevant site. The pathogen, dosing and patient characteristics for each neonate were assessed against published neonatal PK models (Li *et al*., 2013; Fuchs *et al*., 2014; Tang *et al*., 2019; De Cock *et al*., 2012; Tremoulet *et al.*, 2014; Wang *et al*., 2018) on which simulations were run 1,000 times per patient using published population PK models for each antibiotic combination (Rashed *et al.*, 2019).

Target attainment (PTA) was met for a combination when at least one antibiotic reached their MIC-specific PK/PD target value associated with efficacy. Simulated PTA values for each combination were compared against MIC distributions and a PTA achieved in ≥80% of the simulations run, typically associated as threshold for efficacy, was compared to observed overall survival. Additional PTA simulations using the same methodology were performed for meropenem, fosfomycin and colistin, and compared to commonly used antibiotic combinations. Full analysis methods are described in Appendix p5-8.

2.5.2 Frequency of Resistance

Frequency of resistance (FoR) was assessed for antibiotics found to be commonly used in the treatment of neonatal sepsis at BARNARDS sites, as well as antibiotics with

potential to act as therapeutic alternatives due to lower rates of resistance based on MICs carried out during this study. Commonly used antibiotics chosen to include in this analysis comprised of: amikacin, amoxicillin-clavulanate, ampicillin, ceftazidime, and gentamicin. Potential alternative treatments with lower rates of resistance tested included colistin, fosfomycin, meropenem and piperacillin-tazobactam.

Isolates were selected based on availability of antibiotic data and MIC profiles. Species commonly found as sepsis pathogens across multiple sites were chosen to select from, which included *Acinetobacter* spp.; *Burkholderia* spp.; *Enterobacter* spp.; *Escherichia coli; Klebsiella* spp.; *Pseudomonas* spp.; *Serratia marcescens;* and *Ralstonia mannitolilytica.* Isolates of the above common species were assessed and for each species selected for inclusion, phylogenetic groups were divided via clusters/ clades and key sequence types were identified. Representative isolates were then from the filtered list of sensitive isolates, and we selected isolates of different sequence types (STs) from across clinical sites. These were selected proportionally from the BARNARDS dataset, with higher numbers selected of more common species and STs. This process was repeated separately for isolates susceptible to ampicillin, as a lower number of sensitive isolates were available.

Isolates were then filtered for susceptibility towards meropenem, amikacin, fosfomycin, gentamicin and colistin. Isolates that were susceptible to meropenem, amikacin and fosfomycin for which we also had antibiotic therapy data for the neonate were selected. Again, this information was merged with WGS data, and phylogenetic clades were divided, key sequence types (STs) were identified for each species and representative isolates were selected at random for each of the main STs across clinical sites where possible for each species. This was carried out for prevalent susceptible species and included: *Acinetobacter* spp., *Enterobacter* spp., *E. coli*, *K. michiganensis, K. pneumoniae* and other *Klebsiella* spp., *Serratia marcescens*, *Pseudomonas* spp. and *Burkholderia* spp.

Selected isolates were streaked onto Oxoid Ltd. Brilliance™ UTI Clarity™ agar and incubated overnight at 37°C. The following day, 5mL of MHII broth previously made to manufacturer instructions (Sigma-Aldrich®) was added to a 7mL bijoux, one per isolate. Bacterial isolates grown were checked for purity, transferred to a bijou and incubated overnight at 37°C in a shaking incubator at 120 rpm. MHII agar was also made up according to manufacturer instructions, autoclaved and kept overnight at 55°C. The following day, antibiotic stock solutions were made according to the calculation below and correct solvent added:

$$\frac{1000}{potency\ x\ 10}\ x\ Volume\ x\ Concentration = Weight$$

P = potency given by the manufacturer (µg/mg)

V = volume required (mL)

C = final concentration of solution (multiples of 1000) (mg/L)

W = weight of antibiotic in mg to be dissolved in volume V (mL).

MHII agar was cooled to approximately 50°C and appropriate amount of each antibiotic stock solution was added to MHII agar to create two-fold the antibiotics' breakpoint (e.g. amikacin has a breakpoint of growth >8ug/mL inferring resistance, was made at 16µg/mL). The agar with added antibiotic was inverted gently to mix and plates poured into labelled 20mL petri dishes and left to cool until set. Serial dilutions of the MHII broth inoculations were also performed to 10^{-8} with 96-well midi plates. 20uL of each of the 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} dilutions were dropped onto antibiotic free agar plates. For the antibiotic breakpoint plates, four sterile glass beads were placed onto the agar surface of each plate. 100µl of the neat dilution was pipetted over the glass beads of one plate and gently shaken to cover the agar surface. Beads were removed and placed in 70% ethanol. This was repeated for the 10^{-1} and 10^{-2} dilutions. This was done for all isolates that were selected for each

antibiotic. Plates were inverted and incubated overnight at 37°C. The following day, the number of colony forming units (CFU) were counted for each plate. FoR rates were calculated for CFU per mL on antibiotic plates compared to antibiotic free plates.

2.5.3 Pathogenicity indexing

Isolate selection for pathogenicity was based on the isolates which were analysed for FoRs with additional *S. aureus*, *E. coli* and *K. pneumoniae* isolates. The additional isolates were selected based on including a range of sequence types of each species, as well as considering various clinical outcome to give a representation of the pathogens found in BARNARDS. Isolates were additionally chosen based on a range of MICs for AMP-GEN.

Galleria mellonella models were used to determine bacterial pathogenicity for a range of isolates, as well described pathogenicity model (Champion *et al*., 2016; Tsai *et al*., 2016). Isolates were selected across BARNARDS sites, to include a range of species, sequence types (STs) and MIC profiles (full list detailed in Appendix, p. 9-11). Bacterial isolates were first streaked onto UTI agar and incubated at 37°C overnight and checked for purity (any mixed isolates were purified and done at a later date). Pure isolates were inoculated into 10mL of LB broth overnight at 37°C in a shaking incubator at 80 rpm. The following morning, inoculated broths were transferred to a centrifuge tube and centrifuged at 3,600 rpm for 15 minutes. Supernatant was discarded before adding a 10mL sterile saline wash and centrifuged again at 3,600 rpm for 15 minutes. Supernatant was discarded before resuspending the pellet in 3 mL of sterile saline and transferring to a new bijou. All solutions were then standardised using a spectrophotometer at a wavelength of 600nm to an optical density (OD) of 0.700 abs (+/- 0.018 abs) to create a putative 10^9 CFU/mL dilution. 100µl of this dilution was added to 900µl of sterile saline for the 10^8 dilution, this was repeated until a 10^5 dilution were achieved, which was used to inject the *Galleria mellonella* (10^{-7}-10^{-8} dilutions for Enterobacteriaceae; 10^{-7}-10^{-9} for *S. aureus).* Ten viable *Galleria mellonella* larvae were

placed in each petri dish for triplicates of each dilution for each bacterial isolate, alongside sawdust. Prior to injection of *Galleria* and between each isolate, needles were cleaned by dipping into disinfectant (Teknon^{TM} Biocleanse), followed by extracting 70% ethanol and disposing and washing through with sterile saline. *Galleria* were injected with 10µl of each bacterial dilution into the pro-leg and repeated for all 10 *Galleria* replicates and three dilutions. Needles were then washed as above and the process repeated for other isolates. *Galleria mellonella* were kept at 37°C and mortality was recorded at 24, 48 and 72 hours. Pathogenicity indexes (PIs) were calculated for a bacterial isolate from calculating the proportion and time of larvae death in a bacterial dilution.

Three controls were used to enable detection of death from contamination of needles (no solution injected), saline solutions (saline only injected) compared to *Galleria mellonella* supplied (incubated 72 hours, no intervention). In addition to criteria outlined for FoR isolate selection, clinical outcome was also considered.

2.5.4 Virulence Factors

E. coli, *K. pneumoniae* and *S. aureus* for which WGS data was available were analysed by a colleague (Kirsty Sands) for the presence of virulence factors (VFs) using Abricate (v0.9.7) with the VF database (vfdb; updated January 2020) and a cut off ≥98% gene identity (Seemann, 2020; Chen *et al.*, 2005). A presence/absence matrix of all virulence associated factors deposited in vfdb was created to generate a total virulence score. VFs scores produced were then correlated with PIs and both were compared to neonatal outcome.

2.5.5 Costs, antibiotic access and availability

An additional questionnaire was sent to BARNARDS clinical site partner principal investigators by the project principal investigator in 2019, after antibiotic data was collected retrospectively and wide variation between sites noted. This questionnaire aimed to obtain further information on antibiotic availability, data on national public/private health

partnerships and antibiotic costs, whether covered by the hospital/state/government or not. The cost of antibiotic treatment if borne by the patient was also examined and compared with average regional household incomes (Appendix, page 12-13).

2.6 Risk factor analysis of admissions cohort

2.6.1 Questionnaire

As detailed in section 2.1, BARNARDS enrolments consisted of mothers arriving at sites for childbirth and neonates that arrived at clinical sites with suspected sepsis. who were asked a series of 53 questions in a questionnaire by research nurses on site (Appendix, page 1-4). This questionnaire captured background information and socio-demographic data on the mother, information relating to the to the birth and on the neonate to cover a range of potential risk factors for neonatal sepsis, mortality or other factors.

2.6.2 Follow-up

From the follow-up data, neonates with reported mortality were recorded. Neonatal outcome (alive/deceased) was analysed with data captured through the questionnaire. Variables associated with mortality were considered risk factors. Similar analysis was performed for neonates with CCS and pathogen type within the CCS cohort.

2.6.3 Data inclusion/exclusion admissions cohort

Prior to analysis of the admissions cohort, neonates confirmed as inborn had previously been analysed. Data for all other neonates not included in this inborn cohort were assessed to determine cohort. Neonates with place of birth locations alternative to BARNARDS clinical sites recorded, were categorised in the admissions cohort. Where this was not confirmed, additional data collected on the neonates' date of birth (DOB) and date of admission to a clinical site was assessed.

Neonates that were diagnosed with sepsis and admitted to a clinical site over 48 hours from their DOB without a confirmed place of birth were added into the admissions cohort. Neonates that spent >48 hours in a clinical site before the diagnosis of sepsis were not included as this would likely be a nosocomial infection.

Analyses included assessment for risk factors for: the development of sepsis against a small healthy subset of admission neonates; culture confirmed sepsis; mortality, methods for which are detailed from 2.5.3. Neonates with culture confirmed sepsis alongside bacterial Gram-stain results from sites were included in additional analyses to investigate risk factors for CCS infections with GPB vs GNB. Furthermore, these analyses were also carried out on infection with MDR bacteria. Species were also investigated in this cohort, but numbers were too low to perform statistical analyses.

Within the BARNARDS protocol, outborn or returning neonates were to be enrolled onto the study within the admissions cohort when they were presented at a clinical site with signs of sepsis. However, some neonates were mistakenly enrolled when they were brought to the hospital site accompanying the mother, most often when they were admitted to the hospital with postpartum haemorrhage. Neonates without confirmed CDS were not included in the analyses. However, those with notes confirming the neonate as healthy and reason for parental admittance were included in one analysis to assess risk factors for the development of CDS for the admissions cohort.

2.7 Statistical analyses

2.7.1 MICs

Heatmaps were made for MIC values for all bacteria tested against all antibiotics, using Morpheus online software (https://software.broadinstitute.org/morpheus/). Comparisons between continents of resistance profiles were made via chi-squared analyses through an online calculator (https://www.socscistatistics.com/tests/chisquare/default2.aspx).

Comparisons carried out comparing MICs between continents were carried out using an independent samples T-test on SPSS version 26.0. Comparisons of MICs between countries within a continent were carried out with one-way ANOVA followed by post-hoc Tukey's honest significant difference test to differentiate which countries were significantly different from others, again on SPSS, version 26.0. These tests were repeated on various sub-sets of data.

2.7.2 Survival analyses

Survival analyses, which accounted for loss to follow-up of neonates for the four antibiotic combinations assessed were carried out via Cox regression hazard ratios (HR). These were calculated in R v 4.0.2.21 using the 'Survival' (v3.2.7) (Therneau and Grambsh, 2000) 'coxphf' (v 1.13.1). Proportional hazard assumptions were assessed via Schoenfeld residuals showing no patterns over time, unless otherwise stated. An unadjusted Cox regression was first carried out, followed by Cox regression adjusted for multiple potential confounding variables that were found to significantly affect the HR model through an ANOVA. These included cohort (inborn or admissions), gender, premature, whether born via Caesarean section, onset of sepsis (early onset or late onset) and type of sepsis causing organism (Gram-negative or Gram-positive pathogen). Furthermore, a mixed effect model was carried out for the survival analysis with the addition of the 'coxme' (v 2.2.16) (Therneau, 2020) package to account for inter-country variability. 'Survminer' (v 0.4.8) and 'ggfortify' (v 0.4.11) packages were used to create survival plots.

2.7.3 AMR associated with outcome

For MIC values plotted with outcome, 'increased exposure' isolates were categorised as resistant. MIC values were split into three categories per combination: susceptible to both antibiotics in a combination; sensitive one and resistant the other; and resistant to both

antibiotics in a combination and Chi-squared, or Fisher's Exact test compared categories with outcome.

2.7.4 Virulence factors and pathogenicity index correlations

VF and PI R^2 correlations were performed in Microsoft Excel. QQ plots, Shapiro-Wilk and Kolmogorov-Smirnov tests were run in IBM SPSS Statistics version 25 to determine distribution normality and Mann-Whitney U tests demonstrated differences between outcome with increased VF or PI scores separately.

2.7.5 Sensitivity analyses

The impact of categorising untraceable neonates within the 'not reported deceased' category was assessed by repeating analyses with untraceable neonates excluded. While mortality% increased slightly with untraceable neonates excluded due to the reduced denominator, no differences to significant/non-significant results were found (Appendix, pages 14-15).

2.7.6 Frequency of resistance

FoR results were log transformed with an added standard of $1x10^{-10}$ to enable incorporation of zero values for violin plots, created in R v 4.0.2.21 using the ggplot2 package (Therneau and Grambsch, 2000). A one-way ANOVA was carried out in R v 4.0.2.21 to assess differences in growth per ml between the antibiotics tested.

2.7.7 Risk factors admissions cohort

All statistical analyses were carried out in SPSS version 26.0. Variables in the questionnaire were assigned domains for statistical analyses consisting of: Hospital setting (where relevant); mother's previous pregnancies; health of the mother; mother demographics; and information related to the birth and neonate. Early onset sepsis (EOS) was defined as CDS developing within 72 hours from birth and late onset sepsis (LOS) as greater than 72 hours from birth.

2.7.7.1 Development of CDS

This analysis was only carried out for neonates from NK as only this site had high enough numbers of neonates confirmed as healthy enrolled. This analysis was carried out by John Watkins. Chi-squared tests were run separately on all data collected from the questionnaire comparing the 'control' healthy neonates from NK to those that were enrolled with CDS from NK. Any variables that yielded a significant result from Chi-square tests comparing healthy neonates from those with CDS from NK were taken forward to include in a multivariable model. Domains described above were used to split the questionnaire data for multi-variable logistic regression models to get odds-ratios (ORs) for the development of sepsis. Following this, any variables that remained significant in this model were combined into an overall multivariable logistic regression model and ORs recorded.

2.7.7.2 Mortality

Risk factors for mortality were assessed for all admission neonates. Univariate Chi-square analyses were carried out for sites with high enough numbers, including Child Health Research Foundation, Chittagong (BC), St Paul's Millennium Medical College, Addis Ababa, Ethiopia (ESS), Murtala Muhammad Specialist hospital, Kano, Nigeria (NK), National Hospital Abuja, Nigeria (NN) and Pakistan Institute of Medical Sciences, Pakistan (PP). No distinction was made between neonates with CCS and CDS for this analysis.

Chi-squared tests were performed separately on questionnaire data, comparing deceased neonates to those reported alive (at latest follow up) to investigate potential risk factors for neonatal mortality following CDS. All variables that yielded a significant result from Chi-square tests ($p<0.05$) were input into a multivariable logistic regression model for each domain. Following this, all variables retaining significant results from the logistic regression per domain ($p<0.05$) were combined into an overall multivariable logistic regression model. Odds ratios (OR) with 95% confidence intervals (CIs) and p-values of variables that

had significant results in the final model are reported in the results. These analyses were repeated but examining only neonates with CCS.

2.7.7.3 Culture confirmed sepsis

Variables with associations for CCS were assessed for all admission neonates were analysed by comparing those with CCS to neonates with CDS without a positive blood culture. Risk factors were analysed per site, where sample size was sufficient including BC, ESS, NK, NN and PP.

Chi-squared tests were performed separately on each variable from the questionnaire data, comparing neonates with CCS to those with CDS without CCS to investigate potential risk factors for CCS. All variables that yielded a significant result from Chi-square tests ($p<0.05$) were input into a multivariable logistic regression model for each domain. Following this, all variables retaining significant results from the logistic regression per domain ($p<0.05$) were combined into an overall multivariable logistic regression model. Odds ratios (OR) with 95% confidence intervals (CIs) and p-values of variables that had significant results in the final model are reported in the results.

2.7.7.4 Pathogen type

Risk factors for infection with GPB vs GNB were assessed similarly to the methods above. However, only those with culture confirmed sepsis with confirmation of Gram-stain results from sites were included in the analyses. Univariate chi-square tests run on all variables separately. Any variables with significant results GNB vs GPB brought forward into a multivariate model per domain. Significant results per domain were then brought forwards to the final logistic regression model. Omnibus Tests of model coefficients were run on the model. Significant result demonstrated this model is better than the null. Hosmer and Lemeshow Test was run to demonstrate goodness of fit of the model, if not significant, results of the logistic

regression final model interpreted. Additionally, the rates of mortality in neonates infected with GPB were compared against those infected with GNB.

Chi-square tests were used to assess potential risk factors for infection with MDR isolates, comparing those infected with MDR isolates to those infected with isolates that were not MDR. However, only univariate chi-square tests were performed without carrying out a multivariate logistic model, due to the lower sample number as only neonates in the admissions cohort with isolates that had both WGS and MIC data could be included could be included.

Chapter 3: Neonatal sepsis in the BARNARDS study: incidence and aetiology of sepsis.

3.1 Introduction

There is a lack of data from LMICs regarding the burden of neonatal sepsis and aetiology of associated pathogens, despite having the greatest burden (Fleischmann *et al*., 2021). Although there is still a sparsity of data, multiple studies have been carried out in recent years to investigate the rates and aetiology of neonatal sepsis across LMICs.

3.1.1 Rates of sepsis LMICs

A study by Thaver and Zaidi (2009) calculated the rate of sepsis from reviewing 32 studies from a range of developing countries to be 170 per 1,000 live births, with a rate of 5.5 per 1,000 live births for culture confirmed sepsis. A review from Fleischmann *et al*. (2021) calculated global rate of sepsis to be 28.2 per 1,000 live births, with an associated 17.6% mortality rate. However, these figures were predominantly based on studies from middle-income countries and only include two studies from LMICs out of 20.

Rates in Asia have been reported in multiple studies, across countries with variation in reported rates. One study found a rate of 7.1 per 1,000 live births in Kuala Lumpur (Lim *et al*., 1995). Rates of 95.4 CDS and only 1.6 CCS per 1,000 live births were reported in a study across Bangladesh, India and Pakistan (Saha *et al*., 2018). Another review that assessed 101 studies across South Asia, although only 15 studies included rates of sepsis per 1,000 births, mainly in studies from India and overall found neonatal sepsis incidence to be 15.8 per 1,000 live births of culture positive sepsis, associated with a 34.4% mortality rate (Chaurasia *et al*., 2019). Similar rates were found by Viswanathan *et al*. (2010) of 14.8 cases of culture confirmed neonatal sepsis per 1,000 live births at a tertiary care centre in Eastern India., although higher rates of 38 per 1,000 live births (Tallur *et al.*, 2000) were reported in another

study from Southwest India. The DeNIS study (Investigators of the Delhi Neonatal Infection study, 2016) gathered data from three sites in India and found slightly lower rates of 21.8 and 9.5 per 1,000 live births for CDS and CCS, respectively.

A study carried out in South Africa by Velaphi *et al.* (2019) reported a sepsis rate of 39.3 per 1,000 live births, with an associated 12.1% mortality rate. They found positive blood cultures in 8.0% of neonates with clinically diagnosed sepsis and confirmed cases were associated with a 17.2% mortality rate. A review by Seale *et al.* (2009) assessed rates of neonatal sepsis from a combination variety of studies across Africa, however only a small portion of these studies provided the context of rates of sepsis. Within this review, Ojukwu *et al* (2006) reported an incidence of 7.98 per 1,000 live births in Southeast Nigeria, similar to another study in Nigeria reporting 6.5 cases of sepsis per 1,000 live births (Airede, 1999). A study from Zimbabwe reported higher incidence of 21 cases per 1,000 live births (Nathoo *et al*., 1990).

While the previous studies provide some insight into the rates of neonatal sepsis in LMICs, these are mostly single site studies or reviews of multiple single site studies. These all had varied cohorts and methodology, were from varied regions with assorted microbiology facilities to confirm sepsis, therefore it is difficult from this data to extrapolate an average across a continent or country. However, they confirm that the burden of neonatal sepsis is greater in LMICs than in HICs.

3.1.2 Aetiology

A review by Zaidi *et al*. (2005) evaluated studies from Africa, Latin America, Caribbean, Middle east, and central, South and Southeast Asia. This review reported proportions of sepsis caused by Gram-positive bacteria to vary between 31% and 41.7% for a range of LMICs with Gram-negative isolates found to be most common. In this review, overall *Klebsiella* spp. (28.8%) was the most reported species causing sepsis followed by *S.*

aureus (16.3%) and *E. coli* (12.2%). Work undertaken in Iran (Movahedian *et al*., 2006), a middle-income country, reported *Pseudomonas aeruginosa* most commonly (36%), followed by CoNS (20.7%) and *Klebsiella* spp. (17.1%).

In the DeNIS study, *Acinetobacter* spp. was the most commonly found species (22%), followed by *Klebsiella* spp. (57%), CoNS (15%) and *E. coli* (14%) in neonatal centres in Delhi, India (Investigators of the Delhi Neonatal Infection study, 2016). The review by Chaurasia *et al.* (2019) found from the multiple studies that Gram-negative bacteria was the predominant cause of neonatal sepsis and a low occurrence of GBS. In this review, *Klebsiella* spp. was the most common pathogen in both hospital and community settings (23% and 35%, respectively), *S. aureus* was the second main cause in hospital settings (20%) and third main cause in community settings (12%) with *E. coli* as the third most common cause in hospital settings (14%) and second in the community (15%).

A study carried out in Egypt found CoNS to be the leading cause of sepsis followed by *Klebsiella* spp. (Shehab El-Din *et al*., 2015). In a tertiary unit in South Africa, Gram-positive pathogens were found to cause the majority of sepsis cases (68.7%), with CoNS reported as the leading pathogen (53.5%), followed by *K. pneumoniae* (11.6%). However, as previously mentioned CoNS is sometimes treated as a contaminant, in which case the proportion of true GPB pathogens would not have been as high.

3.1.3 Aims and objectives

There are many gaps in data regarding the burden of neonatal sepsis. The aim of this chapter was to assess rates of neonatal sepsis across BARNARDS sites. To achieve this, rates of neonates with sepsis were calculated per 1,000 live births for inborn neonates from all those enrolled. Figures were calculated for all those diagnosed with sepsis and for neonates

with culture confirmed sepsis, as there is some discrepancy in previous studies which group to define as ‘sepsis’.

The second aim was to assess the rates of mortality in these cohorts. To do this, outcome data was assessed, and mortality rates were calculated, again from neonates with CDS, CCS and those without sepsis.

Lastly, aetiology of sepsis was to be investigated due to the lack of reliable, standardised microbiology data from LMICs. For this, whole genome sequencing was carried out to define common sepsis causing pathogens.

We hypothesise that rates of neonatal sepsis and associated mortality will be higher than reported by the current literature as believe this is currently underestimated due to a lack of adequate microbiology facilities at many sites to confirm diagnoses and unregulated reporting systems.

3.2 Results

3.2.1 BARNARDS enrolment and numbers of sepsis

Throughout the BARNARDS enrolment period, 35,016 mothers were enrolled in the study across 12 sites in seven countries. Some mothers had multiple births (twins or triplets); therefore, the total number of neonates enrolled onto the study was 36,285. Enrolments across the 12 sites varied, with highest overall enrolment numbers in PP, Pakistan (7,945) and NK, Kano (7,319). PC, Pakistan has the lowest enrolment (504), followed by IN (1,160), which both joined BARNARDS later than other sites, alongside ESS (Table 3.2).

Overall, 9,874 neonates enrolled were clinically diagnosed with sepsis. Overall rates of sepsis were not calculated, as this included both inborn and outborn neonates which were only enrolled presenting at clinical sites with CDS, which would skew the rates. Culturally confirmed sepsis rates varied by sites, with cases confirmed from those with suspected sepsis ranging from 5.57% in BK to 43.43% in ESS. A total of 2,483 cases of culturally confirmed sepsis were reported overall, accounting for 25.15% of those with a clinical diagnosis (Table 3.1).

Table 3. 1. Overall numbers of babies enrolled in BARNARDS per site with total numbers of suspected per site and numbers of confirmed sepsis (CCS), with percentages of CCS from those with suspected sepsis.

Site	Total neonates enrolled	Clinically diagnosed sepsis	Culturally Confirmed (% of clinically diagnosed)
BC	1,772	1,286	192 (14.93%)
BK	1,707	341	19 (5.57%)
ESS	4,828	1,020	443 (43.43%)
IN	1,160	72	21 (29.17%)
NK	7,319	1,640	315 (19.21%)
NN	1,902	648	228 (14.77%)
NW	2,359	189	62 (32.80%)
PC	504	124	37 (29.84%)
PP	7,945	2,900	767 (26.45%)
RU	1,222	340	65 (19.12%)
RK	2,255	703	240 (34.14%)
ZAT	3,312	611	94 (15.38%)
Total	**36,285**	**9,874**	**2,483 (25.15%)**

Of the 9,874 neonates clinically diagnosed with sepsis, 5,726 were inborn neonates and 3,325 admission neonates, with 823 neonates with clinically diagnosed sepsis not having a birth cohort confirmed. The BARNARDS protocol set out to enrol all inborn neonates and only enrol admission neonates if they presented to hospital sites to suspected sepsis. However, as seen in Table 3.2, 31 enrolments in admission neonates occurred without reported clinically diagnosed sepsis, mainly in NK, presumably due to a misunderstanding of the protocol.

The majority of neonates enrolled were confirmed as born in the hospital sites (31,092 neonates from 30,040 mothers). A further 4,159 neonates were enrolled as admission patients who arrived at the hospital sites presenting with clinical symptoms of sepsis. No data on cohort was available for 1,034 neonates (Table 3.2). All sites had both inborn and non-inborn

admissions patients enrolled with a much higher rate of inborn patients at all sites except for BC where over half of enrolments comprised of confirmed admissions neonates.

Table 3. 2. Neonatal enrolment split by birth cohort (inborn/admission), in addition to inborn/admission cases of clinically diagnosed and culturally confirmed sepsis. 'Missing cohort' refers to neonates where cohort could not be confirmed from data collected.

Site	Neonates enrolled			Clinically diagnosed sepsis			Culturally confirmed sepsis		
	Inborn	Admission	Missing cohort	Inborn	Admission	Missing cohort	Inborn	Admission	Missing cohort
BC	563	986	223	126	955	205	37	118	37
BK	1,469	238	0	107	234	0	4	14	1
ESS	4,187	342	299	479	300	241	183	153	107
IN	1,126	32	2	44	27	1	8	13	0
NK	5,744	1,475	100	582	976	82	94	200	21
NN	1,591	275	36	348	268	32	101	103	24
NW	2,257	87	15	114	67	8	31	27	4
PC	435	68	1	113	10	1	34	3	0
PP	7,206	415	324	2,369	293	238	604	101	62
RU	1,189	28	5	310	26	4	50	8	7
RK	2,074	172	9	541	155	7	149	61	30
ZAT	3,251	41	20	593	14	4	77	5	12
Total	**31,092**	**4,159**	**1,034**	**5,726**	**3,325**	**823**	**1,372**	**806**	**305**

3.2.1.1 Inborn neonates

Clinically diagnosed sepsis (CDS) occurred at a rate of 184.2 per 1,000 live births for inborn neonates, with variation between sites, ranging from 39.1 per 1,000 lives births in IN and 50.5 per 1,000 live births in NW, compared to 328.8 per 1,000 live births in PP (Table 3.4). Despite the large variation between sites, cases of diagnosed sepsis was similar between sites in Asia and those in Africa with 184.85 cases of clinically diagnosed sepsis in inborn neonates per 1,000 live births in Asia and 169.85 cases of clinically diagnosed sepsis in inborn neonates from sites in Africa (Table 3.3). CDS was confirmed with CCS in 1,372/

5,726 (23.96%) neonates with CDS. Rates were not estimated for admission neonates due to a lack of known context of outborn births.

Table 3. 3. Numbers of inborn neonates enrolled and rates of clinically diagnosed sepsis and culturally confirmed sepsis per 1,000 live births enrolled in the study, per site.

Site	**Inborn neonates enrolled**	**Inborn neonates clinically diagnosed with sepsis**	**Clinically diagnosed sepsis from enrolled inborn neonates per 1,000 live births enrolled**	**Inborn neonates culturally confirmed sepsis**	**Culturally confirmed sepsis in inborn neonates per 1,000 live births enrolled**
BC	563	126	223.80	37	65.72
BK	1,469	107	72.84	4	2.72
ESS	4,187	479	114.40	183	43.71
IN	1,126	44	39.07	8	7.10
NK	5,744	582	101.32	94	16.36
NN	1,591	348	218.73	101	63.48
NW	2,257	114	50.51	31	13.74
PC	435	113	259.77	34	78.16
PP	7,206	2,369	328.75	604	83.82
RU	1,189	310	260.72	50	42.05
RK	2,074	541	260.85	149	71.84
ZAT	3,251	593	182.41	77	23.69
Total	**31,092**	**5,726**	**184.16**	**1,372**	**44.13**

Within the inborn cohort, 4,329/31,092 (13.92%) neonates were recorded as premature, with 2,092 (48.33%) of these with CDS. Lower rates of 3,240/23,621 (13.72%) of inborn neonates born on time had CDS and similar rate of 222/1,598 (13.89%) of inborn neonates born late. Furthermore, of those with CDS, 543/2,092 (12.54%) premature neonates had culture confirmed sepsis (CCS), compared to 790/3,240 (24.32%) of inborn neonates born on time and 44/222 (19.82%) neonates with CDS born late. Of the pre-term inborn neonates with a date recorded for CDS, 1431/1851 (77.3%) were diagnosed within 48 hours

of hospital admission as were 2096/2,855 (73.20%) of inborn neonates born at term and 165/222 (74.32%) born late.

Clinical sepsis was diagnosed within 72 hours (EOS) for 4,103/5,726 (71.66%) inborn neonates with CDS and 968/5,726 (16.91%) reported as LOS, and 655 neonates with no data regarding time of diagnosis. Inborns with EOS composed of 2,273/4,103 (55.40%) born on time; 1,513/4,103 (36.88%) premature; 161/4,103 (3.92%) born late and 156 neonates with no time data. Inborn LOS consisted of 582/968 (60.12%) neonates born to term, 338/968 (34.92%) premature, 42/968 (4.34%) late and six with no data.

3.2.1.2 Admissions neonates

Of those reported as admissions neonates (n=4,159), 3,325 had CDS. Of these neonates with CDS, 2,617 (78.71%) were reported as premature, 454 (13.65%) reported as born on time and 217 (6.53%) as born late. Of the 3,325 with CDS, 806 (24.24%) had CCS, similar to the rate of CCS from CDS in inborn neonates.

Clinical sepsis was diagnosed within 72 hours for 813/3,325 (24.45%) admissions neonates, with a higher proportion of LOS (n= 2,358/3,325, 70.92%) than seen for inborn neonates and 154 with no data. Admission neonates with EOS composed of 556/813 (68.39%) neonates born to term; 189/813 (23.25%) born prematurely; 57 (7.01%) born after full term and 11 with no data. Those with LOS composed of 1,945/2,358 (84.49%) neonates born to term; 237/2,358 (10.05%) born prematurely; 152 (6.45%) born after full term and 24 with no data.

3.2.2 Mortality rates

Overall, 1,273 of the enrolled neonates were reported as deceased during the study, of which 875 had recorded CDS and 300 had culturally confirmed sepsis. Of the 1,273 neonates reported as deceased, 881 were recorded as inborn, 307 as admissions and 85 without a defined birth cohort. Mortality rates were higher for neonates with CDS compared to all

neonates enrolled. This was true for all sites, except BC and NW. Mortality rates were then again higher for neonates with CCS compared to those with CDS, the case for all sites except for BK and RU (Table 3.4).

Table 3. 4. Table showing reported mortality rates per site for all neonates enrolled; those with clinically diagnosed sepsis (CDS) and those with culturally confirmed sepsis (CCS). Percentages (%) are also provided with relevant denominators (all neonates; neonates with CDS only; and neonates with CCS only, respectively) to allow comparison between groups.

Site	**Neonates enrolled**	**Reported mortality overall (%)**	**Mortality of those with CDS (%)**	**Mortality of those with CCS (%)**
BC	1,772	65 (3.67%)	46 (3.58%)	14 (7.29%)
BK	1,707	49 (2.87%)	20 (5.87%)	1 (5.26%)
ESS	4,828	184 (3.81%)	131 (12.84%)	67 (15.12%)
IN	1,160	24 (2.07%)	7 (9.72%)	4 (19.05%)
NK	7,319	200 (2.73%)	119 (7.26%)	31 (9.84%)
NN	1,902	137 (7.20%)	116 (17.90%)	45 (19.74%)
NW	2,359	45 (1.91%)	20 (1.58%)	5 (8.06%)
PC	504	32 (6.35%)	22 (17.74%)	11 (29.73%)
PP	7,945	390 (4.91%)	298 (10.28%)	89 (11.60%)
RU	1,222	44 (3.60%)	34 (10.00%)	6 (9.23%)
RK	2,255	25 (1.11%)	22 (3.13%)	9 (3.75%)
ZAT	3,312	78 (2.36%)	40 (6.55%)	18 (19.15%)
Total	**36,285**	**1,273 (3.51%)**	**875 (8.86%)**	**300 (12.08%)**

There were 2,756 premature neonates with CDS overall, with 420 (15.24%) with reported mortality, compared to 409/6,436 (6.35%) of those born at term and 31/470 (6.60%) born late. Of these premature neonates with CDS, 787 had CCS, of which 156 (19.82%) were reported as deceased, compared to with 134/1,539 (8.71%) reported deceased with CCS born at term and 6/91 (6.19%) born late.

Of all neonates with CDS. 5,557/9,874 has EOS, of which 539/5,557 (9.70%) were reported as deceased, compared to 253/3,259 (7.76%) of neonates with LOS reported as deceased. Of the neonates with CCS, 1,429 had EOS, of which 169 (11.83%) were reported as deceased compared to 102/804 (12.69%) with LOS.

3.2.3 Overview of sepsis isolates

Isolates from cases of culturally confirmed sepsis were sent to CU for further analysis, where whole genome sequencing was carried out in addition to minimum inhibitory concentration (MIC) analysis to a range of antibiotics.

Figure 3.8. Distribution of isolates obtained during BARNARDS, with number of isolates that were sent to Cardiff University and underwent agar dilution to test minimum inhibitory concentrations (MIC) and whole genome sequencing (WGS) analyses. Work carried out at Cardiff University highlighted in red outlined boxes. GNB=Gram-negative bacteria; GPB=Gram-positive bacteria.

As seen in Figure 3.1, 2,701 isolates were obtained from 2,483 positive blood cultures. This composed of 1,266 Gram positive bacteria, 1,038 Gram-negative bacteria, 22 fungi, 294 unknown taxonomy and 81 isolates deemed as contaminants.

3.2.3.1 Gram stain results

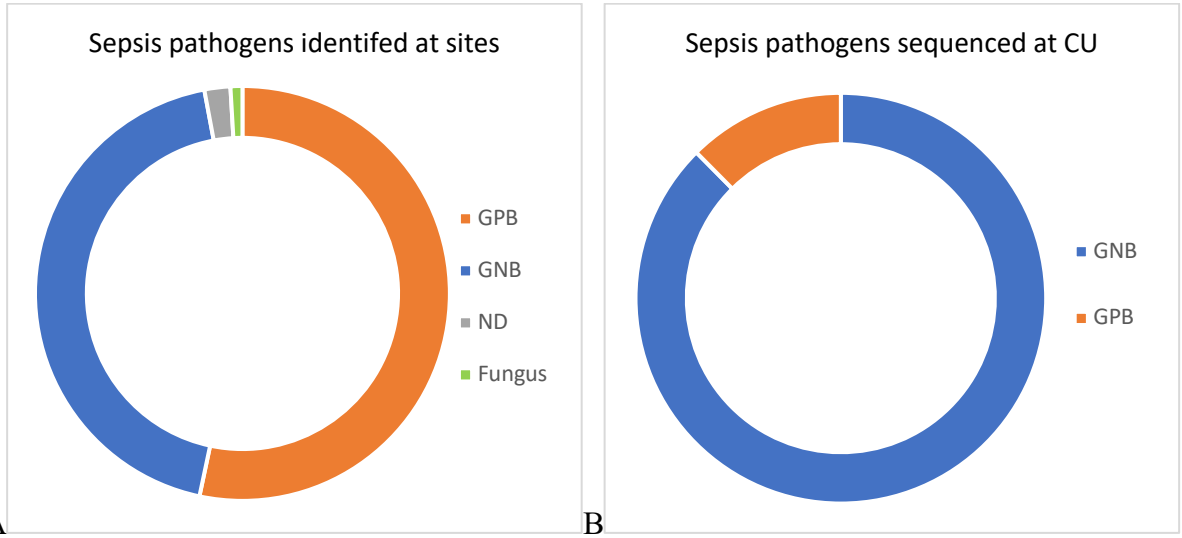
Gram stains were carried out at clinical sites for all bacterial isolates from positive isolates. Of the bacterial isolates tested (n=2,304), 1,266 were found to be Gram-positive and 1,038 were found to be Gram-negative. Most sites had a slightly higher number of Gram-positive isolates, with NK, NN, NW, RK, RU and ZAT having much higher numbers of GPB. Whereas ESS had slightly higher rates of GNB and BC, BK and IN had much higher rates of GNB compared to GPB (Table 3.5)

Table 3. 5. Number of sepsis bacterial isolates identified as Gram-negative (GNB) or Gram-positive bacteria (GPB) by the Gram stain performed at clinical sites and percentage (%) of each per site.

Site	**GNB (%)**	**GPB (%)**
BC	170 (87.18%)	25 (12.82%)
BK	14 (73.68%)	5 (26.32%)
ESS	150 (51.55%)	141 (48.45%)
IN	14 (82.35%)	3 (17.65%)
NK	77 (29.62%)	183 (70.38%)
NN	92 (40.17%)	137 (59.83%)
NW	7 (12.73%)	48 (87.27%)
PC	20 (41.67%)	28 (58.33%)
PP	375 (46.76%)	427 (53.24%)
RU	22 (38.60%)	35 (61.40%)
RK	58 (26.48%)	161 (73.52%)
ZAT	39 (34.82%)	73 (65.18%)
Total	**1,038 (45.1%)**	**1,266 (54.9%)**

3.2.3.2 Isolates analysed at Cardiff University

Gram-positive isolates were originally considered outside the remit of the original BARNARDS study as the study was originally focusing on AMR in GNB, but these were later added into the study, due to the large proportion of sepsis cases caused by GPB (Figure 3.2). Only Gram-positive isolates that had been kept by sites were sent retrospectively later therefore only a subset were received by Cardiff University and processed for further analysis, therefore results are provided for a lower number of GPB. Furthermore, some isolates lost viability and others were extensively mixed when cultured at Cardiff University and therefore the true sepsis causing pathogen could not be defined. Of the isolates received, resistance pattern profiles for 146 Gram-positive isolates were analysed, of which 130 were sequenced. A further 16 GPB isolates had no WGS data (all non-aureus Staphylococci), but species were confirmed via Matrix Assisted Laser Desorption Ionization-Time of Flight Mass spectrometry (MALDI-ToF-MS) identification. The GPB received at CU with WGS data, included 100 *S. aureus*, 8 *S. epidermis*, 9 *S. haemolyticus* and 14 *S. sciuri.*



A B

Figure 3.9. A. Proportions of sepsis pathogens found to be caused by Gram-positive bacteria (GPB) (n=1,266), Gram-negative bacteria (GNB) (n=1,038), fungus (n=22) and those for which no data (ND) was available (n=48) at the sites, using Gram stain. B. Sepsis pathogens sent to Cardiff University (CU) that were processed for WGS included a smaller proportion of GPB (n=130) and GNB (n=916).

3.2.3.3 Common species causing biological sepsis in BARNARDS

Isolates received at Cardiff University were sequenced using the short read Illumina MiSeq platform and 74 distinct species of bacteria were found to cause sepsis. The most common bacteria found were *K. pneumoniae* (n=258), *S. marcescens* (n=152), *K. michiganensis* (n=117), *S. aureus* (n=100) and *E. coli* (n=75) (Figure 3.3).

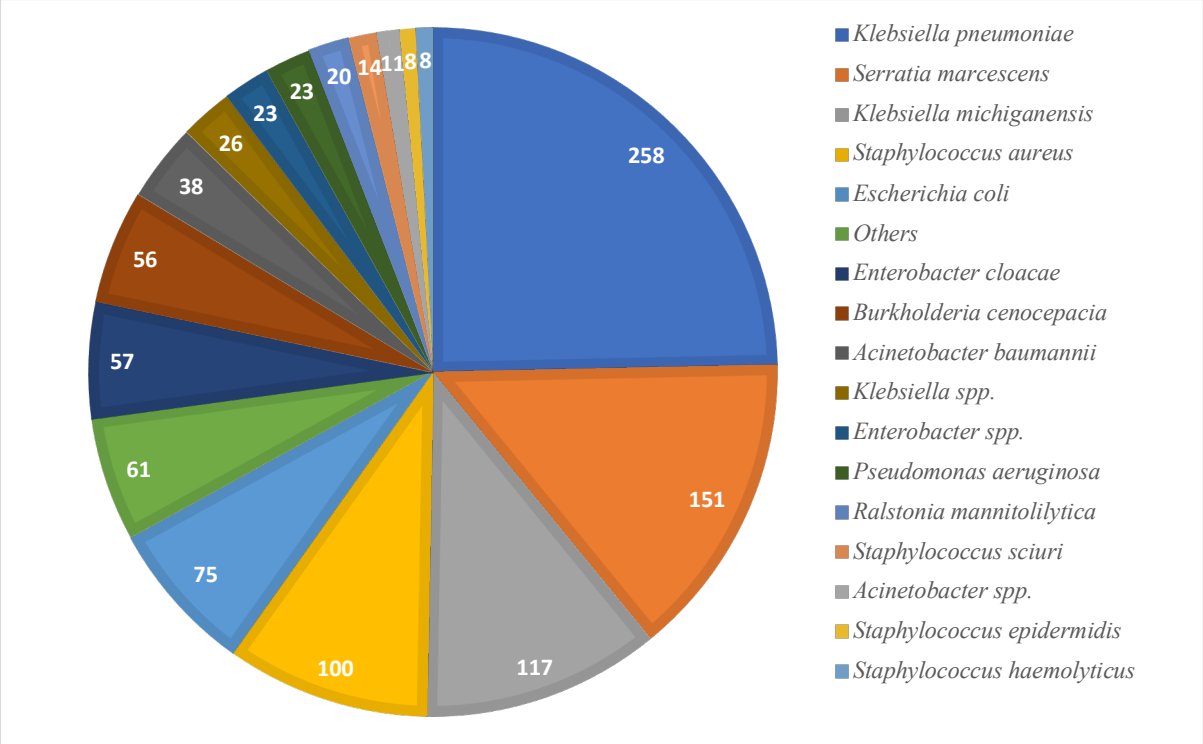


Figure 3.10 Most common sepsis pathogens with whole sequencing data at Cardiff University, with numbers for each species analysed included in white text.

The following results are based only on those isolates characterised at CU by WGS with confirmed species ID and susceptibility profiling (n=883). The top ten Gram-negative aetiological agents of sepsis were shown to cause 783/883 (88.67%) of Gram-negative sepsis cases (Table 3.6).

Table 3. 6. Top ten species causing sepsis across all BARNARDS clinical sites

Species	Number
Klebsiella pneumoniae	253
Serratia marcescens	149
Klebsiella michiganensis	116
Escherichia coli	74
Burkholderia cenocepacia	55
Enterobacter cloacae	46
Acinetobacter baumannii	35
Pseudomonas aeruginosa	21
Ralstonia mannitolilytica	17
Enterobacter hormaechei	17

The top five sepsis causing pathogens included *K. pneumoniae* (n=253), *S. marcescens* (n=149), *K. michiganensis* (n=116), *S. aureus* (n=101), *E. coli* (n=74). *Klebsiella* sp. caused 401 cases of sepsis overall. The most common sepsis causing pathogen: *K. pneumoniae* (n=258) was found in all sites with 17 BC; 2 BK; 95 ESS; 5 IN; 16 NK; 37 NN; 4 NW; 2 PC; 42 PP; 15 RK; 7 RU; 16 ZAT.

K. michiganensis was found to cause 117 cases of neonatal sepsis overall with four in Ethiopia; four in PC; 109 in PP. *Serratia* sp. was found to cause 150 cases of neonatal sepsis, of which *S. marcescens* was responsible for 149 of these cases, with 117 BC; 4 BK; 3 NN; 16 PP; 1 RK; 1 RU; 9 ZAT. *E. coli* caused 75 cases of neonatal sepsis across the BARNARDS sites. This was present as a sepsis causing species in all BARNARDS countries with 3 BC; 0 BK; 11 Ethiopia; 2 IN; 15 NK; 7 NN; 1 NW; 3 PC; 10 PP; 15 RK; 2 RU; 6 ZAT.

Regardless of a retrospective inclusion, *S. aureus* was also found to be within the top five causes of sepsis. *K. pneumoniae, S. aureus* and *E. coli* were found to be the top three causing agents of neonatal sepsis overall when outbreaks were disregarded. These three species occurred in all BARNARDS sites.

Africa and Asia had slightly different common sepsis pathogens. The most common species in Africa was *K. pneumoniae* (n=190), *E. coli* (n=57) and *S. aureus* (n=45). Whereas in Asia, *S. marcescens* was the most common species (n=136), followed by *K. michiganensis* (n=113) and *K. pneumoniae* (n=68) (Figure 3.5). However, *K. michiganensis and S. marcescens* were only common in Pakistan and Bangladesh, respectively and were due to epidemiological cluster, in addition to high number of *K. pneumoniae* in Ethiopia and *Burkholderia cenocepacia* in Pakistan. When major outbreaks were not included, the most common species in Asia were *K. pneumoniae* (n=68), *S. aureus* (n=55) and *Acinetobacter baumannii* (n=21), and the top three species in Africa remained the same (*K. pneumoniae* (n=118), *E. coli* (n=57) and *S. aureus* (n=45).

K. pneumoniae was the leading cause of sepsis in all sites in Africa, except for NK, which included 17 *S. aureus* isolates and 16 *K. pneumoniae* and was joint leading species in ZAT, alongside *S. aureus*. *K. pneumoniae* was also a leading cause of sepsis in IN. However, the leading cause of sepsis in other sites in South Asia included *S. marcescens* in BC, *K. michiganensis* in PP, *S., aureus* plus *K. michiganensis* in PC and *Pseudomonas aeruginosa* in BK (Figure 3.4).

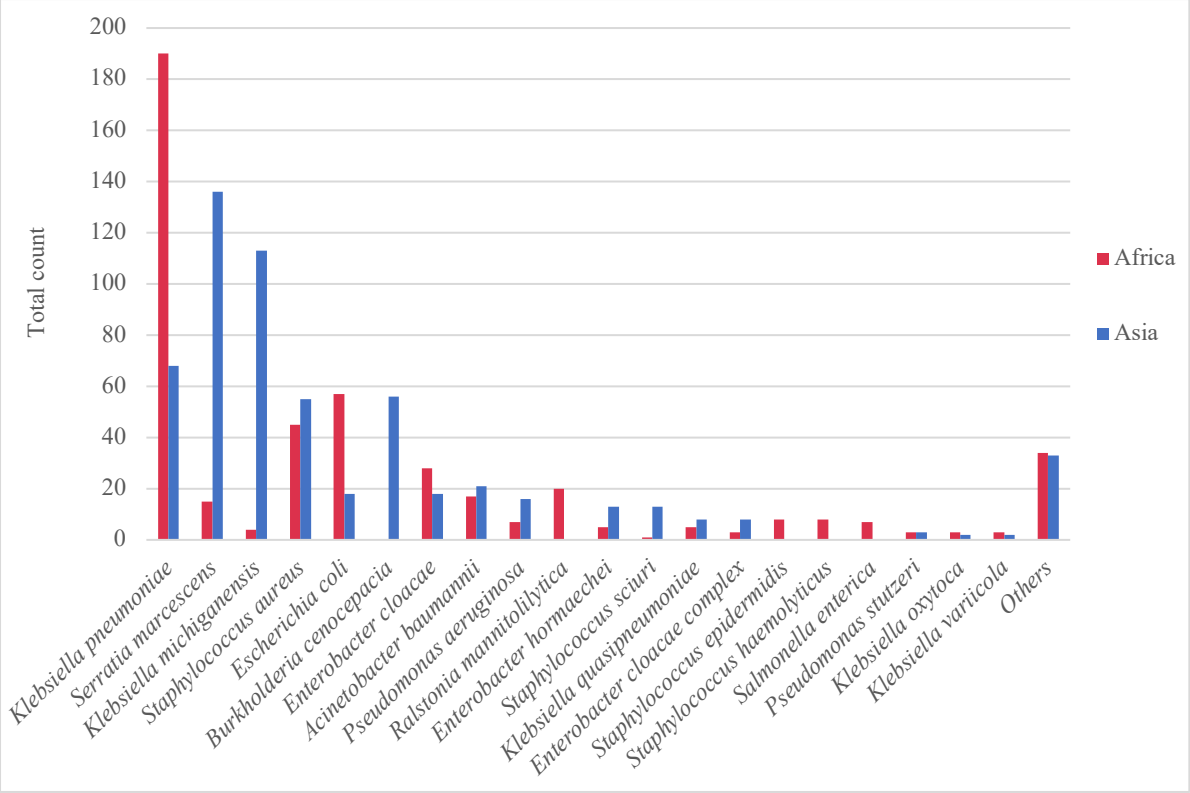


Figure 3.11. Most common species found from blood cultures found to cause five or more cases of sepsis overall, split by continent.

There was some variation when common sepsis causing species were investigated per site for isolates with WGS data, although *K. pneumoniae* was present as one of the three most common species (Table 3.6). Other species that frequently occurred in multiple sites included *E. coli*, *S. aureus*, E. cloacae *A. baumannii* and *S. marcescens* (Table 3.7).

Table 3.7. Most common species found as sepsis causing pathogens per site.

Site	Three most common sepsis causing pathogens
BC	*Serratia marcescens* (n=117) *Klebsiella pneumoniae* (n=17) *Staphylococcus aureus* (n=8)
BK	*Pseudomonas aeruginosa* (n=4) *Serratia marcescens* (n=3) *Klebsiella pneumoniae* (n=2), *Acinetobacter baumannii* (n=2)
ESS	*Klebsiella pneumoniae* (n=95) *Escherichia coli* (n=11) *Acinetobacter baumannii* (n=7)
IN	*Klebsiella pneumoniae* (n=5) *Acinetobacter baumannii* (n=3) *Escherichia coli* (n=2)
NK	*Staphylococcus aureus* (n=17) *Klebsiella pneumoniae* (n=16) *Escherichia coli* (n=15)
NN	*Klebsiella pneumoniae* (n=37) *Ralstonia mannitolilytica* (n=14) *Enterobacter cloacae* (n=9), *Staphylococcus aureus* (n=9)
NW	*Klebsiella pneumoniae* (n=4) *Staphylococcus haemolyticus* (n=3) *Escherichia coli* (n=1)
PC	*Staphylococcus aureus* (n=4), *Klebsiella michiganensis* (n=4) *Escherichia coli* (n=3) *Klebsiella pneumoniae* (n=2)
PP	*Klebsiella michiganensis* (n=109) *Burkholderia cenocepacia* (n=54) *Klebsiella pneumoniae* (n=42), *Staphylococcus aureus* (n=42)
RK	*Klebsiella pneumoniae* (n=38) *Escherichia coli* (n=22) *Staphylococcus aureus* (n=16)
RU	*Klebsiella pneumoniae* (n=7) *Enterobacter cloacae* (n=3) *Escherichia coli* (n=2), *Acinetobacter baumannii* (n=2), *Enterobacter hormaechei* (n=2)
ZAT	*Staphylococcus aureus* (n=16), *Klebsiella pneumoniae* (n=16) *Serratia marcescens* (n=9) *Escherichia coli* (n=6)

3.2.4 Breakdown of three most common species with WGS data

When species that were reported as common in neonatal sepsis were potentially caused outbreaks were discounted, *K. pneumoniae*, *E. coli* and *S. aureus* were noted as the three most common species, occurring across BARNARDS sites.

3.2.4.1 Klebsiella pneumoniae

From isolates with WGS and MIC data, 58 STs were found for *K. pneumoniae*, with one isolate of undetermined ST. The most common ST was ST35, which made up 39 isolates, followed by ST37 (30 isolates) and ST15 (27 isolates) (Figure 3.5).

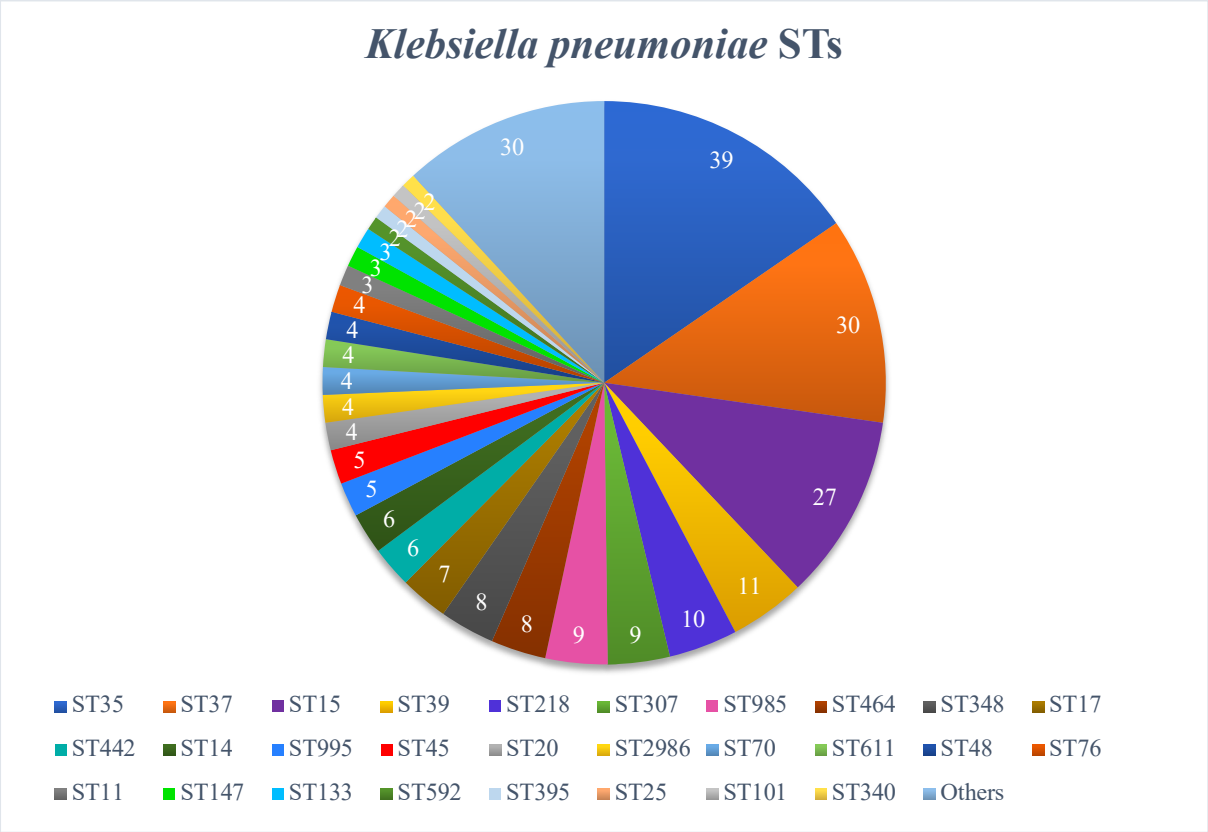


Figure 3.5. Sequence types (STs) found to cause sepsis in Klebsiella pneumoniae isolates. STs are coded by colour and numbers of each ST found are displayed in white text. All STs that only occurred once are grouped together as 'others'.

The most common sequence type, ST35, only occurred in Ethiopia, except for one isolate in RU and was the cause of an outbreak in Ethiopia. Two of these isolates were found in blood cultures taken in June 2017 and one from May and one from March 2017, but most

(n=37) of them were from blood cultures taken in between 11/10/2017 - 20/12/2017 and were found in both inborn and outborn neonates. The isolate from RU had O2v1 O antigen and KL113 K antigen, whereas all the ST35 isolates from ESS had O1v2 or O1/O2v2 O antigens and KL108 K antigens, although they had differing resistance genes. ST37, the second most common sequence type found was found in ESS with one isolate from ZAT and formed another outbreak, again found in both inborn and outborn neonates. Two isolates were found from blood cultures in August 2017, with the rest between 17/10/2017 - 28/11/2017. All ST37 from Ethiopia had 04 O antigen and KL15, whereas the ST37 isolate from ZAT had 03b O antigen and KL14 K antigen. ST37 isolates from ESS were deemed an outbreak, although again, the isolates had differing resistance genes present.

ST15 occurred mainly in South Asia, with one isolate from BC, two from IN, one from PC and 22 from PP, but also one isolate from Africa, in NN. ST39 was found mainly in Africa (two isolates from ESS, one from NK, four from NN, one RK and two ZAT) with one isolate from South Asia (BC).

3.2.4.2 Escherichia coli

E. coli accounted for 75 isolates with WGS data, 74 of which were also assessed for MICs, with 57 cases from sites in Africa and 18 from sites in Asia (Table 3.8). *E. coli* isolates were found to as a sepsis causing pathogen from all BARNARDS sites, except for BK and with highest numbers from in RK (15), NK (15), ES (10) and PP (10) (Table 4.18).

Table 3. 8. Numbers of E. coli isolates assessed from all BARNARDS sites.

Site	**Number *E. coli* isolates**
BC	3
ES	10
IN	2
NK	15
NN	7
NW	1
PC	3
PP	10
RK	15
RU	2
ZAT	6
Total	**74**

Results from WGS found 37 different sequence types from *E. coli* isolates analysed. ST10 was the most common (nine isolates), followed by ST131 (six isolates) and ST410 and ST167, both the sequence type for five isolates (Figure 3.5). ST10 was found in multiple sites, including one in ESS, three in NK, two from NN, one from RK and two from PP. ST131 was only found in Africa, with two isolates from ZAT, two from NN, one from NK and one from RK. ST410, on the other hand was only found in South Asia, including three from PC, one from PP and one isolate from IN. ST167 was found globally, with one isolate from BC, one from IN, one from NK, one from RK and one from RU.

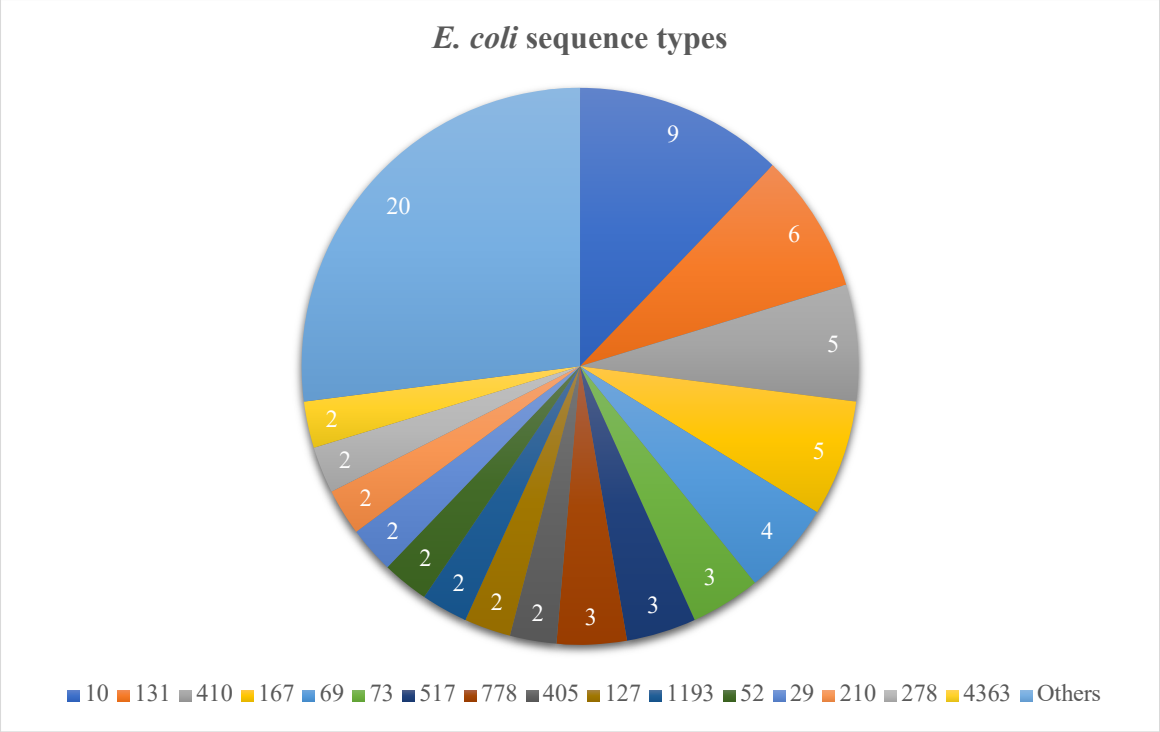


Figure 3.5. Sequence types (STs) found to cause sepsis in E. coli isolates. STs are coded by colour and numbers of each ST found are displayed in white text. All STs that only occurred once are grouped together as 'others'.

3.2.4.1 Staphylococcus aureus

Of the Gram-positive bacteria tested, 100/146 were *Staphylococcus aureus*, consisting of nine from BC, one from BK, two from ESS, 17 from NK, eight from NN, four from PC, 42 from PP, one from RK and 16 from ZAT. From *S. aureus* isolates with WGS data, 18 different STs were found, with ST6 as the most common (18 isolates), followed by ST5 (17), ST152 (15) and ST8 (13) (Figure 3.6). ST6 was mainly found in PP, with one isolate from BC, whereas ST5 was only found in Africa, with six isolates from NK and 11 from ZAT as was ST152, with six from NK, five from NN, three from ZAT and one from ESS. ST8 was then only found in isolates from Pakistan, with ten found in PP and three in PC.

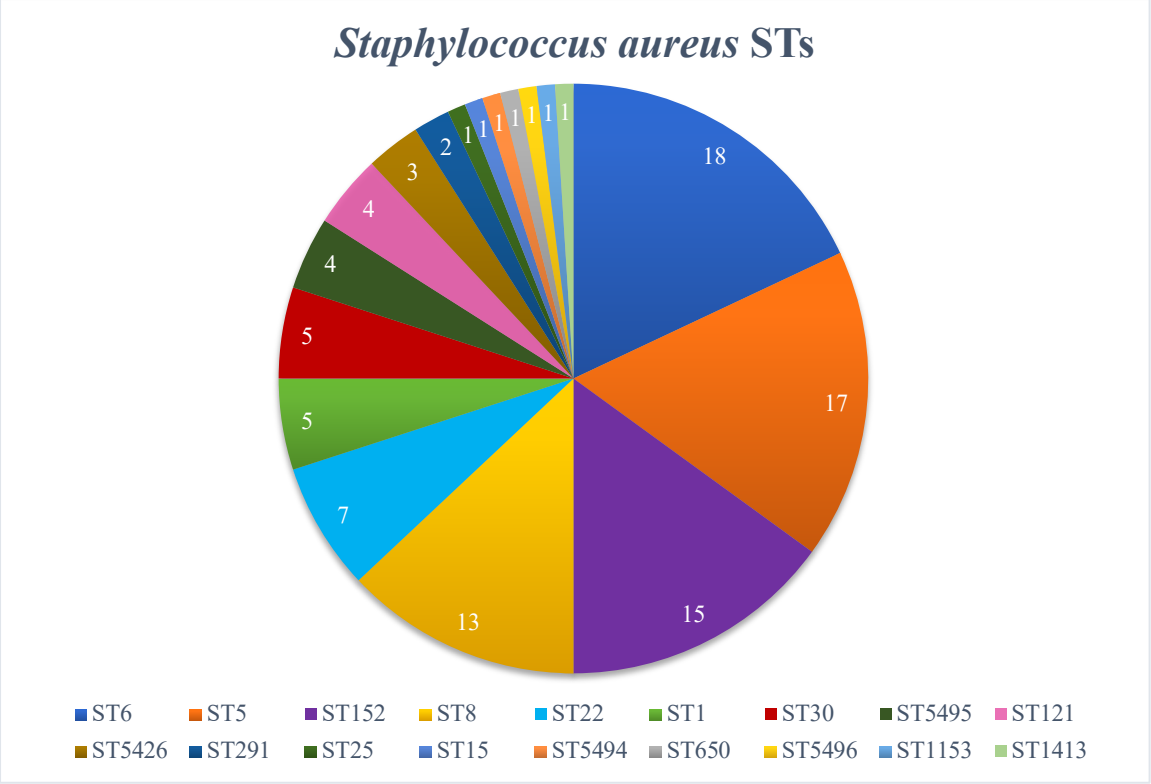


Figure 3.6. Sequence types (STs) of Staphylococcus aureus isolates from BARNARDS sites that were assessed via whole genome sequencing.

3.2.5 Potential outbreak species

Some of the bacterial species were commonly found in multiple sites, however, some bacteria, including *K. michiganensis* and *S. marcescens* were mainly found in one or few sites.

K. michiganensis was not found in all sites or all countries and this species was in the top five sepsis causing isolates due to high prevalence from a potential outbreak in PIMS, Pakistan where it was found to be the cause of 109 out of the 361 of all bacterial sepsis cases for which we have WGS data. Overall, *K. michiganensis* was found to cause 117 cases of sepsis, 112 of which were all ST180 with 109 of them from PP (and a further three from PC). Therefore, we assume this high number of *K. michiganensis* was caused by an outbreak at PP. Looking at the demographic data for the neonates infected with ST180 *K. michiganensis* isolates, 97/103 (94.2%) were isolates from neonates born in the hospital, further suggesting

these may have been nosocomial infections. Mortality related to these isolates was reported in 22/103 (21.36%) neonates for which we had outcome data.

K. pneumoniae accounted for 95 from 120 cases of sepsis with WGS data over the duration of BARNARDS, with 11 different sequence types found (Figure 3.7). High proportional numbers were found of ST35 (n=35) and ST37 (n=29), which were further investigated as potential outbreak strains.

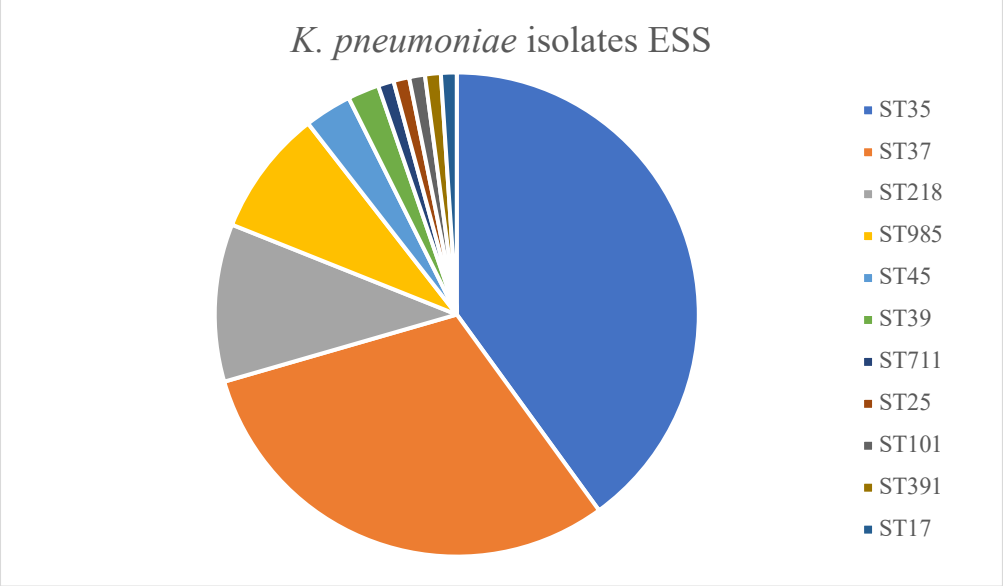


Figure 3.7 Sequence types found in K. pneumoniae isolates assessed from the site in Ethiopia (ESS) (n=95).

All ST35 *K. pneumoniae* isolates found in ESS were isolates between 29th March 2017 to 29th December 2017. ST37 isolates were found between 4th August 2017 to 1st December 2017, although no patterns were seen from the demographic data. Further to this, 11 different sequence types were found in PP, and a high amount of ST15 *K. pneumoniae* was found in PP (n=22/42 *K. pneumoniae* isolates). Of these, 20/22 were born in the hospital.

Serratia marcescens was found in most sites and was put into the top five global causes of sepsis due to an outbreak in BC Bangladesh, where it caused 117 of 169 cases of sepsis. However, no ST data was available with the WGS data and so we cannot determine

whether this would be a singular outbreak or several. Further analyses into outbreak isolates was outside the remit of this thesis and further analyses were carried out by colleagues.

Apart from one case in BC one in PC, *Burkholderia cenocepacia* was mainly found in PP, where it was responsible for 54 cases of neonatal sepsis, 50 of which were caused by ST1621 and appear to be due to an outbreak within the hospital and mainly affected inborn neonates between April 2016 to October 2017.

3.2.5.1 Overview of sepsis causing isolates per cohort

Numbers of inborn and confirmed outborn neonates enrolled in BARNARDS are detailed previously in Table 3.3, with a total of 31,092 inborn and 4,149 confirmed outborn neonates enrolled in addition to 1,034 with no cohort confirmed. Outborn neonates were enrolled with suspected sepsis, although, according to questionnaires only 79.95% of outborn neonates enrolled had clinically diagnosed sepsis (n=3,325), meaning that some outborn neonates were enrolled by mistake (usually enrolled alongside mother when admitted due to maternal postpartum haemorrhage). Within the inborn neonate cohort, 5,726 neonates had clinically diagnosed sepsis (18.42%). Of those with clinical diagnosis of sepsis, 806/3,325 (24.24%) outborn neonates had sepsis confirmed with positive blood culture, a similar percentage as those that were inborn (23.96%, n=1,372/5,726). WGS data was available for 560 isolates from inborn neonates, 115 isolates from neonates without a birth cohort confirmed and 371 neonates confirmed as outborn. The highest number of isolates assessed from inborn neonates were from PP (n=272) and the most outborn isolates were from BC (n=106) (Table 3.9).

Table 3.9. Number of isolates analysed by whole genome sequencing for inborn and outborn neonates at each BARNARDS site.

Site	Inborn	Outborn
BC	33	106
BK	5	10
ES	50	43
IN	4	9
NK	17	72
NN	50	50
NW	4	7
PC	15	2
PP	272	52
RK	41	15
RU	18	2
ZAT	51	3
Grand Total	**560**	**371**

The 560 isolates analysed with WGS from inborn neonates consisted of 44 species, which consisted of 79 GPB and 481 GNB. *Klebsiella pneumoniae* was the most common pathogen (n=131), followed by *Klebsiella michiganensis* (n=79) and *Staphylococcus aureus* (n=62) (Figure 3.8).

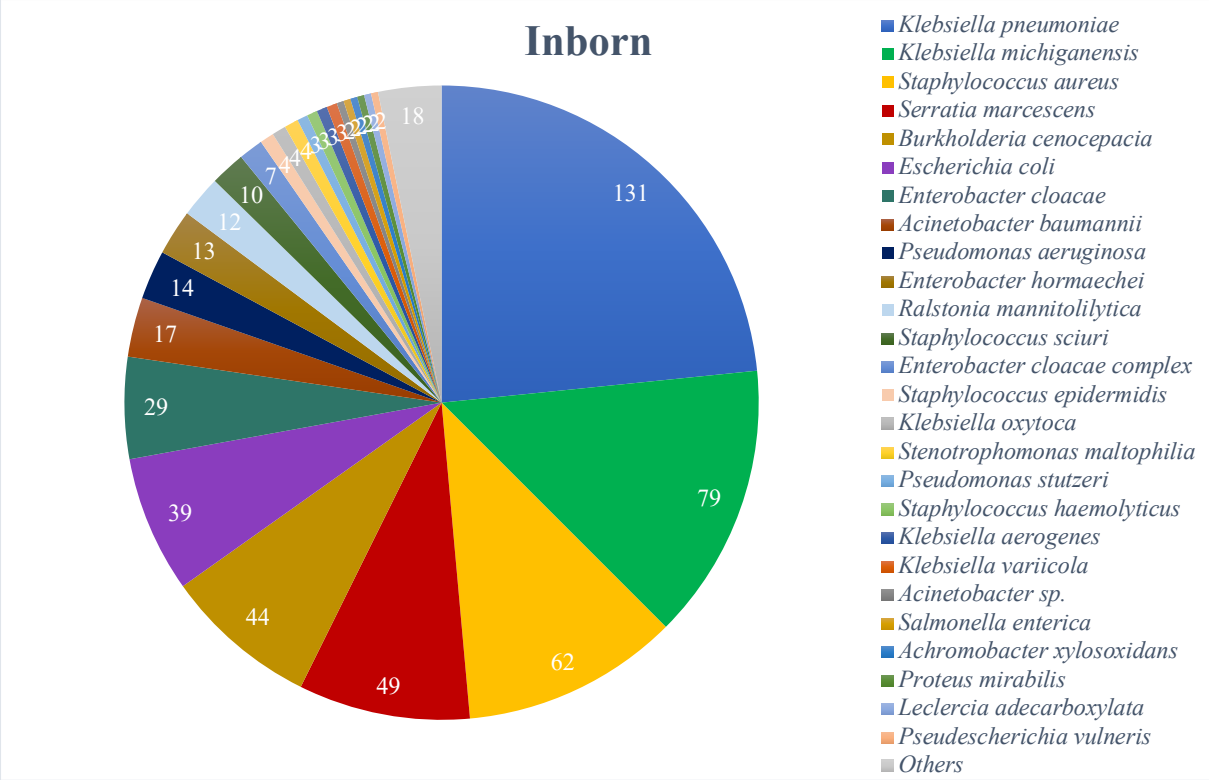


Figure 3.8. Sepsis causing pathogens in inborn neonates assessed via whole genome sequencing (n=560). Numbers in white on the pie chart show numbers of isolates found. Isolates that only occurred once were grouped into 'others'.

A total of 17 different species were found from isolates analysed via WGS for outborn neonates, which composed of 45 GPB and 326 GNB. As with inborn neonates, *Klebsiella pneumoniae* was found to be the most common organism (n=95), followed by *Serratia marcescens* (n=71), *E. coli* (n=33) and *Staphylococcus aureus* (n=33) (Figure 3.9).

Data on onset of sepsis was available for 459/560 cases of inborn sepsis with WGS data. Of these cases, 341/459 (74.29%) were deemed as having EOS (within 72 hours) and 118/459 (25.71%) had LOS. *Klebsiella pneumoniae* was the most common organisms for both EOS and LOS cases. The top five species, detailed in Table 3.10, were similar for EOS and LOS, but *E. coli* was sixth most common in EOS and *B. cenocepacia* was seventh most common in EOS.

Table 3. 10. Most common species causing early onset sepsis (EOS) and late onset sepsis (LOS) and counts within the inborn cohort.

EOS		LOS	
Species	**Count**	**Species**	**Count**
Klebsiella pneumoniae	73	*Klebsiella pneumoniae*	46
Klebsiella michiganensis	42	*Staphylococcus aureus*	17
Serratia marcescens	34	*Klebsiella michiganensis*	12
Staphylococcus aureus	33	*Escherichia coli*	8
Burkholderia cenocepacia	30	*Serratia marcescens*	7

Within the EOS inborn cohort, 153 were recorded as pre-term, 171 as born to full term, seven as late and ten with no data. Details for species causing EOS within the inborn cohort for pre-tern and term neonates are detailed in Table 3.11. Within the inborn cohort with LOS, 58 were categorised as premature, 41 as born to full term, two late and one with no data. Details of LOS pathogens found in premature and full-term inborn neonates are detailed in Table 3.12

Table 3. 11. Most common species causing early onset sepsis and counts for pre-term and term neonates within the inborn cohort.

Pre-term		Term	
Species	**Count**	**Species**	**Count**
Klebsiella pneumoniae	39	*Klebsiella pneumoniae*	23
Klebsiella michiganensis	20	*Serratia marcescens*	22
Burkholderia cenocepacia	16	*Klebsiella michiganensis*	21
Serratia marcescens	12	*Staphylococcus aureus*	21
Escherichia coli	11	*Burkholderia cenocepacia*	14

Table 3. 12. Most common species causing late onset sepsis and counts for pre-term and term neonates within the inborn cohort.

Pre-term		Term	
Species	**Count**	**Species**	**Count**
Klebsiella pneumoniae	26	*Klebsiella pneumoniae*	13
Staphylococcus aureus	7	*Staphylococcus aureus*	4
Klebsiella michiganensis	6	*Klebsiella michiganensis*	4
Serratia marcescens	4	*Enterobacter cloacae*	4
Escherichia coli	3	*Escherichia coli*	4

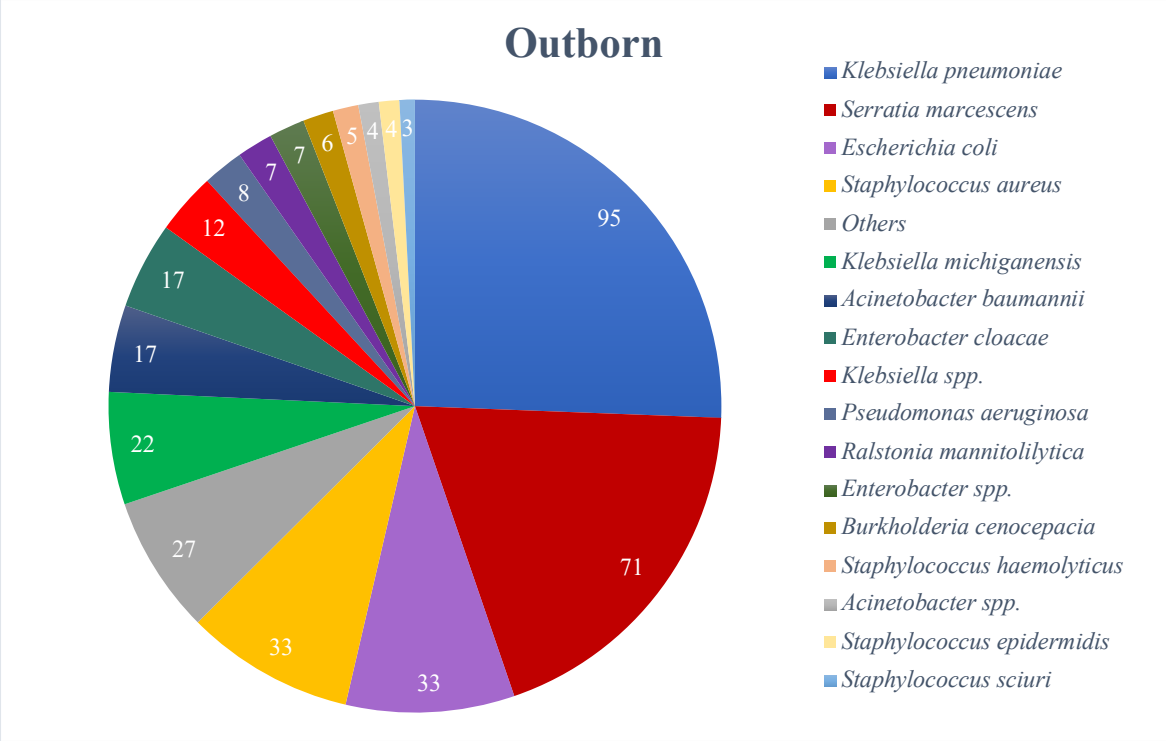


Figure 3.9. Sepsis causing pathogens in outborn neonates assessed via whole genome sequencing (n=3termLOS71). Numbers in white on the pie chart show numbers of isolates found.

As seen in Figure 3.8 and 3.9, a wider range of bacterial species were found in inborn neonates. Both cohorts had mainly the same most common ten species, although in slightly different orders, with *Burkholderia cenocepacia* and *K. michiganensis* being more common in inborn neonates and *S. marcescens* and *E. coli* were more commonly seen in outborn neonates.

3.3 Discussion

3.3.1 Burden of neonatal sepsis

Rates of CDS varied per site with between 39.07 and 328.75 cases in inborn neonates per 1,000 live births, with an average of 184.16 per 1,000 live births. Rates of CCS ranged from 2.72 to 83.82 cases per 1,000 live births, with an overall incidence of 44.13 per 1,000 live births. This was a higher range than found in the literature, which ranged from 1.6 to 39.3 CCS per 1,000 live births (Saha *et al*., 2018; Velaphi *et al*., 2019). A meta-analysis by Churasia *et al.* (2019) collated rates of culturally confirmed sepsis from studies in South Asia and found a rate of 15.8 per 1,000 live births, lower than rates found in this study with an average of 47.50 per 1,000 live births cases of culturally confirmed sepsis in inborn neonates from the sites in South Asia.

A large variation was seen in prevalence of sepsis across BARNARDS sites, presumably due to a multitude of reasons. Study size per site varied hugely per site and some sites had much lower numbers of neonates enrolled, particularly IN, BC, BK, PC and RU, which all had less than 1,500 neonates enrolled. There may have been variation of the level of diagnosis by different medical teams due to the vague symptoms of sepsis (Singh *et al.,* 2022). Although, we attempted to reduce this variance in BARNARDS with standardised definitions and diagnosis checklists, the study involved neonatologists that have plentiful experience diagnosing sepsis and so understandably may not have referred to the standardised checklist. Further variation of rates of neonatal sepsis could be due to differing rates of pre-term new-borns at varying sites, found to be higher in pre-term neonates (48.33% CDS) than those born at term (13.72% CDS), who are at increased risk of infection, due to immature immune systems (Collins *et al.*, 2018). However, lower birth weights and pre-term births, were associated with decreased rates of confirmed sepsis with only 12.54% of CDS cases confirmed with a positive blood culture in pre-term neonates compared to 24.38% of CDS

cases in term neonates, possibly due to difficulties obtaining sufficient blood from the neonate to confirm diagnosis. Busy hospitals and those with limited resources may discharge mothers quickly following birth and not have ample time to check the mother and neonate before symptoms arise, so could have reduced rates of CDS. Alternatively, quieter hospitals may have less sepsis due to a reduction in spread of infections and staff have more time to sterilise instruments properly. Culturally confirmed sepsis rates may vary between sites due to varied microbiology facilities, or a range of skillsets of the microbiology or haematologist staff, although this was reduced in BARNARDS due to standardised consumables and equipment. Uncontrolled discrepancies between sites make it difficult to compare countries or continents regarding the rate of sepsis. However, this standardised data across multiple sites provides advantageous insight into understanding variation of rates and burden of neonatal sepsis across LMICs and provides more reliable data than a collation of various single site studies from different research groups with diverse methodology or cohorts.

The total rate of culturally confirmed sepsis from those clinically diagnosed was 25.15% (n=2,483/9,874). Slightly lower rates of sepsis confirmed with blood culture compared to clinically diagnosed cases were found in a study by Jajoo *et al.* (2018), whereby 1,416 admission neonates enrolled were clinically diagnosed with sepsis, with confirmed culture positive in 13.1% of cases. Reasons for low CCS to CDS ratio could be due to: i) difficulty obtaining enough blood to obtain bacterial growth; or ii) vague symptoms of sepsis may lead to misdiagnosis; iii) There may be fastidious organisms present in a high number of cases. A study carried out in Switzerland found a rate of 19.5% of positive blood cultures from those with suspected sepsis of adult intensive care unit. Therefore, pathogens and the vague symptoms of sepsis may have a larger impact on this comparatively low positive rate compared to the number diagnosed with sepsis in addition to the low volumes of blood extracted from neonates causing large variance between CDS and CCS (Previsdomini *et al*.,

2012). Mortality was lower in those with CDS only compared to CCS, it could be that neonates diagnosed earlier have better outcomes, these may not have had positive blood cultures as the bacteria was at low levels within the blood, or the vague symptoms were associated with an illness associated with reduced rates of mortality, or if sepsis is caused by fastidious bacteria these could be associated reduced mortality.

3.3.1.1 Burden of neonatal sepsis per site

Rates for sepsis of inborn cohort are used for comparison with other studies. While this may skew the data for EOS, we cannot add cases of admissions sepsis to compare rates by assessing per 1,000 live birth results. There were 39.07 cases of CDS per 1,000 live births and 7.10 per 1,000 live births of CCS in India, similar to CDS rates found in the study in India by Tallur *et al*. (2000), although their study had much higher rates of CCS. However, the BARNARDS site in India was brought into the study for only a year of enrolment and had few cases of CCS sepsis during this time and numbers may not be high enough for suitable comparisons. In BARNARDS, CCS rates for Pakistan were 78.16 and 83.82 per 1,000 live births for PC and PP respectively. Another study in Pakistan found much lower prevalence of 5.6 cases of CCS per 1,000 live births (Bhutta and Yusuf, 1997), however, this was only based on 38 neonates and so not suitable for comparison.

Medugu *et al*. (2018) found a sepsis incidence rate of 18.2 from a review of literature, ranging from 7-55 per 1,000 live births, which appear to be based on CCS. This is in line with rates CCS found in BARNARDS from sites in Nigeria, which were slightly higher, ranging from 13.74 to 63.48 per 1,000 live births. Rates for CCS in Ethiopia were 43.71 per 1,000, higher than rates reported by Ghiorgis (1997).

Sepsis incidence rates could not be found from other studies in the literature from Rwanda, the studies found only referred to sepsis cases, without contextualisation of prevalence (Rogo *et al*., 2016; Ndayizeye *et al.,* 2019). Available data from South Africa

demonstrated an incidence rate of 39.3 CDS per 1,000 live births and 3.2 CCS per 1,000 live births from a public hospital in Soweto (Velaphi *et al.*, 2019). This is lower than found in from the BARNARDS site in South Africa, which had 182.4 CDS per 1,000 live births and 23.69 CCS per 1,000 live births. There were limited studies providing data on incidence from South Africa, with most studies focusing on only the sepsis cohort (Motara *et al*., 2005; Pillay *et al*., 2021)

3.3.1.2 Mortality rates

Lower rates of mortality were seen in neonates with CDS (8.86%) compared to those with CCS (12.08%), as supported by Phua *et al.,* (2013), whose study found a mortality rate of 35.9% in the culture negative group compared to 44.0% in the culture positive group, although these rates were higher than found in BARNARDS. Higher mortality rates were also estimated by the review from Fleischman *et al.* (2021), reporting an overall mortality rate of 17.6% of neonates with sepsis; by Chaurasia *et al.* (2019) who found 34.4% mortality of neonates with CCS; and in a study in South Africa of 12.1% in CDS, increasing to 17.2% in CCS. This conveys our underestimation of mortality in the BARNARDS study, which we are aware was a limitation of the study, as any neonate not previously reported as deceased and lost to follow-up during the 60-day period was recorded as 'alive until last follow-up' and categorised as 'not deceased' along with those confirmed as alive throughout the follow-up period.

3.3.2 Sepsis causing pathogens

GPB were estimated at sites to cause over half of all cases of confirmed sepsis. However, the BARNARDS study originally set out to assess the prevalence of Beta-lactamase genes within Gram-negative bacteria and so Gram-positive bacteria was originally outside the remit of the BARNARDS study and only GPB swabs available for retrospective

analysis were able to be included. This hugely skewed WGS data towards GNB and so we have a limited overall picture on sepsis causing pathogens. In HICs, neonatal sepsis tends to be caused more often by Gram-positive bacteria, with Group B streptococcus (GBS) and *Enterococcus* spp. acting as a leading cause of sepsis along with *E. coli* and *Staphylococcus aureus* (Hoffman *et al*., 2008; Simonsen *et al.*, 2014). GBS was not found in this study, although these results are skewed due to the original remit of GPB. There have been mixed findings in the literature regarding prevalence of GBS in LMICs, being reported in a study by Saha *et al.*, (2018) found in 6/102 (5.88%) of blood cultures and 4.8% of CCS in a study in South Africa (Velaphi *et al.,* 2019). However, most of the literature supports the low level of GBS found, reporting 0.17 cases per 1,000 live births (Kuruvilla *et al*., 1999), or none (Daoud *et al*., 1995). Species IDs from sites were not available for most of the GPB isolates that were not sent to site. It may be that a large majority of these could have been contaminants, the majority of which are Gram-positive organisms, most commonly Coagulase negative Staphylococci (Weinstein *et al*., 1997). However, this species and numerous other Gram-positive bacteria can also act as true pathogens and so the WGS dataset is incomplete, and more Gram-positive species may have been leading causes of sepsis.

Although, the top three species reported in this study with WGS data was similar to that found in other studies. This study found *Klebsiella pneumoniae*, *Staphylococcus aureus* and *E. coli* to be leading causes of neonatal sepsis with WGS data throughout different BARNARDS sites, similar to findings reported by Bangi and Devi (2014). *E. coli* and *S. aureus* have also been found to be common causes of bacteraemia in Thailand (Kanoksil *et al*., 2013). Other species that occurred in the top three pathogens per sites included *Pseudomonas aeruginosa* (BK) and *Enterobacter cloacae* complex (RU), both found commonly in a review by Hallmaier-Wacker (2022), *Acinetobacter baumannii* (BK, ESS, IN, RU), also found in literature to be a common cause in India (Viswanathan, 2010). *Ralstonia*

mannitolilytica (NN), rarely found in previous studies, but is emerging as an opportunistic pathogen due to its ability to survive in liquid media and hospital devices as has been found in NICUs previously (Souza *et al*., 2018; Ranjendran *et al.*, 2021). Similarly, *Burkholderia cenocepacia*, commonly found in PP is rarely reported as a sepsis pathogen in neonates but has been isolated from neonatal patients with sepsis previously (Patra *et al*., 2014). However, this was a more common bacteria, due to an outbreak in PP.

3.3.2.1 Other common species due to outbreaks

K. michiganensis was only one of the most common species in PC and PP, due to assumed outbreaks at these sites, as all isolates were from the same ST. *K. michiganensis* has previously been found to cause outbreaks in hospital settings. Chapman *et al.* (2020) found *K. michiganensis* to cause an outbreak as contaminated detergent bottles which ended once they had been removed. Although *K. pneumoniae* was common at all sites, we also believe there was an outbreak of *K. pneumoniae* at ESS, a species also found to commonly cause nosocomial outbreaks (Haller *et al.*, 2015; Zheng *et al*., 2016).

S. marcescens was the most common pathogen in BC, and also found in BK and ZAT but not in other sites. This was mainly a common pathogen overall only due to the high numbers in BC, where we believe an outbreak of *S. marcescens* occurred, although I did not have access to further data on this. Multiple studies have reported outbreaks of *S. marcescens* in hospital settings (Armarsy *et al*., 2020; Mendes and Casado, 2022; Montagnani *et al.*, 2015; Morillo *et al*., 2016; Vetter *et al*., 2016). Although, *S. marcescens* is also commonly found in the environment, associated with soil, water and plants, where it can enhance growth (Gyaneshwar *et al*., 2001) which Abreo and Altier (2019) found rarely cause nosocomial infections, which may be the reason why it was also a common pathogen in outborn neonates in Bangladesh.

B. cenocepacia was also only a common pathogen due to the high prevalence at PP, whereby most isolates were the same sequence type and likely an outbreak. The Centers for Disease and Control and Prevention (2010) include *B. cenocepacia* as a common organism in healthcare settings and has previously been associated with contaminated mouthwash and nasal spray. This species has been found to cause an outbreak in a tertiary care hospital in India amongst patients undergoing chemotherapy (Baul *et al*., 2018) and was found to cause nosocomial infections in a Turkish university hospital, mostly from patients in intensive care units (Dizbay *et al.,* 2009).

3.3.3 Conclusion

Wide variation in rates of sepsis was seen per site and therefore is difficult to extrapolate country averages. This demonstrates the limited application of the single site studies that make up the majority of data from LMICs, although this may be due to a smaller sample number. The range of CCS was higher than that found in previous studies, in accordance with hypotheses, which may be due to limited microbiology facilities or expertise in previous studies. Furthermore, rates of CCS were approximately 25% of those with CDS, demonstrating i) that it is difficult to obtain enough blood to obtain bacterial growth, or ii) Symptoms of sepsis are vague and can lead to misdiagnosis iii) There may be fastidious organisms. Reduced mortality was seen in neonates with CDS, not CCS, it may be that those diagnosed earlier have better outcomes, as these neonates may not have had positive blood cultures as the bacteria was at low levels within the blood. Reasons for reduced rates of CCS might signify that mortality related to neonatal sepsis may be underestimated. In this study, despite efforts to follow up with neonates for 60 days, many could not be traced and therefore mortality was underreported.

Aetiology of sepsis was similar to that found in previous studies, with around equal numbers of cases of neonatal sepsis caused by GPB and GNB. *K. pneumoniae*, *E. coli* and *S.*

aureus consistently prevalent across sites when outbreaks were not considered. Multiple outbreaks, however were witnessed throughout BARNARDS, including a large of *K. michiganensis* in Pakistan in addition to *B. cenocepacia*, *S. marcescens* in Bangladesh and *K. pneumoniae* in Ethiopia. The number of outbreaks seen demonstrate the importance of infection prevention and control (IPC) in hospital settings.

Chapter 4: Resistance profiles of sepsis causing pathogens found in BARNARDS

4.1 Introduction

As previously mentioned in chapter one, high AMR in pathogens causing neonatal sepsis has been reported in a range of studies across various LMIC settings (Li *et al*., 2019; Zaidi *et al*., 2005; Thaver *et al.*, 2009). High resistance against third generation cephalosporins was demonstrated by Li *et al*. (2019) in a range of countries in addition to resistance against carbapenems, which was particularly high in Bangladesh. The DeNIS study (2016), also reported high resistance from three sites in Delhi, India and found that two thirds of sepsis cases were caused by Gram-negative bacteria. This study reported high levels of multi-drug resistance in Gram-negative species tested, including 82% of the *Acinetobacter* spp. isolates, 54% of *Klebsiella* spp., 38% of *E. coli*, 19% of *Pseudomonas* spp. and 50% of *Enterobacter* spp. isolates. The high levels of MDR found in *Acinetobacter* spp. and *Klebsiella* spp. are concerning as these were the most common pathogens to cause sepsis during the study. They also noted methicillin resistance in 61% of CoNS and 38% of *S. aureus* isolates. Although not reported in the study, the high level of *A. baumannii* could potentially be due to an outbreak and associated nosocomial infections, as this species is commonly associated with hospital acquired infections (Garnacho-Montero *et al.,* 2005), which could also increase MDR, as detailed in chapter 3 (section 3.3.3) (Aslam, 2008).

Rates of MDR were found to be incredibly high in a study of 24 *K. pneumoniae* isolates, 96% of which were found to be MDR (Gajul *et al*., 2015). This was supported by Chaurasia *et al.* (2019), who's review noted that 50-70% of Gram-bacteria commonly causing neonatal sepsis in South Asia are MDR. This review also found high rates (at least 65% for GNB and at least 50% of GPB) of resistance against ampicillin, gentamicin,

cefotaxime and ceftazidime across common species in hospital settings. Resistance remained high in isolates from community settings for ampicillin but lowered for most other antibiotics.

A study carried out in a teaching hospital, Peshawar, Pakistan (Rahman *et al*., 2002) reported resistance for the most common species identified: *E. coli*, *S. aureus*, *Pseudomonas* sp., *Klebsiella* sp. and *Proteus* sp. Highest rates of ampicillin resistance was reported in *E. coli* (89%) and *Pseudomonas* sp. (86%) isolates. All species had high resistance towards gentamicin (from 70% in *S. aureus* to 84% in *Klebsiella* sp.). All species had at least 50% resistance against all the cephalosporins tested.

Resistance rates for third generation cephalosporins were around 65% in Enterobacterales and 85% in non-fermenters analysed from a tertiary unit in South Africa (Pillay *et al*., 2021). They found lower rates of MDR isolates (20%) than seen in the studies detailed above across Asia. They also reported rates of methicillin resistant staphylococci (MRSA), at 79%. This study found cases of neonatal sepsis to more often be caused by Gram-positive bacteria (468/681, 68.7%). However, CoNS was reported as the most common pathogen (n=363). If CoNS was removed from this analysis, Gram-positive bacteria would have caused only 105/318, 33% of cases.

A study from a teaching and referral hospital in Southeast Ethiopia that assessed 88 positive blood cultures also reported high levels of CoNS, making up 25% of cases. However, their study found high levels of MDR (72%) (Sorsa *et al*., 2019). Similarly, G/Eyesus *et al*. (2017) found 65% MDR isolates from 120 isolates, with 84.6% resistance against ampicillin, and around 56% against ceftriaxone but low levels of resistance against amikacin and gentamicin.

The above studies are vital for gaining insight into aetiology of sepsis in the countries they are carried out in. However, barring Li *et al.* (2019), the above were based on single sites with low sample numbers, mixed settings and varied methodologies. Furthermore, some

of the studies did not report rates of AMR for specific antibiotics (Dramowski *et al*., 2015) and the data provided was limited. There were more numerous studies from India, but less from other LMICs.

4.1.1 Aims

Within this chapter the aim was to assess the burden of AMR in neonatal sepsis across a range of LMICs. This aim will be achieved through:

- Assessment of the antibiotic minimum inhibitory concentrations of bacteria isolated from cases of neonatal sepsis across BARNARDS sites (2015-2018) via agar dilution.
- Establish associated resistance profiles.
- Compare to corresponding whole genome sequencing (WGS) data to determine the presence of antibiotic resistance genes (ARG) and determine how these may impact resistance.
- AMR in isolates from inborn neonates were also compared to admissions, looking into potential impact of nosocomial infections.

We hypothesise that there will be high levels of AMR as seen in the literature across all BARNARDS sites against a range of antibiotics commonly used in treatment.

4.2 Results

4.2.1 Gram-positive isolates

As previously described in chapter three, only a subset of GPB isolates were received by Cardiff University and processed for further analysis, therefore results are provided for a lower number of GPB. Of the isolates received, resistance pattern profiles for 146 Gram-positive isolates were analysed, of which 130 were sequenced, but species were confirmed via MALDI-TOF identification for the additional 16 isolates.

4.2.1.1 Gram-positive MICs

MICs were performed by agar dilution, as described in the methods section, for a total of 146 Gram-positive isolates, including 100 *S. aureus*, 18 *S. epidermis*, 11 *S. haemolyticus*, 15 *S. sciuri* and two *Enterococcus faecalis*. Growth of the Gram-positive bacteria tested, were mainly inhibited by the lowest concentrations tested for each antibiotic, except for azithromycin, where growth remained for many isolates for the highest concentration tested (8 µg/mL). All antibiotics tested had at least one isolate that demonstrated growth throughout all concentrations tested (Figure 4.1).

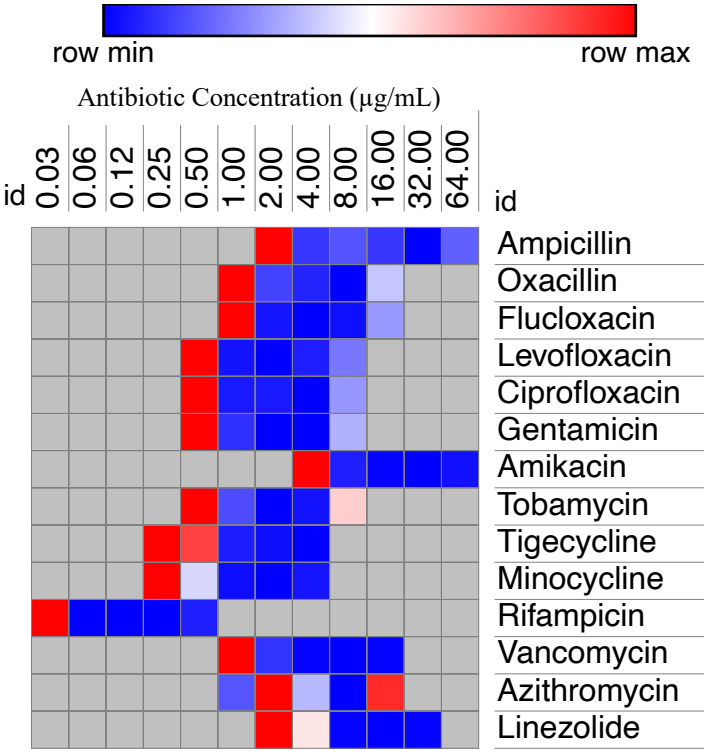


Figure 4.12. Minimum inhibitory concentration (MICs) from agar dilution testing for Staphylococcus spp. isolates tested (n=146). Isolates originated from BC (n=12), BK (n=1), ESS (n=2), NK(n=30), NN (n=20), NW (n=5), PC (n=4), PP (n=54), RK (n=1) and ZAT (n=17). This composes of 100 S. aureus, 18 S. epidermis, 15 S. sciuri, 11 S. haemolyticus and two E. faecalis. Grey squares show concentrations that were not tested. Numbers of isolates that were inhibited at a certain concentration are represented through the colour scale included above the heatmap, 'row min' blue demonstrates the lowest number of isolates with the associated MIC, whereas red shows the highest number of isolates with the associated MIC for each antibiotic. An additional concentration was added for each antibiotic two-fold above the highest concentration tested and all isolates with remaining growth at the highest concentration were included in this concentration. 'Row min' demonstrates the colour that will be shown for the lowest number of isolates with a certain MIC. 'Row max' demonstrates the colour that will be shown for the highest number of isolates with a certain MIC, with the gradient between showcasing decreasing numbers.

MIC_{90} values above the highest concentrations tested were seen for oxacillin, flucloxacin, levofloxacin, ciprofloxacin, gentamicin, tobramycin, and azithromycin. No MIC_{50} values for the Gram-positive isolates were above the maximum concentration tested for any antibiotics (Table 4.1). Oxacillin MIC >2 µg/mL infers methicillin resistance in *S. aureus*.

Table 4. 1 MIC_{50} results show the antibiotic minimum inhibitory concentration (MIC) preventing growth in 50% of isolates and MIC_{90} results show the MIC at which 90% of isolates had stopped growing. These are displayed below for all Gram-positive bacteria tested (n=146), composed of 100 S. aureus, 18 S. epidermis, 15 S. sciuri, 11 S. haemolyticus and two E. faecalis.

Antibiotic	**MIC_{50} (**µg/mL)	**MIC_{90} (**µg/mL)
Ampicillin	2	>32
Oxacillin	1	>8
Flucloxacin	1	>8
Levofloxacin	0.5	>4
Ciprofloxacin	0.5	>4
Gentamicin	0.5	>4
Amikacin	4	8
Tobramycin	0.5	>4
Tigecycline	0.25	0.5
Minocycline	0.25	1
Rifampicin	0.03	0.03
Vancomycin	1	1
Azithromycin	4	>8
Linezolid	2	4

MICs were interpreted into resistance profiles using EUCAST v9.0 guidelines (EUCAST, 2019), and low levels of resistance were seen against many antibiotics (Table 4.2; Figure 4.2). Lowest rates of resistance were seen for amikacin (5.56%), tigecycline (6.16%), rifampicin (4.17%), vancomycin (0.68%), linezolid (1.37%) and a relatively low level of isolates with resistance against minocycline were found (10.27%). Higher rates of resistance were found against oxacillin (36.30%), flucloxacin (25.34%), levofloxacin (23.39%), ciprofloxacin (26.03%), gentamicin (25.00%) and tobramycin (35.42%). Highest resistance was found against azithromycin (52.68%), plus 39.78% requiring increased exposure, the new terminology for isolates previously classes as 'intermediate', with only 7.53% of isolates showing susceptibility with standard doses (Table 4.2; Figure 4.2). Methicillin resistance, determined by oxacillin MIC >2 µg/mL in *S. aureus* was present in 33/100 *S. aureus* isolates. Additionally, 20/46 non-aureus *Staphylococci* spp. isolates had oxacillin MIC >2 µg/mL (seven *S. epidermis*, nine *S. haemolyticus* and two *S. sciuri*, as well as two *E. faecalis*). Overall, 43 isolates were determined as multi-drug resistant (MDR), defined as resistant to at

least one antibiotic in three or more classes (Magiorakos *et al*., 2012). MDR isolates included 25 *S. aureus*, eight *S. epidermis*, eight *S. haemolyticus*, one *S. sciuri* and one *Enterococcus faecalis* from varied sites.

Table 4.2 Number and percentage of Gram-positive isolates that were susceptible, required increased exposure or resistant to antibiotics tested.

Antibiotic	Susceptible (%)	Increased exposure (%)	Resistant (%)
Oxacillin	93 (63.70%)	0 (0.00%)	53 (36.30%)
Flucloxacin	110 (75.34%)	0 (0.00%)	36 (24.66%)
Levofloxacin	112 (76.71%)	0 (0%)	34 (23.29%)
Ciprofloxacin	108 (73.97%)	0 (0%)	38 (26.03%)
Gentamicin	108 (75.00%)	0 (0%)	36 (25.00%)
Amikacin	132 (91.67%)	4 (2.78%)	8 (5.56%)
Tobramycin	93 (64.58%)	0 (0%)	51 (35.42%)
Tigecycline	137 (93.84%)	0 (0%)	9 (6.16%)
Minocycline	123 (84.25%)	8 (5.48%)	15 (10.27%)
Rifampicin	138 (95.83%)	0 (0%)	6 (4.17%)
Vancomycin	145 (99.32%)	0 (0%)	1 (0.68%)
Azithromycin	7 (7.53%)	37 (39.78%)	49 (52.69%)
Linezolid	144 (98.63%)	0 (0%)	2 (1.37%)

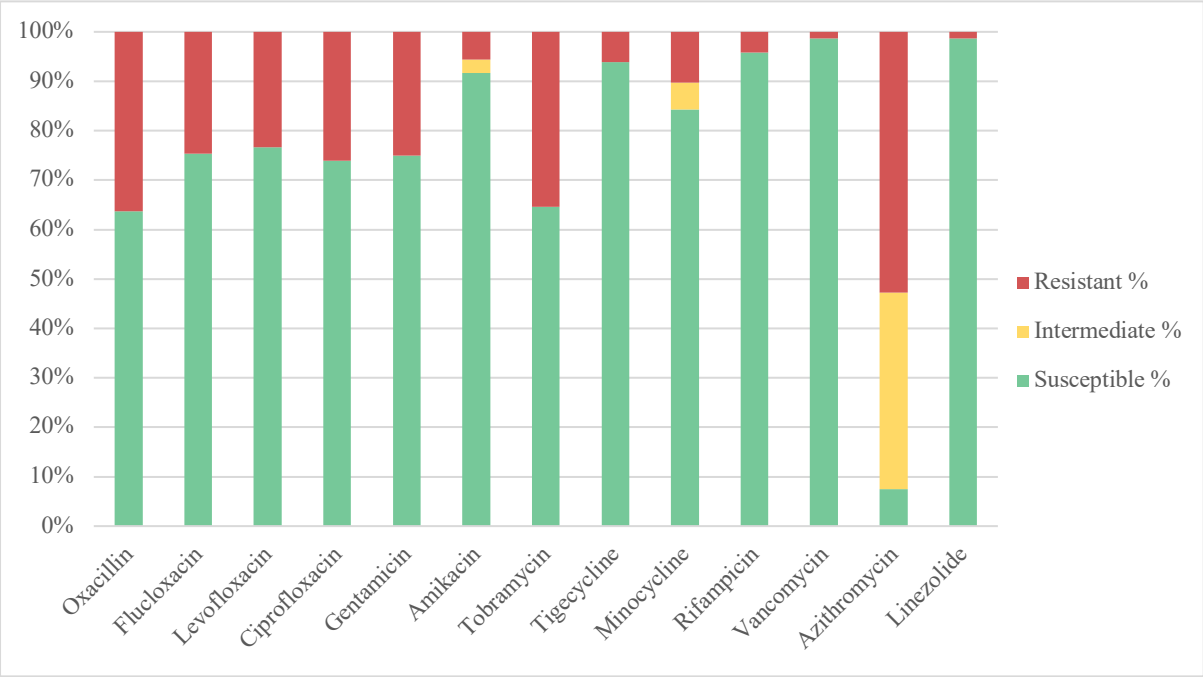


Figure 4.13. Resistance profiles for all Gram-positive isolates tested (n=146) according to EUCAST v9.0. Red sections show the percentage of resistant isolate, yellow shows those that would require increased exposure and green show those determined as susceptible to each antibiotic. Breakpoints for Staphylococcus aureus are not provided by EUCAST for ampicillin and so this was not included in analysis of resistance profiles. Additionally, no formal breakpoints are provided for oxacillin and flucloxacin, although it is stated that oxacillin resistance >2mg/mL infers methicillin resistance and so this was used as the oxacillin and flucloxacin interpretation breakpoint in this study.

4.2.1.2 Gram-positive WGS data

WGS data was available for 130 Gram-positive isolates, including 100 *S. aureus*, eight *S. haemolyticus*, eight *S. epidermis* and 14 *S. sciuri*. Methicillin resistance, determined by oxacillin MIC >2 µg/mL was present in 33/100 *S. aureus* isolates sequenced. This was due to inclusion of *mecA*, generally present on a staphylococcal cassette chromosome mec (SCCmec) and encodes for altered penicillin binding protein (Wielders *et al*., 2002). Four more isolates were deemed as methicillin resistant through phenotypic oxacillin resistance but were not sequenced and another 14 S. *aureus* isolates contained *mec(A)* but did not display phenotypic resistance against oxacillin. No reason for this was revealed through the available WGS data and further work is needed to find out why phenotypic resistance was not seen. Only one isolate of *S. sciuri* had a confirmed SCCmec element. Including non-aureus

Staphylococci, *mecA* was found in 58 isolates (43 *S. aureus*, one *S. sciuri*, six *S. epidermis* and one *S. haemolyticus*).

The gene *blaZ*, encoding for β-lactamase enzyme, was found in 86 isolates (73 *S. aureus*, seven *S. haemolyticus* and six *S. epidermis*). In *S. aureus*, this was present in 34 MRSA isolates, and in 39 MSSA isolates, where it was not associated with increased phenotypic resistance against penicillins tested. Phenotypic tigecycline resistance was found in six GPB; all non-aureus Staphylococci, only two of which contained *tetK*, with no other *tet* analogues identified and none contained *tetX*. In total seven non aureus Staphylococci isolates contained *tetK*, with only two displaying phenotypic tigecycline resistance. One isolate with phenotypic tigecycline resistance was a *Staphylococcus sciuri* that was found to be resistant to both vancomycin and rifampicin but had no known resistance genes identified by WGS, and MICs could have been performed on a contaminant. Minocycline resistance was also found in the same seven non-aureus Staphylococci that displayed tigecycline resistance, in addition to one *E. faecalis* and four *S. aureus* isolates, all from PP. Of the *S. aureus* isolates with phenotypic minocycline resistance two contained *tetM* and all contained tet-38, although *tet-38* was found in all *S. aureus* analysed, acting as a native efflux pump.

Azithromycin was tested against fewer isolates (n=92, all *S. aureus*) due to a batch which failed the quality control checks as control strains were not inhibited at the correct concentrations and an insufficient antibiotic stock to repeat the experiment. High levels of resistance were found against azithromycin with 48 resistant isolates and a further 37 isolates requiring increased exposure (I). We found 29/48 (66.67%) of resistant isolates contained an MLS gene (11 *ermA*; 7 *ermC*; seven *mphC*; and 11 *msrA*), with seven isolates containing both *mphC* and *msrA*. Only 15/37 (40.54%) of 'I' isolates had an MLS gene (with one isolate containing both *mphC* and *msrA*. Two of the eight (14.29%) of sensitive isolates contained

ermA. A range of sequence types (STs) were found across sensitive, intermediate (or requiring increased exposure) and resistant isolates, with no distinct pattern.

Gentamicin resistance was found in 18/100 (18%) *S. aureus* isolates, with 12 of the 18 (66.67%) resistant isolates belonging to ST5 and four (22.22%) were ST8 in addition to one ST152 and one ST5496. Conversely, only 5/81 (6.17%) gentamicin susceptible *S. aureus* isolates belonged to ST5 and 9/81 (11.11%) to ST8, all from Pakistan. A total of 18 STs were found in *S. aureus* isolates, 17 found in gentamicin susceptible *S. aureus,* with ST6 the most common group (n=18/81, 22.22%). All *S. aureus* isolates with amikacin resistance (n=4) belonged to ST8, all had aminoglycoside (Agly) (*aac3*) and two also had *aac6-aph2, and aph3-III*. Three *S. epidermis* displayed amikacin resistance, two of which had corresponding WGS data both containing *aac6*.

Resistance to rifampicin was found in six isolates, three *S. epidermis* 1 *S. sciuri*, and two *S. haemolyticus*. However, no WGS data was available for two *S. epidermis* and two *S. haemolyticus*. No common resistance genes were shared between the remaining two isolates with rifampicin resistance, one of them had no known resistance genes and the other had *blaZ*, *mecA* and *dfrC*. However, data was not accessible to investigate rpoB gene, which often contains mutations responsible for rifampicin resistance.

Only two isolates showed resistance against linezolid, including the one vancomycin resistant isolate (*S. sciuri*) with no identified resistance genes and a *S. aureus* isolate which contained five resistance gene, but none previously associated with linezolid resistance. We found 34 isolates resistant to levofloxacin and 38 to ciprofloxacin, 32 of which were resistant to both levofloxacin and ciprofloxacin. Isolates resistant to both composed of 15 *S. aureus*, nine *S. epidermis* and eight *S. haemolyticus* across sites. Resistance genes were not identified for all these isolates specific for fluoroquinolone resistance, but all isolates with resistance genes determined by WGS had AAc3-Ik, DHA-1 and APH-stph and isolates may harbour

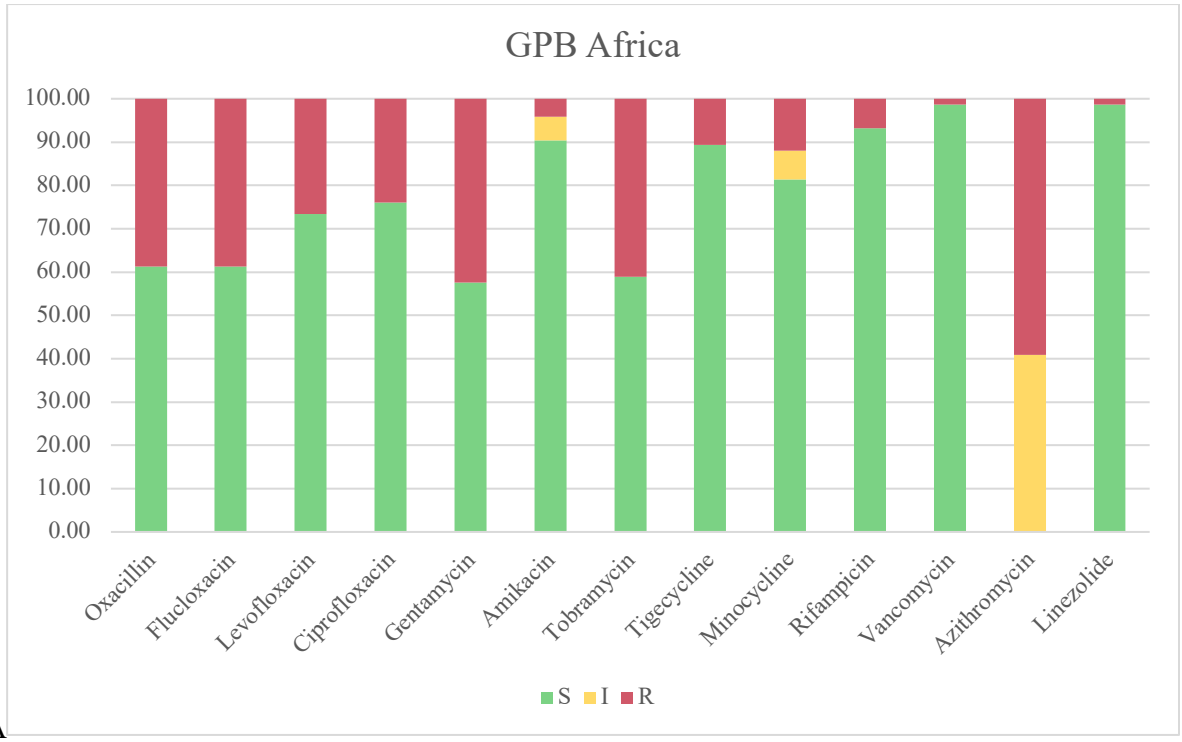
mutations that cause resistance, often caused by conserved mutation in the quinolone resistance determining regions cassette, *gyrA* or *parC* (Pazhani *et al*., 2011; Bagel *et al*., 1999).

4.2.1.3. Comparison between continents

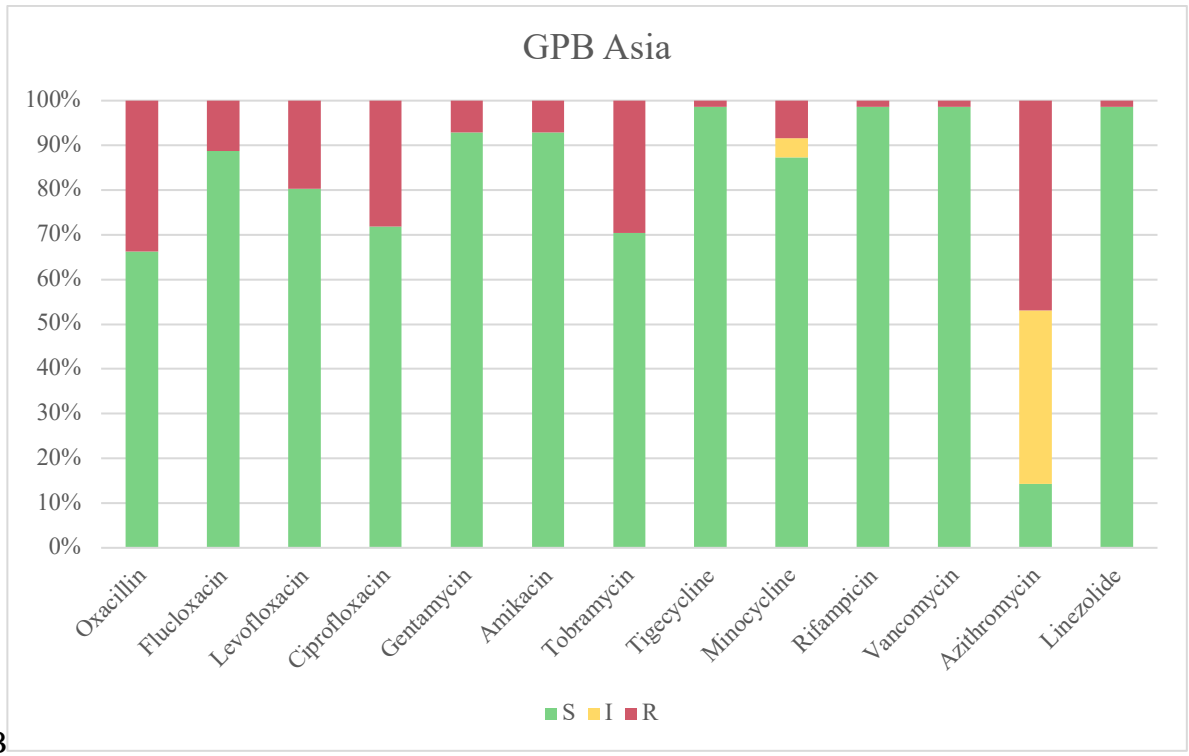
Similar resistance profiles were seen in both Asia and Africa for GPB for several antibiotics (Figure 4.3). However higher levels of resistance were seen in isolates from sites in Africa for flucloxacin (38.67% compared to 11.27% in isolates from sites in Asia, $X^2(1, N=144) = 24.088$, $p=0.000142$), gentamicin (42.47% compared to 7.04% in Asia, $X^2(1, N=144) = 24.088$, $p<0.00001$), and tigecycline (10.67% compared to 1.41% in Asia, $X^2(1, N=144) = 24.088$, $p=0.0200$). Only 7/49 (14.29%) isolates tested from Asia demonstrated susceptibility to azithromycin, with no susceptible isolates found in isolates from sites in Africa (Chi-square analysis not applicable with zero value) (Table 4.3; Figure 4.3).

Table 4. 3 Number and percentage of Gram-positive isolates that were susceptible, required increased exposure or resistant to 13 antibiotics tested split by continent (Africa and Asia)

	Africa			Asia		
Antibiotic	Susceptible (%)	Increased exposure (%)	Resistant (%)	Susceptible	Increased exposure (%)	Resistant (%)
Oxacillin	46 (61.33%)	0 (0.00%)	29 (38.67%)	47 (66.20%)	0 (0.00%)	24 (33.80%)
Flucloxacin	47 (62.67%)	0 (0.00%)	28 (37.33%)	63 (88.73%)	0 (0.00%)	8 (11.27%)
Levofloxacin	55 (73.33%)	0 (0.00%)	20 (26.67%)	57 (80.28%)	0 (0.00%)	14 (19.72%)
Ciprofloxacin	57 (76.00%)	0 (0.00%)	18 (24.00%)	51 (71.83%)	0 (0.00%)	20 (28.17%)
Gentamicin	42 (57.53%)	0 (0.00%)	31 (42.47%)	66 (92.96%)	0 (0.00%)	5 (7.04%)
Amikacin	66 (90.41%)	4 (5.48%)	3 (4.11%)	66 (92.96%)	0 (0.00%)	5 (7.04%)
Tobramycin	43 (58.90%)	0 (0.00%)	30 (41.40%)	50 (70.42%)	0 (0.00%)	21 (29.58%)
Tigecycline	67 (89.33%)	0 (0.00%)	8 (10.67%)	70 (98.59%)	0 (0.00%)	1 (1.41%)
Minocycline	61 (81.33%)	5 (6.67%)	9 (12.00%)	62 (87.32%)	3 (4.23%)	6 (8.45%)
Rifampicin	68 (93.15%)	0 (0.00%)	5 (6.85%)	70 (98.59%)	0 (0.00%)	1 (1.41%)
Vancomycin	75 (100%)	0 (0.00%)	0 (0.00%)	70 (98.59%)	0 (0.00%)	1 (1.41%)
Azithromycin	0 (0.00%)	18 (40.91%)	26 (59.09%)	7 (14.29%)	19 (38.78%)	23 (46.94%)
Linezolid	74 (98.67%)	0 (0.00%)	1 (1.33%)	70 (98.59%)	0 (0.00%)	1 (1.41%)



A



B

Figure 4.14. Resistance profiles split by continent (A. Africa, B. Asia) for Gram-positive bacteria (GBP) tested against 13 antibiotics according to EUCAST v9.0. The green series labelled "S" show percentage of isolates there were susceptible, yellow "I" series show the percentage of isolates that required increased exposure of an antibiotic to be deemed effective and the red "R" displays the percentage of isolates that were determined as resistant to an antibiotic.

4.2.1.4 *Staphylococcus aureus*

Of the Gram-positive bacteria tested, 100/146 were *Staphylococcus aureus*, consisting of nine from BC, one from BK, two from ESS, 17 from NK, eight from NN, four from PC, 42 from PP, one from RK and 16 from ZAT. Most *S. aureus* isolates analysed had MICs at the lowest concentration tested for most antibiotics (Figure 4.4) except for tigecycline and azithromycin.

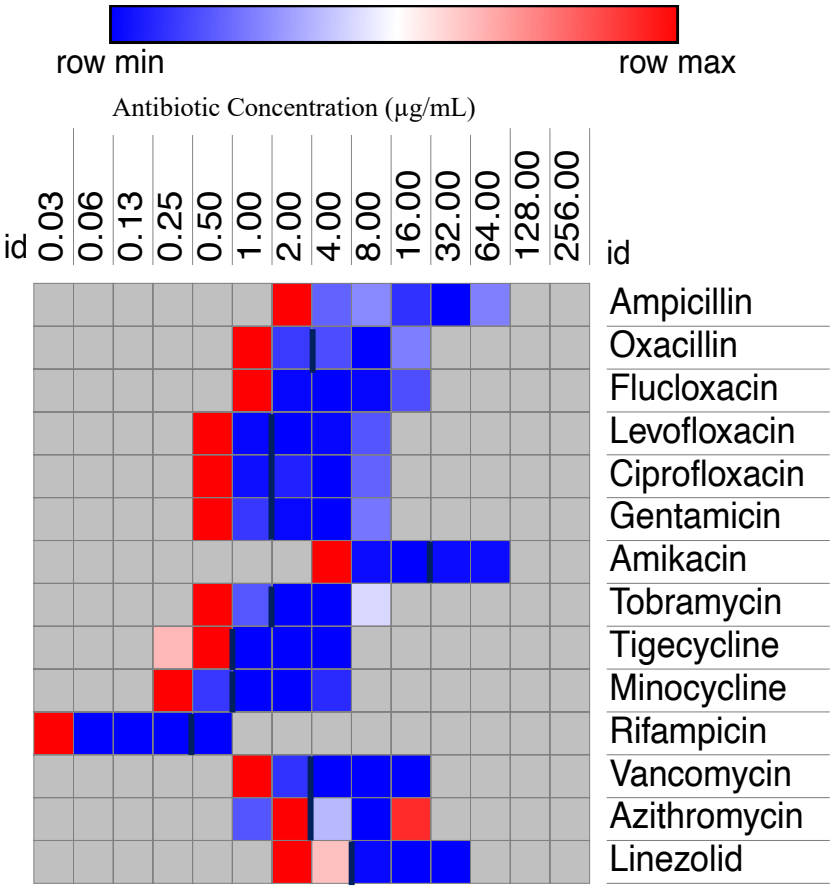


Figure 4.15. Minimum inhibitory concentrations (MICs) for Staphylococcus aureus isolates (n=100 for all antibiotics except azithromycin which had missing data for seven isolates, n=93). Isolates originated from BC (n=9), BK (n=1), ESS (n=2), NK (n=17), NN (n=8), PC (n=4), PP (n=42), RK (n=1) and ZAT (n=16). Grey squares show concentrations that were not tested. Numbers of isolates that were inhibited at a certain concentration are represented through the colour scale included above the heatmap, 'row min' blue demonstrates the lowest number of isolates with the associated MIC, whereas red shows the highest number of isolates with the associated MIC for each antibiotic. An additional concentration was added for each antibiotic two-fold above the highest concentration tested and all isolates with remaining growth at the highest concentration were included in this concentration. 'Row min' demonstrates the colour that will be shown for the lowest number of isolates with a certain MIC. 'Row max' demonstrates the colour that will be shown for the highest number of isolates with a certain MIC, with the gradient between showcasing decreasing numbers.

Resistance breakpoints, where determined by EUCAST (v 9.0), are displayed via black lines in the heatmap, where relevant.

MIC_{50} and MIC_{90} results were similar to those for overall Gram-positive isolates. MIC_{50} results were at the lowest concentration tested for all antibiotics except for azithromycin, which remained true for MIC_{90} results for only amikacin, rifampicin and vancomycin. Ampicillin, oxacillin, flucloxacin, levofloxacin, ciprofloxacin, gentamicin, tobramycin, and azithromycin had MIC_{90} results over the top concentration tested (Table 4.4). Due to the high number of *S. aureus* isolates with high MIC values against oxacillin (n=33), Table 4.4 is repeated split into methicillin susceptible Staphylococcus and methicillin resistant Staphylococcus (Table 4.5).

Table 4. 4 MIC_{50} results show the antibiotic minimum inhibitory concentration (MIC) preventing growth in 50% of isolates and MIC_{90} results show the MIC at which 90% of isolates had stopped growing. MIC_{50} and MIC_{90} results for Staphylococcus aureus isolates (n=100 for all antibiotics except azithromycin which had missing data for seven isolates, n=93).

Antibiotic	**MIC_{50} (**µg/mL)	**MIC_{90} (**µg/mL)
Ampicillin	≤2	>32
Oxacillin	≤1	>8
Flucloxacin	≤1	>8
Levofloxacin	≤0.5	>4
Ciprofloxacin	≤0.5	>4
Gentamicin	≤0.5	>4
Amikacin	≤4	4
Tobramycin	≤0.5	>4
Tigecycline	≤0.5	≤0.5
Minocycline	≤0.25	0.5
Rifampicin	≤0.03	≤0.03
Vancomycin	≤1	≤1
Azithromycin	4	>8
Linezolid	≤2	4

Table 4. 5 MIC_{50} results show the antibiotic minimum inhibitory concentration (MIC) preventing growth in 50% of isolates and MIC_{90} results show the MIC at which 90% of isolates had stopped growing. MIC_{50} and MIC_{90} µg/mL results for methicillin susceptible (MSSA) and methicillin resistant (MRSA) Staphylococcus aureus isolates.

Antibiotic	**MSSA**		**MRSA**	
	MIC_{50} (µg/mL)	**MIC_{90}** (µg/mL)	**MIC_{50}** (µg/mL)	**MIC_{90}** (µg/mL)
Ampicillin	≤2	8	16	>32
Oxacillin	≤1	2	>8	>8
Flucloxacin	≤1	2	1	>8
Levofloxacin	≤0.5	1	≤0.5	>4
Ciprofloxacin	≤0.5	2	≤0.5	>4
Gentamicin	≤0.5	1	≤0.5	>4
Amikacin	≤4	4	≤4	32
Tobramycin	≤0.5	1	>4	>4
Tigecycline	≤0.5	≤0.5	≤0.5	≤0.5
Minocycline	≤0.25	0.5	≤0.25	4
Rifampicin	≤0.03	≤0.03	≤0.03	≤0.03
Vancomycin	≤1	≤1	≤1	2
Azithromycin	2	>8	>8	>8
Linezolid	≤2	4	≤2	4

Resistance profiles for *S. aureus* isolates alone showed no resistance to vancomycin, rifampicin, or tigecycline, which were seen when looking at GPB overall (Figure 4.5, Table 4.6) (present in *S. epidermis*, *S. haemolyticus* and *S. sciuri*). Resistance to azithromycin remained high with 52.69% resistance in addition to 39.78% of isolates requiring increased exposure. Second highest resistance was seen against oxacillin with 33.00% resistant isolates, making them MRSA, followed by tobramycin (29.00%), ciprofloxacin (20.00%) and gentamicin (18.00%), where resistant isolates were mainly the MRSA isolates, but not exclusively.

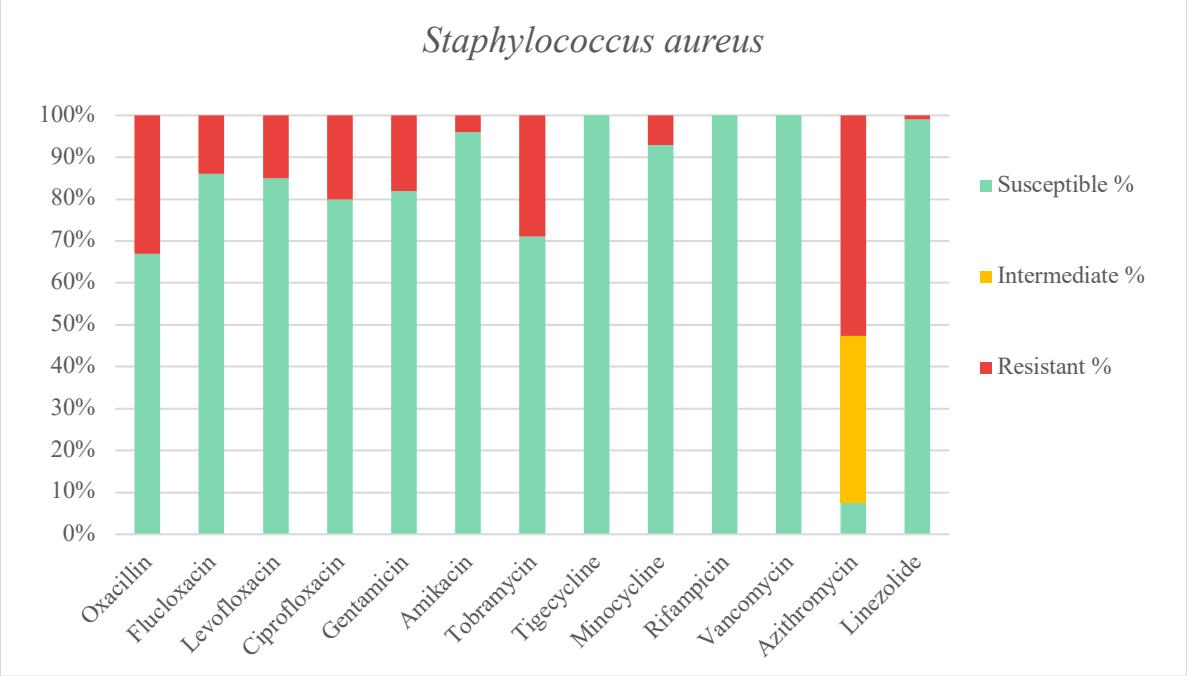


Figure 4.16. Resistance profiles for Staphylococcus aureus only (n=100) according to EUCAST v9.0. Red sections show the percentage of resistant isolate, yellow shows those that would require increased exposure and green show those determined as susceptible to each antibiotic.

Table 4. 6 Number/ percentage of S. aureus isolates that were susceptible, required increased exposure or resistant to antibiotics tested. Only percentages are shown, as numbers equalled 100, except for azithromycin which had n=93.

Antibiotic	Susceptible (%)	Increased exposure (%)	Resistant (%)
Oxacillin	67.00	0.00	33.00
Flucloxacin	86.00	0.00	14.00
Levofloxacin	85.00	0.00	15.00
Ciprofloxacin	80.00	0.00	20.00
Gentamicin	82.00	0.00	18.00
Amikacin	96.00	0.00	4.00
Tobramycin	71.00	0.00	29.00
Tigecycline	100.00	0.00	0.00
Minocycline	93.00	0.00	7.00
Rifampicin	100.00	0.00	0.00
Vancomycin	100.00	0.00	0.00
Azithromycin	7 (7.53%)	37 (39.78%)	49 (52.69%)
Linezolid	99.00	0.00	1.00

4.2.2 Gram-negative isolates

4.2.2.1 Gram-negative MICs

MICs for 1,031 Gram negative isolates were completed. However, IDs were not confirmed for 148 Gram-negative isolates, for which resistance breakpoints consequently could not be determined, therefore MIC results are presented for 883 of the Gram-negative isolates for which identification was confirmed via whole genome sequencing (Table 4.7).

Table 4. 7 Gram-negative species for which minimum inhibitory concentration/ resistance profiles were analysed.

Species ID	**Number of isolates analysed**
Achromobacter sp.	1
Achromobacter xylosoxidans	1
Acinetobacter baumannii	36
Acinetobacter bereziniae	2
Acinetobacter junii	1
Acinetobacter nosocomialis	3
Acinetobacter radioresistens	1
Acinetobacter schindleri	2
Acinetobacter sp.	2
Aeromonas hydrophila	1
Aeromonas sp.	2
Burkholderia cenocepacia	57
Burkholderia gladioli	1
Burkholderia sp.	2
Citrobacter braakii	1
Citrobacter freundii	3
Citrobacter sedlakii	1
Enterobacter asburiae	1
Enterobacter cloacae	46
Enterobacter cloacae complex	11
Enterobacter hormaechei	17
Enterobacter kobei	1
Enterobacter ludwigii	1
Enterobacter sp.	1
Enterobacter xiangfangensis	1
Escherichia coli	74
Franconibacter pulveris	1
Klebsiella aerogenes	3
Klebsiella michiganensis	116

Klebsiella oxytoca	5
Klebsiella pneumoniae	253
Klebsiella quasipneumoniae	13
Klebsiella variicola	5
Morganella morganii	1
Pantoea calida	1
Proteus mirabilis	3
Providencia rettgeri	1
Pseudescherichia vulneris	2
Pseudomonas aeruginosa	21
Pseudomonas alcaligenes	4
Pseudomonas sp.	1
Pseudomonas stutzeri	6
Ralstonia mannitolilytica	17
Raoultella ornithinolytica	1
Salmonella enterica	6
Serratia marcescens	149
Serratia nematodiphila	1
Stenotrophomonas maltophilia	3
Grand Total	**883**

All antibiotics had some isolates with MICs at the top of their respective concentrations tested, or with continued growth at the top concentration (Figure 4.4). Furthermore, MIC or continued growth at the highest concentration tested was the case for the majority of isolates for ten of the 20 antibiotics: ampicillin, amoxicillin/clavulanate, ceftriaxone, cefotaxime, ceftazidime, cefepime, aztreonam, gentamicin, tobramycin and minocycline (Figure 4.4). Antibiotics that demonstrated the highest MICs against GNB isolates included piperacillin-tazobactam, imipenem, meropenem, ertapenem, amikacin, tigecycline, fosfomycin, levofloxacin, ciprofloxacin and colistin (Figure 4.4). The majority of antibiotics (18/20) had an MIC_{90} above the highest concentration tested, including all except imipenem and tigecycline (Table 4.8).

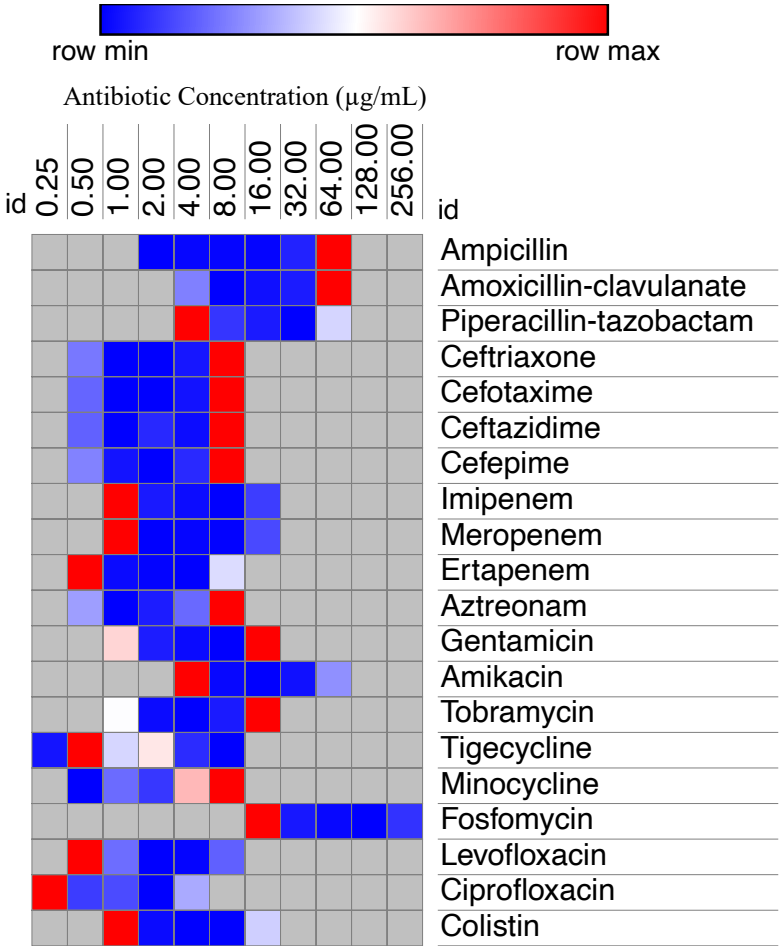


Figure 4.17. Heatmap showcasing minimum inhibitory concentrations (MICs) for sepsis causing Gram negative bacteria isolates (n=883). Grey squares show concentrations that were not tested. The key above demonstrates the number of isolate MICs at each concentration. 'Row min' demonstrates the colour that will be shown for the lowest number of isolates with a certain MIC. 'Row max' demonstrates the colour that will be shown for the highest number of isolates with a certain MIC, with the gradient between. An additional concentration was added for each antibiotic two-fold above the highest concentration tested and all isolates with remaining growth at the highest concentration were included in this concentration.

Table 4. 8 MIC_{50} results show the antibiotic minimum inhibitory concentration (MIC) preventing growth in 50% of isolates and MIC_{90} results show the MIC at which 90% of isolates had stopped growing. These are displayed below for Gram-negative bacteria tested (n=883).

Antibiotic	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Ampicillin	>32	>32
Amoxicillin clavulanate	>32	>32
Piperacillin-tazobactam	≤4	>32
Ceftriaxone	>4	>4
Cefotaxime	>4	>4
Ceftazidime	>4	>4
Cefepime	>4	>4
Imipenem	≤1	4
Meropenem	≤1	>8
Ertapenem	≤0.25	>2
Aztreonam	>4	>4
Gentamicin	>8	>8
Amikacin	≤4	>32
Tobramycin	>8	>8
Tigecycline	1	2
Minocycline	4	>4
Fosfomycin	≤16	>128
Levofloxacin	≤0.5	>4
Ciprofloxacin	0.5	>2
Colistin	≤1	>8

4.2.2.2 Resistance profiles

Resistance profiles could only be determined for species that had a resistance breakpoint determined in the EUCAST guidelines (2019); as these are not defined for minocycline, it has been excluded from resistance analyses. In line with the above results, high levels of resistance were found against multiple antibiotics and a range of antibiotic classes. Resistance ranged from 13.25% against meropenem to 95.36% for ampicillin and resistance was seen in over half of the isolates for nine antibiotics (Table 4.9; Figure 4.5).

Table 4. 9 Resistance found in Gram-negative bacteria from isolates found to be causing neonatal sepsis overall. Total numbers included with resistance % for each antibiotic. Breakpoint data was assessed using EUCAST guidelines v9.0 (2018). Resistance is not determined for minocycline as EUCAST did not determine breakpoint values for any Gram-negative bacteria.

Antibiotic	Resistant (%)	Increased exposure (%)	Susceptible (%)
Ampicillin	842 (95.36)	0 (0.00)	41 (4.64)
Amoxicillin-clavulanate	687 (77.80)	0 (0.00)	196 (22.20)
Piperacillin-tazobactam	249 (28.20)	38 (4.30)	596 (67.50)
Ceftriaxone	706 (79.95)	7 (0.79)	170 (19.25)
Cefotaxime	730 (82.67)	6 (0.68)	147 (16.65)
Ceftazidime	533 (60.36)	105 (11.89)	245 (27.75)
Cefepime	483 (54.70)	82 (9.29)	318 (36.01)
Imipenem	128 (14.50)	41 (4.64)	714 (80.86)
Meropenem	117 (13.25)	50 (5.66)	716 (81.09)
Ertapenem	284 (32.16)	0 (0.00)	599 (67.84)
Aztreonam	517 (58.55)	96 (10.87)	270 (30.58)
Gentamicin	529 (59.91)	9 (1.02)	345 (39.07)
Amikacin	223 (25.25)	17 (1.93)	643 (72.82)
Tobramycin	555 (62.85)	20 (2.27)	308 (34.88)
Tigecycline	132 (14.95)	120 (13.59)	631 (71.46)
Minocycline	NA	NA	NA
Fosfomycin	161 (18.23)	(0.00)	722 (81.77)
Levofloxacin	169 (19.14)	102 (11.55)	612 (69.31)
Ciprofloxacin	377 (42.70)	84 (9.51)	422 (47.79)
Colistin	626 (29.11)	0 (0.00)	626 (70.89)

[1] Ampicillin is active against certain Gram-negative bacteria but inactivated by penicillinases produced by some *E. coli* (BNF, 2019).

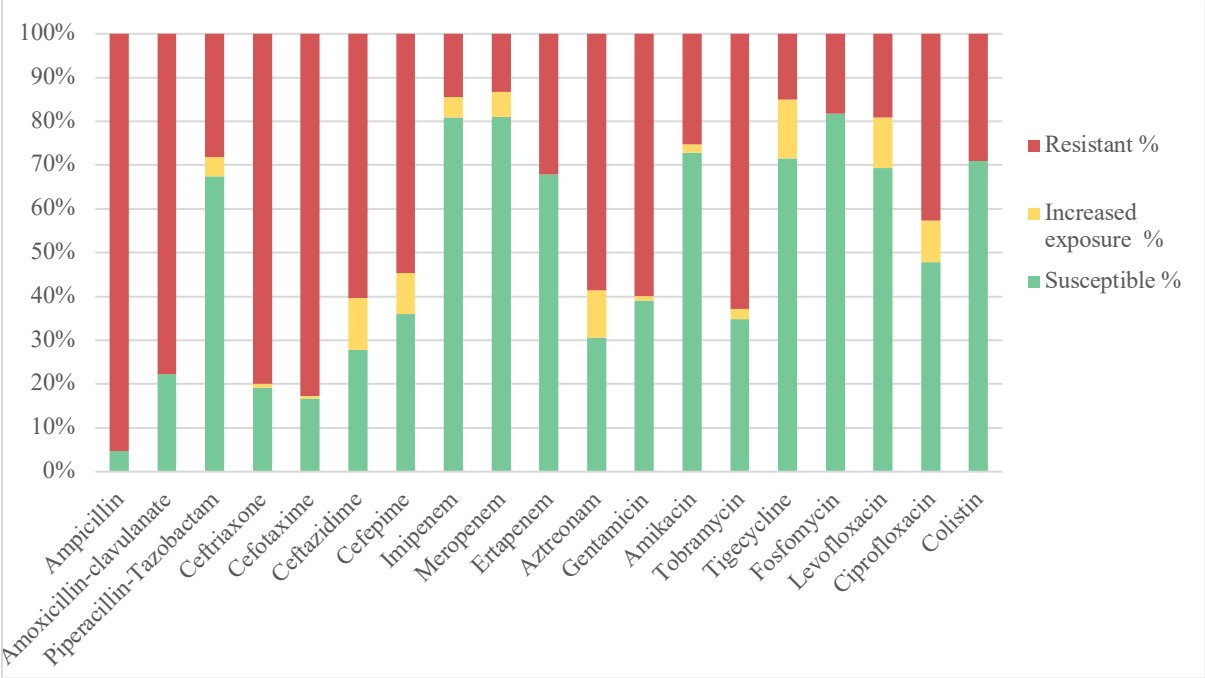


Figure 4.18. Resistance profiles for all GNB tested (n=883). No breakpoints are provided for minocycline as there are no breakpoints determined for Gram-negative bacteria. Resistance was interpreted using EUCAST v9.0 breakpoints (2018). Red sections show the percentage of resistant isolate, yellow shows those that would require increased exposure and green show those determined as susceptible to each antibiotic.

All antibiotics tested had some isolates displaying phenotypic resistance. Particularly high resistance was found for the penicillins: ampicillin (95.36%) and amoxicillin-clavulanate (77.80%); third generation cephalosporins: ceftriaxone (79.95%), cefotaxime (82.97%) and ceftazidime (60.36%), in addition to 54.70% resistance against fourth-generation cephalosporin cefepime. High resistance was also seen against aminoglycosides tobramycin (62.85%) and gentamicin (59.91%), with lower resistance against amikacin (25.25%). Bacteria also exhibited moderate resistance against the quinolone ciprofloxacin (42.70%) and monobactam Aztreonam (58.55%) (Table 4.9; Figure 4.5).

Lowest resistance was recorded against carbapenems meropenem and imipenem, albeit in 13.25% and 14.50% of isolates respectively, but was higher for ertapenem (32.16%). Relatively low resistance rates were also found for tigecycline (14.95%), fosfomycin

(18.23%) and levofloxacin (19.14%) (Table 4.9; Figure 4.5). Resistance profiles with species with intrinsic resistance removed are detailed in Appendix, page 16-18.

Overall, 112 isolates contained *bla*-$_{NDM}$ from a range of countries (20 BC, two BK, seven IN, two NK, 24 NN, one NW, one PC, 53 PP, one RU and one from ZAT). There were 385 isolates in total that contained *bla*-$_{OXA}$, again from all sites (131 BC, 10 BK. 65 ESS, eight IN, 14 NK, 38 NN, two NW, three PC, 77 PP, 20 RK, 11 RU and six ZAT. Of these, there were 75 isolates that had both OXA and NDM, from BC (n=9), BK (n=2), IN (n=4), NK (n=2), NN (n=15), PC (n=1), PP (n=40), RU (n=1) and ZAT (n=1). These were concomitant mainly in *K. pneumoniae* isolates (n=49) and was also found in *E. cloacae* complex (n= 13), *A. baumannii* (n=4), three *S. marcescens*, two *K. quasipneumoniae*, one *K. michiganensis,* one *Providencia rettgeri* and one *E. coli*. Nearly all these isolates displayed resistance or required increased exposure to meropenem, imipenem and ertapenem.

Neonates within the inborn cohort with GNB had similar resistance with pre-term on average resistant to 9.70 antibiotics and those born at term to 9.42 antibiotics. Similarly, Gram-negative pathogens associated with EOS were resistant to an average of 9.73 antibiotics and LOS to 9.23 antibiotics, therefore these were assessed together.

4.2.2.3 Multidrug resistance

Of the Gram-negative isolates tested, 662 (74.97%) were MDR, with five isolates were resistant to eight of nine classes tested from a range of sites. When assessing separate antibiotics, two isolates were resistant to 18 of 19 antibiotics assessed (one *Pseudomonas aeruginosa*, one *Burkholderia cenocepacia* sensitive only to colistin and aztreonam, respectively). Furthermore, 98 (11.09%) of GNB isolates were resistant to 15 or more of the antibiotics tested; 252 cases showed resistance to between one to five antibiotics, with another 252 resistant to six to ten antibiotics; 259 isolates demonstrated resistance to 11-14 antibiotics (mainly *Serratia marcescens, Klebsiella pneumoniae, Burkholderia cenocepacia*

and Ralstonia mannitolilytica). Only 22 isolates show no resistance to any of the antibiotic classes tested, the majority of which were *E. coli* (n=14),with 18/22 from sites in Africa. .

All isolates analysed from India were designated MDR. Only one isolate in RU, BK and NW was not MDR. All sites except for NK had more MDR isolates found than those that were not MDR (Figure 4.6).

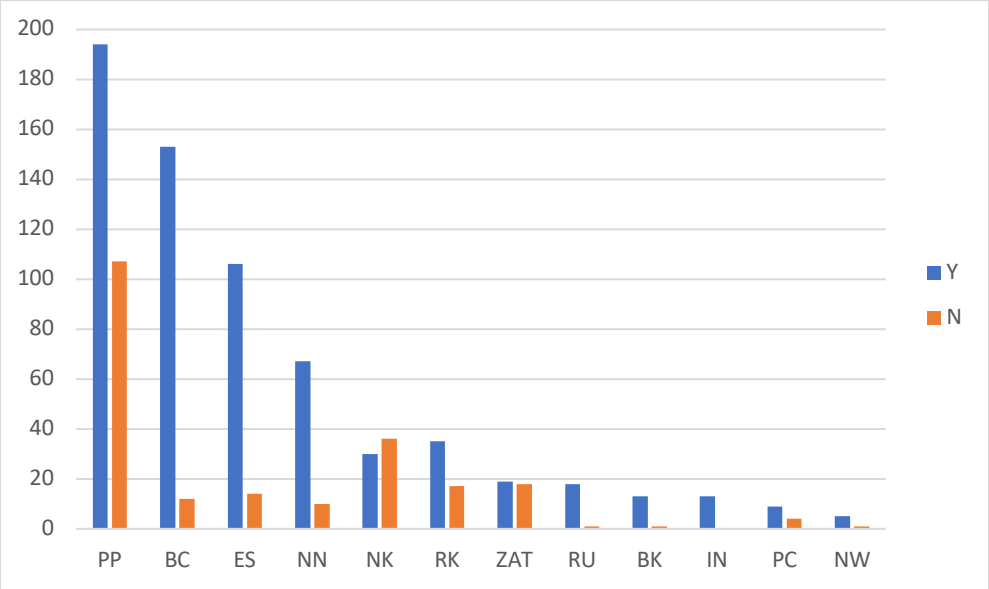


Figure 4.19 Number of Gram-negative isolates tested defined as multidrug resistant (Y) versus those not (N) per site.

A range of Gram-negative species were found to be MDR, including 51 different species in total from all the sites. The most common MDR bacteria were *K. pneumoniae*, followed by *S. marcescens*, *B. cenocepacia*, and *K. michiganensis*, three of which are thought to have caused hospital outbreaks (Figure 4.7).

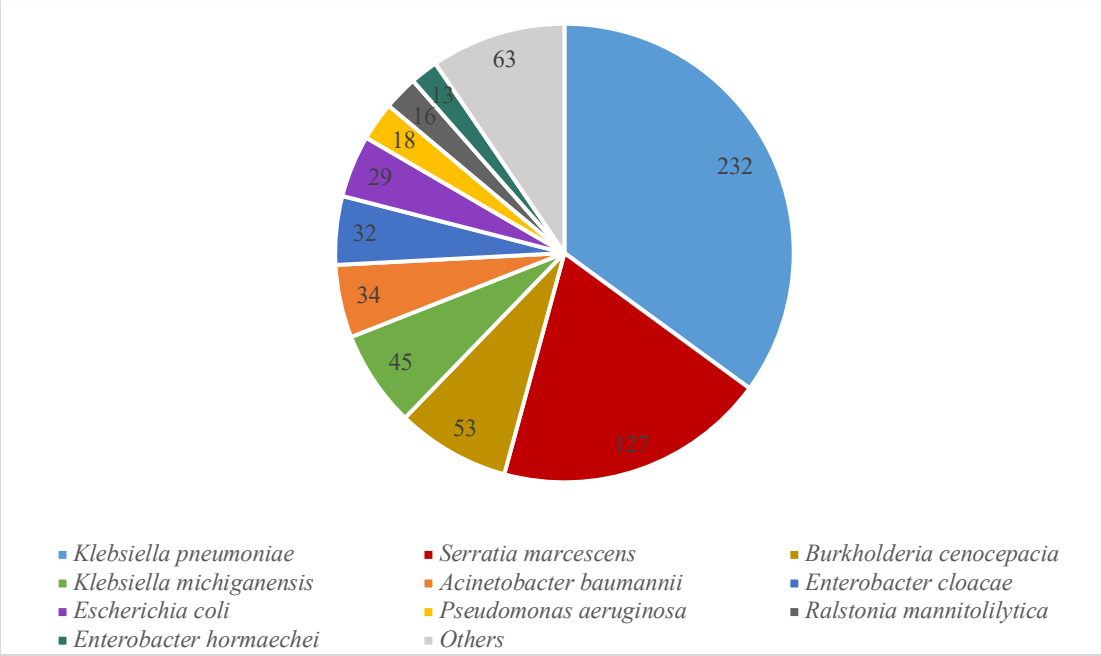


Figure 4.20. Top ten most common Gram-negative multidrug resistant bacteria across BARNARDS sites. Different colours demonstrate different species, as detailed by the key.

4.2.2.4 Comparison of Gram-negative isolates from sites in Africa and Asia

A total of 377 Gram-negative isolates were analysed from Africa, which consisted of 120 from ESS, 66 from NK, 77 from NN, six from NW, 52 from RK, 19 from RU and 37 from ZAT. Isolates from sites in Asia included a total of 506 GNB, consisting of 165 isolates from BC, 14 from BK, 13 from IN, 13 PC and 301 PP. Most common species found to cause neonatal sepsis from each continent are detailed in Table 4.10. These varied between continents, with *S. marcescens*, *K. michiganensis* and *B. cenocepacia* more common in Asia and *R. mannitolilytica* more common in Africa.

Table 4. 10. Top ten Gram-negative bacterial species causing sepsis from sites in Africa and Asia

Africa	Asia
Klebsiella pneumoniae (n=186)	*Serratia marcescens* (n=136)
Escherichia coli (n=56)	*Klebsiella michiganensis* (n=112)
Enterobacter cloacae (n=28)	*Klebsiella pneumoniae* (n=67)
Ralstonia mannitolilytica (n=17)	*Burkholderia cenocepacia* (n=55)
Acinetobacter baumannii (n=15)	*Acinetobacter baumannii* (n=20)
Serratia marcescens (n=13)	*Escherichia coli* (n=18)

Salmonella enterica (n=6)	*Enterobacter cloacae (n=1*8)
Pseudomonas aeruginosa (n=6)	*Pseudomonas aeruginosa* (n=15)
Klebsiella quasipneumoniae (n=8)	*Enterobacter hormaechei* (n=13)
Enterobacter hormaechei (n=4), *Pseudomonas alcaligenes* (n=4), *Klebsiella michiganensis* (n=4)	*Enterobacter cloacae* (n=8), *Klebsiella quasipneumoniae* (n=8)

Similar MICs were found for isolates from sites in Africa compared to Asia overall, although slight variation was seen for some antibiotics (Figure 4.8; Figure 4.11). As seen in Figure 4.11, the majority of isolates (shown in red) for both continents were found to have MICs at or above the highest concentration tested for ampicillin, amoxicillin-clavulanate, all cephalosporins, aztreonam, gentamicin, tobramycin and minocycline. The carbapenems, fosfomycin, levofloxacin, piperacillin-tazobactam, amikacin, ciprofloxacin, tigecycline and colistin prevented growth of most isolates at the lowest concentration tested. More isolates had MICs above the maximum concentration tested for colistin in Asia, due to the high number of *S. marcescens* due to their intrisinc resistance.

Despite this difference in colistin MICs, MIC_{50} and MIC_{90} results were the same between the two continents. MIC_{50} results for GNB from Africa were consistent with MIC_{50} results from Asia, except for piperacillin-tazobactam which increased to 8µg/mL, and ciprofloxacin which reduced to 0.25µg/mL in isolates from Africa. MIC_{90} results were again mainly similar to those found for Asia isolates except for reduced MIC_{90} for imipenem to 4µg/mL meropenem to 8µg/mL and amikacin reduced to 32µg/mL (Table 4.11).

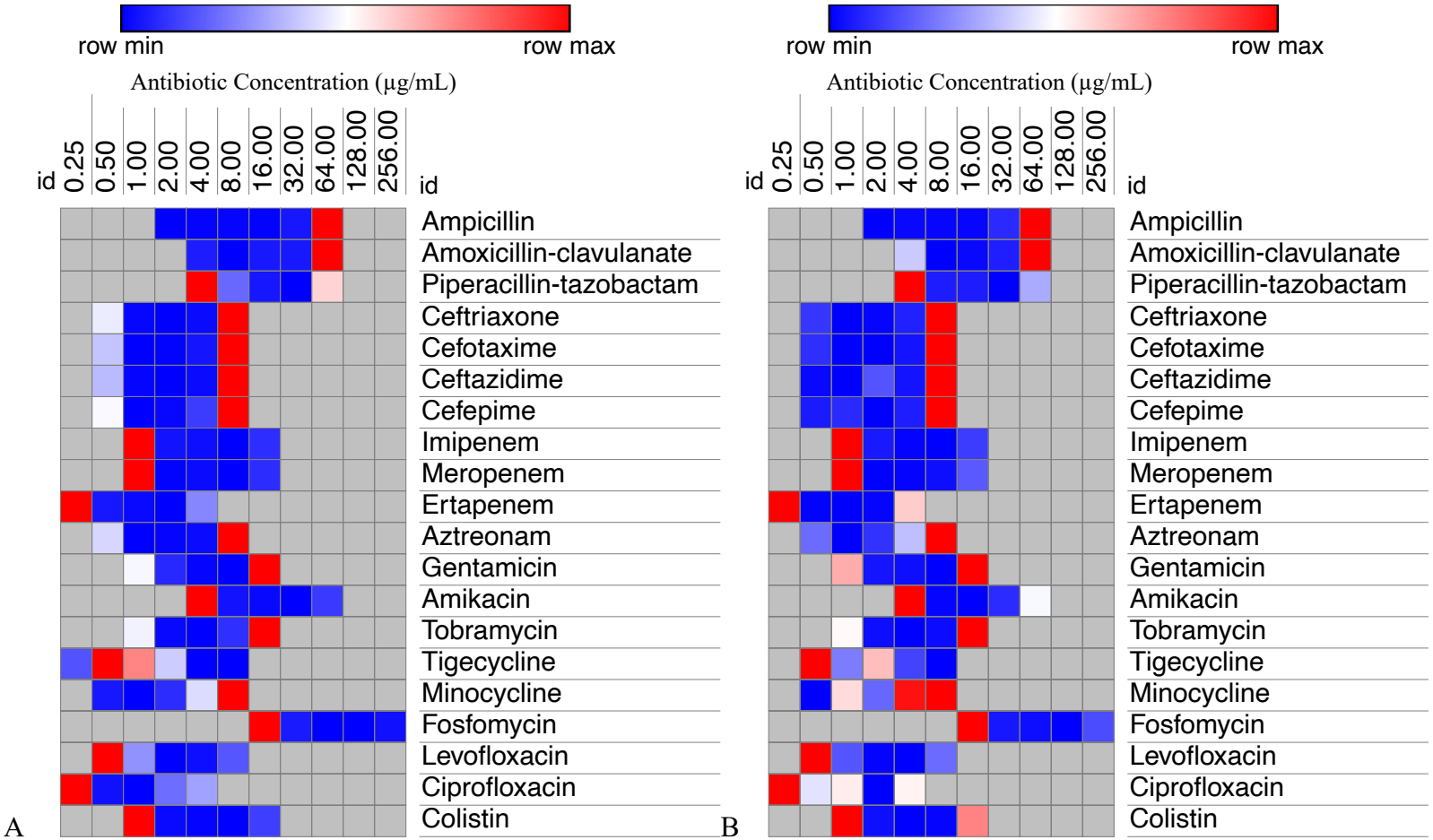


Figure 4.21. Heatmap displaying minimum inhibitory concentrations (MICs) for all antibiotics tested from all sites in A. Africa for Gram negative bacterial isolates causing sepsis. (n=377) and B. Asia from GNB causing sepsis (n=506). Grey squares show concentrations that were not tested. First non-grey squares show when growth was inhibited at the minimum concentration tested, e.g. ampicillin squares at 2 show those with MIC ≤2µg/mL. Isolates with growth remaining at the highest concentrating tested were added to the next concentration up and e.g. for ciprofloxacin, those showing at 4µg/mL should be interpreted as >2µg/mL.

Table 4. 11. MIC_{50} and MIC_{90} results for isolates from sites in Africa (n=377) and from Asia (n=506). MIC_{50} results show the minimum inhibitory concentration (MIC) preventing growth in 50% of isolates and MIC_{90} results show the MIC at which 90% of isolates had stopped growing.

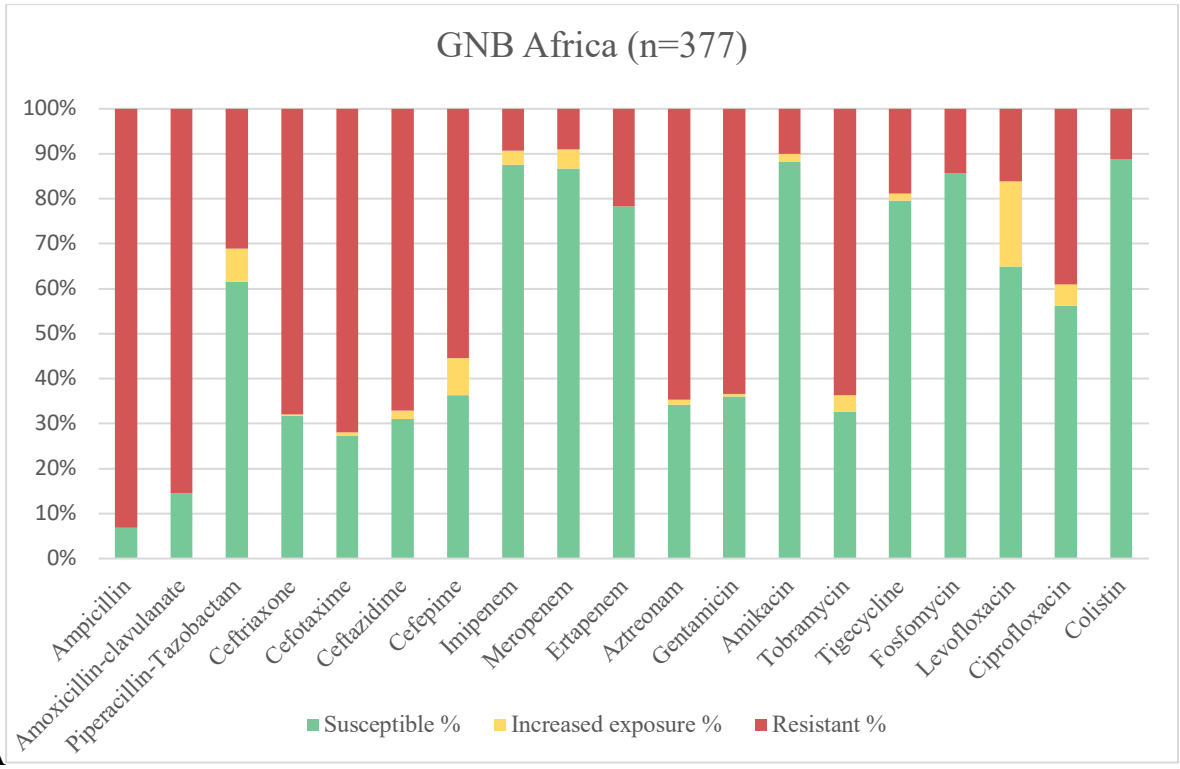
Antibiotic	Africa		Asia	
	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Ampicillin	>32	>32	>32	>32
Amoxicillin clavulanate	>32	>32	>32	>32
Piperacillin-tazobactam	8	>32	4	>32
Ceftriaxone	>4	>4	>4	>4
Cefotaxime	>4	>4	>4	>4
Ceftazidime	>4	>4	>4	>4
Cefepime	>4	>4	>4	>4
Imipenem	1	4	1	>8
Meropenem	1	8	1	>8
Ertapenem	0.25	>2	0.25	>2
Aztreonam	>4	>4	>4	>4
Gentamicin	>8	>8	>8	>8
Amikacin	4	32	4	>32
Tobramycin	>8	>8	>8	>8
Tigecycline	1	2	1	2
Minocycline	>4	>4	4	>4
Fosfomycin	16	>128	16	>128
Levofloxacin	0.5	>4	0.5	>4
Ciprofloxacin	0.25	>2	0.5	>2
Colistin	1	>8	1	>8

Lower levels of resistance were found in isolates from sites in Africa against cephalosporins (ceftriaxone 67.90%, cefotaxime 71.88%, ceftazidime 67.11%, cefepime 55.44%), colistin (11.14%), carbapenems and amikacin (10.08%) compared to those from Asia. However, a higher resistance prevalence was seen for gentamicin (63.40%) compared to isolates from Asia (Table 4.12; Figure 4.9).

As found in isolates from sites in Africa, an overwhelming resistance to ampicillin was again demonstrated in isolates from sites in South Asia (97.04%). Most isolates showed high resistance against two of the third generation cephalosporins tested (ceftriaxone (88.93%) and cefotaxime (90.71%) (Figure 4.10; Table 4.12). High resistance was also found for other cephalosporins ceftazidime (55.34%) and cefepime (54.15%), in addition to amoxicillin-clavulanate (72.13%), tobramycin (62.25%), gentamicin (57.31%) and aztreonam (53.95%) (Figure 4.12; Table 4.11). Over half the isolates (n=295) were resistant to at least ten of the 19 antibiotics assessed. The stark increase of colistin resistance in Asia (42.49%) was largely due to an outbreak of *Serratia marcescens* in the Bangladesh sites (n=120), which is intrinsically resistant to colistin.

Table 4. 12 Resistance data for Gram negative isolates from sites in Africa, presented as number of isolates and percentages.

Antibiotic	Africa			Asia		
	Resistant (%)	Increased exposure (%)	Susceptible (%)	Resistant (%)	Increased exposure (%)	Susceptible (%)
Ampicillin	351 (93.10%)	0 (0.00%)	26 (6.90%)	491 (97.04%)	0 (0.00%)	15 (2.96%)
Amoxicillin-clavulanate	322 (85.41%)	0 (0.00%)	55 (14.59%)	365 (72.13%)	0 (0.00%)	141 (27.87%)
Piperacillin-tazobactam	117 (31.03%)	28 (7.43%)	232 (61.54%)	132 (26.09%)	10 (1.98%)	364 (71.94%)
Ceftriaxone	256 (67.90%)	1 (0.27%)	120 (31.83%)	450 (88.93%)	6 (1.19%)	50 (9.88%)
Cefotaxime	271 (71.88%)	3 (0.80%)	103 (27.32%)	459 (90.71%)	3 (0.59%)	44 (8.70%)
Ceftazidime	253 (67.11%)	7 (1.86%)	117 (31.03%)	450 (55.34%)	98 (19.37%)	128 (25.30%)
Cefepime	209 (55.44%)	31 (8.22%)	137 (36.34%)	274 (54.15%)	51 (10.08%)	181 (35.77%)
Imipenem	35 (9.28%)	12 (3.18%)	330 (87.53%)	93 (18.38%)	29 (5.73%)	384 (75.89%)
Meropenem	34 (9.02 %)	16 (4.24%)	327 (86.74%)	83 (16.40%)	34 (6.72%)	389 (76.88%)
Ertapenem	82 (21.75%)	0 (0.00%)	295 (78.25%)	202 (39.92%)	0 (0.00%)	304 (60.08%)
Aztreonam	244 (64.72%)	4 (1.06%)	129 (34.22%)	273 (53.95%)	92 (18.18%)	141 (27.87%)
Gentamicin	239 (63.40%)	2 (0.53%)	136 (36.07%)	290 (57.31%)	7 (1.38%)	209 (41.30%)
Amikacin	38 (10.08%)	6 (1.59%)	333 (88.33%)	185 (36.56%)	11 (2.17%)	310 (61.26%)
Tobramycin	240 (63.66%)	14 (3.71%)	123 (32.63%)	315 (62.25%)	6 (1.19%)	185 (36.56%)
Tigecycline	71 (18.83%)	6 (1.59%)	300 (79.58%)	61 (12.06%)	114 (22.53%)	331 (65.42%)
Minocycline	NA	NA	NA	NA	NA	NA
Fosfomycin	54 (14.32%)	0 (0.00%)	323 (85.68%)	107 (21.15%)	0 (0.00%)	399 (78.85%)
Levofloxacin	61 (16.18%)	71 (18.83%)	245 (64.99%)	108 (21.34%)	31 (6.13%)	367 (72.53%)
Ciprofloxacin	147 (38.99%)	18 (4.77%)	212 (56.23%)	230 (45.45%)	66 (13.04%)	210 (41.50%)
Colistin	42 (11.14%)	0 (0.00%)	335 (88.86%)	215 (42.49%)	0 (0.00%)	291 (57.51%)



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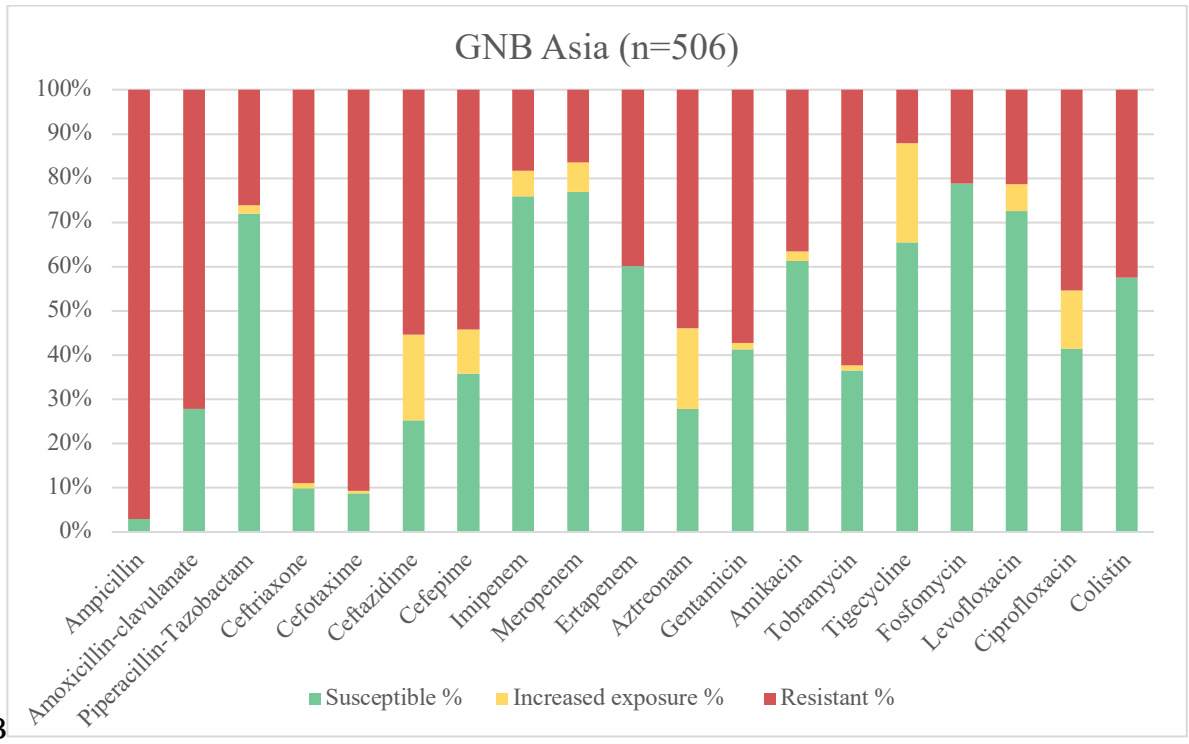


B

Figure 4.22. Resistance profiles for Gram-negative (GNB) isolates analysed from sites in A. Africa (n=377) and B. South Asia (n=506) according to EUCAST v9.0. Resistance profiles are shown as percentages (with red showing percentage of those deemed resistant, yellow those required increased exposure and green the percentage of susceptible isolates).

Of the Gram-negative isolates from sites in Africa, 280/377 (74.27%) were MDR. Resistance to 15 or more of the antibiotics tested was found in 25 isolates, 22 of which were from sites in Nigeria (mainly NHA) and consisted of 16 *K. pneumoniae* in addition to four *Ralstonia mannitolilytica*, three *A. baumannii* and one *Pseudomonas aeruginosa*. Resistance against one to five antibiotics was reported in 97 isolates; 133 isolates were resistant to six to ten antibiotics; 104 isolates resistant to 11-15 antibiotics. Only 18 isolates were not resistant to any of the antibiotics tested, 13 of which were *E. coli* from multiple sites. MDR was seen in 382/506 isolates from Asia, with four isolates showing resistance to all different eight classes of antibiotics tested (one *Providencia rettgeri*, two *Burkholderia cenocepacia* and one *Pseudomonas aeruginosa*). Only three isolates displayed no resistance against any of the antibiotics tested, all from PP, consisting of two *Klebsiella michiganensis* and one *E. coli*).

4.2.2.5 Differences between continents

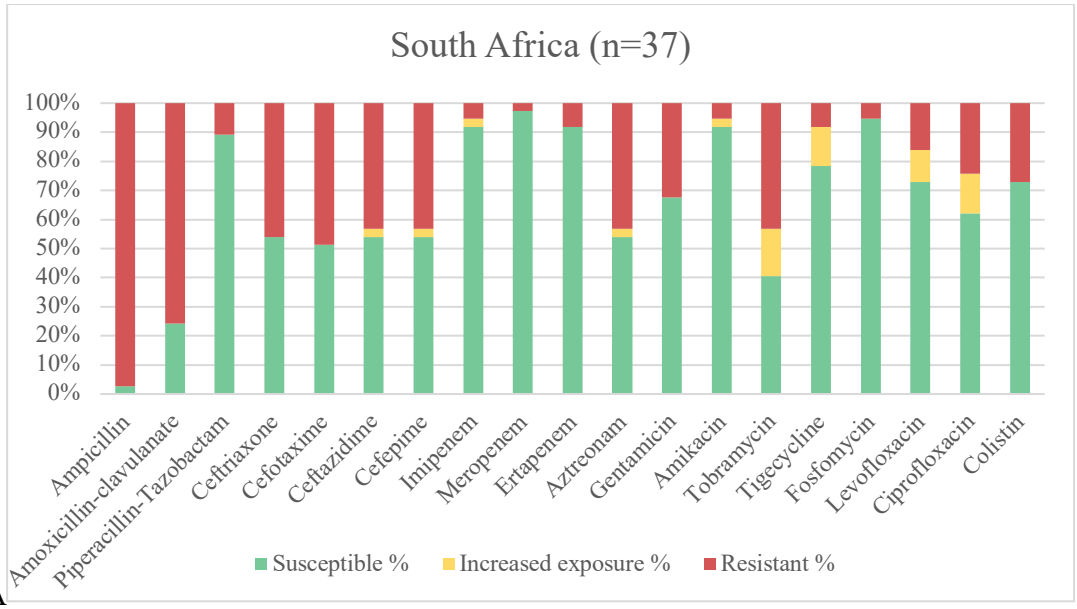
Overall, similar resistance profiles were found between isolates from sites in Asia and those from sites in Africa for GNB, although slightly raised resistance was seen for some antibiotics in South Asia, primarily two of the four cephalosporins tested (ceftriaxone and ceftazidime) but resistance was lower for ceftazidime in Asia compared to Africa and similar in both continents for cefepime. Resistance for the carbapenems tested were approximately double that of isolates from Africa. Resistance against gentamicin was slightly lower in Asia, although higher for amikacin (Figure 4.7, Figure 4.10).

Independent samples T-tests were performed to uncover differences of resistance profiles between continents for GNB isolates. T-tests showed significant differences between MICs of isolates from Asia and Africa for 12 of the antibiotics tested, 10 of which had significantly higher MICs in Asia and two antibiotics had higher MICs in Africa. Higher resistance was found for isolates from sites in Asia for ampicillin ($T(1)= -2.008$, $p=0.045$); ceftriaxone ($T(1)= -6.769$, $p<0.0001$); cefotaxime ($T(1) = -6.990$, $p<0.0001$); imipenem ($T(1)$

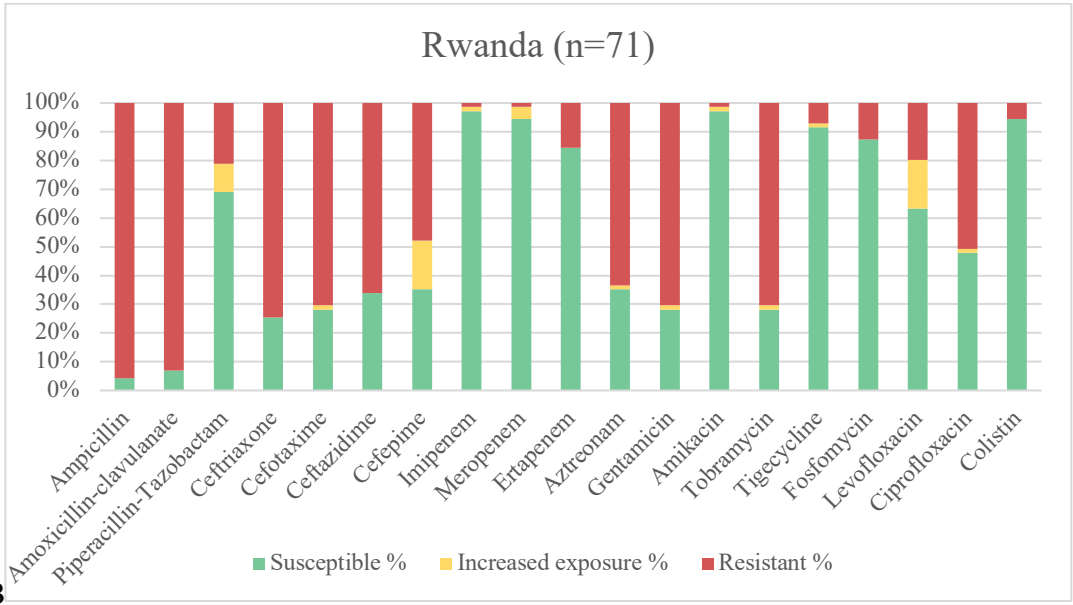
= -4.914, p<0.0001); meropenem (T(1) = -4.951, p<0.0001); ertapenem (T(1) = -5.671, p<0.0001); amikacin (T(1) = -6.109, p<0.0001)); tigecycline (T(1)= -2.303, p=0.022); fosfomycin (T(1) = -3.298, p=0.001) and colistin (T(1) = -5.451, p<0.0001). MICs were significantly higher for isolates from Africa for amoxicillin-clavulanate (T(1) = 2.426, p=0.015) and minocycline (T(1) = 6.826, p<0.0001).

4.2.2.6 Comparison of Gram-negative isolates from countries in Africa

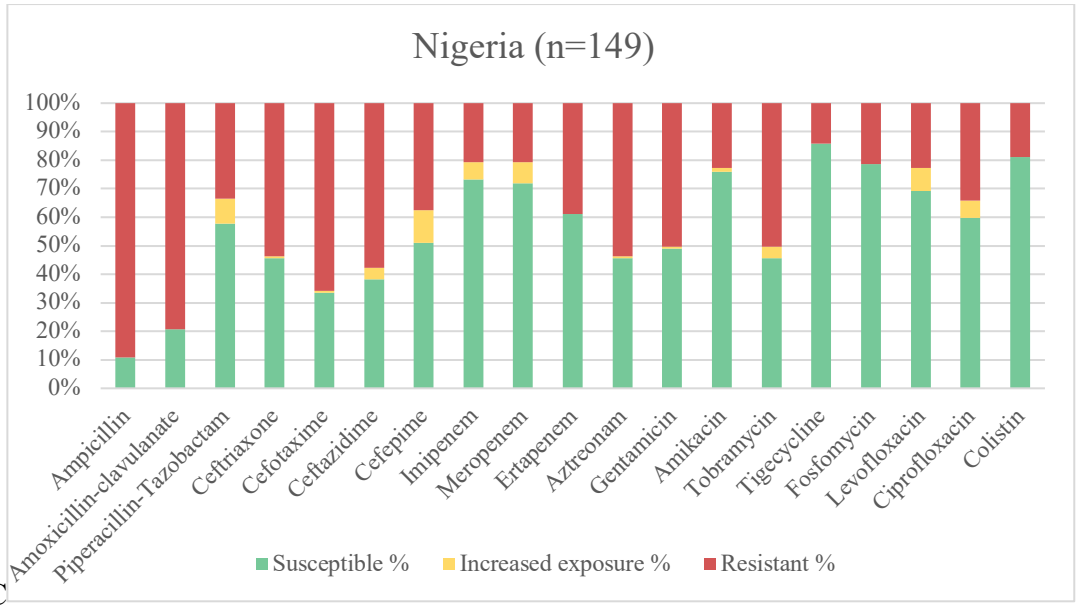
GNB samples from Africa included 37 from South Africa, 71 from Rwanda (split between 52 RK; 19 RU), 149 from Nigeria (including 66 from NK; 77 from NN; 6 from NW) and 120 from Ethiopia. General resistance profiles were similar between the countries where sites were located within Africa, however, slight variations can be seen upon closer inspection (Figure 4.10 A-D). Resistance against ampicillin was >95% for all countries in Africa except for Nigeria, that was just below 90%. South Africa had a lower level of resistance for piperacillin-tazobactam compared to other countries in Africa. Ethiopia had the highest resistance towards the third generation cephalosporins tested (86-88%) and isolates from South Africa the lowest (43-47%). Low resistance was seen against the carbapenems tested for all sites, although this was higher in Nigeria (20.81% for both imipenem and meropenem and 38.93% for ertapenem. Wide variation was seen for the aminoglycosides tested (gentamicin and amikacin), although in all sites, resistance against amikacin was lower than gentamicin. Gentamicin resistance was highest in Ethiopia (85.0%), followed by Rwanda (70.42%), Nigeria (50.34%) and South Africa (32.43%). Ethiopia despite having highest resistance to gentamicin, had the lowest prevalence of resistance against amikacin (0.83%) and Rwanda had the second lowest (1.41%), followed by South Africa (5.41%). Highest resistance towards amikacin was found in isolates from Nigeria (22.82%).


South Africa (n=37)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Ampicillin
Amoxicillin-clavulanate
Piperacillin-Tazobactam
Ceftriaxone
Cefotaxime
Ceftazidime
Cefepime
Imipenem
Meropenem
Ertapenem
Aztreonam
Gentamicin
Amikacin
Tobramycin
Tigecycline
Fosfomycin
Levofloxacin
Ciprofloxacin
Colistin
Susceptible %
Increased exposure %
Resistant %

A


Rwanda (n=71)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Ampicillin
Amoxicillin-clavulanate
Piperacillin-Tazobactam
Ceftriaxone
Cefotaxime
Ceftazidime
Cefepime
Imipenem
Meropenem
Ertapenem
Aztreonam
Gentamicin
Amikacin
Tobramycin
Tigecycline
Fosfomycin
Levofloxacin
Ciprofloxacin
Colistin
Susceptible %
Increased exposure %
Resistant %

B



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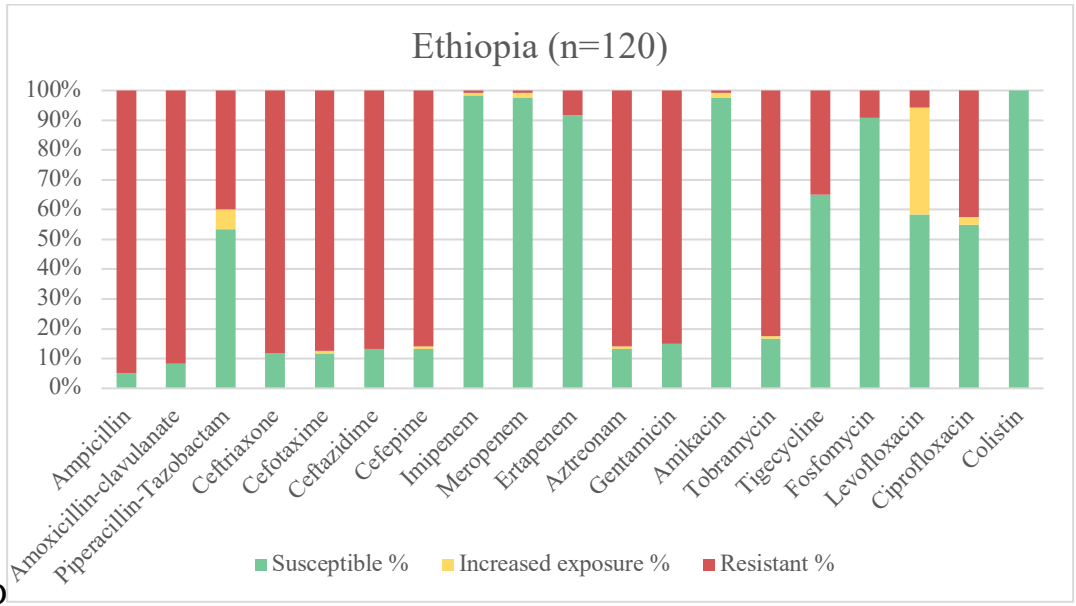


D

Figure 4.23. Resistance profiles per country for sites in Africa for sepsis cases caused by Gram-negative bacteria accoding to EUCAST v9.0. A. South Africa (one site), B. Rwanda (two sites), C. Nigeria (three sites) and D. Ethiopia (one site). Red sections show the percentage of resistant isolate, yellow shows those that would require increased exposure and green show those determined as susceptible to each antibiotic.

Table 4. 13 One-way analysis of variance (ANOVA) results from comparison of minimum inhibitory concentration (MICs) between the four countries within Africa: Ethiopia, Nigeria, Rwanda and South Africa. The countries Ethiopia and South Africa each had one site, Nigeria included three sites (NK, NN and NW) and Rwanda included two sites (RK and RU).

Antibiotic	**One-way ANOVA result**
Ampicillin	F (3) =3.116, p=0.026
Amoxicillin-clavulanate	F (3) =4.232, p=0.006
Piperacillin-tazobactam	F (3) =5.626, p=0.001
Ceftriaxone	F (3) =16.504, p<0.0001
Cefotaxime	F (3) =11.173, p<0.0001
Ceftazidime	F (3) =11.549, p<0.0001
Cefepime	F (3) =17.947, p<0.0001
Imipenem	F (3) =15.476, p<0.0001
Meropenem	F (3) =16.902, p<0.0001
Ertapenem	F (3) =17.201, p<0.0001
Aztreonam	F (3) =1.656, p=0.176
Gentamicin	F (3) =4.020, p=0.008
Amikacin	F (3) =11.010, p<0.0001
Tobramycin	F (3) =11.670, p<0.0001
Tigecycline	F (3) =1.798, p=0.147
Minocycline	F (3) =11.717, p<0.0001
Fosfomycin	F (3) =5.074, p=0.002
Levofloxacin	F (3) =5.430, p=0.001
Ciprofloxacin	F (3) =1.096, p=0.351
Colistin	F (3) =4.650, p=0.003

Comparisons of MICs between countries within Africa demonstrated significant differences between countries for all but three antibiotics (aztreonam, tigecycline and ciprofloxacin), detailed in Table 4.13. For those with significant results from the one-way ANOVA, post-hoc Tukey analyses demonstrated that MICs were found to be higher for ampicillin in Ethiopia compared to Nigeria (p=0.047, 95% C.I. = 0.004 – 20.92). MICs for amoxicillin-clavulanate were significantly lower in South Africa compared to Ethiopia and Rwanda (p=0.030, 95% C.I. = -23.44 – -0.84; p=0.005, 95% C.I. = -27.94 – -3.57, respectively). Similarly, MICs were lower in South Africa compared to Ethiopia and Nigeria for piperacillin-tazobactam (p=0.001, 95% C.I. = -29.48 – -5.51; p=0.021, 95% C.I. = -24.80

– -1.38), in addition to lower MICs in Rwanda compared to Ethiopia (p=0.048, 95% C.I. = -19.13 – -0.04). All third-generation cephalosporins had higher MICS in Ethiopia compared to Nigeria and South Africa (ceftriaxone p<0.0001, 95% C.I. = 1.494 – 3,576; p<0.0001, 95% C.I. = 1.517 – 4.708; cefotaxime p<0.0001, 95% C.I. = 0.868 – 2.907; (p<0.0001, 95% C.I. = 1.330 – 4.456); ceftazidime p<0.0001, 95% C.I. = 0.797 – 2.961; (p<0.0001, 95% C.I. = 1.668 – 4.985) and ceftazidime also had MICs higher in Ethiopia compared to Rwanda (p=0.045, 95% C.I. = 0.021 – 2.662) in addition to higher MICS in Rwanda compared to South Africa (p=0.023, 95% C.I. = 0.197 – 3.773). MICs were also higher in Rwanda compared to Nigeria and South Africa for ceftriaxone (p=0.017, 95% C.I. = 0.182 – 2.630; p=0.016, 95% C.I. = 0.263 – 3.704). Cefepime MICs were higher in Ethiopia compared to all other countries in Africa (p<0.0001, 95% C.I. = 1.667 – 3.776; p<0.0001, 95% C.I. = 0.803 – 3.377; p<0.0001, 95% C.I. = 1.619 – 4.853 for Nigeria; Rwanda; and South Africa, respectively).

MICs for the three carbapenems tested and amikacin in isolates from sites in Nigeria were higher compared to Ethiopia (imipenem p<0.0001, 95% C.I. = 1.47 – 3.69; meropenem (p<0.0001, 95% C.I. = 1.597 – 3.860; ertapenem p<0.0001, 95% C.I. = 0.672 – 1.562; amikacin p<0.0001, 95% C.I. = 10.16 – 31.50); Rwanda (imipenem p<0.0001, 95% C.I. = 1.30 – 3.90; meropenem (p<0.0001, 95% C.I. = 1.377 – 4.038; ertapenem p<0.0001, 95% C.I. = 0.381 – 1.427; amikacin p<0.0001, 95% C.I. = 7.88 – 32.97); and South Africa (imipenem p=0.017, 95% C.I. = 0.24 – 3.56; meropenem (p=0.001, 95% C.I. = 0.752 – 4.141; ertapenem p<0.0001, 95% C.I. = 0.427 – 1.760; amikacin p=0.013, 95% C.I. = 2.87 – 34.84). MICs for gentamicin were higher in Rwanda compared to Ethiopia (p=0.008, 95% C.I. = 4.11 – 38.80) and Nigeria p=0.023, 95% C.I. = 1.79 – 35.21).

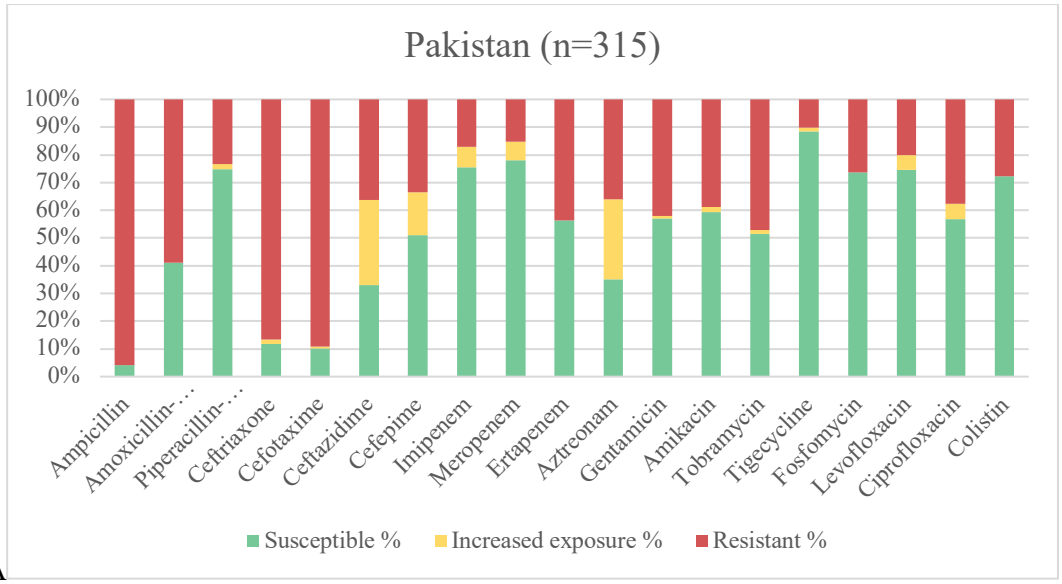
MICs were higher in Nigeria compared to Ethiopia for fosfomycin (p=0.002, 95% C.I. = 7.65 – 45.87), levofloxacin p<0.0001, 95% C.I. = 0.421 –1.935) and colistin MICs higher in South

Africa compared to both Ethiopia (p=0.002, 95% C.I. = 6.72 – 40.30) and Rwanda p=0.030, 95% C.I. = 1.33 – 37.53).

4.2.2.7 Gram-negative isolates from sites in Asia

When resistance profiles were assessed by country within Asia, isolates from India had higher overall resistance, followed by Bangladesh and Pakistan. Resistance against ampicillin was consistently high across the three countries, as was amoxicillin-clavulanate (Table 4.12). India had highest resistance against piperacillin-tazobactam (84.62% compared to 26.82% in Bangladesh ($X^2(1, N=192) = 19.022$, p=0.000013) and 23.25% in Pakistan ($X^2(1, N=377) = 24.627$, p<0.00001)), amikacin (76.92% compared to 29.61% in Bangladesh ($X^2(1, N=190) = 17.570$, p=0.000028) and 38.85% in Pakistan ($X^2(1, N=325) = 11.940$, p=0.000549)), meropenem (38.46%) compared to 15.29% in Pakistan ($X^2(1, N=327) = 4.937$, p=0.0263), but not determined as significant compared to Bangladesh (16.76%) ($X^2(1, N=192) = 3.829$, p=0.0504). Resistance against ertapenem was significantly higher in India (84.62%) compared to 30.17% in Bangladesh ($X^2(1, N=192) = 16.045$, p=0.000062) and 43.63% in Pakistan ($X^2(1, N=327) = 8.464$, p=0.00362). However, analyses only included 13 isolates from India, which may have skewed these results.

Gentamicin and third generation cephalosporin resistance was high in both Bangladesh and India. Colistin resistance was far higher in Bangladesh (70.95% compared to 27.71% in Pakistan and 7.69% in India), but as already discussed this was mainly due to an outbreak of intrinsically resistant *Serratia marcescens*, which accounted for 120/179 BB isolates from Bangladesh (Figure 4.13).



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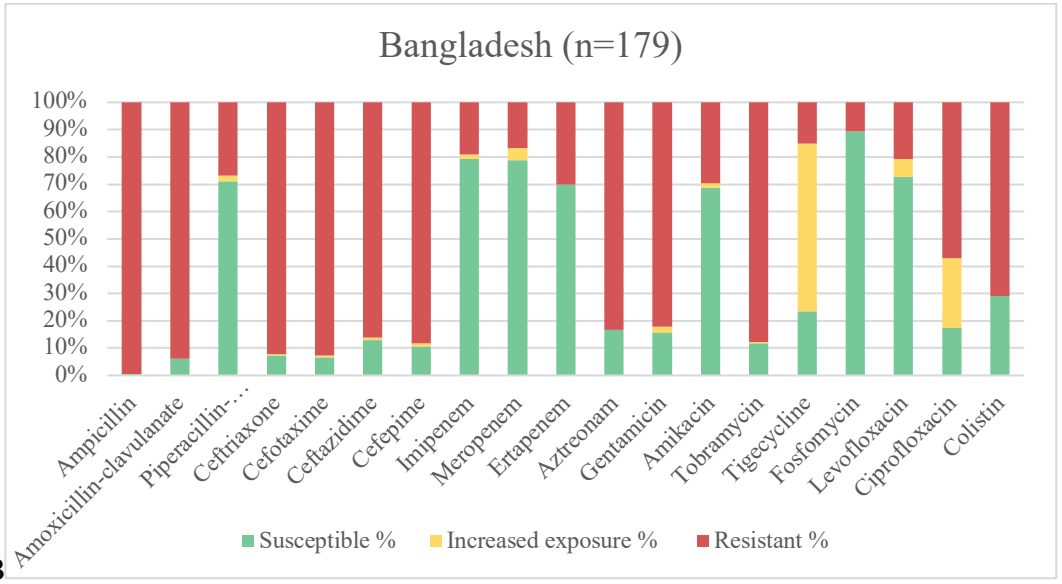

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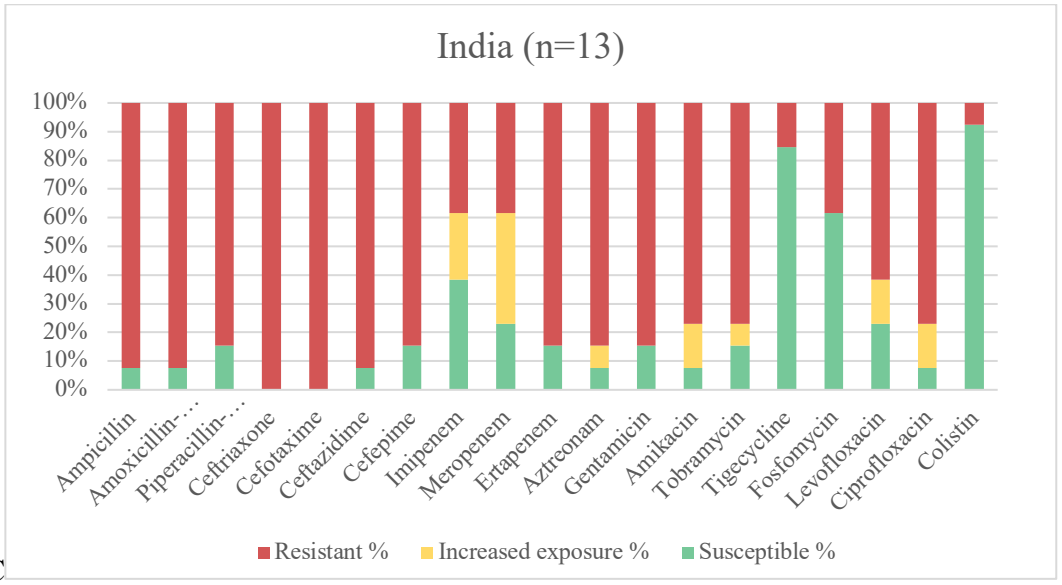


C

Figure 4.24 Resistance profiles per country for sites in South Asia for sepsis cases caused by Gram-negative bacteria, split into sites in (A) Pakistan; (B) Bangladesh; and (C) India. Red sections show

the percentage of resistant isolate, yellow shows those that would require increased exposure and green show those determined as susceptible to each antibiotic according to EUCAST v9.0.

One-way ANOVAs were carried out investigating variance between countries with Asia on MICs. No significant differences were found between countries for imipenem (one-way ANOVA (F (2) =0.503, p=0.605)) or meropenem (one-way ANOVA (F (2) =1.752, p=0.174). Variation between at least two of the three countries within Asia were found to be significantly different for the remaining 18 antibiotics, via one-way ANOVAs carried out, results of which are detailed in Table 4.14, on which Post Tukey HSD analyses were carried and detailed herein.

Table 4. 14 One-way analysis of variance (ANOVA) results comparing minimum inhibitory concentrations (MICs) between countries in Asia.

Antibiotic	**One-way ANOVA result**
Ampicillin	F (2) = 4.488, p = 0.012
Amoxicillin-clavulanate	F (2) = 52.490, p < 0.0001*
Piperacillin-tazobactam	F (2) = 11.986, p < 0.0001*
Ceftriaxone	F (2) = 6.377, p = 0.002*
Cefotaxime	F (2) = 3.817, p = 0.023*
Ceftazidime	F (2) = 66.071, p < 0.0001*
Cefepime	F (2) = 74.012, p < 0.0001*
Imipenem	F (2) = 0.503, p = 0.605
Meropenem	F (2) = 1.752, p = 0.174
Ertapenem	F (2) = 11.916, p < 0.0001*
Aztreonam	F (2) = 40.101, p < 0.0001*
Gentamicin	F (2) = 50.126, p < 0.0001*
Amikacin	F (2) = 7.351, p = 0.001*
Tobramycin	F (2) = 39.025, p < 0.0001*
Tigecycline	F (2) = 57.021, p < 0.0001*
Minocycline	F (2) = 43.293, p < 0.0001*
Fosfomycin	F (2) = 12.871, p < 0.0001*
Levofloxacin	F (2) = 8.446, p < 0.0001*
Ciprofloxacin	F (2) = 9.650, p < 0.0001*
Colistin	F (2) = 58.473, p < 0.0001*

*shows significant result

Tukey's HSD Test for multiple comparisons found the mean value of MICs for ampicillin were significantly higher in Bangladesh compared to Pakistan (p=0.008, 95% C.I. = [0.81-6.75]). Amoxicillin-clavulanate had significantly lower MICs in Pakistan compared to Bangladesh or India (p<0.0001, 95% C.I. = -28.9 - -17.86) and (p=0.012, 95% C.I. = -36.2 - -3.53) respectively). India had higher MICs for piperacillin-tazobactam compared to Bangladesh (p<0.0001, 95% C.I. = 15.32 – 48.31) or Pakistan (p<0.0001, 95% C.I. = 17.55 – 50.06]). Bangladesh had higher MICs than Pakistan for both ceftriaxone (p=0.004, 95% C.I. = 0.188 – 1.238) and cefotaxime (p=0.044, 95% C.I. = 0.011 – 0.989). Pakistan had lower MICs than Bangladesh and India for ceftazidime (p<0.0001, 95% C.I. = -3.490 – -2.268; p<0.0001, 95%, C.I. = -5.284 – -1.591, respectively) and cefepime (p<0.0001, 95% C.I. = -3.761 – -2.521; p<0.0001, 95% C.I. = -4.989 – -1.242, respectively). For ertapenem, India had higher MICs than Bangladesh (p<0.0001, 95% C.I. = 0.977 – 3.322) and Pakistan (p=0.003, 95% C.I. = 0.484 – 2.794), with Bangladesh also lower than Pakistan (p=0.005, 95% C.I. = -0.893– -0.128). MICs were significantly lower in Pakistan for aztreonam compared to Bangladesh and India (p<0.0001, 95% C.I. = -2.796 – -1.605, p=0.003, 95% C.I. = -4.261 – -0.662, respectively), and gentamicin (p<0.0001, 95% C.I. = -7.6 – -4.65, p=0.002, 95% C.I. = -10.83 – -1.93, respectively). Significantly higher MICs were found in India compared to Bangladesh (p=0.001, 95% C.I. = 10.67 – 46.55) and Pakistan (p=0.003, 95% C.I. = 7.05 – 42.40). Tobramycin had lower MICs in Pakistan than Bangladesh (p<0.0001, 95% C.I. = -6.83 – -3.93), and India (p=0.032, 95% C.I. = -9.10 – -0.33), as well as for tigecycline (p<0.0001, 95% C.I. = -1.392 – -0.880), p=0.001, 95% C.I. = -1.943 – -0.395, respectively) and minocycline (p<0.0001, 95% C.I. = -2.791 – -1.631, p=0.001, 95% C.I. = -4.403 – -0.899, respectively). Conversely MICs for fosfomycin were significantly higher in Pakistan compared to Bangladesh (p<0.001, 95% C.I. = 20.42 – 55.76). India had higher MICs for levofloxacin than Bangladesh (p<0.0001, 95% C.I. = 1.339 – 4.961) and Pakistan

p<0.0001, 95% C.I. = 1.253 – 4.822) and for ciprofloxacin (p<0.001, 95% C.I. = 0.643 – 2.554 and p>0.001, 95% C.I. = 0.803 – 2.685, respectively). Bangladesh had the highest MICs for colistin compared to India (p<0.0001, 95% C.I. = 4.94 – 13.91), and Pakistan p<0.0001, 95% C.I. = 5.03 – 7.95), but this was due to their outbreak of *Serratia marcescens* which is intrinsically resistant to colistin.

4.2.3. Species specific resistance profiles

Resistance profiles were assessed for the three most common species found in multiple BARNARDS sites, which included *S. aureus* (previously described), *Klebsiella* spp., mainly *K. pneumoniae* and *E. coli*.

4.2.3.1 *Klebsiella* spp.

A total of 387 *Klebsiella* spp. isolates were recovered from cases of neonatal bacteraemia in BARNARDS, consisting of 258 *K. pneumoniae*, 117 *K. michiganensis*, 13 *K. quasipneumoniae*, five *K. oxytoca*, five *K. variicola*, and three *K. aerogenes*. A large proportion of the *Klebsiella* spp. isolates were from PP (144) and ESS (101), which were largely attributed due to outbreaks of *K. michiganensis* and *K. pneumoniae* at each site, respectively. MICs were done on 380 of these *Klebsiella* spp., which included 253 *K. pneumoniae*, 109 *K. michiganensis*, 12 *K. quasipneumoniae*, five *K. oxytoca*, five *K. variicola*, and three *K. aerogenes* (Table 4.15).

Table 4.15. Numbers of each Klebsiella spp. isolates by site with both whole genome sequencing and minimum inhibitory concentration data.

Site	*K. aerogenes*	*K. michiganensis*	*K. oxytoca*	*K. pneumoniae*	*K. quasipneumoniae*	*K. variicola*	Total
PP		101	2	41	1	1	**144**
ES		4	1	95	1		**101**
NN	1			36	2		**37**
BC				17	5	1	**21**
NK	1			15	2		**18**
RK			2	14		2	**18**

ZAT	1			16			**17**
RU				7		1	**8**
PC		4		2			**6**
IN				5			**5**
BK				2	1		**3**
NW				3			**2**
Total	**3**	**109**	**5**	**253**	**12**	**5**	**387**

4.2.3.1.1 *K. michiganensis*

Only five sequence types were found for K. *michiganensis*, as this species was only prevalent due to an outbreak at PP of ST180, which made up 112/122 *K. michiganensis* isolates, three of which were at PC and 109 at PP. There were also two cases of ST232 in ESS, and one case each of ST108, ST197 and ST268 all from ESS. Most isolates had resistance to three to five antibiotics, but some had higher resistance. Regarding ST180 isolates, all except three of these isolates were resistant to ampicillin and 103/112 were resistant to ceftriaxone with 104/112 displaying resistance against cefotaxime. All except one isolate were susceptible to meropenem and imipenem, with six resistant to ertapenem. There was some variation in resistance against amoxicillin-clavulanate, ceftazidime and cefepime and aztreonam. These isolates were mainly susceptible to the aminoglycosides and other antibiotics tested. Overall, these isolates mainly had CTX-M (n=99) but not many other ARG were identified. As this species was not as common excluding outbreaks, no further assessment has been done on these isolates.

4.2.3.1.2 *K. Pneumoniae* MICs

Klebsiella pneumoniae isolates commonly displayed growth at the highest concentrations tested for a range of antibiotics, including ampicillin, amoxicillin-clavulanate, piperacillin-tazobactam, ceftriaxone, cefotaxime, ceftazidime, cefepime, aztreonam, gentamicin, tobramycin, and minocycline. Only the carbapenems, amikacin, levofloxacin, ciprofloxacin and colistin demonstrated the lowest concentration tested inhibited the majority

of isolates (Figure 4.18). MIC_{90} results were above the highest concentration tested for all antibiotics except for meropenem, tigecycline, fosfomycin and colistin. MIC_{50} results were over the highest concentration tested for ampicillin, amoxicillin-clavulanate, cephalosporins, aztreonam, gentamicin, tobramycin and minocycline (Table 4.16). *K. pneumoniae* MICs per country can be seen on Appendix, page 19-20.

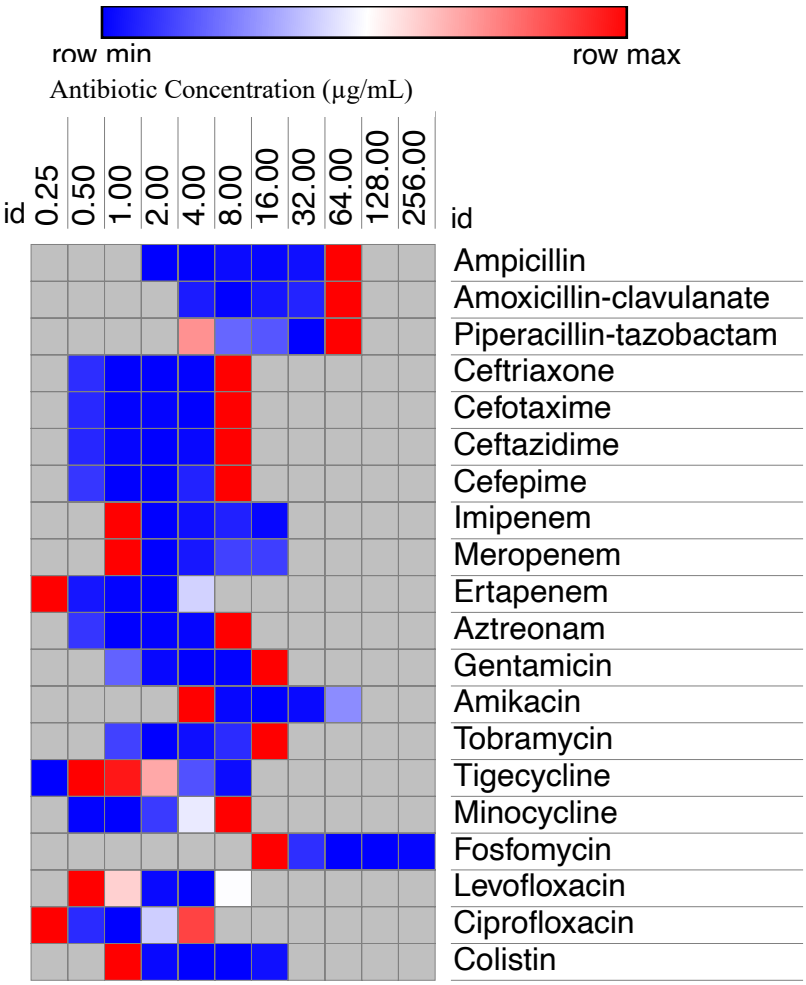


Figure 4.25. Minimum inhibitory concentrations (MICs) for Klebsiella pneumoniae isolates (n=253) for all antibiotics. Each antibiotic was tested two breakpoints above and below respective breakpoint concentrations. Isolates with growth at the highest concentration were recorded as the concentration above for this heatmap. Grey areas show concentrations show concentration not tested.

Table 4. 16. MIC_{50} results show the minimum inhibitory concentration (MIC) preventing growth in 50% of isolates and MIC_{90} results show the MIC at which 90% of isolates had stopped growing. MIC_{50} and MIC_{90} results of Klebsiella pneumoniae isolates (n=253).

Antibiotic	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Ampicillin	>32	>32
Amoxicillin clavulanate	>32	>32
Piperacillin-tazobactam	16	>32
Ceftriaxone	>4	>4
Cefotaxime	>4	>4
Ceftazidime	>4	>4
Cefepime	>4	>4
Imipenem	1	>8
Meropenem	1	8
Ertapenem	0.25	>2
Aztreonam	>4	>4
Gentamicin	>8	>8
Amikacin	4	>32
Tobramycin	>8	>8
Tigecycline	1	2
Minocycline	>4	>4
Fosfomycin	16	32
Levofloxacin	1	>4
Ciprofloxacin	2	>2
Colistin	1	1

High resistance was seen in *K. pneumoniae* isolates for ampicillin (98.02%), although this species is considered intrinsically resistant to ampicillin (EUCAST, 2020). High levels of resistance were also seen against amoxicillin-clavulanate (90.01%), third generation cephalosporins (ceftriaxone, 90.90%, cefotaxime, 91.70%, ceftazidime, 89.72%), aztreonam (88.14%), gentamicin (83.00%), tobramycin (83.79%). Lowest resistance was seen in imipenem (14.62%), meropenem (14.62%) and fosfomycin (3.16%) (Table 4.17; Figure 4.19).

Table 4. 17 Number and percentage of K. pneumoniae isolates that were determined as resistant, requiring increased exposure or susceptible according to EUCAST v9.0 guidelines.

Antibiotic	Resistant (%)	Increased exposure (%)	Susceptible (%)
Ampicillin	248 (98.02%)	0 (0.00%)	5 (1.98%)
Amoxicillin-clavulanate	230 (90.91%)	0 (0.00%)	23 (9.09%)
Piperacillin-tazobactam	127 (50.20%)	17 (6.72%)	109 (43.08%)
Ceftriaxone	230 (90.90%)	1 (0.40%)	22 (8.70%)
Cefotaxime	232 (91.70%)	2 (0.79%)	19 (7.51%)
Ceftazidime	227 (89.72%)	5 (1.98%)	21 (8.30%)
Cefepime	209 (55.44%)	18 (7.11%)	26 (10.28%)
Imipenem	37 (14.62%)	17 (6.72%)	199 (78.66%)
Meropenem	37 (14.62 %)	28 (11.07%)	188 (74.31%)
Ertapenem	74 (29.25%)	0 (0.00%)	179 (70.75%)
Aztreonam	223 (88.14%)	5 (1.98%)	25 (9.88%)
Gentamicin	210 (83.00%)	0 (0.00%)	43 (17.00%)
Amikacin	60 (23.72%)	64(1.58%)	189 (74.70%)
Tobramycin	212 (83.79%)	9 (3.56%)	32 (12.65%)
Tigecycline	78 (30.83%)	0 (0.00%)	175 (69.17%)
Minocycline	NA	NA	NA
Fosfomycin	8 (3.16%)	0 (0.00%)	245 (96.84%)
Levofloxacin	67 (26.48%)	70 (27.67%)	116 (45.85%)
Ciprofloxacin	140 (55.34%)	18 (7.11%)	95 (37.55%)
Colistin	7 (2.77%)	0 (0.00%)	246 (97.23%)

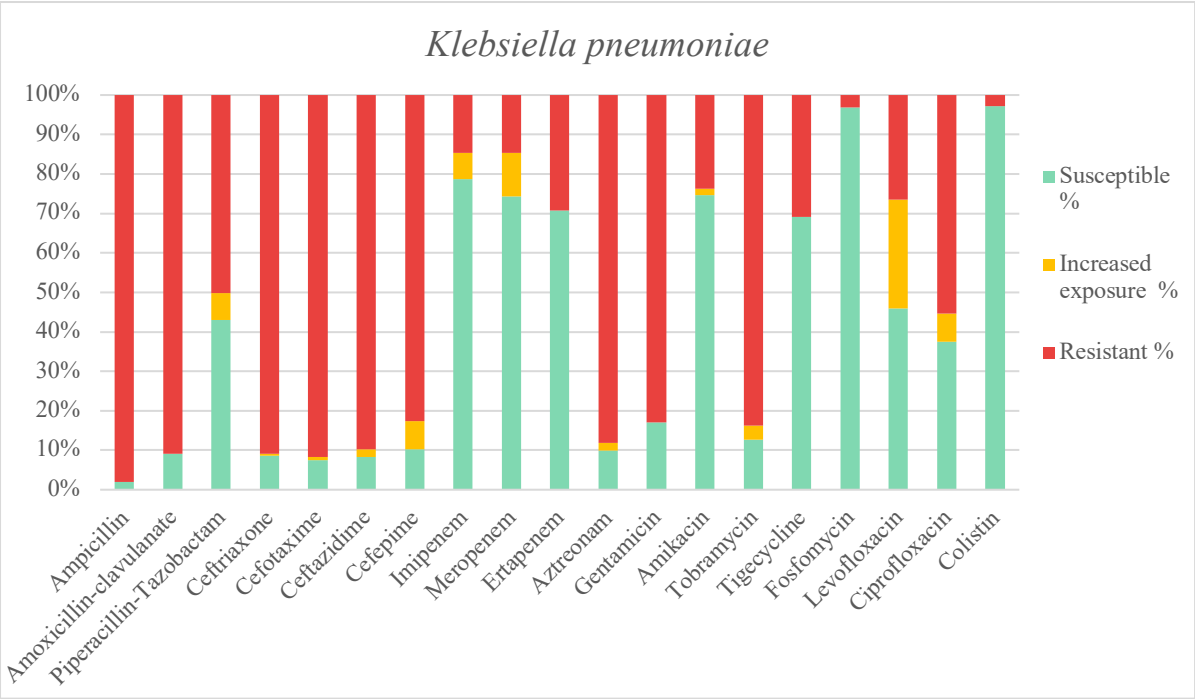


Figure 4.26. Resistance profiles of K. pneumoniae isolates overall, n=253. Red sections show the percentage of resistant isolate, yellow shows those that would require increased exposure and green show those determined as susceptible to each antibiotic according to EUCAST v9.0.

4.2.3.1.3 WGS data *K. pneumoniae*

β-lactamase SHV and TEM were co-expressed in 170 isolates of mainly isolates resistant to amoxicillin-clavulanate but 14 of these were susceptible. Furthermore, 230 isolates were resistant to amoxicillin-clavulanate, so not all resistant co-expressed these. Bla-CTX-M-1 was found in 216/253 isolates, 200 of which were resistant to ceftriaxone and cefotaxime and ceftazidime. Of the 37 isolates demonstrating resistance to imipenem, 34 produced *bla*$_{\text{-NDM-1}}$ (n=24) (16 also producing *bla*-$_{\text{OXA232/181}}$), *bla*$_{\text{-NDM-5}}$ (n=1), or *bla*$_{\text{-NDM-7}}$ (n=9). All these isolates were also either resistant or require increased exposure to meropenem. A higher number of isolates were resistant to ertapenem (n=74), 61 or which produced NDM, with 23 isolates also producing OXA (two *bla*$_{\text{-oxa232}}$, 20 *bla*$_{\text{-oxa181}}$). Only one isolate produced *bla*$_{\text{-OXA232}}$ without NDM. There was one isolate with resistance to imipenem,

meropenem and ertapenem with not recorded NDM or OXA. Overall, NDM was present in 66 *K. pneumoniae* isolates from varied clinical site and was found in a range of sequence types, including ST15 (n=23), ST464 (n=8), ST442 (n=5), ST20 (n=4), ST995 (n=3), ST11 (n=3), ST611 (n=2), ST14 (n=2), ST70 (n=2), ST395 (n=2), ST967 (n=1), ST25 (n=1), ST377 (n=1), ST17 (n=1) and ST147 (n=1). OXA was present in ST15 (n=19), ST14 (n=2) and ST25 (n=1).

4.2.3.2.1 *E. coli* MICs

Unlike *K. pneumoniae isolates* growth above the highest concentration tested for *E. coli* only occurred for most isolates for ampicillin, amoxicillin-clavulanate and minocycline (Figure 4.21). Most antibiotics except for ampicillin and colistin had either a lower MIC_{50} or MIC_{90} MIC compared to *K. pneumoniae* (Table 4.17; Table 4.19). *E. coli* MIC_{50} results were lower for amoxicillin-clavulanate, piperacillin-tazobactam, all cephalosporine, aztreonam, gentamicin, tobramycin, minocycline, levofloxacin and ciprofloxacin. MIC_{90} results were lower for the carbapenems tested, amikacin, tigecycline and fosfomycin (Table 4.18), relative to *K. pneumoniae* results. *E. coli* MICs per country can be seen on Appendix, pages 21-22.

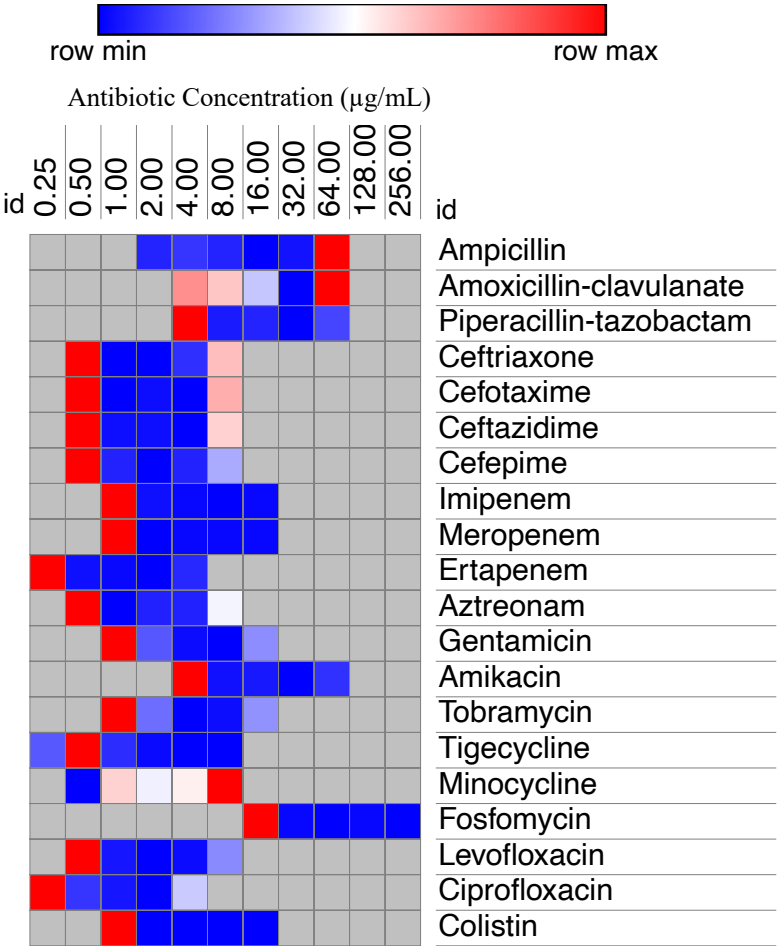


Figure 4.27. Minimum inhibitory concentration (MIC) heatmap for E. coli isolates (n=74). Each antibiotic was tested two breakpoints above and below respective breakpoint concentrations. Isolates with growth at the highest concentration were recorded as the concentration above for this heatmap. Grey areas show concentrations show concentration not tested.

Table 4. 18. MIC_{50} results show the minimum inhibitory concentration (MIC) preventing growth in 50% of isolates and MIC_{90} results show the MIC at which 90% of isolates had stopped growing. MIC_{50} and MIC_{90} results for E. coli isolates tested (n=74).

Antibiotic	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Ampicillin	>32	>32
Amoxicillin clavulanate	16	>32
Piperacillin-tazobactam	4	>32
Ceftriaxone	0.5	>4
Cefotaxime	0.5	>4
Ceftazidime	0.5	>4
Cefepime	0.5	>4
Imipenem	1	1
Meropenem	1	1
Ertapenem	0.25	0.5
Aztreonam	0.5	>4
Gentamicin	1	>8
Amikacin	4	16
Tobramycin	4	>8
Tigecycline	0.5	0.5
Minocycline	4	>4
Fosfomycin	16	16
Levofloxacin	0.5	>4
Ciprofloxacin	0.25	>2
Colistin	1	1

Resistance in *E. coli* was lower for all antibiotics tested compared to *K. pneumoniae* (Table 4.17; Table 4.20). Notably resistance was reduced for ampicillin (81.04%), all cephalosporins (ceftriaxone 41.89%; cefotaxime 39.19%, ceftazidime 33.78%, cefepime 25.68%), carbapenems (imipenem 1.35%; meropenem 2.70%; ertapenem 8.11%), gentamicin (20.27%, amikacin (8.11%) and no resistance to colistin was found (Figure 4.22; Table 4.19)

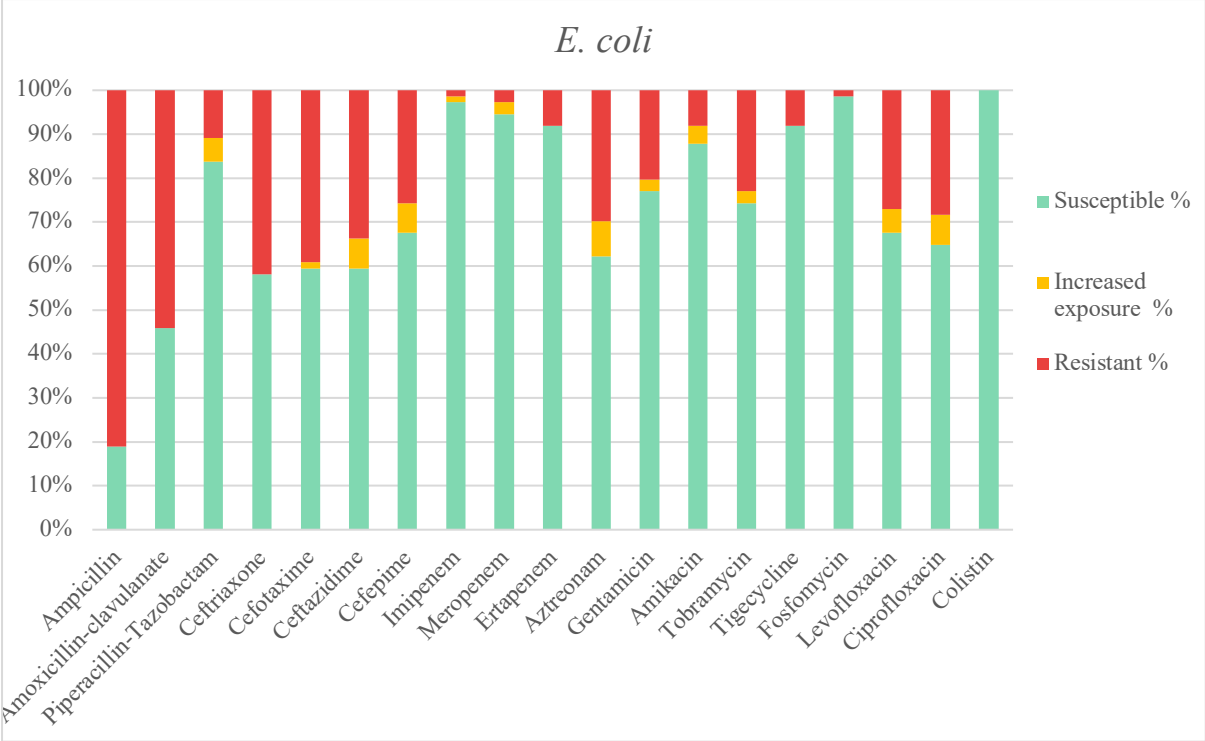


Figure 4.28. Resistance profiles for E. coli isolates from positive blood cultures (n=74). Red sections show the percentage of resistant isolate, yellow shows those that would require increased exposure and green show those determined as susceptible to each antibiotic according to EUCAST v9.0.

Table 4. 19. Numbers and percentages of E. coli isolates defined as susceptible, requiring increased exposure or resistant (n=74).

Antibiotic	Resistant (%)	Increased exposure (%)	Susceptible (%)
Ampicillin	60 (81.08%)	0 (0.00%)	14 (18.92%)
Amoxicillin-clavulanate	40 (54.05%)	0 (0.00%)	34 (45.95%)
Piperacillin-tazobactam	8 (10.81%)	4 (5.41%)	62 (83.78%)
Ceftriaxone	31 (41.89%)	0 (0.00%)	43 (58.11%)
Cefotaxime	29 (39.19%)	1 (1.35%)	44 (59.46%)
Ceftazidime	25 (33.78%)	5 (6.76%)	44 (59.46%)
Cefepime	19 (25.68%)	5 (6.76%)	50 (67.57%)
Imipenem	1 (1.35%)	1 (1.35%)	72 (97.30%)
Meropenem	2 (2.70%)	2 (2.70%)	70 (94.59%)
Ertapenem	6 (8.11%)	0 (0.00%)	68 (91.89%)
Aztreonam	22 (29.73%)	6 (8.11%)	46 (62.16%)
Gentamicin	15 (20.27%)	2 (2.70%)	57 (77.03%)
Amikacin	6 (8.11%)	3 (4.05%)	65 (87.84%)
Tobramycin	17 (22.97%)	2 (2.70%)	55 (74.35%)

Tigecycline	6 (8.11%)	0 (0.00%)	68 (91.89%)
Minocycline	NA	NA	NA
Fosfomycin	1 (1.35%)	0 (0.00%)	73 (98.65%)
Levofloxacin	20 (27.03%)	4 (5.41%)	50 (67.57%)
Ciprofloxacin	21 (28.38%)	5 (6.76%)	48 (64.86%)
Colistin	0 (0.00%)	0 (0.00%)	74 (97.23%)

4.3.2.2 WGS data *E. coli*

Of the 75 *E. coli* isolates with WGS data, seven phylotypes were found through WGS, with most found globally. Phylotype A as the most common (n=22), followed by B2 (n=18), B1 (n=15), D (n=11), C (n=7), and only one phylotype E or F and no phylotype G found (Figure 4.23). The isolate with phylotype E and F were from NK and ZAT respectively. Phylotypes A, B1, C and D were found in sites across both Asia and Africa but phylotype B2 was only found in sites in Africa.

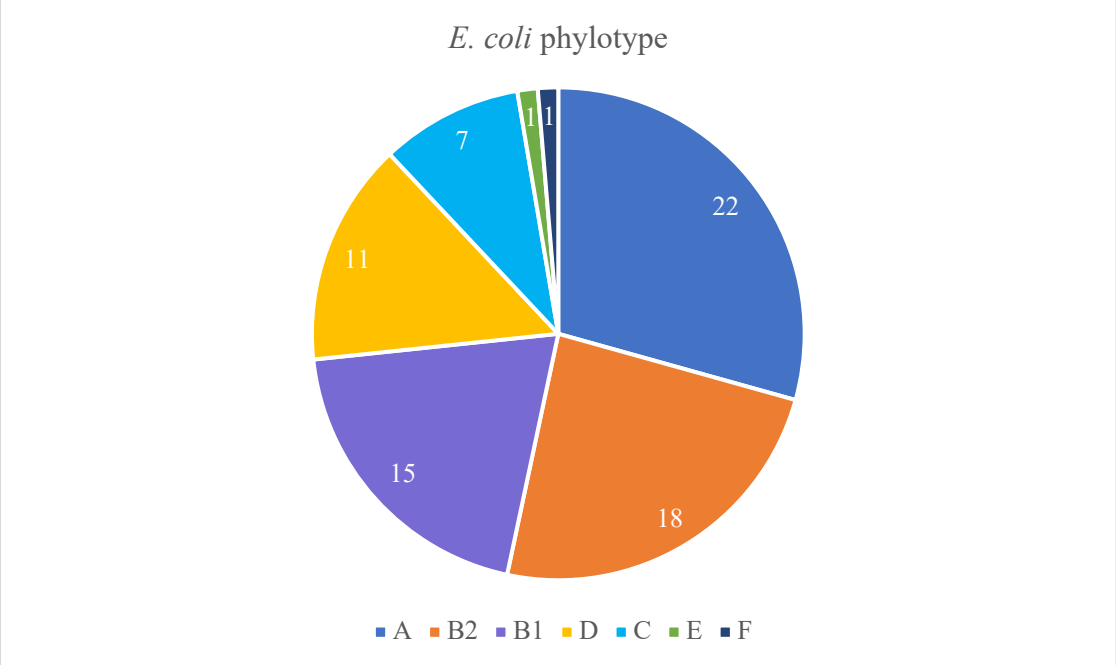


Figure 4.29. E. coli phylotype distribution from isolates with whole genome sequencing data (n=75).

A wide range of O:H antigen variation was found in the E. coli isolates, with few repeats. Most were unique, apart from four O15:H18 and three O31:H4 and O16:H5 although these were isolated at different clinical sites. Three O131:H9 were found, all from PC of which were all ST410 and phylotype C, which may demonstrate a small outbreak. Similarly, two isolates from RK had O160:H19 (both ST517, phylotype B1) could have been transmitted in hospital, with similar blood culture dates.

One isolate, ST410 from PP, was found to have bla-$_{OXA181}$ and was carrying multiple plasmids, including X3, X4 and Y plasmids. This isolate was found to have eight β-lactamase genes, including *bla*-$_{AMPC1}$, *bla*-$_{AMPC2}$, *bla*-$_{AMPH}$, *bla*-$_{CMY}$, *bla*-$_{CTX-M1}$, *bla*-$_{MRDA}$, *bla*-$_{OXA-48-like}$ and *bla*-$_{TEM1D}$. This isolate had high levels of resistance against all antibiotics tested, except tigecycline, fosfomycin and colistin. Two isolates, both ST167 had *bla*-$_{NDM5}$, one from BC and one from IN. These isolates contained multiple β-lactamase genes, including *bla*-$_{AMPC1}$, *bla*-$_{AMPC2}$, *bla*-$_{AMPH}$, *bla*-$_{CTX-M1}$, *bla*-$_{MRDA}$, *bla*-$_{NDM}$ and *bla*-$_{TEM1D}$, although the isolate from IN also had *bla*-$_{OXA-1}$, which also had a Y plasmid detected, unlike the isolate from BC. The isolate from BC was resistant to the majority of antibiotics tested, being susceptible to only imipenem, tigecycline, fosfomycin and colistin. Similarly, the isolate from IN was only susceptible to tigecycline, fosfomycin and colistin.

4.2.4 AMR prevalence in inborn and confirmed outborn neonates with associated mortality

4.2.4.1 GPB inborn vs. outborn MICs

Of the 560 isolates with WGS data from inborn neonates, MIC data was also available for 522 isolates, as were 335/371 isolates from outborn neonates. Additionally, 132 of isolates with MICs did not have a birth cohort confirmed, including 40 from BC, one form BK, 31 from ESS, two from IN, 12 from NK, nine from NN, 36 from PP and one from RK, which will not be included in these following analyses.

A total of 79 GPB sepsis isolates were assessed with WGS for inborn neonates, which all had MIC data and 45 GPB isolates had WGS data from outborn neonates, 43 of which also had MIC data. Distribution for MIC results for all antibiotics tested was found to be non-normal, however due to a larger sample size, T-tests were carried out. MICs for ampicillin, oxacillin and tobramycin were found to be significantly higher in the inborn cohort (T(119.07) = 2.900, p=0.004; (T(105.95) = 2.112, p=0.037; (T(102.648) = 2.408, p=0.018, respectively). MICs for the other antibiotics tested against GPB were not found to be significantly different in isolates from inborn and outborn neonates (p>0.05).

4.2.4.2 GNB inborn vs. outborn MICs

Overall, GNB made up 481/560 isolates analysed with WGS from inborn neonates, for which 460 isolates also had MIC data, and 326/371 of isolates analysed with WGS from outborn neonates with 291 with accompanying MIC data available. For GNB, MICs were higher for isolates from outborn neonates for amoxicillin-clavulanate (T(651.77) = -2.295, p= 0.022), ceftazidime (T(586.93)= -3.020, p= 0.003, cefepime (T(627.47)= -2.824, p= 0.005), tigecycline (T(500.43)= -4.237, p< 0.0001 and minocycline (T(651.57)= -3.450, p= 0.001. MICs were higher in isolates from inborn neonates for aztreonam (T(512.80)= 3.109, p=0.002) and fosfomycin (T(738.93)= 3.212, p=0.001).

Prevalence of AMR was slightly higher in inborn neonates, with an average of resistance against 9.73 antibiotics compared to outborn neonates with an average of 9.39, although this difference was not significant (t-test, p value=0.076). Resistance against both ampicillin and gentamicin simultaneously was more likely in inborn neonates (t-test, p value=0.035). An increase in AMR score was found to be associated with babies delivered by c-section, but this was not found to be significant (t-test, p value >0.05.

4.2.4.3 Mortality in neonates with high vs low resistance

Surprisingly, the results showed similar AMR scores for isolates that caused sepsis in neonates that remained alive (mean=9.14) and those that died (mean=8.51), (n=620 with outcome data) with a slightly lower score in those isolates from neonates that died. This difference was not found to be significant (t-test, p value=0.191). Therefore, there must be other factors in play to predict outcome, or alternatively outcome data collection methods may need to be examined further.

4.3 Discussion

4.3.1 Gram-positive bacteria

In this study overall we found 36.30% of GPB displayed methicillin resistance from phenotypic resistance to oxacillin, or 33% MRSA. A previous study by Shittu *et al.* (2011) found a lower rate of 16% resistance against oxacillin in *S. aureus* from Nigeria. Oxacillin resistance was found to be slightly lower GPB in isolates from sites in Nigeria (32.73%) in BARNARDS and when only *S. aureus* was analysed from Nigeria, as Shittu *et al.* (2011) investigated, only 1/25 isolates showed oxacillin resistance. A larger sample number from Nigeria is needed to enable comparison between studies. Another study from Africa, in Kenya found higher rates of MRSA at 53.4% measured from oxacillin resistance (Wangai *et al.,* 2019). They also found low prevalence of resistance against vancomycin and tigecycline, supporting the results from BARNARDS. A study carried out in Peshawar found similar rates of MRSA to those found in BARNARDS of 36.1% (Ullah *et al*., 2016).

All isolates with oxacillin resistance contained *mecA*, with an additional four isolates that were not sequenced but also presumed to contain *mecA*. However, there were an additional 14 *S. aureus* isolates that contained *mecA* but did not display phenotypic oxacillin resistance. Hososaka *et al*. (2007), also reported oxacillin susceptible *mecA* positive *S. aureus*

isolates in their study of clinical isolates. The oxacillin susceptible *mecA* positive isolates we found showed SCCmec type IVa or V, supported by Conceicau *et al.* (2015), who also found these SCC types associated with oxacillin susceptibility. These isolates from our study were not clonal as included a range of STs across BARNARDS sites.

High resistance (52.69%) was found against azithromycin, with an additional 39.78% isolates requiring increased exposure, however only two neonates were reportedly treated with azithromycin in the BARNARDS study, therefore the high level of resistance does not stem from routine use of azithromycin as a therapeutic for neonates. Azithromycin is commonly used to treat trachoma, an eye infection caused by *Chlamydia trachomatis*, common in infants under five years of age and is hyperendemic in many rural areas in LMICs, including South Asia, with Africa remaining as the most affected continent. In 1998, the WHO launched an initiative to eliminate trachoma in endemic countries by 2020. In 2018 alone, 89.1 million people were treated with azithromycin, the chosen antibiotic, in endemic areas (WHO, 2019). Studies have not found an increase of azithromycin resistance in *C. trachomatis* (West *et al*., 2014), however, temporary increased resistance against azithromycin was seen in other commensal organisms, including *Streptococcus pneumoniae* and *S. aureus* (Ho *et al.*, 2015; Bojang *et al*., 2017). Coles *et al.* (2013) found increased resistance against azithromycin in *S. pneumoniae* in communities provided with mass distribution of azithromycin for trachoma six months following application of the single dose. The trachoma atlas showed high treatment of trachoma in Ethiopia but there was little data displayed for other BARNARDS regions before and during the study period (https://atlas.trachomadata.org/). Azithromycin is also commonly used to treat pneumonia, skin infections and some sexually transmitted infections. This demonstrates the impact wide application of certain antibiotics can have across patients and across different infection and that it is essential to fully evaluate potential widespread impacts of initiatives such as this on

AMR. A study by Bojang *et al.* (2019), also found that the gene *msr(A)* was the main gene in azithromycin resistant *S. aureus* isolates, although they found this in a higher percentage (80%) of isolates than this study. They also found that ST5 *S. aureus* was the most common ST that acquired resistance through the duration of the study. In BARNARDS, 11/13 gentamicin resistant *S. aureus* isolates were ST5. Nine of these gentamicin resistant ST5 isolates were from ZAT, with t045 spa typing and all from inborn neonates, so it is possible this could have been a strain that was present in the hospital long-term may have caused nosocomial infections. *S. sciuri* was more common in Pakistan and did not show high resistance to gentamicin, compared to *S. haemolyticus* and *S. epidermis*, which were only present in Nigeria and had higher rates of resistance.

4.3.2 Gram-negative isolates

High resistance was seen amongst Gram-negative isolates from both Africa and South Asia for many antibiotics tested. In general, isolates from South Asia showed higher prevalence of resistance than isolates from Africa, including ampicillin, but this was >90% in both continents, as supported with other studies (Li *et al*., 2019; Shakiba *et al*., 2019; Doare *et al.*, 2014). This is particularly concerning, as ampicillin is one of the antibiotics suggested by the WHO for use in the empirical treatment of neonates with suspected sepsis. In addition to this, isolates tested also showed high resistance against gentamicin, the second antibiotic suggested to be given alongside ampicillin empirically. Resistance to both ampicillin and gentamicin was seen in 521 of the 883 Gram negative isolates tested (527 if isolates requiring increased exposure are included as non-susceptible). Therefore, the globally suggested empirical therapy will have no effect on 59% of Gram-negative infections.

Conza *et al*. (2014) suggested that co-expression of *bla*-$_{\text{TEM}}$ with -$_{\text{OXA-2-like}}$ or -$_{\text{SHV}}$ induced resistance to amoxicillin-clavulanate. However, we saw no distinct pattern in this co-expression and resistance to amoxicillin-clavulanate. Resistance against ceftriaxone and

cefotaxime, was higher in South Asia, rendering them ineffective in most cases, although both continents showed high resistance against third generation cephalosporins, as supported by Haigh *et al*. (2020) and Doare *et al.* (2014). Highest resistance for all cephalosporins were found in India, Bangladesh and Ethiopia. However, there was only a small number of sepsis cases recorded in India during the BARNARDS study, as this site joined the study late.

Resistance against carbapenems, again had higher frequency of resistant isolates recorded in South Asia where approximately double the resistance was found compared to isolates from sites in Africa. NDM was found at nearly all sites but at lower prevalence compared to sites across Asia. IN had the highest proportion of *bla*-$_{NDM}$ (53.8% of GNB) analysed and PP had the highest proportion of isolates that contained both *bla*-$_{NDM}$ and *bla*-$_{OXA}$ simultaneously (12.7%). India had the highest resistance against carbapenems of all BARNARDS countries with 38.46% resistance to both imipenem and meropenem and 84.62% resistance against ertapenem. Datta *et al.* (2013), found that carbapenem resistance increased from 2% in 2002 to 52% in 2009 in a tertiary care hospital in New Delhi, concurrent with findings of metallo-β-lactamase NDM, discovered in New Delhi (Kumarasamy *et al.*, 2010), found in numerous isolates from a range of species. OXA was more widespread amongst sites in both Asia and Africa, with particularly high numbers in Bangladesh (73.2%) and Ethiopia (54.2%) and a quarter of isolates from NN and PP also contained the gene. This is supported by Pitout *et al*. (2019) whose literature review displayed global dissemination of this carbapenemase.

Jajoo *et al.*, 2018 found that *Klebsiella pneumoniae* and *Acinetobacter baumannii* displayed high levels of multi-drug resistance (78% and 91.3% respectively) with similarly high levels of resistance against last line carbapenems. We found similar results, with *K. pneumoniae* and *A. baumannii* as the most common carbapenem resistant species, followed by *S. marcescens* and *Ralstonia mannitolilytica*. Although there were more *K. pneumoniae* in

Africa, none of the isolates from the outbreaks in Ethiopia, which made up a large proportion of *K. pneumoniae* in Africa, had *bla*-$_{NDM}$ or *bla*-$_{OXA}$.

The resistance for colistin was 29.11% when all Gram-negative bacteria in included, however *Serratia* spp., *Burkholderia* spp. and *Proteus* sp. have intrinsic resistance toward colistin (EUCAST, 2020; Catchpole *et al*., 1997). This is concerning for last resort treatment in cases of MDR infections, which have shown to be prevalent in South Asia and Africa in this study and others (Jajoo *et al.*, 2018; Chaurasia *et al.*, 2019; Zou *et al*., 2021), showcasing the need to retain the efficacy of Colistin.

While many cases were multi-drug resistant, two cases of sepsis were caused by bacteria that was found to be pan-resistant to all antibiotics tested. One of these two cases of pan resistant isolates was found to be *Acinetobacter baumannii*. The neonate was reported as alive during follow-ups, surprising, as their sepsis would not have been treatable by any of the antibiotics tested in this study. However, these tests were only carried out *in vitro* and so antibiotic may have reacted differently *in vivo* and MICs were only carried out on one antibiotic at a time, not taking any synergistic effects into account, which may have occurred in the neonate. It is possible they were treated with an alternative antibiotic combination that was not tested in this study. Alternatively, the isolate may have been a contaminant, as *A. baumannii* has been shown to colonise hospital equipment through building biofilms or colonise the skin (Sebeny *et al.*, 2008), therefore could have been from the skin of the neonate, phlebotomist or microbiologist. The species has shown to be pathogenic (Grupper *et al.*, 2007) therefore, it would be predicted that this case would have ended in mortality. However, this has been shown to be a sepsis causing agent and this is difficult to determine. Some isolates were mixed upon receipt into Cardiff University, it is possible that the strain isolated was from a mixed swab and may not have been the true cause of sepsis. However, QC included checking isolates received against site ID and therefore this is unlikely.

Furthermore, follow-up was only recorded up to 21 days for this neonate, it is possible that they may have died after this time, as follow ups usually occurred for 60 days after enrolment. It is also possible that this outcome could be a mistake from data entry, due to large datasets handled extensively by multiple people, which could potentially have led to errors.

4.3.3 Conclusion

Resistance was seen across Gram-positive isolates against a range of antibiotics. Methicillin resistance was seen in 36% of GPB isolates. Over half GPB isolates were resistant to azithromycin with a further 40% requiring increased exposure. High rates of AMR were seen in GNB across a range of antibiotics, including overwhelming resistance against ampicillin at all clinical sites and 521/883 GNB isolates showcased resistance against ampicillin and gentamicin, the WHO recommended empirical therapy. Furthermore, high resistance was seen against third generation cephalosporins, with >80% resistance against ceftriaxone and cefotaxime due to high prevalence of CTX-M. Resistance against carbapenems was seen in all sites, due to prevalence of *bla*-$_{\text{OXA}}$ and *bla*-$_{\text{NDM}}$. Many GNB isolates were found to be MDR. This data showcased diminishing treatment options for neonatal sepsis when infected with GNB, supported by other studies from LMICs.

5.0. Results: Empirical treatment for neonatal sepsis in LMICs

5.1 Introduction

The World Health Organization (WHO) suggests an empirical therapy of intramuscular or intravenous ampicillin and gentamicin in cases of clinically diagnosed neonatal sepsis, or cloxacillin and gentamicin when *Staphylococcus* sp. infections are suspected. Following positive blood culture, treatment with ampicillin or benzylpenicillin and gentamicin for 7-10 days is recommended, alternatively with IV cloxacillin and gentamicin for 7-10 days where neonates are thought to be at higher risk of Staphylococcal infection (WHO, 2013; Table 5.1). Following initial therapy with ampicillin and gentamicin, additional treatment, usually with third generation cephalosporins are applied when the neonate lacks improvement of symptoms following 2-3 days of initial treatment, and referred to high level care (WHO, 2013).

Table 5.6. WHO guidelines for treatment of infants with suspected sepsis at 0-60 days of life (Fuchs et al., 2016). IM=intramuscular; IV=Intravenous (route of administration).

Reference	Conditions	Antibiotics	Dosing regimen
Pocket book of hospital care for children, 2013	Prophylaxis in neonates with documented risk factors	IM or IV ampicillin and gentamicin for at least 2 days	**Gentamicin** (IM/IV): First week of life : Low-birth-weight infants: 3 mg/kg once a day; Normal birth weight: 5 mg/kg per dose once a day Weeks 2–4 of life: 7.5 mg/kg once a day **Ampicillin** (IM/IV): First week of life: 50 mg/kg every 12 h Weeks 2–4 of life: 50 mg/kg every 8 h **Benzylpenicillin (penicillin G)** (IM): First week of life: 50 000 U/kg every 12 h; Weeks 2–4 and older: 50 000 U/kg every 6 h **Procaine Benzylpenicillin** (IM): 50 000 U/kg once a day **Cloxacillin** (IV): First week of life: 25–50 mg/kg every 12 h; Weeks 2–4 of life: 25–50 mg/kg every 8 h
	Case definition PSBI	IM or IV gentamicin and benzylpenicillin or ampicillin for at least 7–10 days	
	Greater risk of staphylococcus infection	IV cloxacillin and gentamicin for at least 7–10 days	
Managing possible serious bacterial infection in young infants when referral is not possible, 2015	Referral to hospital for young infants with PSBI is not possible	*Option1:* IM gentamicin once daily for 7 days and oral amoxicillin twice daily for 7 days.	**Gentamicin**: IM 5–7.5 mg/kg (for low-birth-weight infants gentamicin 3–4 mg/kg) once daily **Amoxicillin**: Oral 50 mg/kg twice daily oral
		Option2: IM gentamicin once daily for 2 days and oral amoxicillin twice daily for 7 days.	

In recent years, however, sepsis causing pathogens have frequently demonstrated resistance against these treatment options, with common concerns regarding AMR and therapeutic failures with these suggested antibiotics (Zaidi *et al.*, 2005; Fuchs *et al.*, 2018). Resistance rates have also increased against second-line therapy (Viswanathan *et al.*, 2012). This, combined with sub-optimal microbiological support in many LMIC sites has led to empirical use of broad-spectrum beta-lactams, aminoglycoside and even carbapenems as a first-line therapy (Hsia *et al.*, 2019). However, again increased usage of this class of drugs has led to the emergence of pan-resistant infections (Saleem *et al.*, 2010).

Choice of empirical therapy is critical in treating neonatal sepsis and is prescribed when a neonate presents with signs of sepsis, with microbiology results taking >48 hours. Clinical signs of sepsis tend to be vague and may be due to other illnesses (Puopolo *et al*., 2018; Hsieh *et al*., 2014). In LMICs, decisions on treatment are often based on clinical signs of neonatal sepsis due to lack of microbiology facilities in some hospitals. Scarcity of microbiology facilities corresponds to a lack of awareness of frequent sepsis causing pathogens or common local antibiotic susceptibility profiles. In these circumstances, treatment options are based on WHO recommendations or combinations that have previously proved successful. In addition to this obstacle, availability and cost of antibiotics is another factor for antibiotic choice, which are not always accessible, and the costs may have to be borne by the patient (Laxminarayan *et al.*, 2015). Antibiotics are not always covered by the hospital, state or government, or only certain antibiotics covered, potentially affecting patient outcome when patients cannot afford alternative treatments. While access to effective antimicrobials needs to be improved, particularly in LMICs, this needs to be carefully balanced with preventing further overuse which can further drive AMR against currently effective antimicrobials.

Many hospitals within LMICs use varied antibiotics as first line therapy based on previous experience opposed to WHO recommendations. Ineffectiveness has been commonly reported for AMP-GEN (Zaidi *et al.* 2005; Kayange *et al.*, 2010; Viswanathan *et al*., 2012), raising the use of cephalosporins as a first-line empirical therapy (Laxminarayan *et al*., 2016). Resistance rates have therefore increased against third generation cephalosporins, recommended by the WHO as second-line therapy (Viswanathan *et al.*, 2012). This in turn has raised treatment with carbapenems as a first-line therapy (Hsia *et al*., 2019). However, again increased usage of this class of drugs lead to the emergence of pan resistant CRE

infections (Saleem *et al*., 2010). Determining effective antibiotics is becoming rapidly more difficult, with rates of AMR rising rapidly (O'Neil *et al*., 2014).

Access to effective antimicrobials needs to be improved (Laxminarayan *et al*., 2016), alongside preventing overuse. In attempt to limit use of certain antibiotics, the WHO Essential Medicines List for Children classified antibiotics into three categories (Access, Watch and Reserve) (WHO, 2019). The Access group should account for >60% of antibiotic usage, providing adequate coverage for most common infections, including mainly first and second choice empirical therapies, comprising mainly of penicillins and first generation cephalosporins. The Watch group covers antibiotics with broader spectrum with higher resistance potential, recommended for first or second-lime therapy for limited infections. Antibiotics in the watch group include fluoroquinolones, more aminoglycosides, second+ generation cephalosporins, macrolides and glycopeptides among other classes. The Reserve group contains last-resort antibiotics targeted at multidrug-resistant (MDR) infections, including colistin, fosfomycin, fifth generation cephalosporins, tetracyclines and more (WHO, 2019). While use of reserve antibiotics remains low, use of antibiotic regimes other than those recommended in the Access group have been reported in various countries for initial treatment of neonatal sepsis (Laxminarayan et al., 2016). This may represent a lack of antibiotic stewardship, or necessity due to ineffectiveness of antibiotics in the Access group.

WHO's global action plan highlighted a need for AMR surveillance networks and centres to create and strengthen coordinated regional and global surveillance (Rashed *et al*., 2019; WHO, 2014) In 2015, a Gates Foundation funded study entitled Burden of Antibiotic Resistance in Neonates from Developing Societies (BARNARDS) was established to assess the burden of neonatal sepsis and AMR in LMICs.

5.1.1 Aims

This chapter focuses on the assessment of antibiotic therapies after determining a high prevalence of pathogens resistant to AMP-GEN and other antibiotics. The aims of this chapter include:

1. Determine usage of antibiotics at clinical sites for neonatal sepsis
    a. Whether WHO recommended therapies are used as first-line treatment
    b. Determine whether alternatives are used and which these are
    c. Assess dosage of antibiotics prescribed at sites
2. Assess the prevalence of resistance against recommended empirical treatment for neonatal sepsis and associated mortality
    a. Compare antibiotics to resistance profiles
    b. Ascertain rates of mortality with treatment
3. Assess common alternatives used at sites
    a. PK/PD
    b. MICs
    c. Mortality associated with different antibiotic combinations
4. Establish potential alternative treatments
    a. Frequency of resistance experiments to investigate how quickly resistance may arise with increased use of a new antibiotic
    b. Combined with assessment of resistance profiles
    c. Account for cost and availability across sites.
5. Investigate access and availability of antibiotics

5.2 Results

5.2.1. Antibiotics prescribed

From 9,874 neonates clinically diagnosed with sepsis, antibiotic treatment data was available for a total of 5,749 neonates. A multitude of antibiotics were found to be used in treatment courses for neonates, with 29 different antibiotics were reported as first line treatment of sepsis of those with antibiotic data (Table 5.2). However, 157 cases had more than three antibiotics reported in the primary treatment. We do not have data on dates of treatment and so these entries may have accounted for first- and second-line therapies due to slight input errors from sites. These multiple entries have been included in Table 5.2 below, as we cannot establish if they were given as a first-line antibiotic or following failure of a first-line treatment option.

Table 5.7. Number of cases of suspected biological sepsis with antibiotic data recorded for each antibiotic prescribed reported during BARNARDS. Categories from WHO's Access, watch and reserve (AWaRe) categories have been included where listed on the WHO website (WHO, 2019).

Antibiotic	**Number of cases**	**Countries of sites that reported use as a first-line antibiotic**	**AwaRe**
Amikacin	3328	Bangladesh, India, Pakistan, Nigeria	Access
Piperacillin-tazobactam	1598	India, Pakistan, South Africa	Watch
Gentamicin	1547	Bangladesh, Ethiopia, Nigeria, Rwanda, South Africa	Access
Ampicillin	1498	Bangladesh, Ethiopia, Nigeria, Rwanda, South Africa	Access
Ceftazidime	1408	Bangladesh, Pakistan, Ethiopia, Nigeria	Watch
Amoxicillin	718	Bangladesh, Pakistan, Nigeria, Rwanda, South Africa	Access
Vancomycin	276	Bangladesh, Pakistan, Ethiopia, South Africa	Watch
Meropenem	161	Bangladesh, India, Pakistan, Rwanda, South Africa	Watch
Imipenem	121	Pakistan	Watch
Ceftriaxone	48	Bangladesh, Ethiopia, Nigeria, South Africa	Watch

Cloxacillin	47	Bangladesh, Ethiopia, Nigeria, Rwanda, South Africa	Access
Netilmicin	47	India	Watch
Ofloxacin	41	India, Pakistan	Watch
Metronidazole	40	Pakistan, Ethiopia, Nigeria, Rwanda	Access
Tobramycin	36	Pakistan	Watch
Benzylpenicillin	27	Pakistan, Nigeria, Rwanda, South Africa	Access
Cefuroxime	27	Bangladesh, Nigeria, South Africa	Watch
Levofloxacin	22	Bangladesh, Pakistan	Watch
Ciprofloxacin	19	Bangladesh, Pakistan, Nigeria, Rwanda	Watch
Colistin	18	Pakistan, India	Reserve
Cefoperazone	17	Pakistan	Watch
Linezolid	9	Pakistan	Reserve
Azithromycin	7	Bangladesh, Nigeria, South Africa	Watch
Clindamycin	6	Bangladesh, Nigeria, South Africa	Access
Fluconazole	6	Pakistan, South Africa	Anti-fungal
Neomycin	5	Pakistan	Watch
Erythromycin	4	Nigeria	Watch
Tigecycline	3	Pakistan	Reserve
Trimethoprim/sulfamethoxazole	2	Nigeria, South Africa	Access

A range of antibiotics from Access and Watch groups have been reported in use through this data for treatment of neonatal sepsis at some point throughout their illness. Use of Access antibiotics were found to be prescribed 7,213 times and accounted for 9 of the 29 antibiotics as a first line therapy, used by an array of sites. Antibiotics from the watch group were prescribed 3,837 times, accounting for 16 of the 29 antibiotics and were used in multiple sites. Lastly, reserve antibiotics were prescribed as part of the first-line therapy 32 times in sites in Asia and accounted for three of the 29 antibiotics. One antifungal treatment was prescribed six times, five of which alongside antibiotics and once prescribed as a first-line therapy by itself, and later changed to Meropenem.

Use of colistin, linezolid and tigecycline, all classified as reserve antibiotics were reported in first line treatment of neonatal sepsis in Pakistan and India, where resistance rates are high to a wide range of antibiotics, as discussed in chapter three. Colistin was prescribed as a first-line treatment for 18 neonates. However, 13 of these had two or more additional antibiotics and so Colistin may not have been prescribed as a first-line treatment, but recorded incorrectly as this. Similarly, linezolid had two or more additional antibiotics recorded in seven out of nine cases, and so this may only have been used as a first-line treatment in two cases. All reports using tigecycline also had two or more antibiotics reported simultaneously, and so may have been reserved for second- or third-line treatment if this is a data entry error.

Of the 5,749 neonates with antibiotic data available 4,521 (77.42%) were treated with one of four antibiotic combinations at one point during their sepsis episode (data on date or lengths of treatment was not available) (Table 5.3, Figure 5.1). For comparison of antibiotics, these four most commonly prescribed treatments were assessed. These included: Ampicillin and gentamicin (AMP-GEN), administered by almost all sites across Africa and in Bangladesh; ceftazidime and amikacin (CTZ-AMK) used primarily by sites in Bangladesh with minor usage in Nigeria and Pakistan; Piperacillin-tazobactam and amikacin (PIP/TAZ-AMK) used by sites in Pakistan and occasionally by sites in South Africa and India; and Amoxicillin-clavulanate and amikacin (AMC-AMK) used by sites in Nigeria (Table 5.2; 5.3). Other antibiotics commonly reported to be used as one of the top five treatments by sites included cefotaxime, ofloxacin (only in India), benzylpenicillin at one site in Rwanda, meropenem, imipenem in Pakistan, ciprofloxacin in Rwanda and vancomycin (Table 5.3).

Table 5.8. Most frequent antibiotics given per BARNARDS sites. Cells in blue indicate the most frequently prescribed antibiotic therapy combination per site.

Site	**Top empirical antibiotics used**				
BC -Bangladesh	Ceftazidime	Amikacin	Cefotaxime	Ampicillin	Meropenem
BK -Bangladesh	Ampicillin	Gentamicin	Cefotaxime	Ceftazidime	Amikacin
Ethiopia	Ampicillin	Gentamicin	Cefotaxime	Ceftazidime	Vancomycin
India	Piperacillin-tazobactam	Netilmicin	Ofloxacin	Meropenem	Cefixime
NK -Nigeria	Amoxicillin	Gentamicin	Amikacin	Ceftazidime	Cloxacillin
NN -Nigeria	Amoxicillin	Amikacin	Ceftazidime	Ceftriaxone	Cefuroxime
NW -Nigeria	Amoxicillin	Ceftazidime	Ampicillin	Cloxacillin	Gentamicin
PC -Pakistan	Piperacillin-tazobactam	Amikacin	Cefotaxime	Meropenem	Vancomycin
PP -Pakistan	Piperacillin-tazobactam	Amikacin	Cefotaxime	Vancomycin	Imipenem
RK -Rwanda	Ampicillin	Gentamicin	Benzylpenicillin	Cefotaxime	Ciprofloxacin
RU -Rwanda	Ampicillin	Gentamicin	Cefotaxime	Meropenem	Ciprofloxacin
South Africa	Ampicillin	Gentamicin	Meropenem	Piperacillin-tazobactam	Amikacin
Top total 5 antibiotics used	Ampicillin	Gentamicin	Ceftazidime	Amikacin	Piperacillin-tazobactam

While some sites continue to prescribe AMP-GEN at high frequency (BK; ESS; RK; RU; ZAT), some sites already turn to alternative antibiotics more commonly when treating neonatal sepsis (BC; IN; NN; NW; PC; PP), presumably due to concerns over the efficacy of AMP-GEN on common pathogens in these areas.

5.2.2 Cohort analysis

From the 4,521 neonates treated with relevant antibiotic combinations listed above, 1,019 neonates had diagnosed sepsis episodes confirmed with a positive blood culture. A number of these isolates were not analysed at Cardiff University due to original study constraints (as the study was only originally investigating Gram-negative bacteria), loss of viability or isolates grown were deemed contaminants. Neonates for this chapter were

selected from those 1,019 neonates that were treated with one of the four most common antibiotic combinations with positive blood cultures and that had whole genome sequencing data (Figure 5.1), leading to a final dataset comprised of 457 isolates from positive blood cultures of 442 neonates (multiple pathogens were isolated from 15 neonates). Furthermore, 34 neonates were treated with two of the four relevant combinations as first-line and second-line therapy, which were analysed under both prescriptions, to incorporate a total of 476 prescriptions included in analyses.

The 442 neonates included in this subset included those treated with only one combination and those treated with multiple antibiotics as second-line or third-line treatments. From the 442 neonates, 290 neonates were treated with only one of the four first-line common combinations, with no change in therapy following initial treatment. Analyses were repeated on these 290 neonates only to allow assessment of those combinations as an empirical therapy without additional antibiotics (Figure 5.1).

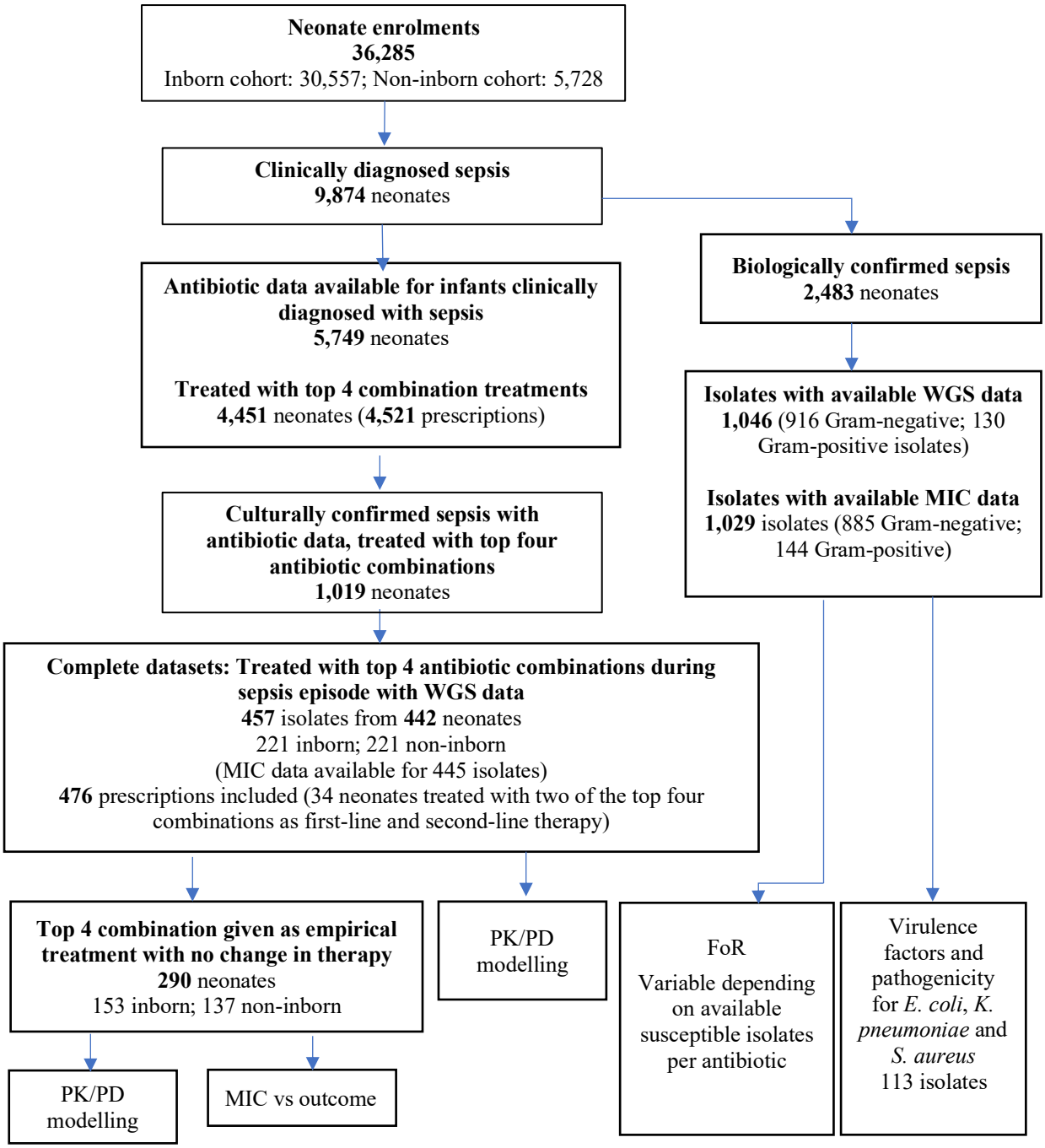


Figure 5.30. Study workflow diagram, demonstrating the process of isolate selection for inclusion in the study. Antibiotic data was recorded for 5,749 neonates enrolled on the BARNARDS study. We found four antibiotics combinations to be used most commonly from these 5,749, with 4,521 neonates treated with one of the four combinations. From the 4,521 clinically diagnosed and underwent treatment with relevant antibiotics, 1,019 had culturally confirmed sepsis at clinical sites. However, due to original constraints of the study, loss of viability or cultured isolated deemed as contaminant, a number of these were not analysed at Cardiff University, UK. After matchups of neonates with confirmed sepsis, relevant antibiotic therapy and whole genome sequencing (WGS) data, a subset of 442 neonates were selected. Due to recorded growth of multiple pathogens in blood cultures of some neonates, this led to inclusion of 457 isolates. Treatment of two from the four antibiotic therapies was reported in 34 neonates, where they had been treated with one combination as a first line therapy, followed by another of these four combinations as a second line therapy. These neonates have been

analysed under each of the two combinations prescribed, leading to a total of 476 prescriptions. Isolates from confirmed sepsis cases were sent to Cardiff University, UK where Minimum inhibitory concentration (MICs) was assessed via agar dilution, of which 12 of the sub-set had undetermined results. However, these neonates were left in the subset due to low numbers and could contribute to other analyses. Pharmacokinetic/pharmacodynamic (PK/PD) modelling was carried out on the above isolates. Virulence factors and pathogenicity indexing were performed on a selection based on varied bacterial species, sequence types, outcomes and resistance profiles from a range of BARNARDS sites. Frequency of resistance (FoR) isolates were chosen similarly but restrained to isolates recorded as sensitive during MICs separately for each antibiotic.

The cohort of neonates included both inborn and non-inborn neonates and also those with early onset sepsis (EOS) or late onset sepsis (LOS), with varied genders and a range of birth factors detailed in Table 5.4. Rates of these varied between antibiotic combinations and were not controlled due to the prospective nature of the study and this sub-set was based on the maximum number of neonates with relevant available information.

Table 5.9. Numbers included in this sub-study, including all isolates and all prescriptions from each country, with information on associated cohort and clinical variables for 476 prescriptions. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin. EOS=Early-onset sepsis; LOS=Late onset sepsis; GNB= Gram-negative bacteria; GPB=Gram-positive bacteria as the infecting pathogen.

		Antibiotic therapy				Total	Missing data
		AMP-GEN	CTZ-AMK	AMC-AMK	PIP/TAZ-AMK		
Total number enrolled		111	172	78	115	476	-
Cohort	Inborn	69	34	37	102	242	-
	Non-inborn	42	138	41	13	234	
Gender	Male	46	116	44	46	252	70
	Female	43	52	34	25	154	
Premature	Yes	65	24	36	59	184	4
	No	42	148	42	56	288	
Caesarean	Yes	44	70	33	73	220	7
	No	60	102	45	42	249	
Onset of sepsis	EOS	61	44	20	53	178	45
	LOS	49	121	54	29	253	
Type of organism	GNB	102	163	62	91	418	-
	GPB	9	9	16	24	58	

BARNARDS countries were represented in this subset, with the exception of India. However, antibiotic combinations were dominated by certain sites (Table 5.5). AMP-GEN

was the most widely distributed antibiotic combination, including neonates in this subset from five countries, being more commonly prescribed in three (Ethiopia, Rwanda and South Africa). Neonates treated with CTZ-AMK were predominantly from Bangladesh, with a notable number from Nigeria; those treated with AMC-AMK were mainly enrolled at sites in Nigeria and PIP/TAZ-AMK was mainly used in Pakistan with a few prescriptions in South Africa (Table 5.5).

Table 5.10. Number of each combination per country for 476 prescriptions. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin.

Antibiotic combination	Country						Total
	Bangladesh	Ethiopia	Nigeria	Pakistan	Rwanda	South Africa	
AMP-GEN	6	29	2	0	51	23	111
CTZ-AMK	157	0	14	1	0	0	172
AMC-AMK	0	0	75	3	0	0	78
PIP/TAZ-AMK	0	0	0	108	0	7	115

5.2.2.1 Empirical only subset

As with the overall dataset of 476 prescriptions, a range of clinical presentations were included in the 290 subset of neonates that received only one of the four antibiotic prescriptions, with no change in therapy, although this included low numbers of GPB (Table 5.6). The associations of certain combinations per country is also clearer in this subset due to the lower numbers, although neonates treated with AMP-GEN were included across multiple countries. Within this subset, AMC-AMK was only given to neonates at sites in Nigeria and PIP/TAZ-AMK only in Pakistan (Table 5.7).

*Table 5.*11. *Numbers included in this sub-study, including all isolates and all prescriptions from each country, with information on associated cohort and clinical variables for 290 neonates treated only with one antibiotic combination with no change in therapy. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin. EOS=Early-onset sepsis; LOS=Late onset sepsis; GNB= Gram-negative bacteria; GPB=Gram-positive bacteria as the infecting pathogen.*

		Antibiotic therapy				Total	Missing data
		AMP-GEN	CTZ-AMK	AMC-AMK	PIP/TAZ-AMK		
Total number enrolled		78	109	27	76	290	-
Cohort	Inborn	44	22	19	68	290	-
	Non-inborn	34	87	8	8		
Gender	Male	33	69	12	31	236	54
	Female	25	37	15	14		
Premature	Yes	36	11	13	31	286	4
	No	38	98	14	45		
Caesarean	Yes	23	47	18	48	283	7
	No	48	62	9	28		
Onset of sepsis	EOS	47	29	8	37	272	28
	LOS	31	75	18	27		
Type of organism	GNB	76	102	20	59	290	-
	GPB	2	7	7	17		

Table 5.12. Number of prescriptions for each combination per country for 290 subset of neonates that only received one antibiotic combination with no change in treatment. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin.

Antibiotic combination	Country						Total
	Bangladesh	Ethiopia	Nigeria	Pakistan	Rwanda	South Africa	
AMP-GEN	5	29	0	0	40	4	78
CTZ-AMK	107	0	1	1	0	0	109
AMC-AMK	0	0	27	0	0	0	27
PIP/TAZ-AMK	0	0		76	0	0	76

5.2.3 Sensitivity analyses

Analyses carried out to compare the main subset (n=476) with the overall BARNARDS dataset found that the proportion of neonates reported deceased in this subset was similar to those with confirmed sepsis overall from BARNARDS ($X^2[1]=1.18$, p=0.278).

All neonates from this subset reported as deceased within 60 days of life were included (eight out of 74 of deceased neonates were reported over 10 days following infection), as death after an extended time within the 60-day period could not be discounted as being caused by the sepsis episode. Four neonates that were reported as deceased did not have the associated date available.

Sensitivity analyses carried out to determine whether removal of 'untraceable' neonates for statistical analyses had a significant impact on statistical analyses of differences of mortality rates (%) for the different antibiotic combinations found similar p-values. Similar results were also seen when untraceable neonates were removed for MIC vs outcome (Appendix, pages 14-15).

5.2.4 Antibiotics prescribed and outcome

Neonates treated with CTZ-AMK had the lowest reported mortality (9.3%, n=16/172) from the four antibiotic combinations assessed, followed by AMP-GEN (16.2%, n=18/111), AMC-AMK (24.4%, n=19/78) and PIP/TAZ-AMK (27.8%, n=32/115) (Table 5.8). Analyses were repeated on neonates treated only with one of the top four antibiotic combinations with no antibiotic changes following the initial treatment (n=290). Reported mortality remained lowest when treated with CTZ-AMK (8.2%, n=8/109), followed by AMP-GEN (10.3%, n=8/78), PIP/TAZ-AMK (22.4%, n=17/76) and highest mortality for this subset was seen in neonates treated with AMC-AMK (29.6%, n=8/27) (Table 5.9). However, these percentages included those lost to follow-up and therefore may under report mortality rates, as those lost to follow-up were included as 'not reported deceased'. In order to reduce this issue, survival analyses were undertaken, which accounts for neonates lost to follow-up.

Table 5.8. Number of cases for which each antibiotic combination was prescribed. Mortality rate for each of these combinations has also been reported.

Antibiotic treatment	Number of prescriptions including treatment	Mortality rate (%)
Ampicillin + Gentamicin	111	16.2
Amoxicillin + Amikacin	78	24.4
Ceftazidime + Amikacin	172	9.3
Piperacillin-tazobactam + Amikacin	115	27.8

Table 5.9. Number of cases each empirical therapy combination was prescribed with no change in antibiotics reported to have been prescribed following initial therapy combination. Mortality rate for each of these combinations has also been reported. Cases where additional antibiotics were reported to be prescribed simultaneously were excluded.

Empirical therapy	Number of cases	Mortality rate (%)
Ampicillin + Gentamicin	78	10.3
Amoxicillin + Amikacin	27	29.6
Ceftazidime + Amikacin	109	8.2
Piperacillin-Tazobactam + Amikacin	76	22.4

5.2.5 Survival analyses overall subset (n=476)

5.2.5.1 Overall subset unadjusted

Unadjusted survival analyses carried out via Cox regression were undertaken without taking any neonatal factors into consideration. Graphical inspection of the Schoenfeld residuals plot showed wide standard error, although the slope was around zero and the Schoenfeld test was not significant with a p-value of 0.680, signifying that the assumption proportional hazards in the Cox regression was not violated (Appendix, page 23).

The unadjusted Cox regression analysis showed significantly better survival for neonates treated with CTZ-AMK compared to those treated with AMP-GEN (HR=0.338, 95% CI= 0.169-0.675, p=0.002). No significant differences in survival was seen in neonates treated with AMC-AMK or PIP/TAZ-AMK compared to AMP-GEN (HR=1.281, 95% CI= 0.666-2.467, p=0.458; HR=1.669, 95% CI= 0.851-3.275, p=0.136, respectively) (Table 5.10, Figure 5.3).

Table 5.10. Cox regression proportional hazards results for overall data, n=476 for unadjusted. HR=Hazards-ratio; CI=Confidence interval; AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin.

	HR	95% CI		P-value
		Lower	Upper	
AMP-GEN				
CTZ-AMK	0.338	0.169	0.675	0.002
AMC-AMK	1.281	0.666	2.467	0.458
PIP/TAZ-AMK	1.669	0.851	3.275	0.136

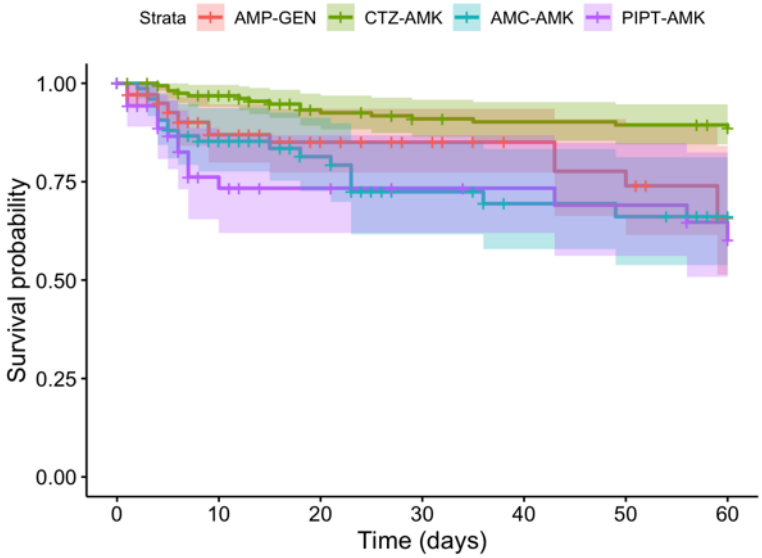


Figure 5.31. Cox regression proportional hazards results displayed as graphs for overall data, n=476 per antibiotic therapy given for unadjusted Cox regression. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin. Shading displays confidence intervals.

5.2.5.2 Overall subset Adjusted

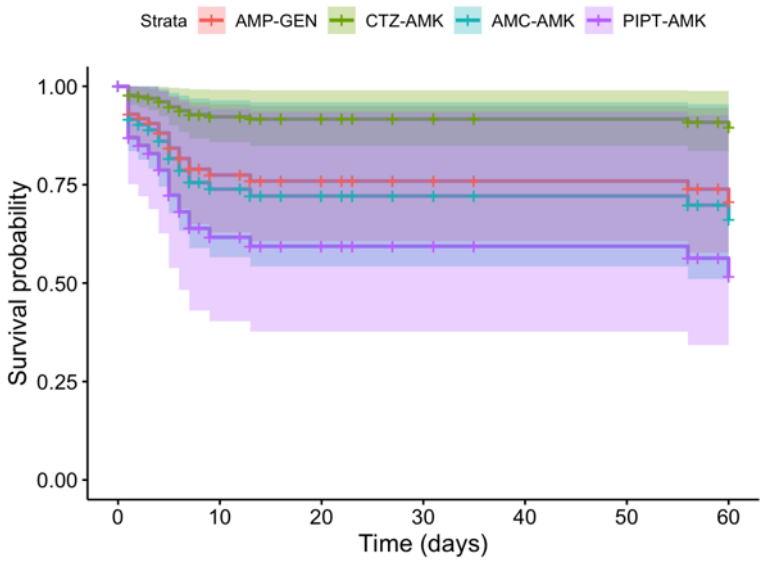
Clinical and neonatal factors adjusted for are detailed in Table 5.4 and included whether the neonate was: born in the hospital, premature, birth via Caesarean section, gender, the type of sepsis causing pathogen and whether the neonate was diagnosed with EOS or LOS. Schoenfeld residuals carried out for the adjusted Cox regression took all of these factors into consideration and we found that EOS/LOS violated the assumption of proportional hazards, due to the concept of this, as EOS were all under seven days and LOS

above seven days of age. Therefore, to overcome this, EOS/LOS was stratified in the overall model. When this was carried out, the assumption of proportional hazards was not violated for the overall model (p=0.067), or for any other factors (Appendix, page 23).

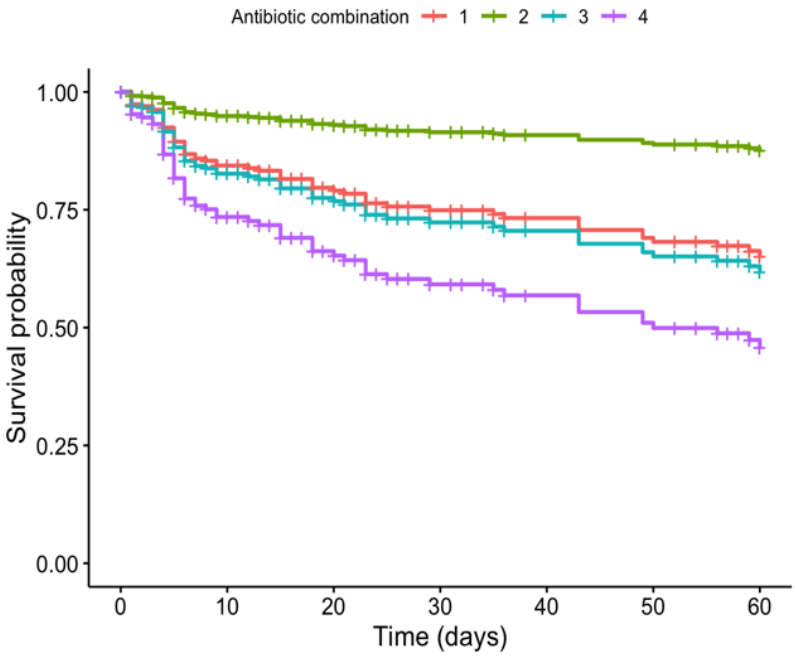
Similarly, to the unadjusted analysis, the adjusted Cox regression analysis showed that mortality was significantly decreased in those treated with CTZ-AMK compared to AMP-GEN (HR=0.309, 95% CI=0.136-0.701, p=0.005). There was no significant difference between AMP-GEN and AMC-AMK (HR=1.122, 95% CI=0.563-2.236, p=0.744) or PIP/TAZ-AMK (HR=1.816, 95% CI=0.846-3.898, p=0.126) (Table 5.11; Figure 5.5).

Table 5.11. Cox regression proportional hazards results for overall data, n=476 for adjusted model as per clinical information provided in Table 4.4. Adjusted analysis n=368 (108 observations deleted due to missingness), number of events=63. EOS/LOS was stratified in this model to ensure proportional hazard assumptions were met. HR=Hazards-ratio; CI=Confidence interval; AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin.

	HR	95% CI		P-value
		Lower	Upper	
AMP-GEN	Reference combination			
CTZ-AMK	0.316	0.139	0.718	0.006
AMC-AMK	1.186	0.595	2.237	0.628
PIP/TAZ-AMK	1.894	0.883	4.063	0.101



A.



B.

Figure 5.32. Survival analysis for neonates treated with each antibiotic therapy carried out through Cox regression hazard ratio, adjusted for clinical factors, n=476. A. contains standard error displayed as shading. The key shows neonates treated with AMP-GEN=ampicillin and gentamicin; CTZ-AMK=ceftazidime and amikacin; AMC-AMK=amoxicillin and amikacin; and PIP/TAZ-AMK=piperacillin-tazobactam and amikacin. B. In the legend above, antibiotic combination 1=AMP-GEN; 2=CTZ-AMK; 3=AMC-AMK; and 4=PIP/TAZ-AMK. Clinical variables considered included cohort; gender; type of pathogen (GNB/GPB); whether the neonate was delivered via Caesarean section; and whether the neonate was premature. For the purpose of this survival curve, clinical variables were set at the following: gender: male, cohort: inborn, Type of sepsis: EOS, sepsis pathogen type: GNB, C-section: no, premature: no. Survival curves were made in R Studio, with the survival and survminer packages.

5.2.5.3 Overall Mixed-effect model

Mixed effect models were performed to account for country level variation for comparison of survival between treatments. However, results from the mixed-effect models are not reliable as the Cox proportional hazards assumption could not be met with most Schoenfeld results reading N/A for birth factors, presumably due to the dispersion of the antibiotic combinations per site, which were heavily favoured by a particular country, with little or no distribution between sites, except for AMP-GEN (Appendix, page 24). Furthermore, no CI intervals could be obtained in the mixed-effect Cox regression results (Table 5.12) due to the poor fit of the model. A larger sample number with better distribution of antibiotic combinations between sites would be needed to enable this to be added to the analyses.

Table 5.12. Cox regression proportional hazards results for overall data, n=476 for mixed-effect model adjusted per clinical information provided Table 4.4 with country added as a mixed-effect. N=368 (108 observations deleted due to missingness), number of events=63. EOS/LOS was again stratified in this model. No confidence intervals could be obtained for the mixed effect model accounting for country variation due to the poor fit of the model. HR=Hazards-ratio; CI=Confidence interval; AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin.

	HR	95% CI		P-value
		Lower	Upper	
AMP-GEN	Reference combination			
CTZ-AMK	2.092			0.450
AMC-AMK	2.760			0.270
PIP/TAZ-AMK	0.342			0.190

5.2.6 Survival analysis, empirical only subset (n=290)

5.2.6.1 Empirical only unadjusted Cox regression

Survival analyses were repeated on the smaller empirical only treatment subset of neonates (n=290). Schoenfeld residual plot for the unadjusted Cox regression again showed a wide standard error, but the proportional hazard assumption was found to be met (p=0.546) (Appendix, page 25).

For the subset of neonates treated only with one antibiotic combination (n=290), unlike the analysis for the overall subset (n=476), while a lower rate of mortality was seen in neonates treated with CTZ-AMK compared to AMP-GEN, this was not significant (HR=0.572, 95% CI 0.211-1.566, p=0.279). Unlike results for the overall subset, a significantly higher mortality was associated with neonates treated with AMC-AMK compared to AMP-GEN (HR=2.900, 95% CI= 1.050-8.011, p=0.279). No significant difference was seen in survival for neonates treated with PIP/TAZ-AMK compared to AMP-GEN (HR=2.002, 95% CI=0.701-5.713, p=0.195) (Table 5.13, Figure 5.8).

Table 5.13. Cox regression proportional hazards results for empirical dataset, n=290 for unadjusted. HR=Hazards-ration; CI=Confidence interval; AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin.

	HR	95% CI		P-value
		Lower	Upper	
AMP-GEN	Reference combination			
CTZ-AMK	0.572	0.211	1.566	0.279
AMC-AMK	2.900	1.050	8.011	0.040
PIP/TAZ-AMK	2.002	0.701	5.713	0.195

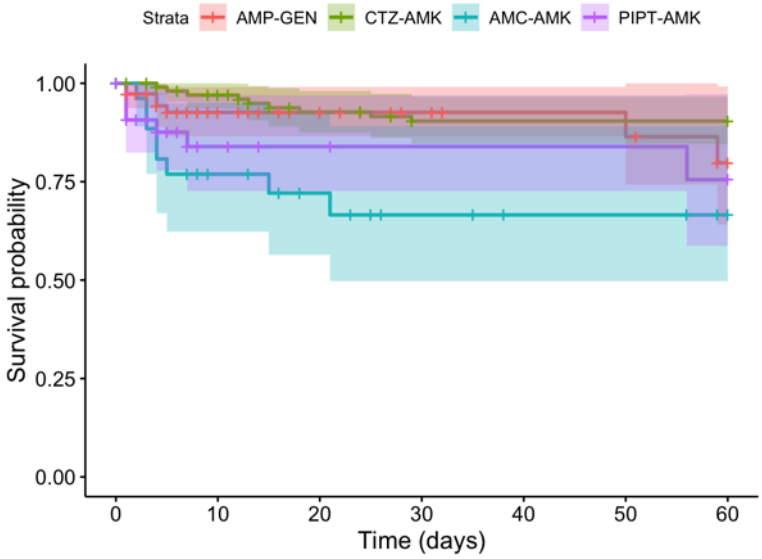


Figure 5.33. Unadjusted Cox regression proportional hazards results displayed as graphs for empirical dataset with survival, n=290 per antibiotic therapy given. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin. Shading displays confidence intervals.

5.2.6.2 Empirical only adjusted Cox regression

As with the overall subset of 476, the Cox regression for the empirical only subset was also stratified by EOS/LOS to maintain a good fit and ensure that the model fit into the proportional hazards assumption, with an overall statistic of p=0.716. All other factors were considered to also have proportional hazards in this model, with p values >0.05 (Appendix, page 26).

No statistically significant difference in reported mortality between AMP-GEN and CTZ-AMK (HR=0.576, 95% CI=0.175-1.902, p=0.365) or PIP/TAZ-AMK (HR=2.904, 95% CI=0.898-9.390, p=0.075) was found for neonates with the subset of neonates that had no change in therapy following initial treatment (n=290). However, mortality with AMC-AMK was higher than AMP-GEN (HR=5.557, 95% CI=1.707-18.803, p=0.004). Confidence intervals were wider due to the reduced number of neonates included in the adjusted analysis, as some neonates had data missing for one or more of the confounding variables, which were excluded by the software for the adjusted model (Table 5.14; Figure 5.10).

Table 5.14. Cox regression proportional hazards results for empirical dataset, n=290 for unadjusted and adjusted models as per clinical information provided in Table 4.4. Adjusted model n=210 (80 observations deleted due to missingness), events, n=28). AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin. *Early-onset / late onset sepsis was stratified in this model to ensure proportional hazard assumptions were met.*

	HR	95% CI		P-value
		Lower	Upper	
AMP-GEN (1)	Reference combination.			
CTZ-AMK (2)	0.511	0.155	1.687	0.271
AMC-AMK (3)	5.557	1.707	18.083	0.004
PIP/TAZ-AMK (4)	3.018	0.928	9.813	0.066

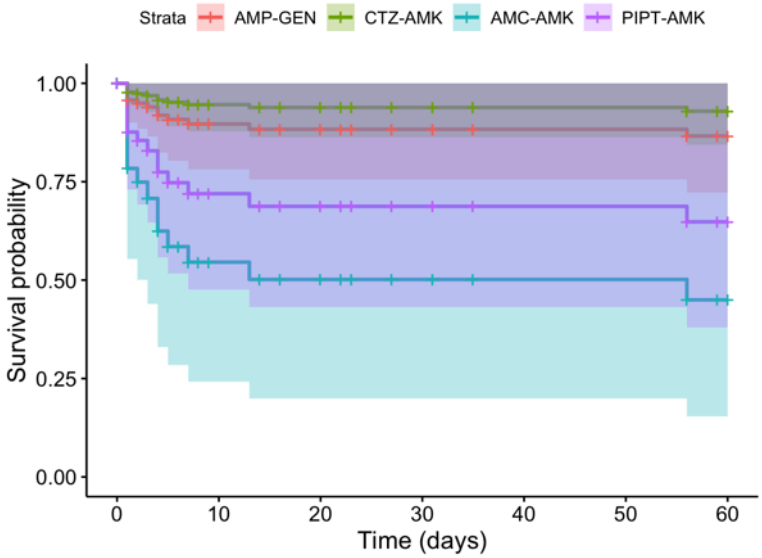


Figure 5.34. Cox regression proportional hazards results for empirical dataset, n=290 for unadjusted and adjusted models as per clinical information provided in Supplementary Table 12. Adjusted model n=210 (80 observations deleted due to missingness), events, n=28). Early-onset / late onset sepsis was stratified in this model to ensure proportional hazard assumptions were met. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin. Shading displays confidence intervals.

5.2.6.3 Mixed-effect model

A mixed-effect model was carried out using the adjusted Cox regression for the empirical therapy only subset of neonates with the addition of country as a mixed-effect, to investigate whether differences in outcome could be due to country opposed to antibiotic

therapy. When neonates were nested by country within this model however, many of the Schoenfeld plots for confounding variables reached a test result of N/A demonstrating a poor fit of the model Appendix, page 26).

Following on from the poor fit of the Schoenfeld residuals, no confidence intervals were able to be provided due this poor model fit. This was due to the small sample size and because antibiotic combinations were not prescribed evenly by different countries, with many being predominanlty prescribed within one site/ country. Therefore, the HR and P-values provided in Table 5.15 are not reliable.

Table 5.13. Cox regression proportional hazards results for empirical dataset, n=290 for mixed effect model as per clinical information provided in Table 4.4. Adjusted model n=210 (80 observations deleted due to missingness), events, n=28) nested per country. HR=Hazards ratio; CI=confidence interval. Early-onset / late onset sepsis was stratified as per adjusted model.

	HR	95% CI		P-value
		Lower	Upper	
AMP-GEN	Reference combination			
CTZ-AMK	0.511			0.270
AMC-AMK	5.557			0.004
PIP/TAZ-AMK	3.018			0.066

5.2.7. Site variation

As previously discussed, different antibiotic combinations were prescribed at varying rates at BARNARDS sites, causing an inability to incorporate the effect of country into the survival analyses effectively. Therefore, differences in site antibiotic prescriptions and mortality have been looked at further by site for both the empirical only (Table 5.16) and overall subsets (Table 5.17).

Antibiotic combinations within this subset, as previously discussed in this chapter were not consistently prescribed at different sites. Mortality rates were assessed per site firstly for the empirical only subset (n=290), which was found to vary significantly between sites (X^2(N=290, df=9) =22.912, p=0.06). Highest mortality was seen in ZAT (n=2, 50.00%),

although there were only four neonates included, followed by NN (n=8, 30.77%), PC (n=4, 30.77%), and PP (n=14, 21.88%). Mortality for sites using AMP-GEN was similar for ESS (n=3/29, 10.34%) and RK (n=3/35, 8.57%), although numbers for other sites using AMP-GEN were too low for comparison. PIP/TAZ-AMK was only used at the sites in Pakistan. Higher reported mortality was seen at PC (n=4/13, 30.77%) than PP (n=13/63, 20.63%), although the PC had quite a low number of neonates (n=13) and so this percentage is not reliable (Table 5.16). Reported mortality for more than one antibiotic combination at a site could not be compared for any of the sites as none had comparable numbers. Numbers in this empirical subset were low or zero for some sites (e.g. NK) and so this analysis was repeated for the overall cohort (n=476).

Table 5.16. Numbers of neonates treated with each antibiotic combination for the empirical only subset of neonates (N=290) and the number of neonates with reported mortality within this subset per site. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin.

Antibiotic therapy	Sites used	Mortality per site
AMP-GEN	BC (1); BK (4); ESS (29); RK (35); RU (5); ZAT (4)	ESS (3); RK (3); ZAT (2)
CTZ-AMK	BC (106); BK (1); NK (1); PP (1)	BC (9); PP (1)
PIP/TAZ-AMK	PC (13); PP (63)	PC (4); PP (13)
AMC-AMK	NK (1); NN (26)	NN (8)

In the overall subset, mortality (%) was again found to vary between sites (X^2(N=476, df=10) =41.433, p<0.001). However, again some sites still had low numbers, including BK (6), NK (3), and NW (1) and so these sites have been discounted from this analysis. PC had the highest mortality (50%), followed by ZAT (30.0%), PP (26.6%), RU (25.0%) and NN (24.1%). Other sites with mortality <20% for CCS included ESS (10.3%), RK (8.6%), and BC (7.0%). Again, no sites had comparable numbers for more than one antibiotic combination. However, when looking at outcome of neonates treated with AMP-GEN, we

can see differences between sites, with lowest mortality reported at RK (n=3/35, 8.57%), followed by ESS (n=3/29, 10.34, RU (n=4/16, 25.00%) and ZAT (n=8/23, 34.78%) (Table 5.17). However, numbers for RU and ZAT were low and so this is still not particularly reliable and therefore statistical analyses were not performed.

Table 5.17. Numbers of neonates treated with each antibiotic combination for the overall subset of neonates (N=442) and the number of neonates with reported mortality within this subset per site. AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin.

Antibiotic therapy	Sites used	Mortality per site
AMP-GEN	BC (1); BK (5); ESS (29); NN (1); NW (1); RK (35); RU (16); ZAT (23)	ESS (3); RK (3); RU (4); ZAT (8)
CTZ-AMK	BC (156); BK (1); NK (1); NN (13); PP (1)	BC (11); NN (4); PP (1)
PIP/TAZ-AMK	PC (16); PP (92); ZAT (7)	PC (7); PP (24); ZAT (1)
AMC-AMK	NK (2); NN (73); PC (2); PP (1)	NN (17); PC (2)

5.2.8 Treatment changes

Treatment changes from each of the four combinations when used as a first-line treatment to other antibiotics were assessed for the 442 neonates. PIP/TAZ-AMK was found to be changed the least number of times after being prescribed as a first-line treatment (7.2%), followed by AMP-GEN (30.28%) and CTZ-AMK (31.0%). Neonates treated with AMC-AMK were found to have the greatest frequency of change in therapy following its use as a treatment (58.46%) (Table 5.18). When antibiotic failure of first-line treatments were assessed by the change of antibiotic therapy or by neonatal death, PIP/TAZ-AMK remained 7.2% but failure for all other combinations increased. Again, AMC-AMK had greatest rate of failure (70.77%), followed by AMP-GEN (38.53%), CTZ-AMK (36.71%) and PIP/TAZ-AMK (7.2%) (Table 5.19).

Table 5.18. Number of cases with top four combinations prescribed as empirical first-line treatment and number of times each therapy combination needed to be changed following original therapy. Cases where additional antibiotics were reported to be prescribed simultaneously were excluded.

Antibiotic first line treatment combination	Total number	Number of times therapy was changed from original
Ampicillin + Gentamicin	109	33 (30.28%)
Amoxicillin + Amikacin	65	38 (58.46%)
Piperacillin-Tazobactam + Amikacin	83	6 (7.2%)
Ceftazidime + Amikacin	158	49 (31.0%)

Table 5.19. Number of cases with top four combinations prescribed as empirical first-line treatment and rates of antibiotic failure as the number of times each therapy combination needed to be changed following original therapy in addition to neonates reported deceased that had no change in therapy. Cases where additional antibiotics were reported to be prescribed simultaneously were excluded.

Antibiotic first line treatment combination	Total number	Antibiotic failure: Change from original or neonate reported deceased
Ampicillin + Gentamicin	109	42 (38.53%)
Amoxicillin + Amikacin	65	46 (70.77%)
Piperacillin-Tazobactam + Amikacin	83	6 (7.2%)
Ceftazidime + Amikacin	158	58 (36.71%)

Changes from a range of antibiotics that were used at any point including first-line to fifth-line treatment were also assessed for the overall subset of neonates for any antibiotics prescribed over ten times. AMC-AMK was again changed most commonly (58.82%), followed by vancomycin (53.85%), although this was only prescribed 13 times. Levofloxacin and meropenem were also commonly changed once prescribed (52.38% and 50.00%, respectively) (Table 4.25). Meropenem and levofloxacin were prescribed only ten times but was not changed. Ciprofloxacin was prescribed 16 times and only changed once (6.25%) (Table 5.20). Including mortality as failure in addition to antibiotic change, failure increased for all antibiotics. Vancomycin had highest rate of failure (69.23%), followed by AMC-AMK (67.65%), levofloxacin (57.14%) and meropenem (52.94%) (Table 5.21).

Table 5.20. Antibiotics used in treatment line of neonatal sepsis across multiple sites. This table denotes whether an antibiotic therapy change was recorded following treatment with each antibiotic/ combination. These include first line to fifth line treatment, and whether a change occurred following prescription of each antibiotic/ combination below. Antibiotics included if prescribed in >=10 cases of neonates included in this study. Cases where additional antibiotics were reported to be prescribed simultaneously were excluded.

Antibiotic prescription (singular or combination)	**Total number treated with antibiotic**	**Number of cases reported antibiotic change**	**Antibiotic change %**
Ceftazidime + Amikacin	164	52	31.71%
Ampicillin + Gentamicin	111	36	32.43%
Piperacillin-Tazobactam + Amikacin	88	15	18.07%
Amoxicillin (clavulanate) + Amikacin	68	40	58.82%
Meropenem	34	17	50.00%
Levofloxacin	21	11	52.38%
Cefotaxime	16	3	18.75%
Vancomycin	13	7	53.85%
Ciprofloxacin	16	1	6.25%
Meropenem + Vancomycin	19	7	36.84%
Meropenem + Levofloxacin	10	0	0.00%

Table 5.21. Antibiotics used in treatment line of neonatal sepsis across multiple sites. This table denotes 'antibiotic failure' due to either an antibiotic therapy change recorded following treatment with each antibiotic/ combination or reported mortality of a neonate within the overall subset (n=442). These include first line to fifth line treatment, and whether a change occurred following prescription of each antibiotic/ combination below. Antibiotics included if prescribed in >=10 cases of neonates included in this study. Cases where additional antibiotics were reported to be prescribed simultaneously were excluded.

Antibiotic prescription (singular or combination)	**Total number treated with antibiotic with no additional reported simultaneously**	**Number of cases reported antibiotic failure**	**% antibiotic failure**
Ceftazidime + Amikacin	164	62	37.80%
Ampicillin + Gentamicin	111	45	40.54%
Piperacillin-Tazobactam + Amikacin	88	25	29.41%
Amoxicillin (clavulanate) + Amikacin	68	46	67.65%
Meropenem	34	18	52.94%
Levofloxacin	21	12	57.14%
Cefotaxime	16	5	31.25%
Vancomycin	13	9	69.23%
Ciprofloxacin	16	3	18.75%
Meropenem + Vancomycin	19	8	42.11%
Meropenem + Levofloxacin	10	1	10.00%

Rates of antibiotic failure can provide a rough gauge of response of neonates to antibiotics, with the idea that changes to an antibiotic treatment will indicate a lack of improvement in the neonate. However, those not in the main four combinations were often second line to fifth-line treatments, and therefore given following varied antibiotics before them and at different times during the sepsis episode. Additionally, the above antibiotics were prescribed by different sites which will affect prescribed changes in therapy and outcome. Furthermore, many neonates were untraceable and so rates of antibiotic failure may not be reliable.

5.2.9 Antibiotic resistance

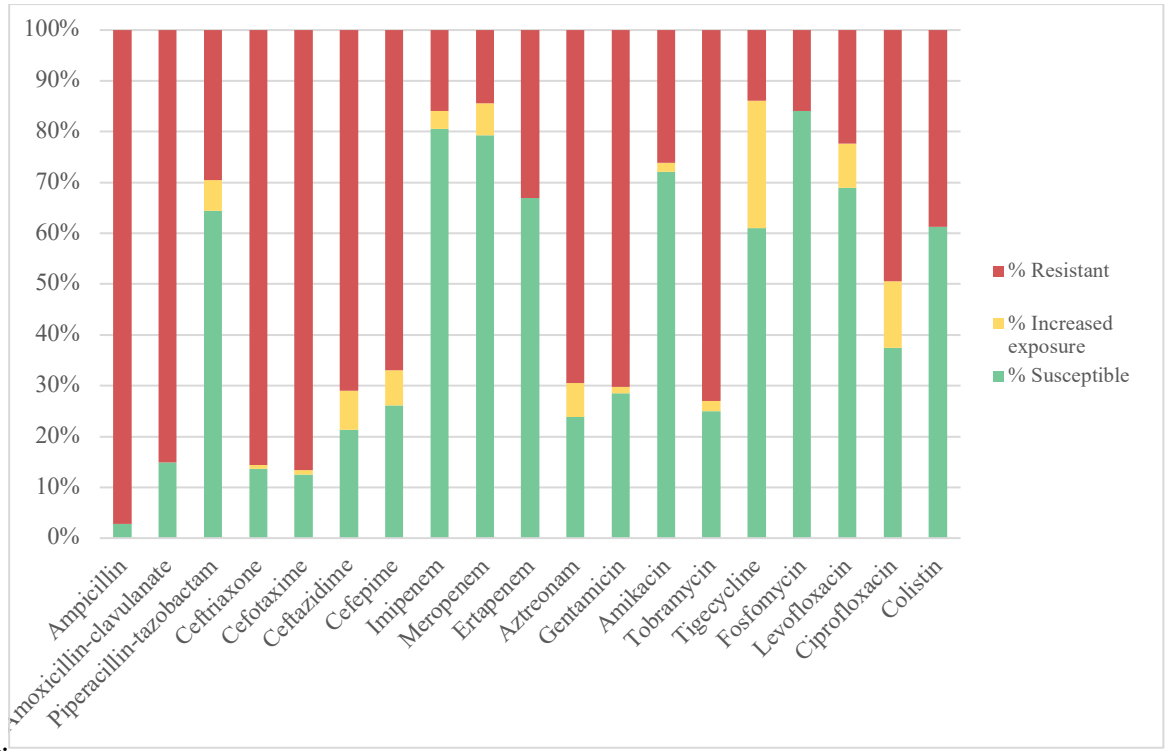
As with the overall data seen in the previous chapter, Gram-negative isolates for this cohort (n=390) displayed a high-level of resistance to ampicillin (97.2%, n=379/390) and

gentamicin (70.3%, n=274/390) (Figure 4.12A). Amikacin had lower resistance (25.9%, n=101/390) compared to other aminoglycosides. Amoxicillin-clavulanate also had a high prevalence of resistance of 85.13%, while resistance to piperacillin-tazobactam was much lower with 29.49% resistant Gram-negative isolates. Low resistance rates were observed for Fosfomycin (15.6%, n=61/390), imipenem (15.9%, n=62/390) and meropenem (14.4%, n=56/390).

When considering Gram-negative isolates for the four combinations commonly used, the lowest coverage (susceptibility to at least one antibiotic in combination) was provided by AMP-GEN (28.5%, n=111/390), with higher coverage from AMC-AMK (73.3%, n=286/390); CTZ-AMK (77.2%, n=301/390) and PIP/TAZ-AMK (80.0%, n=312/390). Resistance rates provided include intrinsically resistant bacteria, colistin resistance was notably lower in Gram-negative isolates when intrinsically resistant isolates were excluded from analysis, demonstrating 9.1% resistance, opposed to 39% resistance inclusive of bacteria with intrinsic bacteria, such as *Serratia marcescens,* prevalent in blood cultures from Bangladesh.

Gram-positive isolates (n=55) generally showed lower levels of resistance than the Gram-negative bacteria overall against antibiotics tested (Figure 5.12B) (Appendix, page 27, 29). Resistance against ampicillin is not depicted in Figure 5.12B due to EUCAST (2018) stating that most staphylococci are resistant to this antibiotic as are penicillinase producers. Resistance against other antibiotics tested were 36% or lower, with the exception of azithromycin, which had 51.56% resistance with an additional 45.5% determined as increased exposure. MIC_{50} and MIC_{90} values for the dataset are detailed Appendix, page 27.

A.



B.

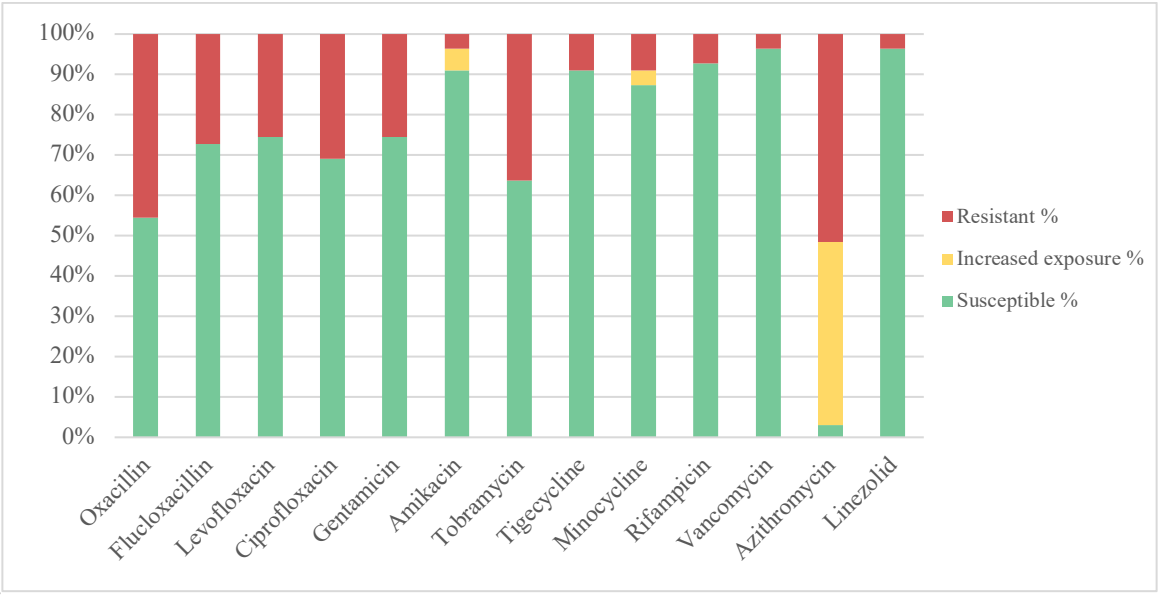


Figure 5.35. Antibiotic resistance profiles according to EUCAST v9.0 (2019). A: Gram-negative isolates (n=390) were tested against 20 antibiotics (minocycline is not shown, as there is no defined breakpoint in EUCAST for Gram-negative species). B: Gram-positive isolates (n=55, 33 for Azithromycin) were tested against a panel of 14 antibiotics. Resistance profiles for 12 of 14 antibiotics tested against Gram-positive bacteria are shown. Breakpoints for oxacillin and flucloxacillin are based on the assumption that isolates with minimum inhibitory concentration >2mg/L are resistant due to mecA/C. These are also evaluated as methicillin resistant S. aureus (45.5% of isolates). No EUCAST breakpoints supplied for S. aureus against ampicillin, as most Staphylococci are penicillinase producers making them resistant to ampicillin. Continent breakdown can be seen Appendix, page 29. Red sections show the percentage of resistant isolate, yellow shows those that would require increased exposure and green show those determined as susceptible to each antibiotic.

5.2.10 Antibiotic resistance and outcomes

MIC values and consequential resistance profiles for the most common four antibiotic combinations prescribed during BARNARDS were compared to reported outcomes for neonates with no change in therapy following relevant empirical treatment (n=290). Associations were not as anticipated between resistance found in pathogens and infant outcome, with no statistically significant difference between resistance of infecting pathogen and outcome following treatment of neonate with each of the four combinations. (RS HR=1.279, 95% CI=0.248-6.603, p=0.769; RS HR=2.781, 95% CI=0.348-22.240, p=0.335; RS HR=0.372, 95% CI=0.083-1.669, p=0.196; and RS HR=1.732, 95% CI=0.383-7.825, p=0.475 for AMP-GEN; CTZ-AMK; AMC-AMK; and PIPT-AMK, respectively) (Figure 5.13; Appendix, page 30-33). Survival analyses for this assessment were based on unadjusted Cox regressions. Proportional hazard assumptions were not met for all adjusted analyses, presumably due to the lower number of isolates assessed in adjusted analyses due to missing data of some confounding variables, as the model removes isolates with missing data in any of the covariates.

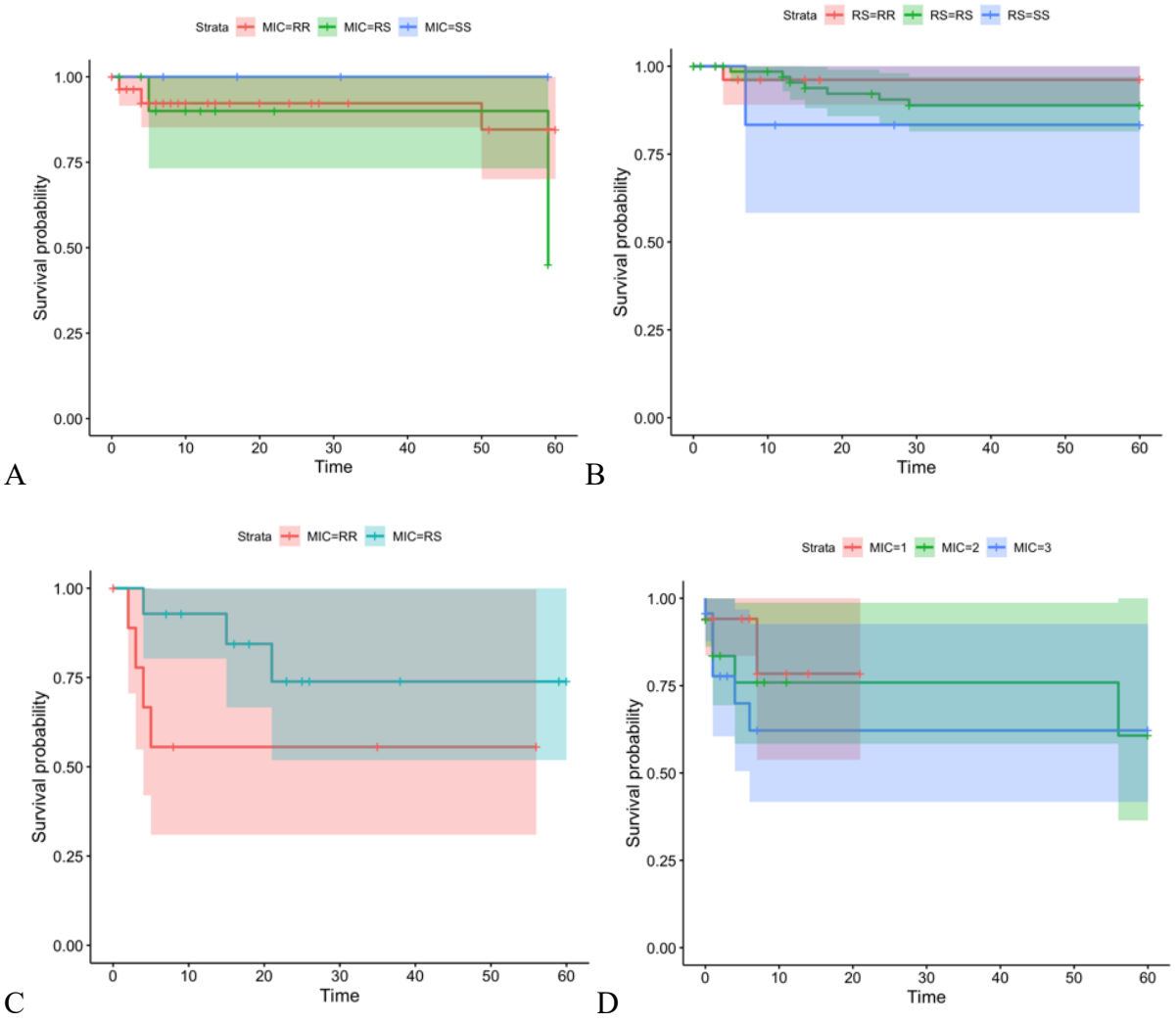


Figure 5.36. Minimum inhibitory concentration (MIC) vs outcome survival curves for differing resistance profiles (resistant to both antibiotic in a given combination=RR; resistant to one of the two antibiotics given=RS; susceptible to both antibiotics given= SS) for each treatment combination with outcome for neonates treated only with one empirical therapy, n=290. A. AMP-GEN; B. CTZ-AMK; C. AMC-AMK; D. PIP/TAZ-AMK. Unadjusted graphs displayed due to limited data, which was lowered when survival curves were adjusted for clinical presentations due to missing data of certain variables. No neonates with infecting pathogens resistant to both PIP/TAZ-AMK were followed up past 21 days, therefore this line in the graph does not continue for the entire 60-day follow-up period. Cox regression results are displayed Appendix, pages 30-33. Shading displays confidence intervals.

No neonates were reported as deceased when the isolate was susceptible to both AMP-GEN (Figure 5.14) (HR=0.001), this was only the case for four isolates. No isolates were found to be resistant to gentamicin but susceptible to ampicillin, due to the high rates of resistance against ampicillin.

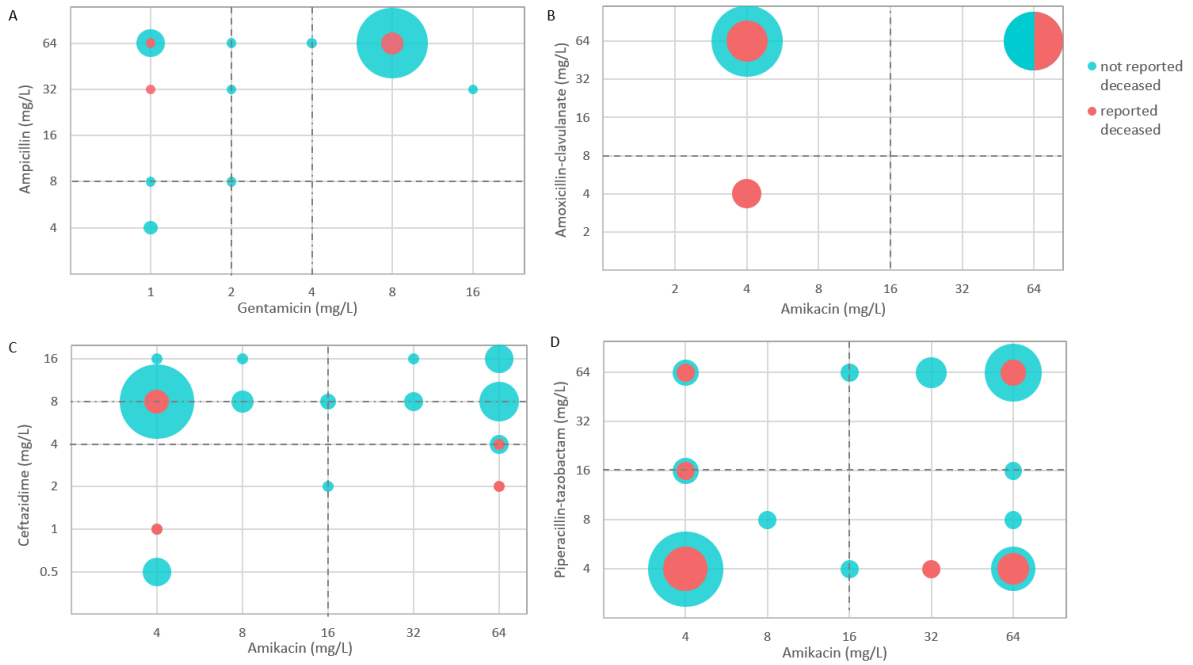


Figure 5.37. Minimum inhibitory concentration (MIC) outcome graphs showcasing dispersion of combined MIC values for the top four most commonly applied treatment combinations with outcome for neonates only treated with each of the first empirical therapy. A: Ampicillin and gentamicin (AMP-GEN), n=76 (MIC values not available for 2 isolates), B: Amoxicillin-clavulanate and amikacin (AMC-AMK), n=24 (MIC values not available for 3 isolates), C: Ceftazidime and amikacin (CTZ-AMK), n=107 (no MIC values for 2 isolates), and D: Piperacillin-tazobactam and amikacin (PIP/TAZ-AMK), n=76. Size represents the number of isolates with MIC value combinations. Isolates were split according to outcome after follow-up for 60 days following birth or admission into the hospital. Dotted lines represent breakpoint values according to EUCAST v9.0 (EUCAST, 2019) for Enterobacteriaceae and Pseudomonas species where relevant. MICs were tested only at concentrations around their respective breakpoints. Maximum MICs given are the concentration above the highest concentration tested. MICs are provided for non-fermenting bacteria up to 16µg/ml and Enterobacteriaceae up to 8µg/ml to cover resistance breakpoints. NB. Only non-fermenters were tested at concentration 8mg/L for Ceftazidime. Antibiotic therapies in this analysis were the primary empirical treatment given to neonates upon clinical diagnosis of sepsis. The four charts are all set to a scale of 100 and therefore the sizes of the bubbles are relative to the number of isolates included in each treatment combination.

5.2.11 Species impact on outcome

5.2.11.1 Top ten isolates

A range of species was included in the overall subset of 457 isolates included in this chapter and the top ten causes of biological sepsis accounted for 398/457 (87.09%) of sepsis

cases. The most common species overall was *Serratia marcescens* (n=110), *K. pneumoniae* (n=100), *S. aureus* (n=100), *E. coli* (n=34) and *K. michiganensis* (n=32) and *Enterobacter cloacae complex* (n=32) (Table 5.22).

Table 5.22. The overall subset (n=457 isolates) included in this study included 401/457 (87.75%) Gram-negative species and 56/457 (12.25%) Gram-positive species. Top ten species are detailed.

Species ID	**Number /457**
Serratia marcescens	110 (24.07%)
Klebsiella pneumoniae	100 (21.88%)
Staphylococcus aureus	38 (8.32%)
Escherichia coli	34 (7.44%)
Klebsiella michiganensis	32 (7.00%)
Enterobacter cloacae complex	32 (7.00%)
Acinetobacter baumannii	18 (3.94%)
Burkholderia cenocepacia	13 (2.84%)
Ralstonia mannitolilytica	12 (2.63%)
Pseudomonas aeruginosa	9 (1.96%)
Other species	59 (12.91%)

Most species included in this analysis were found across a range of sites and countries, with the exception of *Ralstonia mannitolilytica,* found only in one site in Nigeria (NN). *Burkholderia cenocepacia* was only found in sites in South Asia, with the majority of isolates in Pakistan. *Klebsiella michiganensis* was only found in Ethiopia and Pakistan. This was mainly (n=26) due to an outbreak at PP, Pakistan potentially explaining the higher mortality rate. Of the top ten species included in this overall subset, nine occurred more than ten times.

5.2.11.2 Mortality associated with common species

High mortality rates were associated with sepsis related to *Klebsiella michiganensis* outbreak isolates (56.3%); *Klebsiella pneumoniae* (23.6%)*; S. aureus* (22.5%)*; Enterobacter cloacae* (21.4%); and *E. coli* (20.0%) (Table 5.23). From this data, it seems that higher overall resistance in a particular species did not necessarily correlate with mortality, such as

with the comparatively lower rates of resistance in *E. coli* despite relatively high mortality rates, and vice versa with *A. baumannii*.

Table 5.23. Mortality reported for each species included within this cohort, per country.

Species	Associated mortality (%)	Countries with species
Acinetobacter baumannii	0%	Bangladesh (8); Pakistan (4); Rwanda (4); South Africa (2)
Burkholderia cenocepacia	7.0%	Bangladesh (1); Pakistan (12)
Enterobacter cloacae complex	21.4%	Bangladesh (2); Nigeria (8); Pakistan (9); Rwanda (9)
E. coli	20.0%	Bangladesh (3); Ethiopia (3); Nigeria (4); Pakistan (8); Rwanda (15); South Africa (2)
Klebsiella michiganensis	56.3%	Ethiopia (2); Pakistan (30)
Klebsiella pneumoniae	23.6%	Bangladesh (14); Ethiopia (24); Nigeria (35); Pakistan (11); Rwanda (14); South Africa (12)
Ralstonia mannitolilytica	7.7%	Nigeria (13)
Serratia marcescens	7.1%	Bangladesh (102); Nigeria (3); Pakistan (3); Rwanda (1); South Africa (3)
Staphylococcus aureus[1]	22.5%	Bangladesh (6); Nigeria (7); Pakistan (18); Rwanda (1); South Africa (8)

[1]*S. aureus* was not collected throughout the study and so site prevalence may depend more on collection and recording by each site.

5.2.11.3 Mortality associated with common species per four antibiotic combinations

To further evaluate effect of species on outcome, sepsis causing pathogens were broken down per antibiotic therapy, for the five most common species found to be the cause of sepsis for neonates treated with each combination. *Klebsiella* spp. and *Staphylococcus* spp. appeared as one of the most common species infecting neonates treated with all four combinations. *E. coli* was found to be a common cause for all combinations AMK. *Burkholderia* sp. and *Ralstonia* sp. were only found for neonates treated with PIP/T-AMK and AMC-AMK, respectively.

Enterobacter spp. was associated with no reported mortality for neonates infected with this species that were treated with AMP-GEN, but higher reported mortality in neonates treated with AMC-AMK (figure 4.15 A, C). *E. coli* was associated with some reported

mortality for all combinations that it was treated with, with highest mortality when treated with AMC-AMK. *K. pneumoniae* had some level of reported mortality associated with all treatment options, again with highest mortality reported with AMC-AMK treatment. *S. aureus* had highest mortality with treatment of AMP-GEN, followed by PIPT-AMK, with low mortality associated with treatment of AMC-AMK and none with CTZ-AMK (Appendix, page 34-35).

5.2.11.4 Resistance and outcomes related to common species

Resistance rates varied between species, with high levels of resistance in *K. pneumoniae* and *Acinetobacter baumannii* isolates where present. *Acinetobacter* sp. had resistance in >60% of isolates against all antibiotics tested, except for tigecycline (58.3%) and colistin (16.7%). All species had relatively low resistance (<30%) against the carbapenems tested, except for *Acinetobacter* sp. and *Ralstonia mannitolilytica* isolates with >60% resistance against the carbapenems tested. *K. michiganensis* isolates had overall lower resistance compared to *K. pneumoniae* isolates. *E. coli* had the lowest overall rates of resistance when compared to the other Gram-negative species, including lowest resistance against ampicillin, although this was still high at 85.3%.

Levels of resistance found per species did not align with outcomes in all cases, as seen in 4.2.7. *Acinetobacter baumannii* for example was related to low reported mortality in all treatment combinations, despite high levels of resistance common from Acinetobacter isolates (Appendix p34-35). *Acinetobacter* sp. were found at low levels in most countries with the exception of Ethiopia. Conversely, *E. coli* had one of the lower overall rates of resistance but was associated with one of the highest mortality rates (20%).

5.2.11.5 Other factor relating to common species

C-section births were not found to be associated with higher mortality rates in these cases (X^2(N=476, df=2) =3.597, p=0.166). Species of bacteria causing sepsis were also not found to alter dependent on C-section (X^2(N=476, df=78) =48.606, p=0.996).

Species in the overall subset between neonates diagnosed with EOS or LOS were also assessed. Chi-square analysis was carried out for all species in the overall subset (n=457 isolates) and the difference of species between EOS and LOS was significant (X^2(N=457, df=39) =70.451, p=0.002), however many of the species included only occurred once. Therefore, the analysis was repeated only including species that occurred at least five times (Table 5.24) and was again found to be significant (X^2(N=376, df=13) =38.573, p<0.0001).

Table 5.24. Common species with that occurred over five times in the overall subset of neonates (n=457 isolates, 442 neonates) split into neonates with early onset sepsis (EOS) or late onset sepsis (LOS). No data on onset of sepsis was available for 44 isolates, which were excluded from this analysis.

Species ID	**EOS**	**LOS**	**Total**
Acinetobacter baumannii	10	8	18
Burkholderia cenocepacia	5	5	10
Enterobacter cloacae	10	14	24
Escherichia coli	14	17	31
Klebsiella michiganensis	16	4	20
Klebsiella pneumoniae	40	56	96
Klebsiella quasipneumoniae	1	6	7
Pseudomonas aeruginosa	4	4	8
Ralstonia mannitolilytica	10	2	12
Serratia marcescens	30	72	102
Staphylococcus aureus	14	19	33
Staphylococcus epidermis	1	4	5
Staphylococcus haemolyticus	0	5	5
Staphylococcus sciuri	4	1	5

5.2.10 PK/PD modelling and outcome

Probability of target attainment (PTA) values ≥80% were determined by collaborators from Leiden Academic Centre for Drug Research, Leiden University, Leiden, Netherlands for each of the four antibiotic combinations for neonates with only empirical therapy reported (n=290). These took MIC profiles for isolates and standard dosing schedules given by each country into account. For each antibiotic combination evaluated, only isolates that were treated with that specific combination were assessed. Figure 5.16 A-D demonstrates numbers of isolates that were calculated to achieve PTA ≥80% (green) relative to resistance of the organisms to the treatment antibiotics. AMP-GEN had the highest number of isolates that would not reach PTA ≥80%, mainly due to resistance of the pathogen to both antibiotics in the treatment combinations. This can also be seen for AMC-AMK and PIP/TAZ-AMK. However, some isolates that were resistant to both reached PTA >80% for CTZ-AMK, which will be due to the dosing schedule used.

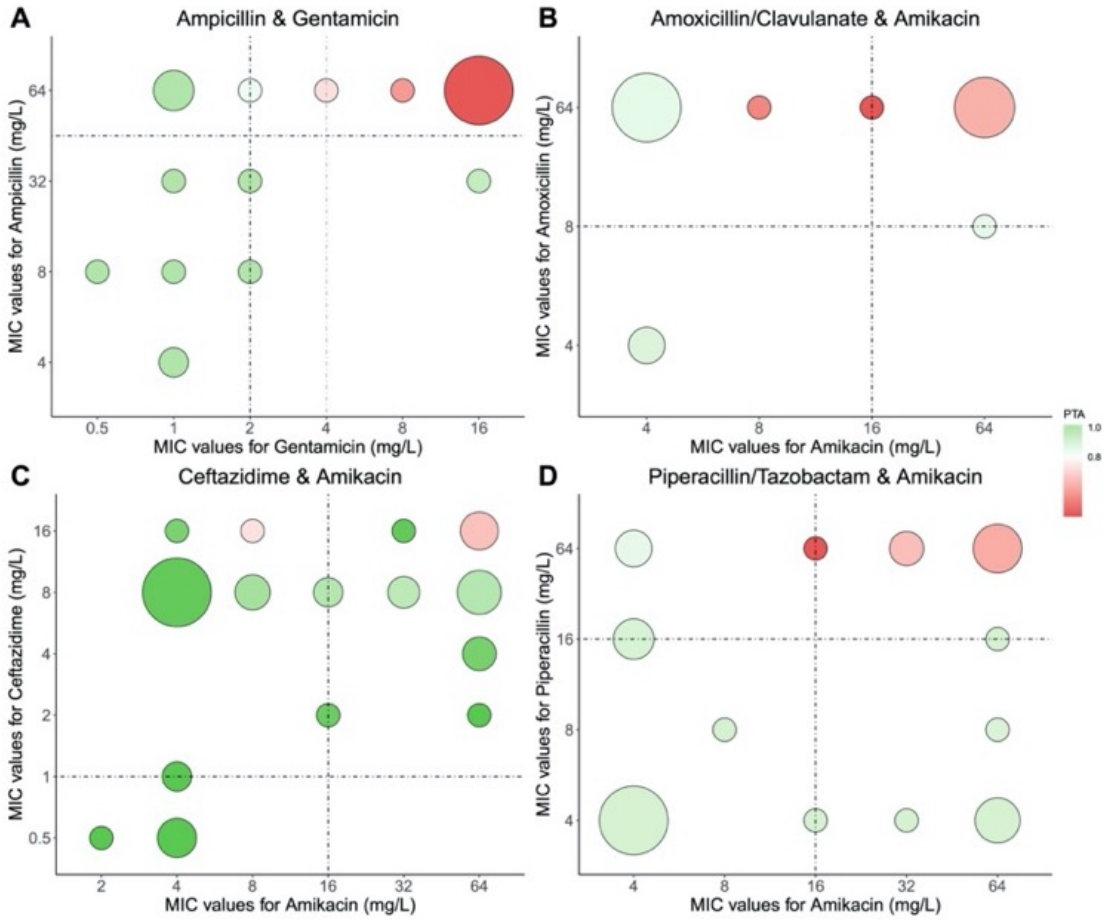


Figure 5.38. A-D: Relationship between simulated probability of target attainment (PTA) values and MIC values for four combination therapies for neonates treated empirically with no following change in treatment (n=290). Vertical and horizontal lines represent (ranges of) minimum inhibitory concentration (MIC) breakpoints according to EUCAST. Source: Thomson et al., 2021)

Major differences in dosing schedules and resulting PTA values existed across countries, where these were recorded to be prescribed in sites in more than one country (Figure 4.17). AMP-GEN was the therapy prescribed by the largest number of sites/ countries, and was collated for Bangladesh, Ethiopia, Nigeria, Rwanda and South Africa. Large discrepancies between countries were seen, in terms of both mg/kg prescribed and frequency of doses, although this appeared to be low in all countries. Ampicillin was given in doses similar to that recommended by WHO (50mg/kg every 12 hours) in most sites but was increased in Rwanda (50mg/kg every six hours) and Nigeria, where 75mg/kg was prescribed every 12 hours. Dosage of gentamicin appeared to be lower than recommended (7.5mg/kg

once a day) in all countries except Ethiopia, prescribing a half dose every 12 hours (Figure 4.17). Dosing for CTZ-AMK and PIP/TAZ-AMK was more even between countries. Pakistan prescribed lower dosages of AMC-AMK than Nigeria (Figure 5.17).

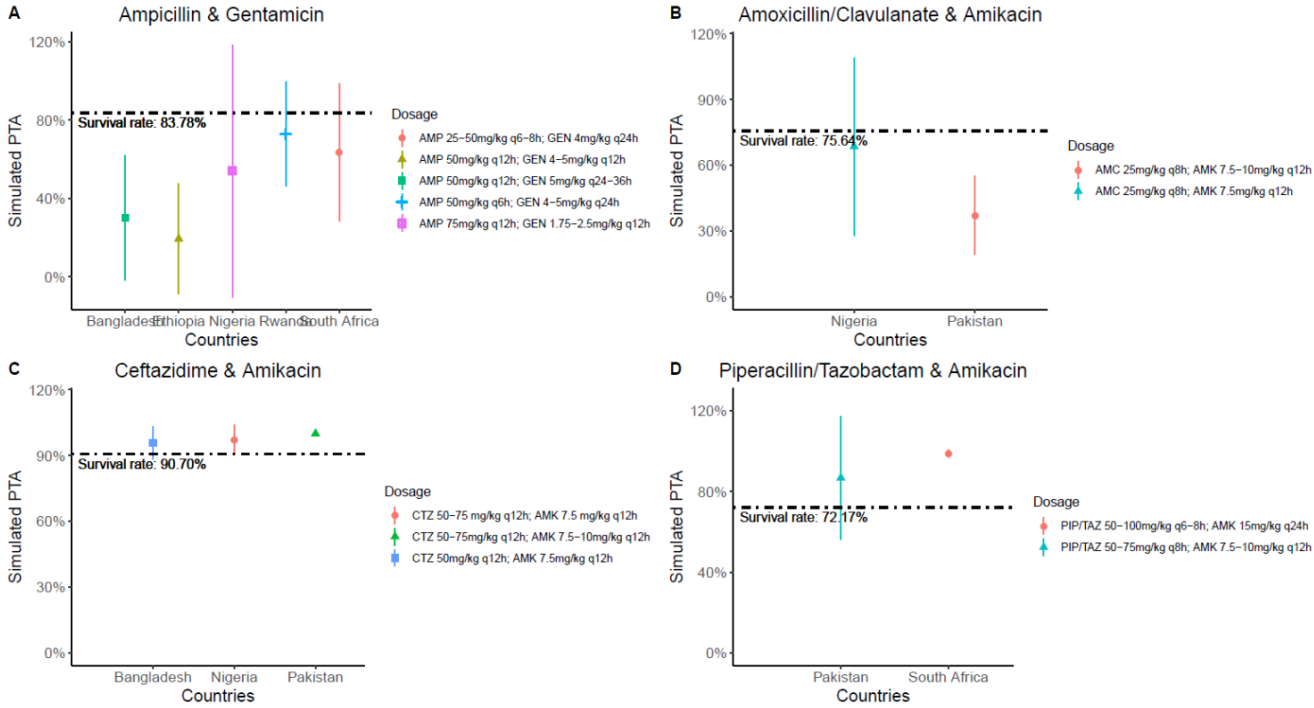


Figure 5.39. Simulated probability of target attainment (PTAs) for different site (country)-specific dosage regimens for the most commonly used four antibiotic combination therapies (n=476 patients). Source: Thomson et al., 2021.

Observed survival was aligned with PTA ≥80% for the four antibiotic combinations, and potential alternative antibiotics (Figure 5.18). The number of neonates without reported mortality (observe survival) matched the number of neonates that were calculated to reach PTA≥80% for those treated with AMC-AMK. Observed survival for neonates treated with CTZ-AMK was approximately 5% higher than expected from the number of isolates obtaining ≥80% PTA. Observed survival was slightly lower (approximately 15%) than expected for neonates treated with PIP/T-AMK. However, many site clinicians have told us that PIP/TAZ-AMK is usually used in higher risk patients, potentially with additional risk factors (such as prematurity), and so we would expect the observed survival rate here to be

lower than that predicted via only MIC and dosing schedules. These were all quite close to PTA estimation, however the observed survival for AMP-GEN was much higher than expected through ≥80% PTA. According to this analysis, 34.6% of these neonates would have been expected to survive taking the pathogen MIC profile and hospital dosing schedules into account, however, neonates treated with AMP-GEN related to 89.7% observed survival (not reported deceased).

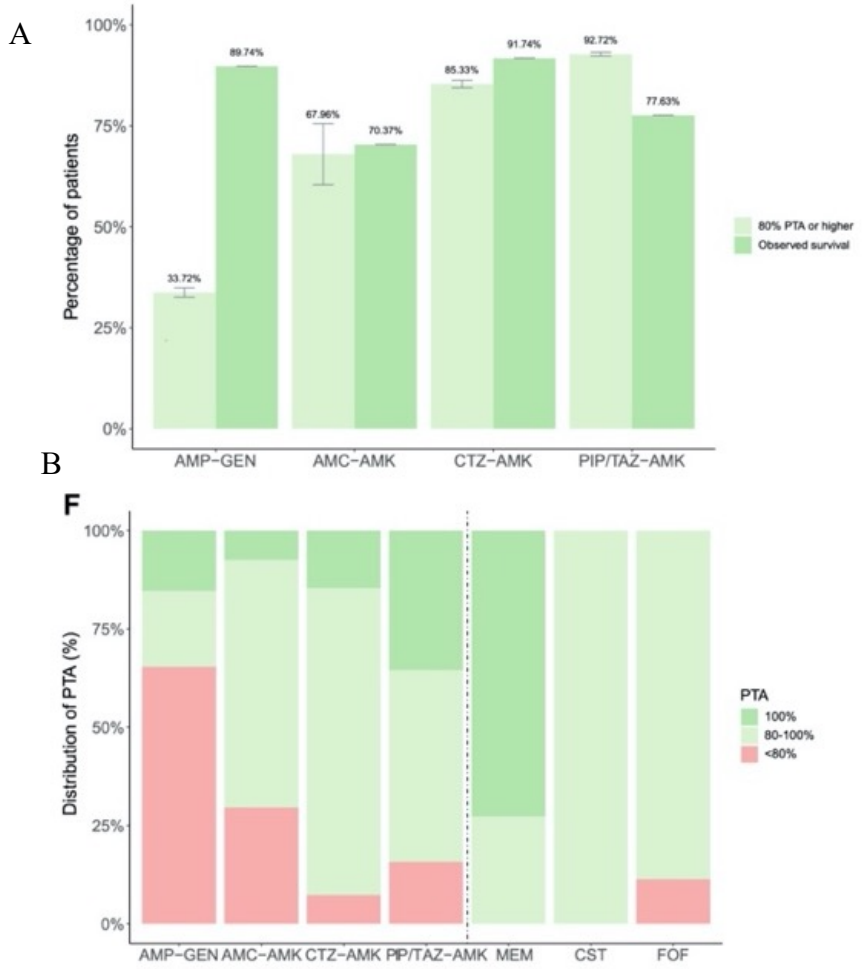


Figure 5.40. A: Comparison of simulated probability of target attainment (PTA ≥80% and observed survival rate AMP-GEN: Neonates treated with ampicillin and gentamicin; CTZ-AMK: Neonates treated with ceftazidime and amikacin; AMC-AMK: Neonates treated with amoxicillin and amikacin; and PIP/TAZ-AMK: Neonates treated with piperacillin-tazobactam and amikacin. B: Simulated PTA values for the four combination therapies, in comparison to meropenem (MEM) (10mg/kg every 8 hours), fosfomycin (FOF) (200 mg/kg every 12 h) and colistin (CST) (5 mg/kg per day), based on observed minimum inhibitory concentration distributions for these antibiotics. (Source: Thomson et al., 2021).

In addition to PTA values ≥80% for the four common antibiotic therapies described, this was also carried out for potentially appropriate alternative therapies for treatment of neonatal sepsis, with low resistance rates (meropenem, fosfomycin and colistin) (figure 4.18F). All isolate analysed reached PTA ≥80% for Meropenem and Colistin, with 89% attainment for Fosfomycin, showing that these antibiotics could be potential alternatives.

5.2.11 Synergy

Due to lower-than-expected rates of reported mortality for neonates treated with AMP-GEN with resistant pathogens and low PTAs, synergy between AMP-GEN was tested on a small subset of 16 isolates of varied bacterial species, with a range of outcomes and MIC profiles against AMP-GEN via checkerboard microbroth dilution (Table 5.25).

Table 5.14. Details of isolates tested in synergy experiments. Minimum inhibitory concentration (MIC) for each antibiotic is provided, alongside fractional inhibitory concentration index (FICI) calculations. $FICI_{min}$ and $FICI_{max}$ were calculated from FIC_A and FIC_B, providing the range of the FICIs found. FICI values ≤0.5 represent synergy; >0.5-1 represent Additivity; 1-4 show indifference; and >4 demonstrate antagonism between the antibiotics, as according to Saiman (2007).

Isolate ID	**Species**	**Resistance**	**Outcome**	**MIC (ug/mL) alone**		**FICI**		**Synergy**
				AMP	**GEN**	$FICI_{min}$	$FICI_{max}$	
RK-BB973	*E. coli*	SS	Not deceased	8	1	0.75	1.125	Additive to no effect
BK-BB749	*P. aeruginosa*	RS	Not deceased	>256	2	1.25	1.5	No effect
ESS-BB440	*K. michiganensis*	RS	Deceased	>256	64	2	2	No effect
RK-BB86	*A. baumannii*	RS	Not deceased	32	2	1	1.125	No effect
RK-BB2000	*S. aureus*	RS	Not deceased	16	1	0.75	0.75	Additive to no effect
ZAT-BB102-I1	*K. pneumoniae*	RS	Deceased	64	0.5	1.125	1.125	No effect
RK-BB64	*E. coli*	RI	Not deceased	>256	1	1.0625	1.0625	No effect
ZAT-BB2180a	*S. aureus*	RR	Not deceased	128	64	1.25	1.5	No effect
BK-BB838	*A. baumannii*	RR	Not deceased	>256	64	2	2	No effect
ESI-BB1044b	*K. pneumoniae*	RR	Not deceased	>256	64	2	2	No effect
RK-BB1495	*E. coli*	RR	Deceased	>256	128	1.5	1.5	No effect

BK-BB1099	*P. aeruginosa*	RR	Not deceased	>256	2	1.25	1.25	No effect
ZAT-BB1784-I2	*A. baumannii*	RR	Not deceased	>256	>256	2	2	No effect
ZAT-BB658	*S. aureus*	RR	Not deceased	128	128	0.75	1.0625	Additive to no effect
RU-BB83	*K. pneumoniae*	RR	Deceased	>256	128	1.5	1.5	No effect
RK-BB1384	*E. coli*	RR	Not deceased	>256	64	2	2	No effect
ATCC 25922	*E. coli*	NA/ SS	NA	8	0.5	1.5	1.5	No effect
ATCC 27853	*P. aeruginosa*	NA/ RS	NA	>256	2	1.5	1.5	No effect

According to fractional inhibitory concentration index (FICI) calculations, although some additive effects were witnessed, no synergy was found between AMP-GEN for any of the species tested. Two of the three *S. aureus* isolates, and one of five *E. coli* isolates tested demonstrated AMP-GEN to have an additive effect. However, no synergistic effect was found for any isolates *in vitro* and most isolates showed no effect.

5.2.12 Neonates lost to follow-up

Another reason observed survival, assessed as those not confirmed deceased for neonates treated with AMP-GEN may have been much higher than those estimated to reach >80% PTA due to the high number lost to follow-up. Of neonates treated with AMP-GEN, 93/111 were not reported as deceased. These neonates had an average follow-up time of 29.74 days, and 25.82 days when age at diagnosis was subtracted from age at outcome with no data on age at outcome available for five neonates. Neonates treated with CTZ-AMK had 156/172 not reported deceased. These had a much higher average for days to outcome of 58.01 and 49.19 when ages at diagnosis of sepsis was subtracted from age at outcome. However, no data on follow-up age was available for 50 neonates (32.05%). Neonates treated with PIP/TAZ-AMK has 83/115 neonates not reported deceased, which had an average follow-up age of 43.05 days and 37.13 days for age of outcome minus age of diagnosis However, there were a high number of neonates with no data on age at outcome (n=42,

50.60%). Neonates treated with AMC-AMK composed of 59/78 neonates that were not reported deceased. These neonates had an average follow-up time of 32.35 days, or 25.75 days when time to sepsis was subtracted from age at outcome, with no data for two neonates (3.39%).

A one-way ANOVA determine that there were significant differences in follow-up ages between treatments ($F(3, 317) = 51.505$, $p<0.0001$). Post hoc Tukey analyses found treatment with CTZ-AMK had a significantly longer follow-up time than all other treatments (AMP-GEN ($p<0.0001$, 95% CI = [21.55 - 34.52]); PIP/TAZ-AMK ($p<0.0001$, 95% CI = [6.56 – 23.37]); AMC-AMK ($p<0.0001$, 95% CI = [18.22 - 33.11])). Follow-up time of AMP-GEN was also significantly lower than follow-up time for PIP/TAZ-AMK ($p=0.001$, 95% CI = [-22.01 - -4.13]) and that neonates treated with PIP/TAZ were followed up longer than those treated with AMC-AMK ($p=0.023$, 95% CI = [1.03 – 20.36]).

5.2.13 Frequency of resistance

Following collection of data on resistance profiles, frequency of resistance experiments were carried out against a range of antibiotics. This was performed as a preliminary assessment of of how quickly resistance could be likely to develop against those currently used with high resistance rates already present and how quickly resistance may rise with increased usage of alternative antibiotics with lower current levels of resistance. Development of resistance was defined by *in vitro* growth of isolates previously determined as sensitive when challenged by the antibiotic at two times breakpoint concentration. Through this method, resistance was found to occur most frequently on previously susceptible isolates when grown in the presence of fosfomycin (68.4%, n=78/114), colistin (57.3%, n=55/96) and gentamicin (53.0%, n=62/117), followed by piperacillin-tazobactam (34.3%, n=35/102), Amoxicillin-clavulanate (33.3%, n=8/24), ceftazidime (32.7%, n=17/52), ampicillin (16.7%, n=1/6), amikacin (7.7%, n=9/117), with no growth of isolates when

challenged with meropenem (0%, n=0/117) (Figure 5.19). However, when CFU per ml was assessed, gentamicin had higher growth per ml than other antibiotics ($4.45x10^{-3}$) (one-way ANOVA, $p<0.005$; Post-hoc Tukey test $p<0.005$ for growth per ml for all other antibiotics tested). Isolates grown on amoxicillin-clavulanate agar had the second highest growth ($2.50x10^{-3}$), followed by colistin ($4.17x10^{-4}$), amikacin ($3.75x10^{-4}$), fosfomycin ($2.59x10^{-4}$), piperacillin-tazobactam ($1.97x10^{-4}$), ceftazidime ($1.92x10^{-4}$), ampicillin ($6.67x10^{-7}$) and no growth for meropenem. However, the results for ampicillin are not very reliable, as only based on six isolates that were sensitive to ampicillin.

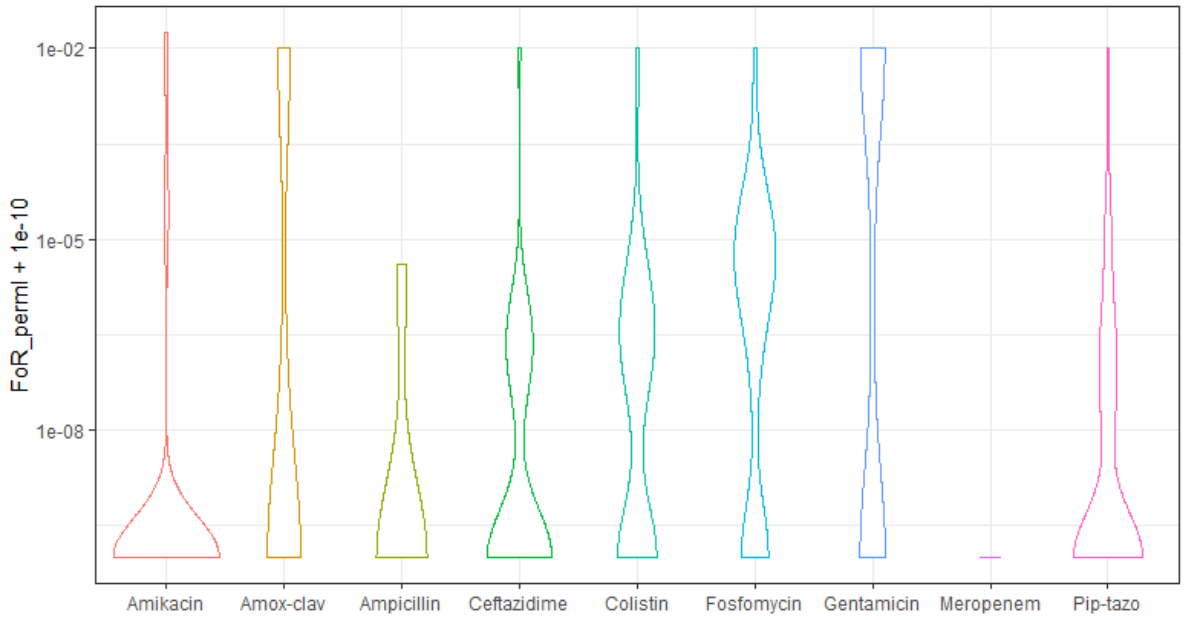


Figure 5.41. Frequency of resistance (FoR) for Gram-negative isolates. Numbers included in the analysis for the following antibiotics were: Amikacin n=117; amoxicillin-clavulanate n=24; ampicillin n=6; ceftazidime n=52; colistin n=96; fosfomycin n=114; gentamicin n=117; meropenem n=117; piperacillin-tazobactam n=102. Numbers differ across antibiotics due to susceptibility patterns, with only sensitive bacteria suitable for FoR determination. Data is presented per ml and the FoR calculated from growth at a lower dilution on control plates free of antibiotics. Results have been log transformed with a standard of $1x10^{-10}$ added to enable incorporation of zero values. This standard was chosen, as the lowest rate of FoR found was $1x10^{-9}$.

FoRs were tested on a range of species and sequence types from across clinical sites where possible. *E. coli* isolates displayed lower FoR overall than *K. pneumoniae* isolates for most antibiotics tested (Figure 5.20; 5.21). *E. coli* demonstrated development of resistance

against amikacin, ceftazidime, gentamicin, piperacillin-tazobactam and amoxicillin-clavulanate at low levels. No development of resistance was seen in *E. coli* isolates against ampicillin, colistin, fosfomycin or meropenem. Like *E. coli*, there was no development of resistance in *K. pneumoniae* to meropenem. Unlike *E. coli* (n=19 tested), no development of resistance was seen against amikacin in *K. pneumoniae* isolates tested (n=52 tested). No *K. pneumoniae* isolates were tested against ampicillin, as there were no originally susceptible isolates. *K. pneumoniae* isolates did not develop resistance against amikacin or meropenem, although did against all other antibiotics tested, at similar or higher levels than *E. coli*.

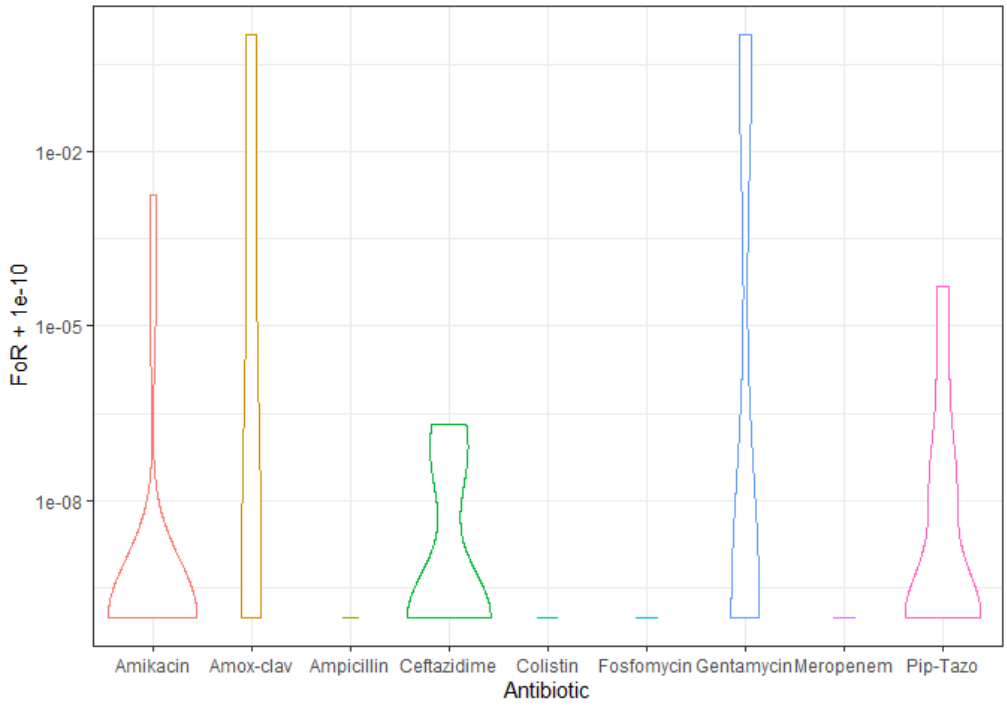


Figure 5.42. Frequency of resistance (FoR) occurring in E. coli isolates tested against Amikacin (n=19); Amoxicillin-clavulanate (n=6); Ampicillin (n=4); Ceftazidime (n=12); Colistin (n=19); Fosfomycin (n=19); Gentamycin (n=19); Meropenem (n=19) and Piperacillin-tazobactam (n=18). Data is presented per ml. Results have been log transformed with a standard of $1x10^{-10}$ added to enable incorporation of zero values. This standard was chosen, as the lowest rate of FoR found was $1x10^{-9}$.

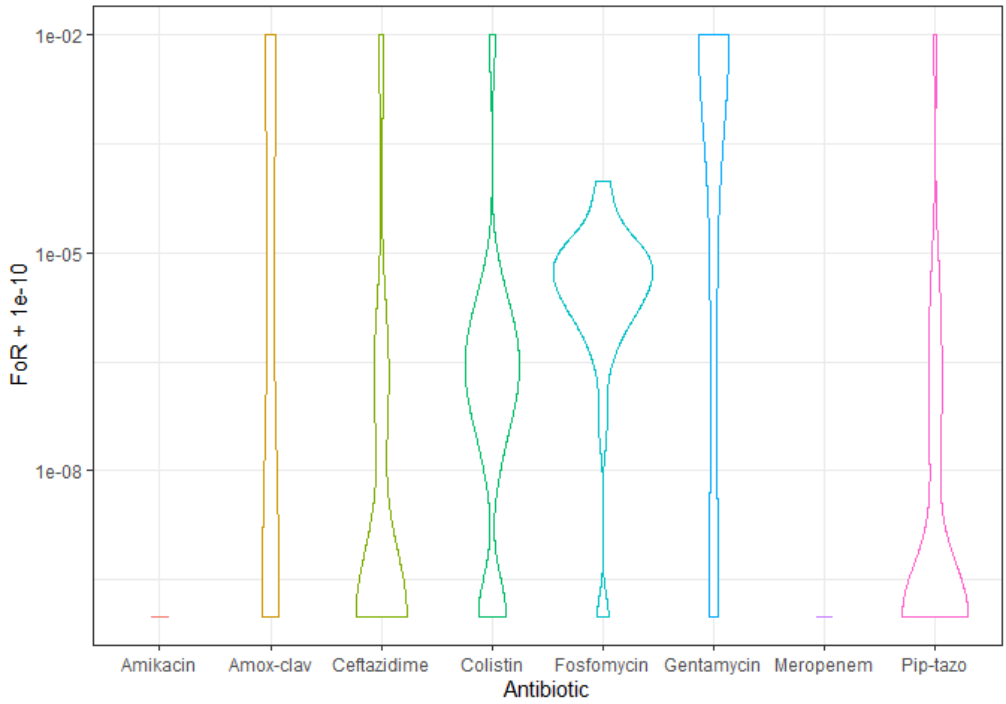


Figure 5.43. Frequency of resistance (FoR) occurring in K. pneumoniae isolates tested against Amikacin (n=52); Amoxicillin-clavulanate (n=13); Ceftazidime (n=19); Colistin (n=49); Fosfomycin (n=49); Gentamycin (n=52); Meropenem (n=52) and Piperacillin-tazobactam (n=44). No isolates were tested against Ampicillin, as K. pneumoniae is intrinsically resistant to this antibiotic. Data is presented per ml. Results have been log transformed with a standard of $1x10^{-10}$ added to enable incorporation of zero values. This standard was chosen, as the lowest rate of FoR found was $1x10^{-9}$.

In addition to *E. coli* and *K. pneumoniae*, other Gram-negative species tested included *A. baumannii*, *E. cloacae*, *E. hormaechei*, *Klebsiella michiganensis*, *Pseudomonas aeruginosa* and *Serratia marcescens* (Appendix, pages 36-38), demonstrating varied FoRs to each antibiotic (Resistance against amikacin developed only in *E. coli*, *K. michiganensis* and *S. marcescens* isolates. *E. coli* was the only species that did not show growth with colistin (*A. baumannii* and *S. marcescens* were not tested with colistin due to intrinsic resistance/ lack of sensitive isolates). Growth was seen at lower levels in the presence of fosfomycin in all species apart from *E. coli*, with slightly higher rates found in *Enterobacter* sp. and *K. pneumoniae* isolates. Growth with gentamicin was seen at higher levels, consistent amongst all species, except for *P. aeruginosa* isolates which showed no development of resistance. Likewise, growth was seen in all species against piperacillin-tazobactam (*A. baumannii* not

tested due to lack of sensitive isolates) except for *S. marcescens*. No species demonstrated resistance against Meropenem (Figure 4.22).

The least number of different species developed growth against amikacin. All other antibiotics tested (colistin, fosfomycin, gentamicin, and piperacillin-tazobactam) had growth from all species tested against it bar one species, which varied between antibiotics. *E. coli* lacked resistance against colistin and fosfomycin, whereas no resistance developed in *P. aeruginosa* isolates when challenged with gentamicin or *S. marcescens* for piperacillin-tazobactam (Figure 5.22). Therefore, the best antibiotics to reduce development of resistance would depend on local common pathogens.

Variation between species was not tested for ampicillin, as only six isolates were tested, four of which were *E. coli*, one *A. baumannii* and one *Enterobacter cloacae*. Similarly, Amoxicillin-clavulanate was only tested against 24 isolates, 13 of which were *Klebsiella* spp and six were *E. coli*, without comparative numbers of other species.

Only *Staphylococcus* spp. was analysed in terms of Gram-positive bacteria. None of the *Staphylococcus* spp. isolates demonstrated development of resistance against amikacin or fosfomycin. Growth was seen for flucloxacin and gentamicin, with 31.58% (n=6/19) of isolates developing growth in the presence of flucloxacin, with an average growth of 5.30×10^{-7} CFU per ml and 10.53% (n=2/19) in the presence of gentamicin with an average growth of 7.78×10^{-7} CFU per ml (Figure 5.23).

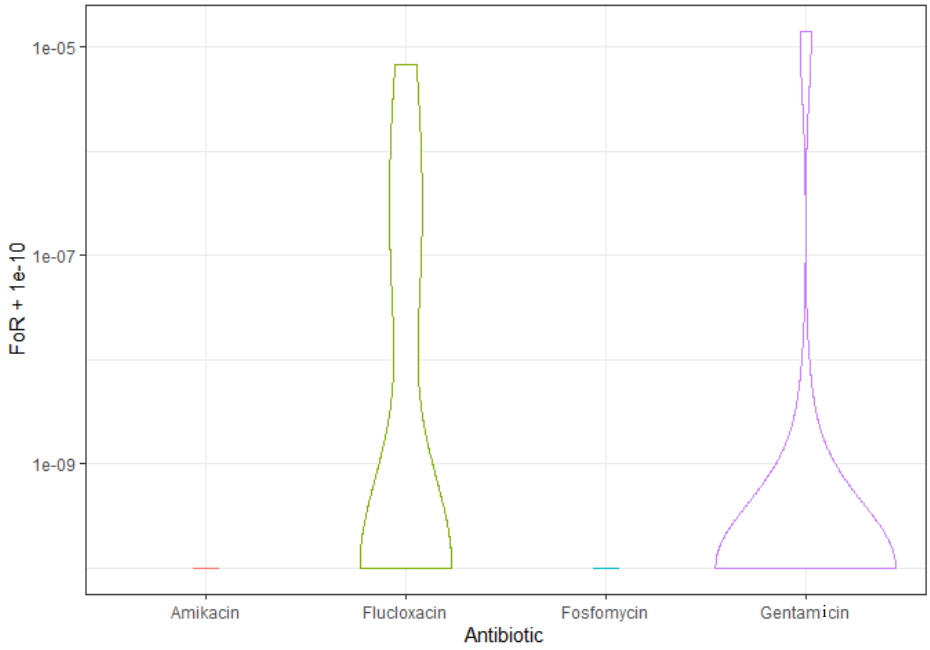


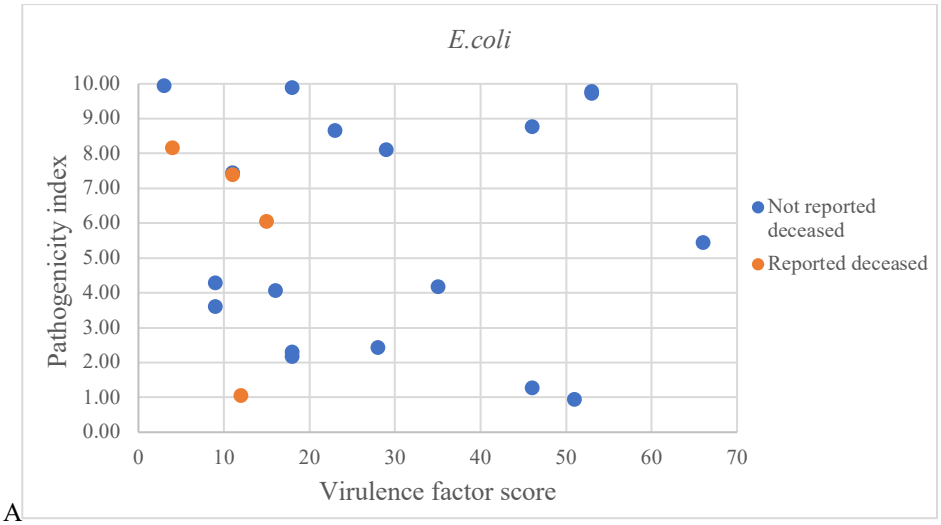
Figure 5.44. Frequency of resistance (FoR) occurring in Staphylococcus aureus isolates tested (n=19) against amikacin, flucloxacin, fosfomycin and gentamicin. Data is presented per ml. Results have been log transformed with a standard of $1x10^{-10}$ added to enable incorporation of zero values. This standard was chosen, as the lowest rate of FoR found was $1x10^{-9}$.

5.2.14 Pathogenicity index and Virulence factors correlated with outcomes

Pathogenicity was determined from a *Galleria mellonella* model on 155 isolates. This included five *Acinetobacter* spp., two *B. cenocepacia*, 27 *E. coli*, 20 *Enterobacter* spp., 16 *K. michiganensis*, 56 *K. pneumoniae*, and three other *Klebsiella* spp., five *P. aeruginosa*, one *R. mannitolilytica*, and 40 *S. marcescens*. Overall, PI ranged from 0.06 to 10 (maximum possible score). Wide variation was also seen within species, detailed for those with ≥5 isolates tested. PIs for *E. coli* isolates ranged from 0.94-9.78, averaging 5.78. *Enterobacter* sp. had one isolate with a PI score of 0.06, with other scores up to 9.92, averaging 5.64. A lower average PI of 2.56 was found for *K. michiganensis*, which ranged from 0.33-9.17. *K. pneumoniae* isolates had an average of 6.77, ranging from 0.94-10.00. *Acinetobacter* sp. had an average PI of 6.23 (1.56-10.00). PIs for the *S. marcescens* were the highest, with an average of 9.71 (3.39-10.00). and *P. aeruginosa* (9.98, ranging 9.89-10.00). 34 *S. aureus*

isolates were also tested, which had lower PIs than seen for Gram-negative isolates, ranging from 1.94-7.56, with an average PI of 4.22.

Virulence factors were also assessed for *K. pneumoniae*, *E. coli* and *S. aureus* isolates via VF databases. This was only done for these species, due to insufficient databases for many other species in and that these species are top causes of neonatal sepsis. Surprisingly, no correlations were found between PIs and VF scores for *K. pneumoniae* (R^2=0.14); *E. coli* (R^2=0.00); or *S. aureus* (R^2=0.10), therefore VFs and PIs were analysed separately against outcomes. Lower VF scores in *E. coli* were found to be associated with higher rates of reported mortality (U=12.50, p=0.042), but no association was found between PI and outcome (U=33.00, p=0.837). No significant association between VFs or PIs and outcome were found for *K. pneumoniae* (U=188.0, p=0.663; U=178.50, p=0.517) or for *S. aureus* isolates (U=113.0, p=0.954; U=128.0, p=0.630, respectively) (figure 5.24).



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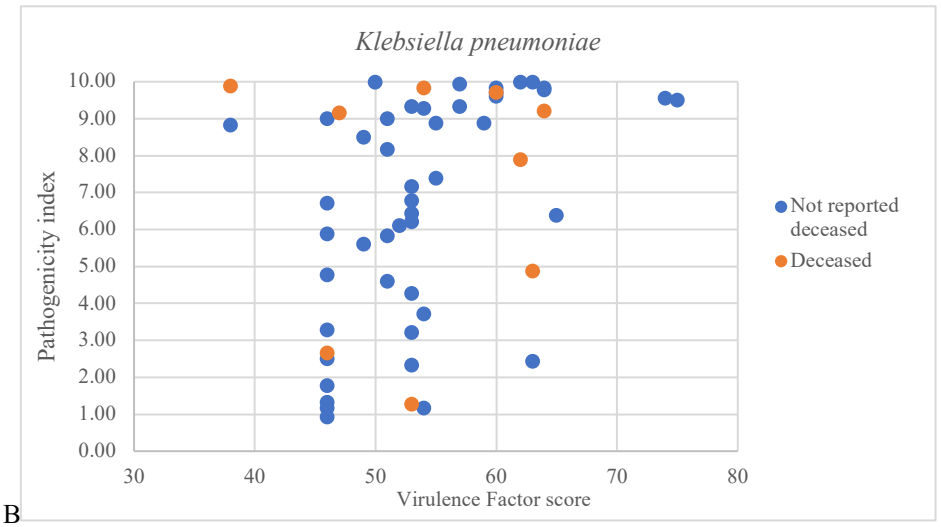

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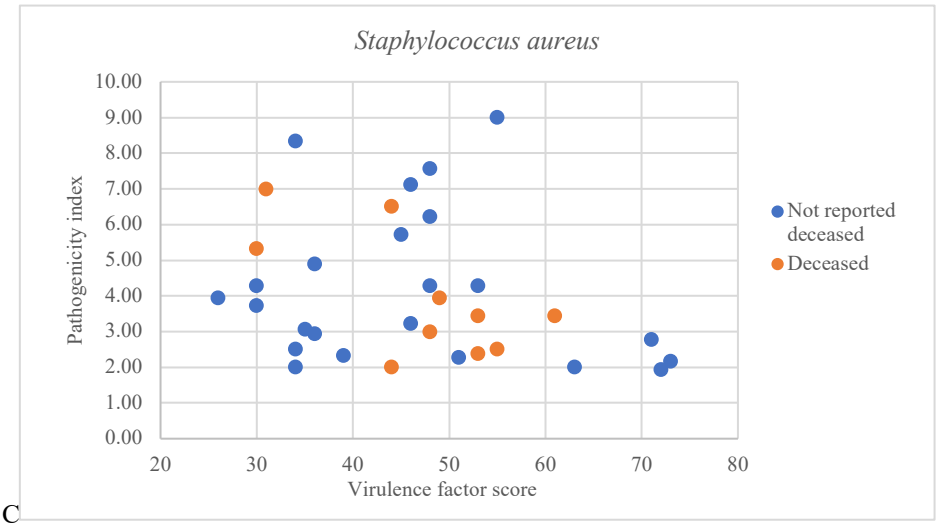

C

Figure 5.45. Pathogenicity index (PI) calculated from pathogenicity of isolates injected into G. mellonella models have been plotted against Virulence factor (VF) scores obtained from sequencing data for A. E. coli (n=24), B. K. pneumoniae (n=55) and C. S. aureus (n=34) isolates. The data was split into two groups for comparison ('reported deceased' and 'not reported deceased'). Any untraceable neonates have been put into the 'not reported deceased' category. These bacterial species were selected for this analysis, as were found to be common causes of neonatal sepsis across BARNARDS sites and have extensive literature available on clinically relevant virulence factors.

5.2.15 Availability of antibiotics

To better understand neonatal sepsis treatment practices, questionnaires were completed by the PI for each site regarding antibiotic costs, availability, and average monthly salaries in respective countries (Table 5.31). Average monthly salary varied from $98 in India to $316 in Pakistan. Payment of antibiotics varied between sites, and all sites except for sites in Nigeria offered AMP-GEN free of charge for patients. Bangladesh and Pakistan covered most antibiotics, except for colistin, piperacillin-tazobactam (for Bangladesh), meropenem (for Pakistan) and tigecycline. The clinical sites in India and South Africa reported all antibiotics to be covered by their governments, with patients not having to pay for any antibiotics prescribed. Information was limited on this for Rwanda, as the clinical lead asked stated that >80% are on private insurance and had little information on those without insurance. Private healthcare insurance appears to be widespread in Bangladesh, India, Rwanda and to a lesser extent South Africa. Healthcare in Ethiopia, Nigeria and Pakistan does not appear to depend as much on private insurance.

Cost of antibiotics varied wildly between different antibiotics, and the cost of a particular antibiotic fluctuated between countries. Costs of antibiotics were commonly cheaper in India, Pakistan, and Bangladesh. Ampicillin and gentamicin were the two cheapest antibiotics in all countries ($0.15 in India-$1.50 Nigeria and $0.20 in Bangladesh and India-$1.00 in Nigeria). Amikacin was the next cheapest antibiotic and was available in all countries, except for Ethiopia, followed by ceftazidime which was available in all countries questioned. Amoxicillin-clavulanate was not available in all countries and cost more than previously listed antibiotics. Piperacillin-tazobactam was not available in Ethiopia and Rwanda and ranged in priced from $2.60 in India to $24.60 in Bangladesh. Colistin was a similar price bracket ($6.00-9.00) and was only available in four countries. Meropenem was available in all countries included in BARNARDS, but this varied drastically between

countries from $3.50 in South Africa to $14.00 in Rwanda. Tigecycline was the most expensive antibiotic, ranging between $27.00-45.00 and was only available in India, Pakistan, and South Africa. Large variations were reported in the cost coverage of antibiotics by local governments, role of private healthcare, prices of antibiotics and availability between countries making different choices in antibiotic therapy more feasible for patients in some countries compared to others. Cost of antibiotic was also confirmed by sites in six of the seven countries to potentially influence treatment of neonatal sepsis (Table 1). This is an important factor to bear in mind when analysing antibiotic treatments and potential alternatives. Tigecycline had a high cost, the cost of which often borne by the patient. In addition to this, tigecycline was not available in four from seven countries, making this an inappropriate treatment alternative in many cases, in addition to being on the reserve list. Sites in six of the seven countries confirmed that cost of the antibiotic would influence treatment of neonatal sepsis (Table 5.26).

Table 5.26. Healthcare matrix consisting of average salaries, costs of antibiotics and coverage by the government across different BARNARDS countries. Site acronyms are provided where more than one clinical site has participated in BARNARDS in a country. Clinical sites included Kumudini Women's Medical College (BK) and Chittagong Ma O Shishu Hospital (BC), Bangladesh; St. Paul's Hospital Millennium Medical College, Ethiopia; Institute of Post Graduate Medical Education and Research, Kolkata; Murtala Mohammad Specialist hospital, Kano (NK), Wuse District Hospital, Abuja (NW), National Hospital Abuja (NN), Nigeria; Pakistan Institute of Medical Sciences (PP), Community health centre, Bhara Kahu (PC), Pakistan; University Central Hospital of Kigali (RK), Kabgayi Hospital (RU), Rwanda; and Tygerberg Academic Hospital, Cape Town, South Africa. Costs are provided in US dollars for ease of comparison, with exchange rates calculates through www.XE.com, March 2020.

	Bangladesh	**Ethiopia**	**India**	**Nigeria**	**Pakistan**	**Rwanda**	**South Africa**
Average monthly salary	$228 (BK); $663 (BC)	$142	$98	$81 (NK); $204 (NW); $274 (NN)	$316 (PP); $100 (PC)	$250 (RK); $102 (RU)	$274
Who pays for the antibiotics?	Antibiotics (colistin, Piperacillin-tazobactam, tigecycline) not supplied by the hospital are paid by the patient.	Only Ampicillin and Gentamicin are available through the public system.	Cost of antibiotics for neonates are borne by the West Bengal government.	Cost of all drugs are mainly borne by patients. Some support of government employees.	Colistin, meropenem and tigecycline will be paid for by the patient.	80% of the population have insurance. Little data on those that cannot afford insurance.	Government cover the cost of all antibiotics.
Role of private insurance?	Yes. But only for those with high incomes.	Availability to only a few. The poor are not covered.	Widely used in India but not needed for our hospital.	Availability to only a few. The poor are not covered.	Poor patients are covered by the Government. Private insurance covers only <1% of the population.	Of those with insurance, companies cover 85% of treatment costs.	20% of country uses private health care.
Local cost of Antimicrobials/day (percentage of average daily wage based on 30day/month (%))							
Ampicillin	$0.35 (1.5;4.6)	$0.50 (11)	$0.15 (4.5)	$1.50 (16.5-56)[1]	$0.50 (1.2;4.8)	$0.50 (6;15)	$0.60 (6.6)
Gentamicin	$0.20 (1;2.6)	$0.30 (6.3)	$0.20 (6.1)	$1.00 (11-38)	$0.60 (1.4;5.7)	$0.50 (6;15)	$0.40 (4.4)
Ceftazidime	$3.00 (1.4;40)	$3.50 (74)	$2.50 (76)	$3.50 (38-130)	$2.30 (5.6;22)	$2.00 (24;59)	$1.80 (20)
Amikacin	$0.50 (2.2;6.6)	Not available	$1.00 (30)	$3.00 (33-111)	$0.50 (1.2;4.8)	$2.00 (24;59)	$0.40 (4.4)

Amoxicillin-Clavulanate	Not available	Not available	$8.00 (242)	$10.00 (110-370)	Not available	Not available	Not available
Piperacillin - Tazobactam	$24.60 (111;309)	Not available	$2.60 (79)	$20.00 (219-741)	$9.00 (22;86)	Not available	$7.20 (79)
Meropenem	$10.00 (45;132)	$11/day (234)	$6.40 (194)	$12.5/day (137-463)	$6.50 (62;197)	$14.00 (169;412)	$3.50 (38)
Colistin	$8.00 (36;105)	Not available	$9.00 (272)	Not Available	$8.00 (19;76)	Not available	$6.00 (66)
Tigecycline	Not available	Not available	$45.00 (1363)	Not Available	$30.00 (73;286)	Not available	$27.00 (297)
Does antibiotic cost influence accessibility?	Yes	Yes	Yes	Yes	Yes	Yes	No

5.3 Discussion

5.3.1 Antibiotics prescribed

A wide range of antibiotics were recorded to be used as first-line treatments, with no countries adhering strictly to one or two combinations. This diversity of antibiotic prescriptions and divergence from WHO recommended treatment is supported by Jackson *et al.* (2019) whose study found a range of 59 combinations and 19 monotherapies prescribed to neonates with sepsis in LMICs. Despite WHO guidelines recommending AMP-GEN, amikacin was the most prescribed antibiotic followed by PIP/TAZ overall, with gentamicin and ampicillin prescriptions occurring third and fourth most regularly, respectively, as first-line treatments. This may be indicative of concerns at sites regarding the empirical use of AMP-GEN, potentially due to reduced response in neonates or findings of resistance in previous sepsis causing isolates. Furthermore, they found similarly to this study, that only 185/824 (22.5%) neonates were prescribed the WHO recommend first-line therapy. Jackson *et al.* (2019) found that meropenem was prescribed more commonly (15.9%) than AMP-GEN in LMICs, although in our study, meropenem was prescribed modestly (2.80%). In this study, antibiotics within the access group were prescribed most often (7,213 prescriptions), although watch antibiotics were also commonly used (3,837). Similar use of access and watch antibiotics were found by Hsia *et al.* (2019) in countries in Africa and Asia, although as would be expected, use of access antibiotics varied between countries assessed.

5.3.2 Assessment of antibiotics

As seen in the previous chapter, high AMR was witnessed in the subset included in these analyses. Resistance to ampicillin was extraordinarily high, with 97.2% resistance in Gram-negative isolates. While ampicillin is effective against Group B streptococcus, a common cause of neonatal sepsis in higher income countries (Stoll *et al.*, 2011; McCraken, 1973) this was not found to be a sepsis causing pathogen in BARNARDS. Similarly, Downie

et al. (2013) only found GBS to cause only 2% of neonatal sepsis cases from a meta-analysis of LMICs. This demonstrates that guidelines used for high income countries should not automatically be applied to LMICs, due to the disparity of sepsis causing pathogens and resistance profiles. Despite differences between HICs and LMICs, therapy deemed successful in HICs is applied to LMICs, due to the lack of data available for LMICS (Fuchs *et al*., 2018; Huynh *et al*., 2015). Furthermore, many studies assessing antibiotic therapy focus on neonates with clinically diagnosed sepsis, with no culture data. Therefore, outcomes in these studies may be unrelated to a sepsis episode and the vague clinical signs diagnosed as sepsis may have been due to other issues. Ampicillin is effective against pathogens common in HICs, such as group B streptococcus and Listeria, although these species were not reported in this study.

A high level of resistance was also found in Gram-negative isolates against gentamicin with 70.2% resistance. From MIC data, AMP-GEN was found to cover only 28.5% of Gram-negative cases. Amikacin showed greater activity (74.0% susceptibility) and when paired with ceftazidime (on WHO Watch list [WHO, 2019]) provided 77.1% coverage against Gram-negative isolates, suggesting that this combination could potentially replace AMP-GEN in LMICs. A concern with the use of amikacin as an empirical therapy is the potential nephrotoxicity and ototoxicity in neonates, particularly in premature neonates <34 weeks gestational age (Adelman *et al.*, 1987; Langhendries, *et al*., 1993; Engler, 2014). However, some studies have found no adverse side effects with amikacin treatment (Flidel-Rimon *et al.*, 2006). Neonatal dosages would need to be monitored carefully, alongside serum concentrations to ensure accurate dosing (Cristea *et al.*, 2017) particularly for premature neonates that should also be monitored more closely for signs of nephrotoxicity.

All BARNARDS sites except in Ethiopia had access to amikacin. Although more expensive (ranging $0·40-3·00/day compared to $0·15-1·50/day for gentamicin), this was a

small increase compared to alternative antibiotics. In all BARNARDS sites, ceftazidime was more expensive ($1·80-3·50/day) than ampicillin ($0·15-0·60/day) which may affect the choice of empirical use. While reported mortality was low among neonates treated with AMP-GEN, it was found to be significantly lower for CTZ-AMK (n=476), although this was negated when country-specific analysis was applied. The mixed effect model was not able to be assessed due to high levels of skew per country, meaning that effects of site/country differences could not be distinguished from antibiotic effect. Antibiotic combinations were dominated by one country, except for AMP-GEN, which was used by sites in multiple countries. CTZ-AMK was mainly used by sites in Bangladesh, which had higher levels of outborn neonates and *S. marcescens* was the most common species, opposed to *K. pneumoniae* for AMP-GEN, AMC-AMK and PIP/TAZ-AMK. Therefore, it may be due differences in pathogens and associated resistance. Numbers were not high enough to assess species separately but would be useful exercise for future work with higher sample numbers. Furthermore, Bangladesh had the lowest rate of premature births which likely would have impacted outcome. However, mortality did not seem to be associated with premature birth in this country. Different countries and clinical sites have different facilities, training and demands on staff, in addition to prevalence of resistance, bacterial strains and patient characteristics. Some sites may be exposed to counterfeit antibiotics (Delepierre *et al*., 2012), which could also alter outcome due to suboptimal dosing. This is interesting preliminary work but outcome is due to many variables within site, let alone between hospital sites, which must be considered when assessing this data and further work is needed for assessing appropriate alternative treatment recommendations.

5.3.3 Effect of resistance

Results that compared resistance to antibiotic treatment with survival were not as expected, with no significant association found between survival and pathogen resistance to

the treatment given. However, as this was based on only a subset of 290 neonates, and split per antibiotic therapy and per resistance profile, this may be due to the resulting low sample size in addition to loss to follow-up.

Despite high levels of resistance (14/20 resistant to their respective treatment antibiotics), *A. baumannii* was not associated with any reported mortality. This may be due to follow-up issues. Of the 14 resistant isolates, 10 were followed up <4 days four followed up 5-21 days following outcome. Another explanation could be that this were contaminants to the blood culture, transferred from healthcare worker gloves (Morgan *et al*., 2010; Qi *et al*., 2016).

5.3.4 PK/PD

PK/PD testing carried out to gain further insight into the PTA of AMP-GEN and the other three antibiotic combinations combined MICs with dosing schedules. PTA levels ≥80% for were low for AMP-GEN (33·7% [SD±0·59]) due to high levels of resistance, compared to other treatment combinations CTZ-AMK (92·7% [SD±0·24]), PIP/TAZ-AMK (85·3% [SD±0·47]) and AMC-AMK (68·0% [SD±3·84]). Simulated PTA variation was small for all combinations, except for AMC-AMK, where significant inter-individual variation was observed. This study did not determine patient-specific antibiotic concentration. However, variation in dosing schedules across sites/countries is an important factor to consider when comparing differences in treatment outcomes. The high reported survival rate relative to AMP-GEN PTA values could arise from under-reporting of mortality, as a high rate for neonates not reported deceased treated with AMP-GEN were followed up <10 days (59·14% AMP-GEN opposed to 23·72% of those treated with CTZ-AMK). Follow-ups are particularly difficult in LMIC settings, where mothers may live far away from clinical sites, or not have a contact number.

These results also showed that reported mortality was lower than expected for AMP-GEN. The literature used for AMP-GEN PK/PD modelling was based on two separate models: one for gentamicin (Fuchs *et al*., 2014) and the other for ampicillin (Tremoulet *et al*., 2014). There were no models for AMP-GEN in combination, which is surprising as it is the recommended empirical therapy for neonatal sepsis. In addition to this, many PK/PD studies concentrate on adults, with gaps in literature to cover neonates (Hartman *et al*., 2020). Synergy tests were also undertaken to ascertain why reported mortality in AMP-GEN was lower than expected with high MICs and low PTA. While potential additivity was displayed for three isolates, no synergy was found between the two antibiotics *in vitro*. The low reported mortality rate for neonates treated with AMP-GEN may be due to a lack of sufficient follow-up data and further data needs to be collected.

Patients in many LMICs have reduced access to prenatal care and may have reduced accuracy of gestational age (Alliance for Maternal and Newborn Health Improvement, 2021), which would also impact the PK/PD modelling carried out. LMICs also have limited access to creatine clearance/ albumin levels in neonates (George *et al*., 2017), making assessment of PK/PD modelling in neonates from LMICs challenging. Lack of accurate gestational age may be an issue particularly with amikacin as this is a strong determiner, alongside bodyweight for renal clearance (Allegaert *et al*., 2007)

There is limited PK/PD data on most antibiotics in neonates, particularly from LMICs. A review carried out by Shafiq *et al*. (2017) presents the PK/PD studies carried out on neonates, demonstrating wide variation regarding optimal dosing, many of which were based on gestational age which may not be accurate in LMIC settings. They showed two studies investigating piperacillin-tazobactam, one of which suggested 44.44/5.56 mg/kg every eight hours (Li *et al*., 2013) and another study suggested three varied dosing regimens of 100mg/kg every eight hour for postmenstrual age <30 weeks, 80mg/kg every six hours at 30-

35 postmenstrual age or 80mg/kg every four hours at 35-49 weeks (Cohen-Wolkowiez et al., 2012). Even gentamicin, a recommended first-line antibiotic for neonates has limited data and multiple dosing strategies (Rastogi *et al*., 2002; Fuchs *et al*., 2016). It may be that results from the simulations would differ for AMP-GEN if a different dosing schedule was used. As individual dosing strategies were not available, this was based on a hospital policy so any discrepancies from this dosing schedule would not have been accounted for. From the variance in the literature in addition to variance found at different clinical sites for some antibiotics, it is important that further research is carried out and optimal dosing recommendations are standardised.

5.3.5 Pathogenicity and outcome

Associations were not found between pathogenicity or VFs and outcome, except for low VF scores in *E. coli* associated with higher reported mortality in neonates (U=12·5, p=0·042). It is possible that while an *E. coli* may have numerous VF genes, these may not be expressed or regulated. Discrepancies between outcome and microbiological data may be partly due to numbers of neonates lost to follow-up. AMR can affect bacterial virulence, but is mechanism dependent, which could possibly explain the lack of correlations.

5.3.6 Potential alternative therapies

Due to the high level of resistance against AMP-GEN and poor PTA, alternative antibiotics were assessed for FoR to provide an indication of the speed at which bacteria may develop resistance. This was an important initial assessment that supports potential alternative antibiotics; however, this should be carried out on a wider range of isolates and stability of resistance assessed before suggesting an alternative empirical therapy. While FoR against ceftazidime was higher than ampicillin, ampicillin was only tested against six isolates, as there were only this number susceptible to ampicillin. However, amikacin showed a much lower FoR compared to gentamicin in both Gram-negative and Gram-positive bacteria,

suggesting this could be a suitable alternative recommendation. This is supported by Pollock *et al.* (1985), whose study showed no negative side effects for treatment of neonates with ceftazidime and demonstrated no emergence of ceftazidime resistant bacteria in the baby unit within which they tested ceftazidime treatment over 12 months. Regarding cost, CTZ-AMK was a relatively cheaper alternative compared to other alternative options to AMP-GEN and was available in all countries except for Ethiopia. Amikacin was found to have less resistance against all species all except for *P. aeruginosa*, and so gentamicin may be a better alternative where this is known to be the most common cause of sepsis or during a *P. aeruginosa* outbreak. CTZ-AMK however, would not be very effective against *S. aureus*, one of the most common sepsis causing pathogens in this study. Of the third generation cephalosporins, ceftazidime possesses higher efficacy against *P. aeruginosa* but combined with reduced efficacy against *S. aureus* than earlier cephalosporins and not against MRSA (Richards and Brogden, 1985).

Amoxicillin/clavulanate was not available in 5/7 countries and piperacillin-tazobactam was expensive (ranging from $2·60-24·60/day) (table 1). A high level of mortality associated with PIP/TAZ-AMK (27·8%), despite high coverage (79.9%. for Gram-negative isolates). This may be due to confounding factors, as this treatment is often used for nosocomial infections, where neonates were already hospitalised; however, severity of neonatal sepsis was not recorded. This may again represent country effect, as 108/115 PIP/TAZ-AMK prescriptions were from sites in Pakistan. Outcomes may be poorly affected where patients bear the cost of antibiotic therapies and may not have been able to afford full antibiotic courses.

Although no resistance was developed against fosfomycin when assessing Gram-positive bacteria, FoR rates seen in Gram-negative bacteria suggest that resistance could arise quickly against Fosfomycin. However, growth per ml was similar to other antibiotics,

potentially portraying unstable mutations, perhaps due to fitness costs, although Campos *et al*. (2020) found resistance to have no/minor effects on bacterial fitness in *E. coli* strains; further work needs to be carried out investigating stability of Fosfomycin resistance across bacterial species. Fosfomycin is associated with high sodium load, associated with heart failure. This will have greatest impact on neonates in the first hours of life when a lower level of sodium is required (Brien and Walker, 2013) and pre-term infants before 32-36 weeks when glomerular are still developing (Hinchcliffe *et al*., 1991).

Colistin has previously been found to be associated with high levels of nephrotoxicity and neurotoxicity (Koch-weser *et al*., 1970). However, this has recently been debated in papers stating that the antibiotic is safer than previously thought (Cheng *et al*., 2010; Durakovic, *et al*., 2011; Iosifidis, *et al*., 2010), although Alan *et al.* (2014) found acute kidney injury in 19% of preterm infants and that electrolyte supplementation was often required during colistin therapy. Colistin would not be a suitable empirical treatment for neonatal sepsis in Bangladesh where *S. marcescens* was found to be a common pathogen, with intrinsic resistance against colistin (EUCAST, 2021) diminishing potential use of this antibiotic. Further work is needed to determine whether this could be an effective treatment for neonatal sepsis, however, it's high cost and low availability (only available in four of seven countries in this study) will still preclude it from acting as a reasonable alternative empirical treatment. Furthermore, colistin is a last-resort antibiotic in the WHO reserve category (WHO, 2019) reserved for MDR infections.

FoR results, combined with low resistance rates demonstrated that meropenem could be a suitable alternative with potential to be used as a monotherapy due to no development of resistance in FoR analyses. However, this is a 'last-resort' carbapenem within the WHO watch category and much more expensive than alternatives, the use of this as an empirical therapy for neonatal sepsis is questionable.

5.3.7 Site variation

Large discrepancies between sites could be due difference to a wide range of factors related to the antibiotics prescribed, common pathogens, local resistance patterns, hospital and residential circumstances in each area. The sites included in BARNARDS consisted of a range of hospital types between tertiary and secondary care with vast differences between sites in terms of experience of doctors and nurses, the number of nurses/ doctors onwards, patient demand, laboratory facilities along with antibiotic availability and facilities in the NICU or maternity wards.

5.3.8 Conclusion

Various antibiotics were used by sites as first-line treatment. Many sites veered from ampicillin and gentamicin, which composed of only 22.5% of prescriptions due to known prevalence of resistance, as supported by other studies in LMICs. This combination was found to only provide coverage for 28.5% of Gram-negative infections assessed in this study. Ceftazidime and amikacin were commonly prescribed to neonates as a first line treatment and found to provide a coverage of 77.1% GNB based on resistance profiles. This was associated with improved outcome data in neonates, and a reduced FoR was seen for amikacin compared to gentamicin. We found variation in access and availability of different antibiotics across BARNARDS sites, with some antibiotics not available or too expensive to be considered as a standard empirical treatment option. The empirical therapy AMP-GEN recommended by the WHO for treatment of neonatal sepsis is based on data from HICs which have different aetiology and associated resistance. This treatment recommendation needs to be urgently reassessed for LMICs due to the high rates of resistance.

6.0 Results: Admissions cohort and risk factors

6.1 Introduction

UNICEF/WHO (2021) estimated that in 2014-2020, 33% of births globally did not take place in a healthcare facility. However, this number fluctuates per location, with higher rates in West and Central Africa (45%), Eastern and Southern Africa (34%) and South Asia (16%). A study by Montagu *et al*. (2011) found that a mother's socio-economic status played a role in the place of birth of their child, with 77.7% of 'poorest' mothers giving birth at home in Sub-Saharan Africa compared to 22.4% of those considered the richest, with similar findings in South and Southeast Asia. This study found similar trends for Latin America and Caribbean, North Africa and the Middle East, although with a lower proportion of mothers giving birth at home.

In contract, low numbers (<1%) of births outside a healthcare facility were reported throughout most of Europe (Euro-Pristat project, 2013) and the USA (Gregory *et al*., 2021). Higher rates are seen in the Netherlands, however here and in other high-income countries, homebirths are usually attended by a certified midwife (Evers *et al*., 2010; UNICEF/WHO, 2021). On the other hand, 40% of births in West and Central Africa, 31% in Eastern and Southern Africa and 23% in South Asia were not reported to have a skilled birth attendant present. Montagu *et al.* (2011) reported higher estimations of unattended births with up to 54% of births and up to 41% with an alternative traditional birth attendant present. Garces *et al*. (2012) report that 80% of birth assistants had less than one month formal training and most had no basic equipment. Births not assisted by a skilled attendant have been reportedly related to increased risk of neonatal and perinatal mortality (Darmstadt *et al.,* 2009).

In the BARNARDS network, the facility-based birth cohort contributed to over three-quarters of the total dataset (Milton *et al*., 2022). Within the facility-born cohort the factors

found to be associated with clinically suspected sepsis, laboratory confirmed sepsis and all-cause mortality included maternal hypertension, previous maternal hospitalisation within 12 months, average or higher monthly household income, ward size (>11 beds), living in a rural environment, preterm birth, perinatal asphyxia, and multiple births (Milton *et al*., 2022). Results described herein focus on associations between birth characteristics and socio-demographics of the mother and neonatal mortality and culture confirmed sepsis (CCS) among the admissions cohort: those presenting with clinically diagnosed sepsis (CDS) at BARNARDS clinical sites that likely developed sepsis outside of the hospital setting. Further analyses of the microbiology of these neonates are also undertaken, including risk factors for development of GNB compared to GPB and infection with MDR bacteria. Lastly, potential risk factors for development of a likely nosocomial infection are also assessed.

This chapter aimed to provide further insight on the admissions cohort of neonates for BARNARDS, as there is limited data on these neonates. This aimed to: i) assess risk factors for mortality following CCS and CDS. Ii) to compare specific pathogens with outcomes and assess whether infection with GPB, GNB altered outcome; ii) Assess whether infection with MDR bacteria was associated with poorer outcomes.

It was hypothesised that: i) Risk factors for mortality following CDS and CCS will be similar and that this will involve some birth related and sociodemographic related factors; ii) GNB will be associated with worse outcomes compared to GPB, due to higher increased pathogenicity and prevalence of antibiotics resistance; iii) MDR bacteria will be associated with worse outcomes.

6.2 Results

6.2.1 Enrolment

During BARNARDS 35,107 mothers/guardians were enrolled alongside 36,285 neonates. Of these neonates, 30,557 were included in the BARNARDS facility-based birth cohort detailed in Milton *et al*. (2022). Of the 5,728 neonates not confirmed as the facility-based inborn cohort, 2,096 could not be included within the admissions cohort due to exclusion criteria or missing data (detailed in Figure 6.1), therefore a total of 3,632 neonates with CDS were included in the admission cohort analysed in this herein.

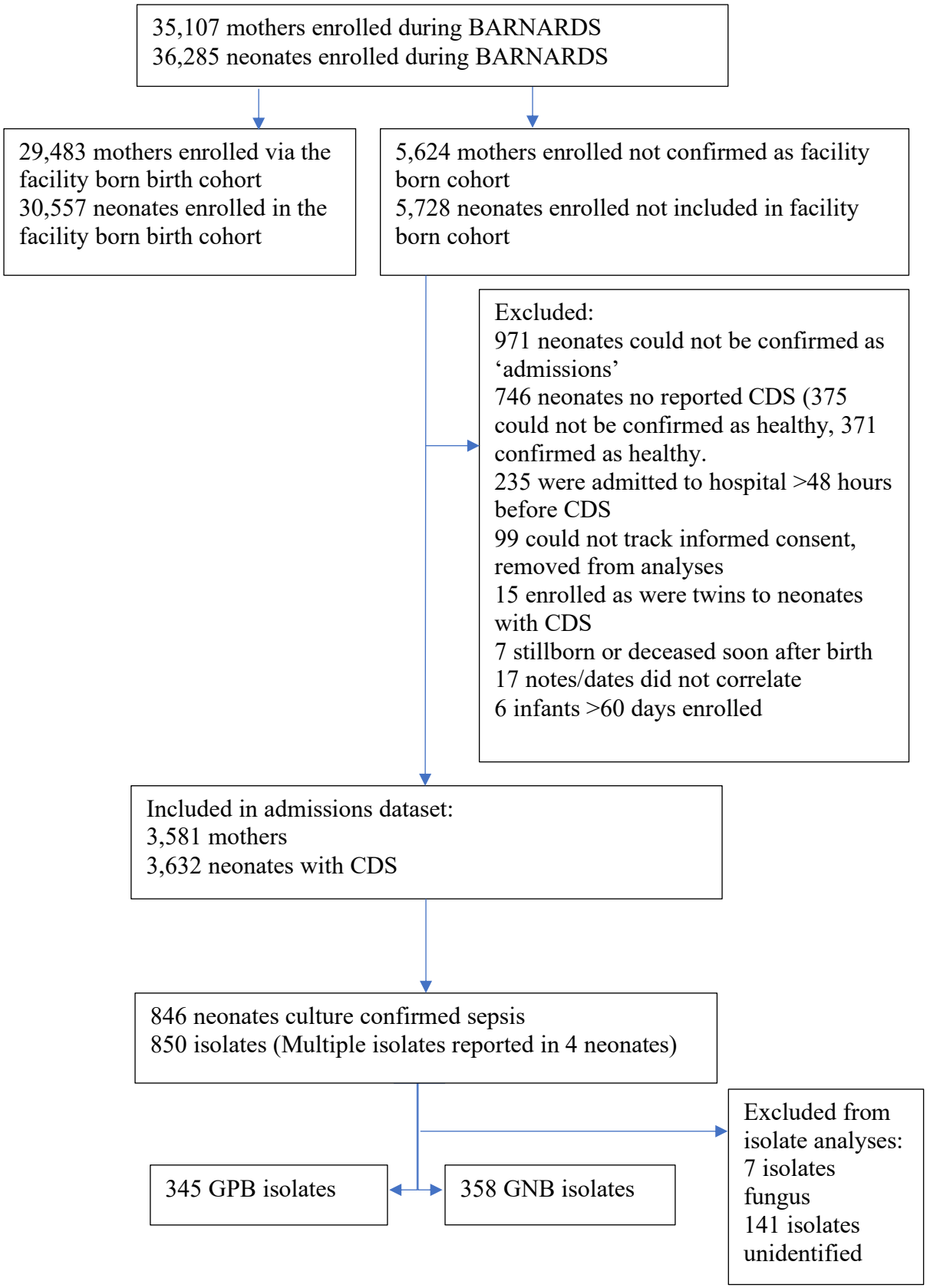

Figure 6.46. Flow diagram. Flow diagram detailing inclusion and exclusion criteria for the admissions cohort analysed in this paper. For further information regarding the inborn cohort, please refer to Milton et al., 2022. CDS=clinically diagnosed sepsis; GPB=Gram-positive bacteria; GNB=Gram-negative bacteria.

6.2.2 Incidence rates of CCS and mortality in admissions cohort

Within the admissions cohort, numbers of enrolments varied widely per site, ranging from nine enrolments in India (IN) to 1,096 enrolments in BC. Of the total number enrolled with CDS, 846 (23.3%) neonates had CCS. Rates of CCS from those with CDS ranged from 5.1% in BK to 51.3% in ESS (Table 1). One bacterial isolate was cultured from nearly all CCS cases, except for five cases where two bacterial pathogens were purified from a single blood culture (one case in ESS; three cases in NN).

Overall, mortality was reported for 294/3,632 neonates, accounting for 8.1% of those with CDS with rates again varying between sites from 3.4% (n=37/1,096) mortality reported in BC to 18.9% (n=50/264) mortality reported in NN. Reported neonatal mortality was lower for CDS compared to CCS, with a mortality rate of 5.2% (n=187/2,786) in neonates with CDS and not CCS. Of those with CCS, overall reported mortality was 12.7% (n=107/846), although there was no reported mortality for neonates with CCS from BK or PC (Table 1). Incidence of all variables are listed in Table 6.1.

Table 6.15. Numbers of neonates enrolled per site included in the admissions cohort and incidence of clinically diagnosed sepsis (CDS), culture confirmed sepsis (CCS) and mortality per site.

Country	**Site**	**Neonatal admissions clinical sepsis**	**CCS (% of those with CDS)**	**Mortality**		
				All admissions CDS	**CDS no CCS (%)**	**CCS (%)**
Bangladesh	BC	1,096	147 (13.41%)	37 (3.38%)	27 (2.46%)	10 (6.80%)
	BK	293	15 (5.12%)	14 (4.78%)	14 (4.78%)	0 (0.00%)
Ethiopia	ESS	316	162 (51.27%)	47 (14.87%)	20 (6.33%)	27 (16.67%)
India	IN	9	3 (33.33%)	1 (11.11%)	0 (0.00%)	1 (33.33%)
Nigeria	NK	1,048	207 (19.75%)	94 (8.97%)	68 (6.49%)	26 (12.56%)
	NN	264	92 (34.85%)	50 (18.94%)	27 (10.23%)	23 (25.00%)
	NW	72	26 (36.11%)	11 (15.28%)	9 (12.50%)	2 (7.69%)
Pakistan	PC	12	3 (25.00%)	1 (8.33%)	1 (8.33%)	0 (0.00%)
	PP	317	109 (34.38%)	31 (9.78%)	16 (5.05%)	15 (13.76%)
Rwanda	RK	164	65 (39.63%)	2 (1.21%)	1 (0.61%)	1 (1.54%)
	RU	14	7 (50.00%)	3 (21.43%)	2 (14.29%)	1 (14.29%)
South Africa	ZAT	27	10 (37.04%)	3 (11.11%)	2 (7.41%)	1 (10.00%)
Total		**3,632**	**846 (23.29%)**	**294 (8.09%)**	**187 (5.15%)**	**107 (12.65%)**

*Neonates admitted to hospital >48 hours before CDS were not included in this cohort to reduce potential nosocomial infections. This excluded 235 neonates from analyses.

6.2.3 Admission cohort characteristics

Most of the mothers enrolled (n=2,269/3,581, 63.4%) had been pregnant previously. One or two previous pregnancies was commonly reported (n=811/3,581 (22.6%); n=505/3,581, 14.1%, respectively), with up to 11 previous pregnancies reported (n=42, 1.2%). During this study, 85/3,581 (2.4%) of pregnancies were multiple, 51 of which had both twins enrolled with suspected sepsis.

Overall, 1,510/3,581 (42.2%) of mothers reported visiting private healthcare within three months, and 228/3,581 (6.4%) of mothers had been hospitalised in the previous 12

months. The most commonly reported health condition in mothers was malaria (n=575/3,581, 16.1%), followed by hypertension (n=240/3,581, 6.7%) and infection (n=152/3,581, 4.2%), all associated with increased numbers of hospitalisation ($p<0.0005$); $p<0.0005$; $p=0.001$, respectively) and attendance to private healthcare was associated with hypertension ($p=0.010$) and infection ($p<0.0005$). Antibiotic usage within three months prior was reported for 552/3,581 mothers (15.4%), with usage associated with hospitalisation within the previous 12 months ($p<0.0005$). β-lactams were reported most often when mothers reported use of antibiotics (n=380/552, 68.8%), followed by metronidazole (n=361/552, 65.4%), with 275/552 of these mothers reporting taking both.

Average household income was often reported by participants (n=1,262/3,581, 35.2%), with a similar number of participants reporting over two times below average household income (n=1,022/3,581, 28.5%). Education to secondary school level was common (n=2,044/3,581, 57.1%), with 842/3,581 (23.5%) reporting limited education, 382/3,581 (10.7%) reporting no education and 307/3,581 (8.6%) reported education to university level.

Participants often described their residence area as urban (n=1,891/3,581, 52.8%) or rural (n=1,285/3,581, 35.9%), with 400/3,581 (11.2%) describing residence in semi-rural areas. Most mothers/guardians did not have running water in their house (n=2,620/3,581, 73.2%), and pit latrine was a common toilet type (n=1,868/3,581, 52.2%), associated with rural residence (n=1058 pit latrine of 1,302 living in a rural area, 81.3%). Nearly all mothers reported to have access to soap (n=3,472/3,581, 97.0%).

Home births accounted for 1,020/3,632 (28.1%) neonates, while those born in different hospitals and referred to a BARNARDS clinical site or those that were returning to a BARNARDS clinical site accounted for 1,939/3,632 neonates (53.4%), and 618/3,632 neonates (17.0%) were born in healthcare clinics. Premature rupture of membrane was reported in of mothers in 171/3,632 births (4.7%), although due to most births occurring outside of the

reporting hospital, this may be underreported. A quarter of births were by Caesarean section (n=832/3,632, 22.9%), of which 591/832 (71.0%) were classified as emergency C-sections. Only 99/3,32 (2.7%) of neonates were recorded as breech during birth and 607/3,632 (16.7%) had perinatal asphyxia. The majority of enrolled neonates (n=2,861/3,632, 78.8%) were full term deliveries (37-42 weeks), with 489/3,632 (13.5%) premature and 242/3,632 (6.7%) post-mature. Gender was recorded for 3,094 (85.2%) of neonates and were more often males (n=1,935/3,094, 62.54%) compared to females (n=1,159/3,094, 37.46%) (Appendix, page 39-44).

6.2.4 Risk factors for CDS

A total of 746 neonates without CDS were mistakenly enrolled in the admissions cohort of the BARNARDS study, mainly due to arrival at hospital sites alongside the mother when she was admitted, commonly for postpartum haemorrhage according to notes. From these, 371 neonates had notes which confirmed they were healthy. Due to the nature of enrolling admissions neonates, this occurred for no or low numbers of neonates at most sites apart from NK where 347 admission neonates confirmed as healthy were enrolled (Table 6.2), therefore comparisons of admissions cohort with CDS compared to healthy admissions enrolments were only carried out for those enrolled at NK.

Table 6.16 Enrolments of neonates categorised in the admissions cohort that were confirmed as healthy.

Country	**Site**	**Admission 'control' neonates**
Bangladesh	BC	0
	BK	0
Ethiopia	ESS	0
India	IN	0
Nigeria	NK	347
	NN	2
	NW	7
Pakistan	PC	0
	PP	0
Rwanda	RK	7
	RU	2
South Africa	ZAT	6
Total		**371**

The logistic regression model comparing CDS from NK with neonates confirmed healthy from NK, 15 variables were found to be significantly associated with the development of CDS (Table 6.2). Variables that appear to have the biggest impact on increasing the risk of CDS included place of birth in a hospital compared to home (OR=4.02, 95% CI=2.60-5.34, p<0.0005). Previous private healthcare visits were also linked to higher rates of CDS (OR=1.63, 95% CI=1.15-2.71, p=0.010).

Drinking water source appeared to impact development of sepsis, although the confidence interval ranges for these results are wide, due to a small widely dispersed sample size (due partly to multiple options dispersing data), accordingly this result is unreliable. House served by wastewater network appeared to be associated with increased rates of CDS and stagnant sewage within 100m of the house was associated with reduced risk of CDS according to the statistics. However, these variables were also associated with other variables that were associated with reduced numbers of CDS, including living in a residential area.

More bedrooms than one was associated with a higher risk of sepsis, detailed in table 6.3. Chi-square analyses showed that the number of bedrooms was related to increased people residing ($X^2(8, n=3,632)=1,563.820, p<0.0005$). This variable was also related to multiple other domestic variables and so is likely a confounding factor or possibly due to lower numbers for each category as the variable was split into five categories.

Variables that were associated with the lowest risk of sepsis included houses with access to running water, allowing for increased sanitation. However, again this variable was split into five categories and related to multiple other domestic variables and so again it is likely a confounding variable of lifestyle and living area and possibly due to low numbers per category. Neonates born at term had a lower risk of CDS,

Table 6.17. Significant risk factors for development of clinically diagnosed sepsis from neonates in NK. Only results significant from logistic regression included.

Variable	**Odds ratio**	**95% confidence interval**	**P-value**
Mother visiting private healthcare in past three months	1.763	1.148 – 2.708	0.010
Mother taking β-lactam antibiotics past three months	0.561	0.380 – 0.830	0.004
Semi-rural residence compared to rural	0.245	0.135 – 0.445	<0.0005
Number of bedrooms compared to 1:			
6 bedrooms	4.268	2.207 – 8.251	<0.0005
4+ bedrooms	2.017	1.097 – 3.710	0.024
Drinking water compared to municipal network:			
Communal taps	9.152	1.134 – 73.838	0.038
Sachet or bottled	32.530	2.981 – 354.962	0.004
Ground water	12.689	1.114 – 144.520	0.041
House has access to running water one day a week compared to none	0.096	0.011 – 0.862	0.036
House has electricity supply at least some of the time compared to none	0.264	0.151 – 0.462	<0.0005
Solid waste within 100 metres of the home	2.552	1.712 – 3.805	<0.0005
Stagnant sewage within 100 metres of the home	0.549	0.370 – 0.815	0.003
House served by a wastewater network	1.687	1.117 – 2.545	0.013
Multiple birth	0.257	0.137 – 0.482	<0.0005
>24 hours water breaking to birth	0.206	0.137 – 0.482	<0.0005
Female compared to male neonate	0.657	0.469 – 0.919	0.014
Place of birth hospital compared to home	4.022	2.595 – 5.234	<0.0005
Born at term compared to premature	0.165	0.049 – 0.553	0.004

6.2.5 Risk factors for neonatal mortality following CDS

6.2.5.1 Overall

Statistically significant associations noted from the final logistic regression model are detailed in Table 2. Houses served by a wastewater network (compared to those not served by a wastewater network) were associated with increased odds of mortality for neonates diagnosed with CDS (OR=1.70, 95% CI=1.25-2.30, p=0.001,). However, upon further investigation when analysed per site (p>0.05) suggests site differences may account for the overall significance (Table 6.4).

Premature birth of the neonate and premature rupture of membrane (PROM) was associated with increased neonatal mortality. Birth between 18:00-08:00 was found to be associated with increased neonatal mortality when compared to 08:00-18:00. This was significant for neonates born in hospitals (p=0.001) and home births (p=0.029). Less neonates born in healthcare centres had time of birth reported. Assisted delivery was associated with reduced chances of mortality from CDS, and assisted delivery was associated with birth in a hospital or healthcare clinic (p<0.0005) (Table 6.4). CCS was associated with increased mortality ($\chi^2(2, n=3107)= 30.611$, p<0.0005).

Table 6.18. Overall risk factors associated with significant differences in the odds of reported mortality

Variable	Odds ratio	95% confidence interval	P-value
Mother taken metronidazole within three months*	0.550	0.343 – 0.881	0.013
Number of people residing in house 7+ compared to 1-3*	1.551	1.099 – 2.190	0.013
House connected to wastewater network*	1.699	1,253 – 2.304	0.001
Birth time 18:00-08:00 compared to 08:00-18:00	1.542	1.171 – 2.032	0.002
Full term delivery (37-42 weeks) compared to pre-term delivery	0.471	0.322 – 0.688	<0.0005
Premature rupture of membrane	1.909	1.181 – 3.086	0.008
Assisted delivery	0.718	0.531 – 0.970	0.031

*Not significant when investigated at site level.

6.2.5.2 Per site

Risk factors for mortality were assessed for sites BC, ESS, NK, NN and PP. Univariate analyses demonstrate various significant results for BC, ESS, NK, PP, and no significant results found for NN. However, when significant results were combined for respective multivariate models, no significant results were seen, due to low sample numbers accounting for any missing variable data (BC n=680; ESS n=56; NN n=263). Chi-square analyses found that CCS was related to poorer outcome in BC ($\chi^2(1, n=680) = 3.891$, $p=0.049$), NK ($\chi^2(1, n=902) = 5.328$, $p=0.021$) and PP ($\chi^2(1, n=166) = 13.141$, $p<0.0005$). A higher number of bedrooms in the household had a significant association with mortality for BC ($\chi^2(3, n=680) = 9.467$, $p=0.024$) and NK ($\chi^2(3, n=902) = 16.623$, $p=0.001$). Furthermore, a residence with a greater number of bedrooms was associated with higher rates of CCS in BC ($p<0.05$). Gestational term of the neonate when born was significant for ESS and NK with higher rates of mortality associated with pre-term neonates compared to those born at term ($\chi^2(2, n=260) = 8.607$, $p=0.014$ and ($\chi^2(2, n=900) = 15.802$, $p<0.0005$, respectively).

6.2.6 Risk factors for CCS

6.2.6.1 Overall

Of all admissions neonates enrolled with CDS, 846 neonates had CCS. A total of six variables were found to be significantly associated with CCS from the final logistic regression with data from all sites combined (Table 6.5). No birth related factors were found to be associated with risk of CCS when analysing statistics for all sites combined.

Table 6.19 Variables with significant p-values from final logistic regression model for risk factors associated with increased rates of culture confirmed sepsis.

Variable	Odds ratio	95% confidence interval	P-value
Mother taken metronidazole within previous three months*	0.550	0.343 – 0.881	0.013
Maternal hypertension*	1.532	1.127 – 2.081	0.006
2 household bedrooms compared to none	0.435	0.272 – 0.696	0.001
3 household bedrooms compared to none	0.526	0.323 – 0.857	0.010
4+ household bedrooms compared to none	0.555	0.339 – 0.910	0.020
Primary drinking water from sachets or bottles than municipal network*	0.570	0.410 – 0.792	0.001
Sit down with flush toilet compared to no toilet at home	0.442	0.255-0.767	0.004
Squat with flush toilet compared to no toilet at home	0.444	0.258-0.764	0.003
Pit latrine compared to no toilet at home	0.380	0.232-0.623	<0.0005

*Not close to significant p-value when site variation accounted for.

Type of toilet was also associated with many other demographic factors. Mothers with no toilet in the home also reported washing their hands less often (72/78, 92.31%), compared to those with various toilets that reported 2,221/3,523 (63.04%) less frequent hand washing. As expected, those with flushing toilets were connected to a waste water network, and those with different toilets had lower numbers connected to a wastewater network and those with no toilets were not.

6.2.6.1 Per site

Neonatal numbers were not large enough to run multivariate analyses per site to assess risk factors for CCS and so univariate analysis was carried out for sites, where possible – BC, ESS, NK, NN, PP and RK. No variable was significant for all sites; however, significant results across two or more different sites were determined. Residential area was associated with CCS in BC ($\chi^2(2, n=380, p=0.017)$ with lowest rates reported in semi-rural settings and higher rates reported in those living in rural settings. An association between CCS and residential area was

also seen in NK (χ^2(2, n=902) =5.292, p=0.071), but with highest rates of CCS associated with those living in rural areas, with similar rates for urban/semi-rural. Stagnant sewerage within 100m of the home had an association with CCS in BC and ESS; however, only four mothers reported this factor in ESS, so these numbers are too low for statistical consideration.

6.2.7 Risk factors for mortality following CCS

Of the 846 neonates with culture confirmed sepsis, 107 (12.65%) were reported as deceased during the study. Due to the relatively low numbers, logistic regression models were not carried out and results are based on univariate chi-squared analyses. Variables with significant results showing potential associations with mortality following CCS are presented in Table 6.6 below.

Table 6.20 Variables with potential association for mortality following CCSculture confirmed sepsis (CCS) according to chi-square analyses

Variable	**Chi-square (χ^2) result**	**Potential association**
Mother visited traditional healer in past three months	(χ^2(1, n=728) =4.359, p=0.037)	Mothers that visited a traditional healer were associated with reduced neonatal mortality following CCSCCS
Income*[1]	(χ^2(5, n=723) =11.239, p=0.047)	Mothers with higher income associated with lower neonatal mortality following CCSCCS
House served by a wastewater network	(χ^2(1, n=728) =3.955, p=0.047)	Houses served by wastewater network increased reported mortality.
EOS/LOS	(χ^2(1, n=729) =3.925, p=0.048)	EOS was associated with higher rates of reported mortality than LOS following CCS
Term of baby at delivery	(χ^2(2, n=724) =11.662, p=0.003)	Neonates born pre-term associated with higher risk of mortality following CCS
PROM*[2]	(χ^2(1, n=729) =11.451, p=0.001)	Mothers reporting PROM associated with higher rates of neonatal mortality following CCS

*[1] Income had a significant p-value (p=0.047), but there was no distinct pattern and included zero values for some results so may need to be considered with caution.

Birth time out of hours was an association for CDS to mortality. Those born out of hours in the CCS cohort had a higher reported mortality (44/259, 16.99% compared to 34/298, 11.41%), although this did not produce a significant chi-square result ($\chi^2(1, n=557) = 3.581$, $p=0.058$). Similarly, as with CDS, assisted births had a slightly lower rate of reported mortality (12.32% in assisted births vs 15.60% none assisted births), however this was not significant for those with CCS ($\chi^2(1, n=667) = 1.427$, $p=0.232$).

6.2.8 Gram-negative and Gram-positive prevalence of sepsis pathogens

Within the admissions cohort, there were 846 cases of culture confirmed sepsis. Of these, Gram stain data was not available for 110 isolates. Furthermore, seven cases were identified as being fungus so were not subject to further microbiological analyses. A single isolate was grown in the majority of these blood cultures, with five blood cultures yielding two bacterial strains. Microbiology analyses were carried out per isolate, therefore any neonates that had two strains identified in a single blood culture were analysed separately to assess risk factors per pathogen. From isolates identified via the Gram stain at sites (n=733), 378 (51.6%) were identified as gram-negative and 355 (48.4%) identified as gram-positive (Figure 6.2). Only those with Gram stain data are taken forward for analyses in this section.

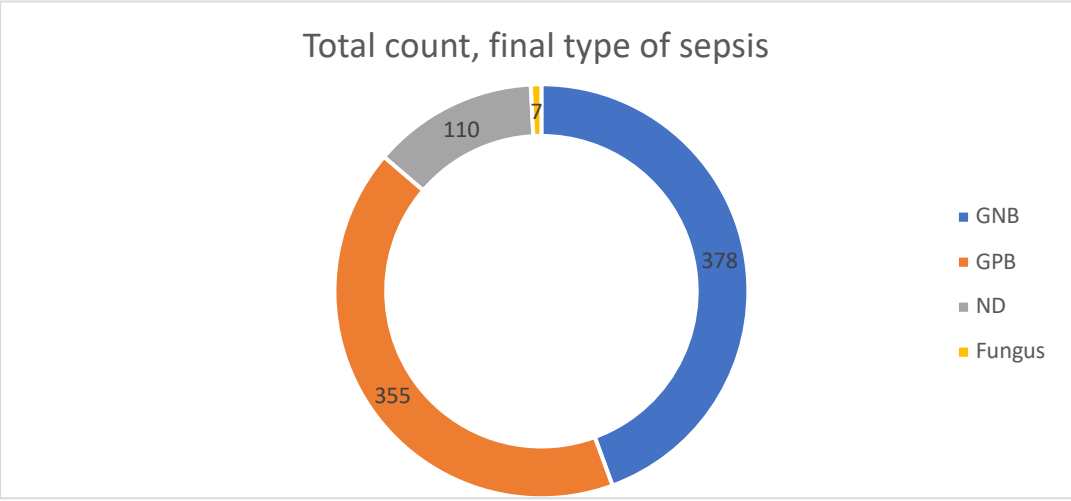

Fig 6.47. Gram stain. A. Number of isolates identified as Gram-negative (GNB) (n=378) and Gram-positive (GPB) (n=356), as well as fungus and those with no data (ND) at clinical sites using the Gram-stain method.

Of those neonates with culture confirmed sepsis, a higher rate of GPB was found in sites in Africa (n=287, 62.26%) compared to Asia (37.96%). Within Africa, higher rates of GPB vs GNB were seen in all clinical sites, except in South Africa, where there was an equal distribution of GNB and GPB. All sites in Asia had a higher rate of GNB, except PC (33.33%), however the numbers of culture confirmed sepsis in the admissions cohort at PC were low (Table 6.7). However, rates of GPB may have varied per site due potential differences in the consideration of contaminants.

Table 6.21. Gram-negative bacteria (GNB) and Gram-positive bacteria (GPB) numbers causing sepsis per site. Number of culture confirmed sepsis isolates with Gram-stains identification at clinical sites. Distribution of GPB and GNB provided per site and per continent.

Continent	**Country**	**Site**	**GNB (%)**	**GPB (%)**	**Total**
Asia	Bangladesh	BC	123 (84.83%)	22 (15.17%)	145
		BK	10 (66.67%)	5 (33.33%)	15
	India	IN	2 (100%)	0 (0.00%)	2
	Pakistan	PC	1 (33.33%)	2 (66.67%)	3
		PP	67 (62.62%)	40 (37.38%)	107
Total Asia			203 (74.63%)	69 (25.37%)	272
Africa	Ethiopia	ESS	52 (48.15%)	56 (51.85%)	108
	Nigeria	NK	60 (35.71%)	108 (64.29%)	168
		NN	36 (40.45%)	53 (59.55%)	89
		NW	4 (18.18%)	18 (81.82%)	22
	Rwanda	RK	15 (26.32%)	42 (73.68%)	57
		RU	3 (42.86%)	4 (57.14%)	7
	South Africa	ZAT	5 (50.00%)	5 (50.00%)	10
Total Africa			175 (37.96%)	286 (62.26%)	461

6.2.9 Risk factors GPB vs GNB

Multiple variables were found to have a significant association with neonates infected with GPB vs GNB from a logistic regression model accounting for questionnaire/ birth data. Residential area was found to have an effect, with higher odds of GPB compared to GNB from semi-rural residential areas (OR=3.88, p<0.001, 95% CI= 1.85-8.10) and higher rates in urban areas (OR=2.06, p=0.22, 95% CI=1.11-3.82) compared to those living in rural areas that had lower rates of GPB and higher rates of GNB. Rural areas were also linked to toilet type (p<0.0005), reduced wastewater network (p<0.0005) and reduced handwashing frequency (p=0.044). Rural areas were also associated with reduced running water, with 1,079/1,913 (56.40%) mothers in urban, 332/411 (80.78%) in semi-rural and 1,241/1,302 (95.31%) in rural areas reporting no household supply. As the number of people residing in the house increased, the chances of a GNB infection increased. Odds of GPB infections were reduced (OR=0.521) and GNB infections increased with 4-6 people living in the house compared to 1-3 people (p=0.006, 95% CI = 0.33-0.83).

Primary drinking water source was a significant variable, with higher chance of GPB vs GNB infections from water vendors / sachet water (OR=3.45, p=0.14, 95% CI=1.28-9.30/ OR=2.26, p=0.036, 95% CI=1.05-4.85) vs municipal network. Mothers reporting no education delivered neonates with a higher chance of GNB infections compared to GPB (p=0.023), although education was related to many other variables and demographics of the mothers which may not be accounted within this study. Female neonates were associated with 1.597 higher odds of GPB vs GNB bacteria (p=0.022, 95% CI=1.07-2.38) whereas males were found to have a higher risk of GNB sepsis compared to GPB sepsis. Males were associated with higher rates of illnesses reported in the mother as well as perinatal asphyxia, associated with 0.499 lower rate of GPB infections vs GNB infections (p=0.003, 95% CI=0.31-0.79). Regarding reported

mortality, Chi-square analysis revealed that neonates infected with a GNB were more likely to be reported deceased compared to those infected with a GPB ($\chi^2(1, n=472) = 11.076$, $p=0.001$).

6.2.10 Bacterial species

From neonates with positive blood cultures, 389 isolates had WGS data from 387 neonates (two isolates were recovered from two neonates), therefore the following analyses refer to 389 isolates. Of the isolates with WGS data, 39 different bacterial species were identified. The most common species found was *Serratia marcescens* (n=89), followed by *Klebsiella pneumoniae* (n=83), *Staphylococcus aureus* (n=33), *Escherichia coli* (n=32) and *Klebsiella michiganensis* (n=28) (Figure 6.3). *K. pneumoniae*, *S. aureus* and *E. coli* were common across all BARNARDS sites. *S. marcescens* was predominantly found in BC (82/89, 92.1%) and *K. michiganensis* was mainly found in PP 25/28, 89.3%). However, as with the overall dataset, WGS data for GPB isolates is disproportionately underrepresented as were only collected retrospectively.

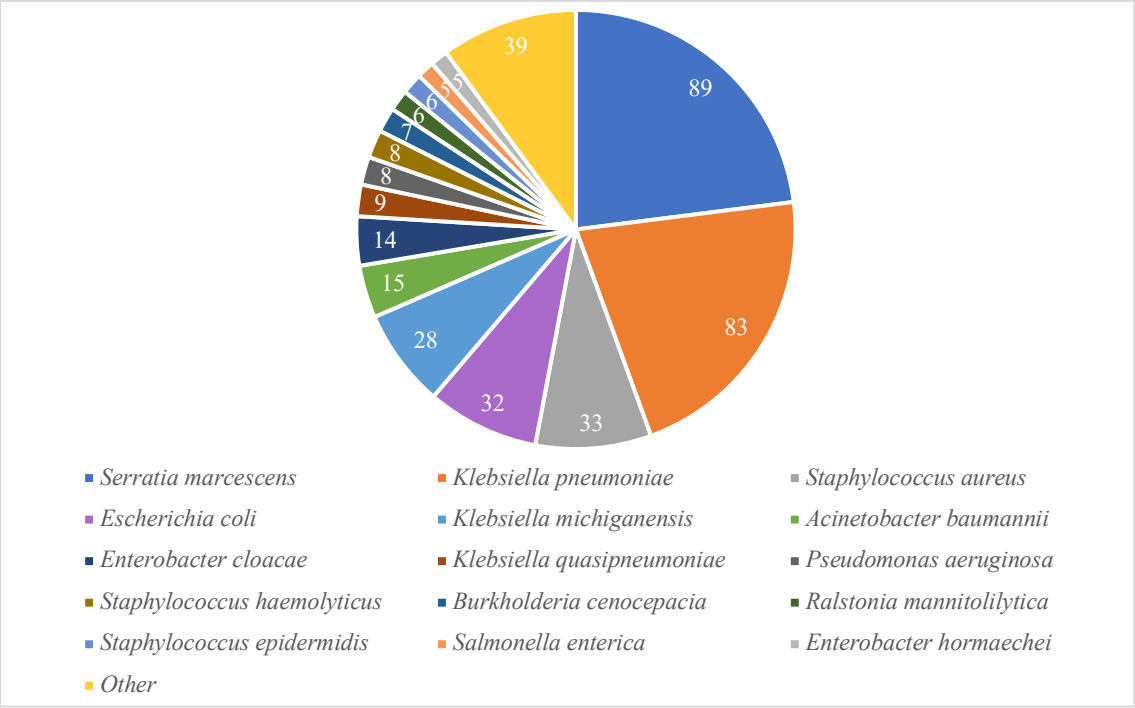

Figure 6.48 Common sepsis causing bacteria in admission neonates. Top 15 most common bacteria sequenced shown with identification confirmed by whole genome sequencing (n=387).

6.2.10.1 Species found in EOS vs LOS

Similar common species were found in neonates with EOS and LOS (Figure 6.4) from those with WGS data, although at varied ratios. *K. pneumoniae* was most common in EOS (n=35), followed by *K. michiganensis* (n=16), *S. aureus* (n=10), *S. marcescens* (n=8) and *E. coli*. S. marcescens. In cases of LOS, the most common species was *S. marcescens* (n=70), although this was skewed by the high proportion of LOS cases from BC where *S. marcescens* is common. This was followed by *K. pneumoniae* (n=41), *E. coli* (n=22), *S. aureus* and *Acinetobacter baumannii* (n=13). Common species were similar to those found in EOS, except for a higher rate of *S. marcescens* and *K. michiganensis*, common to PP, was replaced with *Acinetobacter baumannii* (Figure 6.4).

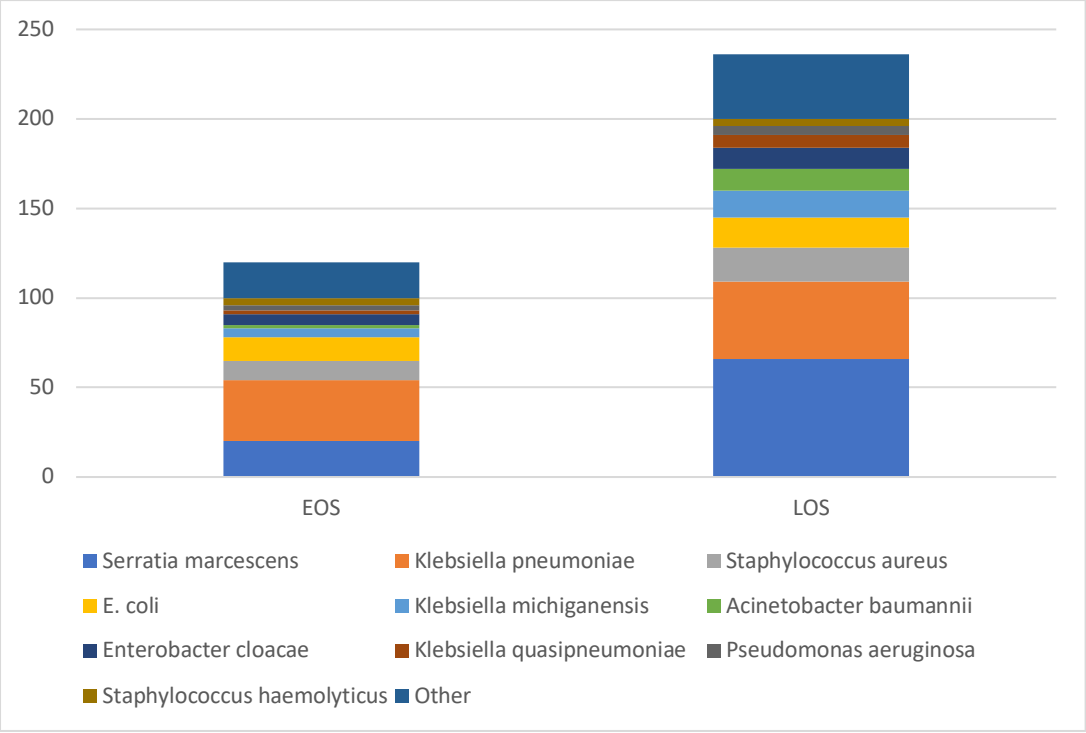

Figure 6.49. Top ten most common bacterial species identified by whole genome sequencing split by onset of sepsis (Early onset sepsis (EOS) ≤72 hours and late onset sepsis (LOS) >72 hours). Of those with WGS data, EOS was confirmed for 106 neonates and LOS confirmed for 231 neonates. Data for onset of sepsis was not available for 50 of these neonates.

6.2.10.2 Potential demographic effects on species prevalence

Descriptive results are reported here for any variables that showed a pattern with species identification. Neonates of mothers reporting other health conditions had higher rates of *K. michiganensis* (85% of cases), *K. pneumoniae* (71.08%) and *S. marcescens* (84.27%), compared to 63.65% of *S. aureus* and 56.25% of *E. coli* cases. Cases of *K. michiganensis* (60.71%) and *S. marcescens* (66.29%) were also higher when mothers reported attending private healthcare within the past three months compared to those that did not.

Other health conditions reported in the mother was related to increased hospitalisation reported from mothers ($\chi^2(1, n=383)=8.419$, $p=0.006$), with high rates of *K michiganensis*, *K. pneumoniae* and *S. marcescens*. Those infected with *K. pneumoniae, K. michiganensis, S. aureus, E. coli* were often from urban settings (57.8%, 62.1%, 54.6%, 57.6% respectively) Mothers reporting a lower education status tended to have higher rate of *K. pneumoniae* (44.44% of cases had no or limited education).

6.2.11 Risk factors for sepsis infection with MDR bacteria

MIC data was available for 311 GNB isolates within this cohort, for which it was possible to determine whether isolates were MDR. The five most common species included in this analysis were *S. marcescens* (n=87), *K. pneumoniae* (n=75), *E. coli* (n=28), *K. michiganensis* (n=27) and *E. cloacae* complex (n=19). A total of 224/311 (72.03%) of isolates were classified as MDR, including a range of bacterial species from different clinical sites. The most common MDR species were *S. marcescens* (n=84), *K. pneumoniae* (n=62) and *A. baumannii* (n=10). *E. coli* (n=21), *K. michiganensis* (n=19) and *K. pneumoniae* (n=13) were the most common species not determined as MDR (Appendix, page 45-46). MDR was seen in all sites, and the highest number in BC, due to the high number of *S. marcescens* (Figure 6.5)

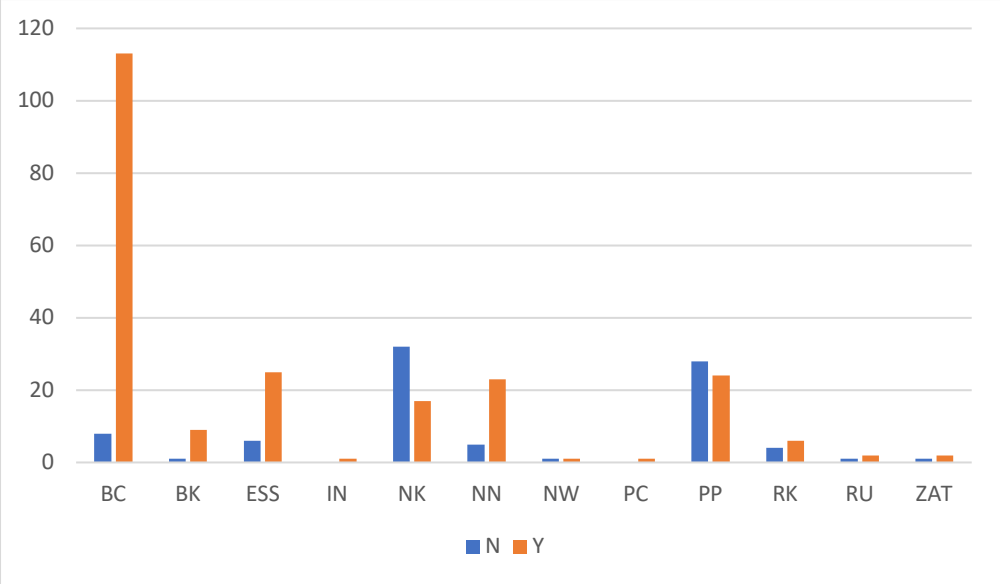

Figure 6.50 Number of bacteria categorised as multidrug resistant (Y) or not (N) within the admissions cohort at each site.

From these isolates, only 12 were fully sensitive for the antibiotics tested, including intrinsic resistance. This analysis did not include GPB, as infection with GPB bacteria may have different risk factors to infection with GNB bacteria and so these were excluded not to counteract analyses. GPB analysis was not carried out separately as only a low number of isolates in this cohort had MIC data available (n=45), only 15 of which were classified as MDR (five from sites in Bangladesh, nine from sites in Nigeria and one from Pakistan).

Chi-square analyses demonstrated significant associations of MDR with 12 variables (Table 6.6). Mother's first pregnancy was associated with increased rate of MDR (p=0.001), first time mothers were also more likely to have an assisted birth ($\chi^2(1, n=297) = 14.768$, $p<0.0005$), which was associated with increased rates of MDR (p=0.001). Neonates born by Caesarean section also had increased rate of MDR infections, although this was not found to be statistically significant (p=0.063). Premature neonates had highest rates of infections with MDR bacteria (p=0.001). Maternal use of antibiotics was associated with a decrease in MDR. However, only 28 mothers reported previous (within three months) antibiotic use (p=0.004). Many neonates with mothers reporting not taking antibiotics had infections with *S. marcescens*

and *K. pneumoniae*, which are common nosocomial infection that often demonstrate high levels of AMR/MDR. Associations to a wastewater network and the type of toilet were significant with rates of MDR demonstrating that those with a flushing toilet, connected to a wastewater network were linked to lower rates of MDR opposed to pit latrines (p=0.001) (Table 6.8).

Table 6.22 Variables with significant chi-square (χ^2) results for infection of the neonate with a multidrug resistant (MDR) pathogen.

Variable	**Effect of variable**	**χ^2 results**
Mother's first pregnancy	Higher levels of MDR in sepsis isolates associated with first pregnancy (82.26%, n=102/124) compared to 65.36% (n=119/182) in mothers with previous pregnancies	p=0.001
Other health conditions increased rate MDR	63% (n=53/84) MDR no other health conditions 75.34% (n=168/223) MDR other health conditions	p=0.025
Visited traditional healer in prior three months	Higher rates of MDR when the mother reported recently visiting a traditional healer (83.54% MDR, n=66/79) compared to not (68.28% MDR, n=155/227)	p=0.009
Reported taking antibiotics in prior three months	MDR was 50.00% (n=14/28) in patients reported recently taking antibiotics and 75.29% (n=195/259) in those that had not.	p=0.004
β-lactam	Prevalence of MDR was reduced in those reported taking B-lactams, however on 8 reported this and so numbers may not support this.	P=0.011
Maternal education*	Prevalence of MDR bacteria increased as mother education increased. MDR was present in 43.90% (n=18/41) of neonates with mothers reporting no education, 76.47% (n=52/68) of those reporting limited education,	P<0.0005

	75.61% (n=124/164) with mothers reporting secondary education and 80.65% (25/31) in university educated mothers.	
Number of people in household	MDR decreased with 7+ people living in the house (75.58% (n=65/86) 1-3 people, 77.44% (n=103/133) 4-6 people, 60.22% (n=53/88) 7+ people)	p=0.014
Type of toilet at residence	The most common toilet associated with MDR was pit latrine (125/221) Of those with this toilet, MDR isolates were recorded in 80.13% (n=125/156) of isolates. The most common toilet with fewest MDR isolates was squat with flush (n=38/85) Of people with this toilet, 40% (n=43/81) had MDR isolates.	P=0.001
Residence connected to wastewater network	No connection to a wastewater network had higher rates of MDR (82.94%, n=141/170) than those connected (58.82%, n=80/136)	p<0.0005
Neonate born by Caesarean section	Higher rates of MDR were seen when neonates were born by C-section (80.00%, n=68/85) compared to those that were not (69.34%, n=147/212)	p=0.063
Assisted birth	Higher rates of MDR were seen in assisted births (80.52% MDR when assisted [n=124/154]) compared to 63.63% MDR when births were no assisted (n=91/143).	P=0.001
Term of neonate	Premature neonates had a higher level of MDR (84.31%, n=43/51), compared to neonates born on time (71.31% MDR, n=169/237) and late neonates had lowest rates of MDR (37.5%, n=6/16)	p=0.001

* Significance thought to be due to site variation

6.2.12 Place of birth admissions cohort

Within the admissions cohort, there were varied places of birth recorded, including some neonates that were born at a clinical site and returned at a later date with CDS, those born at other hospitals, healthcare centres, home, or other, such as in the car on the way to hospital. This data was available for 3,604/3,632 neonates in the admissions cohort. Places of birth were categorised as 'hospital' (including those born at other hospitals or previously at a clinical site), 'healthcare centre' or 'home', with most neonates born in a hospital, followed by home births (Figure 6.6).

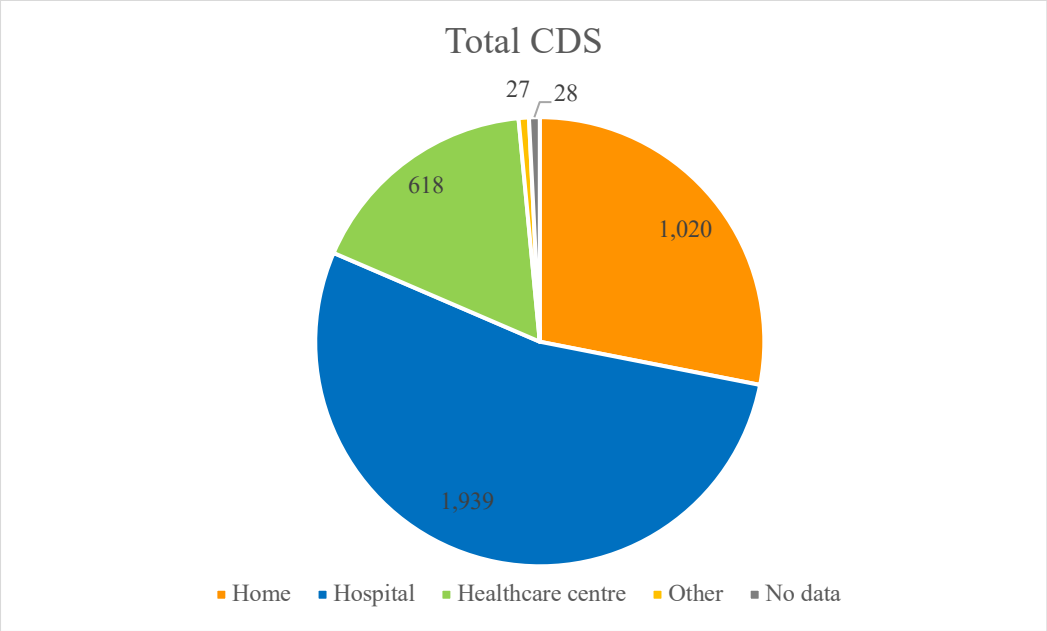

Figure 6.51 Numbers of place of birth reported for neonates within the admissions cohort with clinically diagnosed sepsis (CDS).

The incidence of reported mortality for each place of birth was assessed and similar rates were found for births at hospital (7.79%) and healthcare centres (7.93). Slightly higher rates were reported for home births (8.73%) and higher rates for those with place of birth categorised as 'other' (11.11%) (Table 6.9)

Table 6.23. Rates of reported clinically diagnosed sepsis (CDS), culture confirmed sepsis (CCS) and mortality for neonates with different places of birth reported. Percentages for CCS and mortality are also provided from the total number of neonates enrolled with CDS at each type of birthplace to provide comparison.

Place of birth	Total CDS	CCS (%)	Reported mortality (%)
Home	1,020	216 (21.18%)	89 (8.73%)
Hospital	1,939	418 (21.56%)	151 (7.79%)
Healthcare centre	618	185 (29.94%)	49 (7.93%)
Other	27	10 (37.04%)	3 (11.11%)
No data	28	17 (60.71%)	2 (7.14%)

Rates for place of births varied per site. RK, IN and ESS had the highest proportion of births in healthcare centres and NK had the highest proportion of births at home, followed by BK, whereas BC, NN, NW, ZAT, RU, PC and PP had the highest proportion of births in hospitals (Table 6.10).

Table 6.24 Categorised place of birth and numbers for each per clinical sites within the admissions cohort

Site	**Home (%)**	**Hospital (%)**	**Healthcare centre (%)**	**Other (%)**	**No data (%)**	**Total**
BC	269 (24.54%)	760 (69.34%)	66 (6.02%)	1 (0.09%)	0 (0.00%)	1,096
BK	112 (38.23%)	93 (31.74%)	88 (30.03%)	0 (0.00%)	0 (0.00%)	293
ESS	30 (9.49%)	58 (18.35%)	204 (64.56%)	5 (1.58%)	19 (6.01%)	316
IN	0 (0.00%)	2 (22.22%)	6 (66.67%)	1 (11.11%)	0 (0.00%)	9
NK	536 (51.15%)	415 (39.60%)	91 (8.68%)	6 (0.57%)	0 (0.00%)	1,048
NN	29 (10.98%)	200 (75.76%)	33 (12.5%)	1 (0.38%)	1 (0.38%)	264
NW	9 (12.50%)	45 (62.50%)	16 (22.22%)	2 (2.78%)	0 (0.00%)	72
PC	2 (16.67%)	10 (83.33%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	12
PP	23 (7.26%)	279 (88.01%)	11 (3.47%)	3 (0.95%)	1 (0.32%)	317
RK	9 (5.49%)	45 (27.44%)	103 (62.80%)	7 (4.27%)	0 (0.00%)	164
RU	0 (0.00%)	14 (100.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	14
ZAT	1 (3.70%)	18 (66.67%)	0 (0.00%)	1 (3.70%)	7 (25.93%)	27
Total	1,020 (28.08%)	1,939 (53.39%)	618 (17.02%)	27 (0.74%)	28 (0.78%)	3,632

Demographic data was compared for neonates born at home, hospitals or healthcare centres, which accounted for 3,577 neonates. Variables with significant chi-square results and obvious patterns are listed in Table 6.11. Place of birth did not have a significant correlation with reported mortality ($X^2(2, N=3,056)=0.474$, $p=0.789$), and similar rates were seen for all categories, with slightly increased rate at healthcare centres, but again this was not significant (Table 6.12)

Table 6.25. Variables that had significant differences and distinct patterns for neonates born at different locations prior to admission with clinical sepsis.

Variable	Chi-square (X^2) result	Pattern of difference
Mother attended private healthcare in past 3 months	$X^2(2, N=3,568) = 265.604$, $p<0.0005$	More mothers gave birth in a hospital when they had previously attended private healthcare
Mother attended traditional healer in past 3 months	$X^2(2, N=3,573) = 157.187$, $p<0.0005$	Mothers that had not visited a traditional healer more often gave birth in a hospital
Previous hospitalisation prior 3 months	$X^2(2, N=3,518) = 30.556$, $p<0.0005$	Mothers that had been previously hospitalised less likely to give birth at home
Previous antibiotic consumption prior 3 months	$X^2(2, N=289) = 31.673$, $p<0.0005$	Mothers that had not previously taken antibiotics more likely to give birth at healthcare centres than those that had
Mother previous education	$X^2(6, N=3,572) = 332.779$, $p<0.0005$	The proportion of births at home decreased as education levels of mothers increased.

Table 6.26. Number and percentages of reported mortality for neonates at each categorised place of birth.

Place of birth	Not reported as deceased	Reported deceased
Home	831	89 (10.71%)
Hospital	1500	151 (10.07%)
Healthcare centre	436	49 (11.24%)

Place of birth had a significant association with the type of sepsis pathogen that was obtained from their positive blood culture when admitted to the sites with CDS ($X^2(2,$

N=710)=14.292, p=0.001). Hospitals had considerably more Gram-negative infections, whereas healthcare centres had more Gram-positive cases of sepsis (Table 6.13). However, this may be due to variation between sites opposed to risk factors from place of birth, as births in healthcare centres were more common in ESS and RK, where Gram-positive isolates were quite common, whereas hospital births were more common in BC and PP, where Gram-negative bacteria were common. Furthermore, these hospitals both had outbreaks of Gram-negative bacteria and so this could have been a factor.

Table 6.27. Gram stain results for isolates from neonates born at different locations (data was available for n=710).

Place of birth	Gram-positive	Gram-negative
Home	94 (51.65%)	88 (48.35%)
Hospital	162 (42.30%)	221 (57.70%)
Healthcare centre	87 (60.00%)	58 (40.00%)

6.3 Discussion

Although neonatal sepsis and mortality in LMICs is a key focal health area, there are few publications that have investigated outborn neonatal sepsis. Of the 37 articles found from the past five years, none combined epidemiological, demographic, genomic and outcome data. Some of these studies were undertaken in the same countries as BARNARDS sites but few sought to address the home environment as factors for neonatal sepsis and mortality or link these to sepsis microbiology (Cavallin *et al*., 2020 Meshram *et al*., 2019).

Home births are more common in Africa and Asia compared to Western countries (Montagu *et al*., 2011; UNICEF/WHO, 2021; European-Pristat project, 2014), and whilst there are cultural reasons for mothers selecting to give birth at home, it is also likely that some home births occur because of practical limitations including access to and cost of healthcare. Home births accounted for 1,020/3,604 (28.30%) of neonates within the admissions dataset with place of birth data available. This was in line with previous findings from the overall global data from Hernandez-Vasquez *et al*. (2021). Their review reported rates of home births were above this average, reporting rates of 34% in Pakistan, 50% in Bangladesh, 59% in Nigeria and 73% in Ethiopia, with lower rates were reported in South Africa and Rwanda. The low rates of home births were in line with findings from BARNARDs. We also found higher rates in Bangladesh and in NK, Nigeria, but lower for other sites. However, we would expect some variation in our results from other averages and we may not be able to extrapolate the data collected to provide percentages of home births in each country, as estimates are based on few sites. Furthermore, only mothers that attended clinical sites presenting with a neonate were captured. It is therefore likely that this is a skewed dataset, as it will be skewed to mothers familiar with the care centre or to those that gave birth in the clinical site. There is a tendency for mothers undergoing home births to be less likely to seek medical care for new-borns, due to cultural beliefs, a lack of access or affordability or due to lack of permission from husbands or elders (Lassi *et al*., 2019;

Owais *et al*., 2011), acting as the reason for both home birth and reduced care seeking for ill neonates. Therefore, home births will be underreported in this study.

Herein, we found that assisted births were associated with decreased neonatal mortality, suggesting the birth was attended by skilled healthcare staff, and that neonates born 08:00-18:00 in hospitals were associated with lower mortality than those born 18:00-08:00 when reduced care would be available, supporting the importance of attendance by skilled healthcare workers (WHO, 2004; Ljungblund *et al*., 2019). Higher mortality was also seen for neonates with home births between 18:00-08:00, potentially partly due to closed medical centres and additional barriers to accessing healthcare out of hours have been found by Essendi *et al*. (2011) to include safety concerns, higher price of taxis, and lack of access to public transport.

Various risk factors were found to be associated with the development of sepsis when comparing the small subset of neonates confirmed as healthy to those with CDS in NK, with the greatest association including place of birth in a hospital compared to home, potentially as neonates would have a higher chance of diagnosis. However, most healthy neonates enrolled were enrolled alongside mothers when mothers were admitted into hospital with postpartum haemorrhage. Therefore, many of the variables appearing as associations with the development of sepsis may be risk factors for postpartum haemorrhage, as healthy mothers with healthy neonates were not later admitted in the clinical sites. Therefore, these analyses should not be considered when investigating risk factors for CDS and within BARNARDS, only the inborn data from BARNARDS should be considered when assessing risk factor for CDS.

This study found an association between increased neonatal mortality in neonates born pre-term, observed for the overall admissions dataset, and when analysed per individual sites, in concurrence with previous studies (Afroza, 2006; Veloso *et al*., 2019; Daemi *et al*., 2019; Mercer *et al*., 2006). Premature neonates with a CCS had higher rates of MDR, which may be

related to multiple factors such as hospitalisation prior arrival at a BARNARDS clinical site and/or increased vulnerabilities to nosocomial infections. However, only 51 neonates with MIC results were categorised as pre-term and therefore, this finding should be interpreted with caution, although pre-term neonates were also found to have higher rates of MDR infections in a study by Ballot *et al.* (2019). We report an increased risk of neonatal sepsis caused by a MDR pathogen following assisted birth, including Caesarean sections. Assisted births were more common in hospital births. This suggests that MDR bacteria may have been transmitted from medical equipment, as nosocomial infections are often prone to MDR (Berberian *et al*., 2019). This emphasises the need to understand current infection prevention control policies in hospitals to minimise bacterial transmission between neonates and the clinical environment. Further work is needed to understand transmission dynamics within LMIC hospitals; however, comprehensive environmental sampling and surveillance requires intensive resources.

Multiple associations were found between healthcare related risk factors before or during birth and neonatal sepsis caused by *S. marcescens. S. marcescens* is a well-known opportunistic nosocomial pathogen, commonly found in healthcare environments (Khanna *et al*., 2013) with infections found to stem from a range of contaminated surfaces (Solkalski *et al*., 2022; Gupta *et al*., 2014; Rabier *et al*., 2008; Uduman *et al*., 2002). During our study *S. marcescens* was predominantly recovered from one site in Bangladesh (BC), with our previous work (Sands *et al*., 2022) indicating that over an 18-month period, a large epidemiological cluster of *S. marcescens* was recovered from BC, with over 90% of all *S. marcescens* neonatal sepsis in BARNARDS isolates from this hospital site.

Mortality was higher for neonates with CCS compared to CDS only. Some neonates initially diagnosed with sepsis, may not have had a bloodstream infection but may have been suffering from another non-infectious clinical syndrome, as supported by Phua *et al*. (2013) due to the non-specific signs of sepsis in neonates. It is also possible that antibiotic therapy had

been administered to neonates prior to having a blood sample taken, inhibiting bacterial growth resulting in a negative blood culture and false-negative CCS classification. Alternatively, these sepsis cases could have been diagnosed early, before the bacteria had reached detection levels and was therefore easier to treat (Im *et al*., 2022). Furthermore, CCS negative neonates could have been infected with fastidious bacteria that may have not been detected under standard laboratory conditions (Doern, 2000).

We found multiple associations between CCS and MDR with various environmental factors. Having access to a toilet within the house was associated with a reduced incidence of neonatal CCS. Moreover, we found higher rates of neonatal sepsis caused by an MDR bacteria with mothers/families having pit latrine toilets home compared to lower rates observed with those mothers having flushing toilets and a connection to a wastewater network. This association echoes data from other studies whereby high levels of MDR bacteria were cultured from pit latrine samples in a study by Beukes *et al*. (2017). It is likely that individuals will empty sewage and waste products themselves which, alongside risk of flooding in wet seasons, exposes them to an increased risk of colonisation with MDR bacteria (Kwiringira *et al*. 2016). Sewage and related stagnant wastewater may remain around home environments for an extended time attracting insects that can act as disease vectors (Nakagiri, *et al*., 2016), which may contribute to the higher rates of MDR in those with pit latrines and also the higher rates of CCS in those with no toilet in the home, in addition to the indication that these people may face additional obstacles. We also found that neonates with CCS living in rural areas had a higher chance of sepsis caused by GNB compared to GPB. Enterobacterales are often detected in environmental areas linked with human waste, and rural areas were less likely to be connected to a wastewater network, and were associated with pit latrine toilets, which some participants emptied themselves. Mothers from rural areas also reported washing their hands less frequently, partly due to reduced handwashing facilities, such as running water.

Various risk factors for mortality following CDS were not significant upon single site analyses and therefore, may be due to site variation. Single site analyses were not possible to assess mortality following CCS and so it may be that odd findings, such as mothers connected to a wastewater network were associated with decreased neonatal sepsis may be due to site variation, as this risk factor was significant for overall mortality following CDS, but not when single site analyses were undertaken. Term of a new-born at delivery and premature rupture of membrane were the only two risk factors found to have an association with mortality following both CDS and CCS and retained significance following per site analyses in the CDS cohort, as supported by multiple other studies (Al-Sheyab *et al*., 2020; WHO, 2022; Chowdhury *et al*., 2010; Desalew *et al*., 2020)

6.3.1 Conclusion

BARNARDS was undertaken to understand the risk factors for neonatal sepsis and mortality and is one of the few studies combining multiple epidemiological datasets with genomic sequencing. Interestingly, the facility-based birth cohort CDS associations were mainly associated with maternal and/or neonatal aspects (e.g. maternal hypertension, previous maternal hospitalisation, preterm birth, perinatal asphyxia, and multiple births) and only living in a rural environment was strongly associated. Our findings, whilst also identifying similar risk factors also had household hygiene factors highlighting the role of living environment and demographics in neonatal sepsis and mortality. Future studies designed to evaluate risk factors between neonates born within the hospital and neonates born outside of the hospital will be valuable to identify potential sepsis interventions. Similarly, a study with a larger sample size per hospital will allow for meaningful analyses at a site level to explore localised nuances. Furthermore, future research should integrate anthropological and health economic approaches to perform a deep dive on responses generated from survey or questionnaire data.

7.0 Discussion

7.1 Sepsis rates

Overall, there were 9,874 cases of CDS and 2,483 cases of CCS reported from 36,285 neonates enrolled throughout BARNARDS, averaging at 184 CDS and 44 CCS per 1,000 live births for inborn neonates. High variation was seen in BARNARDS regarding rates of sepsis per site, ranging from 39 to 261 CCS per 1,000 live births at different sites. This may be partly due to the vague symptoms of sepsis and natural variation between sites. Results from admissions neonates found an association between various demographic factors, such as having a non-flushing/ pit latrine toilet at home to be associated with increased rates of CDS in the neonate. Sites will have variations in the local demographic area, regarding factors such as this, e.g. access to wastewater networks.

Previous studies have shown both higher rates previously discussed in chapter 3, including higher rates of CCS found by a study in India by Tallur *et al*., (2000) compared to rates in BARNARDS from India. Conversely, lower rates have also been seen such as in the meta-analysis of rates in South Asea by Churasia *et al*. (2019) had lower rates than the BARNARDS sites in South Asia.

Many of the studies from LMICs are from single sites, which are unreliable to compare when assessing rates within a country, as there are many site differences. For example, even with standardised diagnosis, microbiology consumables and equipment, we still found wide variation between BARNARDS sites within a single country, for CDS and CCS. Furthermore, many studies focus on the sepsis cohort only, without contextualising rates of neonatal sepsis. Each of the studies were independent studies or reviews of multiple independent studies and there would have therefore been multiple discrepancies in the types of sites with assorted microbiology facilities, expertise of staff in addition to varied enrolment

cohorts in addition to natural variation between sites. However, unanimously, rates reported within BARNARDS and from other studies across LMICs are considerably higher than seen in HICs. Cailes *et al*. (2018) reported rates of 6.9 CCS per 1,000 live births across 30 neonatal units in the UK and 1.08 CCS in a study of over 200,00 neonates in America (Stoll *et al*., 2020).

A much greater number of neonates were diagnosed with sepsis than those with culture confirmed sepsis (25.15%). Slightly lower rates of confirmed sepsis were found by Jajoo *et al*. (2018). Disparity between CCS and CDS may be due to a range of reasons, including collection of insufficient blood samples to detect bacterial colonies particularly when taking samples from pre-term neonates. Additionally, variabilities in clinical presentation, and vague symptoms of sepsis are not universally defined. Symptoms include tachycardia or bradycardia, increased breathing rate and a low/high temperature and feeding problems which could portray a range of other illnesses or issues, such as dehydration, congenital heart disease, or acute renal failure (Maayan-Metzger *et al*., 2003; Baruteau *et al*., 2016; Agras *et al*., 2009). An expert meeting on neonatal and paediatric sepsis by the European Medicines Agency (European Medicines Agency, 2010) stated that a diagnosis of neonatal sepsis should be based on at least two clinical symptoms and at least two laboratory signs, such as white blood cell count, c-reactive protein levels or platelet counts. However, many of these laboratory-based tests are absent in LMICs and they rely solely on clinical signs before starting empirical treatment. Many healthcare facilities lack microbiology facilities to confirm diagnoses of sepsis (Yadav *et al*., 2021).

7.2 Sepsis as a contributor to neonatal mortality

Overall mortality from CDS was 8.86% and increased to 12.08% following CCS. Lower rates seen in neonates with CDS without a positive blood culture may be due to neonates not having had sepsis but may have been diagnosed due to vague symptoms as

discussed above. Additionally, they may have been diagnosed early in their infection when there were not ample bacteria in the blood to be detected in the small sample taken, making treatment less complex.

Overall, within our study, 1,273 of all enrolled neonates were reported deceased (3.5%). This number was greater than those with reported mortality following CDS (n=875), demonstrating additional risks for mortality besides from sepsis for an additional 398 neonates. It may be that they had sepsis that was not diagnosed, but other causes for mortality are likely, including intrapartum related event, pre-term birth complications (WHO, 2019). Additionally, of the 575 of those that died following CDS did not have CCS and these may also have died from other complications. We can assume that 300/1,273 (23.56%) deaths in enrolled neonates were likely attributable to sepsis, this is higher that estimations of proportion of deaths in neonates due to sepsis (WHO, 2019). Another aspect to consider is, even though 300 neonates with CCS died, the mortality may have been due to other reasons. For example, pre-term neonates are at higher risk of developing sepsis and those with longer hospital stays and the reason they were admitted into hospital may be the cause of death, and sepsis was a compounding issue. Results from the admissions dataset found that PROM and term of baby to have the greatest association with mortality of neonates with both CDS and CCS. Association of PROM and mortality may demonstrate mortality may have been caused by intrapartum related events, supporting WHO (2019).

These overall rates of mortality are lower than estimates from a study by Bangi *et al*. (2014) in India, who found a 46.7% mortality rate from neonates with CDS. The DeNIS study (2016) also reported higher rate of mortality for neonates with CCS compared to CDS, although the rates of mortality they reported were also higher, more in line with Bangi *et al.* (2014) at 26% mortality for those with CDS and 48% for those with CCS. It is most probable that rates of mortality within BARNARDS is underreported due to insufficient follow-up data

for many neonates and according to statistical protocols, any neonates that were lost to follow-up or untraceable were categorised as alive until last follow-up while only those confirmed as deceased during the follow-up period were reported as such. While sepsis is named as one of the leading causes of neonatal mortality, it is likely that the accompanying burden is underreported.

There is considerable variation in published estimates of deaths cause by neonatal sepsis (Roth *et al*., 2018; Ranjeva *et al*., 2017; UN IGME, 2019; Liu *et al*., 2015). Such high variation is seen in estimates due to gaps in available data, with many studies only looking at single sites (Fleischmann *et al.,* 2021), with some countries having no available data due partly to a lack of formal monitoring or reporting processes at many healthcare centres, particularly regarding cause of death (Naz *et al.*, 2012; Turner *et al.*, 2021; Yadav *et al*., 2021), and it is likely that rates of neonatal mortality and the impact of sepsis on neonatal mortality will be underestimated in LMIC settings.

Furthermore, many births take place outside of hospitals, due to reduced awareness of risks associated with birth, or a lack of access or affordability to take the neonate to healthcare facilities and associated mortality or sepsis will not be reported (Montagu *et al*., 2011). Within this study, the rate of mortality relating to admissions neonates was 23.29% for those with CDS and 12.65% for neonates with CCS. This was higher, particularly for those with CDS compared to the overall mortality, which included inborn neonates at 8.86% for CDS and 12.08% in neonates with CCS. This may be associated with delays in care seeking, as studies have found that mothers that underwent home births were less likely to seek medical care for new-borns, due to cultural beliefs, a lack of access or affordability or due to lack of permission from husbands or elders (Lassi *et al*., 2019; Owais *et al*., 2011).

Another potential reason for higher mortality rates in admissions neonates may be due to a lack of skilled medical staff at the birth. Owais *et al.* (2011), reported that 47% of women

did not plan to have a birth attendant present during their home birth. Additionally, Bucher *et al.* (2016) report that attendants that are present can be unskilled or use outdated methods. This is in line with our findings that within the admissions cohort, those that were born without assisted delivery were associated with increased mortality. Furthermore, neonates born between 18:00-08:00 also had an increased rate of mortality following CDS, presumably due to lack of access to care.

There is little data on outborn neonatal sepsis, which make up a large proportion of births in LMICs as many women give birth at home or in primary healthcare centres. A study carried out by Montagu *et al*. (2011) found that 70% of births in South Asia and over 82% of births in Sub-Saharan Africa in the lower two wealth quintiles occurred at home. They found this to be lower in women from wealthier families in the same area, but still with around half of births occurring at home.

7.3 Aetiology and resistance

Common bacteria found from cases of CCS during the BARNARDS study mainly aligned with previous findings from LMICs. Studies have found similar rates of GPB and GNB sepsis to the prevalence found at sites with 54.9% sepsis caused by GPB and 45.1% by GNB (Shah *et al.,* 2012). This was also similar to rate of each in the admissions cohort (51.6% GPB: 48.4% GNB). Although, a wide variation in rates of GPB: GNB was seen per site, with a high proportion of GNB sepsis in sites in Bangladesh and India. This may have been due to the outbreak of *S. Marcescens* in Bangladesh and numbers in India were small, so ratios are not reliable. Conversely, a high proportion of GPB was seen in the sites in Nigeria, Rwanda and South Africa. Ethiopia may not have had a higher proportion of GPB due to the outbreak of *K. pneumoniae.*

Some common species were due to outbreaks, including *K. michiganensis* and *S. marcescens* but these were not common to all sites. When outbreaks were disregarded, *K.*

pneumoniae, *E. coli* and *S. aureus* were the most common species isolates from sepsis cases, in line with previous studies (Medugu *et al*., 2018; Waters *et al*., 2011).

K. pneumoniae was the most common species overall and was one of the three most common species for all sites, commonly found in other studies in LMICs (Viswanathan *et al*., 2010; Wen *et al*., 2021). Having *K. pneumoniae* as a common sepsis agent is concerning as when assessed separately, *K. pneumoniae* isolates had high levels of resistance against multiple antibiotics; higher than seen in *E. coli* across all antibiotics. This finding was supported by Navon-Venizi *et al*. (2017) with data from the European Antimicrobial Resistance Surveillance Network, who showed that rates of resistance in *K. pneumoniae* isolates increased at a much faster rate than seen in relatively stable *E. coli* isolates over a ten-year period. We also found in this study that a higher number of *K. pneumoniae* isolates displayed higher FoRs for multiple antibiotics than *E. coli* isolates. Navon-Venizi *et al*. (2017) also characterised 52 resistance plasmids from *K. pneumoniae* isolates that can readily transfer various resistance genes to other bacteria. In BARNARDS, the majority of isolates (49/74, 66.2%) containing both *bla*-$_{\text{NDM}}$ and *bla*-$_{\text{OXA}}$ were *K. pneumoniae.* The high resistance rates across multiple antibiotics, observed rapid increase of resistance aligned with numerous mobile resistance elements found in *K. pneumoniae* in addition to its hypervirulence potential and ability to occur on hospital surfaces make this pathogen a major concern. Research has recently commenced to design vaccines against *K. pneumoniae*, which could be given to pregnant women to provide neonatal protection via transplacental antibodies (Assoni *et al*., 2021).

No cases of GBS were found to cause neonatal sepsis throughout BARNARDS. However, Gram-positive was not originally a focus in this study and therefore, many Gram-positive isolates were only identified at site and species was not confirmed via WGS. Furthermore, some Gram-positive bacteria was only identified to Gram stain level. There are

few reports of GBS from South Asia in the literature (Edmond *et al*., 2012; DeNIS, 2016). There are mixed reports from Africa, with some studies reporting low rates of GBS (Medugu *et al*., 2011), whereas other studies report this to be a leading cause of neonatal sepsis (Sigauque *et al*., 2009; Milledge *et al.,* 2013). Overall, previous studies found that GBS is less common in LMICs that HICs, where it is frequently reported as the most common cause of sepsis (Zea-Vera and Ochoa, 2015).

AMR was high across GNB for a range of antibiotics, with some resistance seen against all antibiotics tested and 75% were MDR. Highest resistance seen against ampicillin (95.36%), amoxicillin-clavulanate (77.80%), ceftriaxone (79.95%), cefotaxime (82.67%). These included WHO recommended first-line (ampicillin and gentamicin) and second line (third generation cephalosporins) treatments.

7.4 Outbreaks and IPC

IPC is required in hospitals to prevent outbreaks, as with substandard IPC, pathogens from other patients can be transferred onto medical equipment and passed onto others if not properly cleaned, or via healthcare workers hands between patients. Patients are often immunocompromised and/or undergoing procedures that disrupt their skin and so are vulnerable to infections with these nosocomial organisms (Sood and Perl, 2016). Sterile, intrusive biomedical devices are coated with proteins and glycoproteins upon use, which contain binding ligands for bacteria, attracting bacterial cells, creating a biofilm. Soon after attachment, cell up-regulate cell signal molecules which enhance cell virulence factors and recruit more bacteria (Bryers, 2008). Cells embedded in a biofilm are difficult to eliminate compared to individual cells, protected from antibiotics (Aslam, 2008) and this creates an optimum environment for sharing resistance plasmids. However, overuse of biocides may increase AMR (Jone and Joshi, 2021).

Two species of *Klebsiella* spp. (*K. pneumoniae* and *K. michiganensis*) were found to cause outbreaks in Ethiopia and Pakistan, respectively, along with *Serratia marcescens* in Bangladesh and *Burkholderia cenocepacia* in PP, Pakistan. *Klebsiella* spp. has been found on thermometers, oxygen saturation probes, disinfectant and incubator humidifiers (Macrae *et al.*, 2001; Reiss *et al*., 2000; Jeong *et al.*, 2001). *S. marcescens* outbreaks have previously be associated with contaminated soap, baby formula and by the hands of healthcare workers (Archibald *et al.*, 1997; Fleisch, *et al*., 2002; van Ogtrop *et al*., 1997). This depicts the importance of appropriate IPC practices within hospitals as outbreaks are commonly due to continued contamination of wards or instruments, which effective IPC practices would reduce (Haley *et al.*, 1985). Standard IPC measures include hand hygiene, personal protective equipment, respiratory protective equipment, safe management of laundry, sterilisation of equipment, cleaning of the hospital environment, patient placement and aseptic techniques. Hand hygiene compliance monitoring is considered as a key IPC performance indicator, as well as healthcare associated infection surveillance (WHO, 2016).

Isolates matching outbreak strains accounted for 371/1,046 (35.47%) of confirmed sepsis cases with WGS data. A lack of good infection prevention and control (IPC) can enable outbreaks in hospitals, commonly seen globally, with HAIs estimated to cause 2.5-14.8% of infections in Africa, with up to 45.8% from surgical wards, compared to 7.1% in Europe (Nejad *et al*., 2011; European Centre for Disease Prevention and Control, 2008). Implementing effective IPC strategies to reduce the numbers of infections within hospitals is vitally important in addition to ensuring optimum antibiotic treatment.

7.5 Current Empirical treatment

Empirical treatment guidelines for neonatal sepsis suggested by the WHO are guided by studies from HICs, where infections from Gram-positive bacteria dominate, with the leading pathogens reported to be GBS, Streptococci, CoNS and Enterococci in addition to *E.*

coli and fewer other Gram-negative species, predominant species of which were shown to still have high susceptibility to penicillin and gentamicin (Stoll *et al*., 2011; Hoffman *et al*., 2008; Weston *et al.*, 2011; Bizzarro *et al*., 2005). In this study, however, we found different pathogens with *Staphylococcus aureus* as the most common Gram-positive bacteria and a higher prevalence of Gram-negative pathogens with high levels of resistance. Ampicillin is much more effective against streptococcus than *S. aureus*, the most common Gram-positive pathogen found in the BARNARDS study, which is commonly resistant to ampicillin, as they are penicillinase producers (EUCAST, 2019). Furthermore, ampicillin is effective in HICs against Gram-negative bacteria as there is low resistance (Cailes *et al*., 2017). However, in BARNARDS, ampicillin was found to be the antibiotic to which isolates had highest resistance, an overwhelming 95% in GNB, with nearly all isolates demonstrating resistance across all sites making it ineffective in many cases. Further to this, >60% GNB showed resistance against gentamicin in both continents. The combination of ampicillin and gentamicin was found to provide coverage for only 28.5% GNB isolates causing sepsis. Furthermore, two of the three third generation cephalosporins, recommended as second-line treatment had around 80% resistance.

It is obvious from this data that the recommended empirical first-line treatment suggested by the WHO for neonatal sepsis (ampicillin and gentamicin) is not a suitable empirical therapy in the LMICs included in this study, where antibiotic resistance is widespread, particularly for Gram-negative sepsis. Additionally, second-line treatment may need to be reconsidered, or specify which cephalosporins to use. Many healthcare facilities in LMICs lack microbiology facilities to gain knowledge of local common pathogens or resistance profiles, therefore rely heavily on empirical recommendations. Furthermore, blood cultures take 24-48 hours, with a further 24 hours to determine antibiotic susceptibility profiles.

Different countries need to be focused on for their own empirical treatment options aligned with principal causes of sepsis and local antibiotic resistance profiles. When antibiotic resistance in isolates from sites in Asia were compared to those in Africa, slight differences were seen, but overall, similar levels of resistance were seen from each continent. Therefore, the larger site/ country variation seen may be partly due to smaller sample sizes. While I believe that there is veracity for some variance in antibiotic resistance between countries, i.e. there were higher levels of resistance in Gram-negative bacteria isolates from neonates in India, overall resistance profiles may be more similar if a higher sample number were used. Defining an empirical therapy per country may be a better solution, taking additional barriers into account, such as antibiotic licensing, access, and affordability.

7.6 Antibiotic usage for CDS and potential alternatives

Due to known high levels of resistance in sepsis causing pathogens, various sites did not follow the WHO recommendation of ampicillin and gentamicin, supported by other studies (Jackson *et al.*, 2019). Sites mainly prescribed antibiotics from the access group, but antibiotics from the watch group were also commonly used. Antibiotics from the WHO reserve list (WHO. 2021), such as carbapenems were reported to be used modestly, and were only reported to be used in Pakistan although other studies have found this use to be higher (Jackson *et al*., 2019). Antibiotic usage data for neonates was collected retrospectively in the BARNARDS study and was not available for all neonates with CDS, therefore these figures may be underreporting the use of some antibiotics.

The four most common first-line antibiotic therapies for neonatal sepsis at BARNARDS sites were ampicillin and gentamicin; amoxicillin-clavulanate and amikacin, piperacillin-tazobactam and amikacin; and ampicillin and gentamicin. Despite high resistance found against ampicillin and gentamicin and accompanying low modelled PTA 80% levels, observed survival was higher than expected. Synergy was tested with the two antibiotics

using broth microdilution, but no synergy was found. It may be that neonates at lower risk were given ampicillin and gentamicin. However, follow-up of neonates on this antibiotic was low and mortality may be underreported. Piperacillin and tazobactam, commonly prescribed in Pakistan was associated with higher mortality than expected from the low resistance against these antibiotics. However, the site PI told us this is usually prescribed in neonates that are already admitted into hospital and may be of higher risk. This demonstrates that patient survival is complex and may involve confounding variables. Ceftazidime and amikacin displayed 77.1% coverage against GNB and was associated with lowest mortality. However, ceftazidime has reduced efficacy against *S. aureus* isolates (Richards and Brogden, 1985). Further to this, amikacin in ineffective against certain strains of *S. aureus* (Aronson, 2016). Therefore coverage of this species, found to be one of the leading causes of sepsis, may be somewhat impaired with this combination.

As discussed in chapter five, country effects could not be distinguished from antibiotic effects, as certain antibiotics were favoured by certain sites. Certain antibiotic combinations were used by different sites. For example, AMP-GEN was used more commonly across Africa, CTZ-AMK used mainly in Bangladesh, AMC-AMK in Nigeria and PIP/TAZ-AMK in Pakistan. Neonates treated with CTZ-AMK in Bangladesh for example, had *S. marcescens* was the most common pathogen, opposed to *K. pneumoniae* as the most common species in neonates undergoing other treatment combinations in other countries. They were also more often outborn and different patient populations, alongside other site variables, such as standard of care that will impact outcome. Country and site effects must be considered, therefore further work is needed on alternative antibiotics on a larger sample size within a standard care setting and matched patients (i.e. similar pathogens and prematurity) and additional clinical information.

FoRs were carried out for commonly used antibiotics and potential alternatives with low resistance to determine how quickly resistance may arise if use increased. High FoR was found for fosfomycin in GNB. FoR for amikacin was lower than for gentamicin, showing this could be a potentially beneficial substitution. Although no resistance developed against meropenem during FoR experiments and a low prevalence of resistance was found, in combination with low prevalence of resistance, and is also supported by Bielicki and Sharland (2020) to provide high coverage in LMICs. However, this is categorised as a reserve antibiotic by the WHO (WHO, 2019) and is reserved as a last resort treatment. Increased use of this drug may increase rates of widespread resistance due to increased integration of *bla*-$_{NDM}$, which we already found in 112 isolates from multiple sites. Furthermore, meropenem is expensive, particularly in Rwanda ($14 per day), Nigeria ($12.50 per day) and Bangladesh ($10 per day).

Taking all the data collected into consideration, ceftazidime and amikacin appears to be a suitable potential alternative empirical therapy to replace ampicillin and gentamicin. This combination provided vastly improved coverage for GNB compared to ampicillin and gentamicin and FoR results showed these antibiotics to be relatively stable. Furthermore, these antibiotics were second cheapest options following ampicillin and gentamicin, making it an affordable option for patients/ hospitals and they were both available in all countries, except amikacin was reportedly not available in Ethiopia. Further to these findings, previous usage has been shown in neonates, suggesting safety, supported by previous studies (Mulhall and Louvois, 1985), although there are mixed findings regarding amikacin (Hughe *et al*., 2017; Flidel-Rimon *et al*., 2006), which may require close monitoring for signs of nephrotoxicity (Cristea *et al*., 2017).

It may be that a risk-based approach could be a suitable option for assessing empirical therapy. Opposed to prescribing the same antibiotic therapy empirically to all neonates, their

relative risk could be assessed before deciding upon treatment. Neonates that are assessed as more at risk, for example if they are premature or with more severe signs of sepsis, could be prescribed an escalated regimen, whereas those deemed at a lower risk, with less progressed symptoms and a higher birthweight could be given AMP-GEN. This could reduce potential use of lesser studied/ more toxic antibiotics that themselves may bring slightly higher risk to the neonate in neonates unless considered necessary in a septic infant that could not afford the time to have an initial ineffective antibiotic treatment. This would also reduce as much of a broad use and have a lesser impact on potential increased prevalence of resistance to chosen alternative treatment.

7.7 Lack of access or affordability to antibiotics

Although AMR is estimated to be causing numerous deaths per annum, the review by Laxmanirayan *et al.* (2016) stated that a lack or delays in access to antibiotics still kill more people worldwide than AMR. Therefore, reducing availability of antibiotics is not a practical solution to prevent AMR. Within this study, we found multiple variation in access and affordability to antibiotics: 1. Not all antibiotics were available in all countries. This was mainly relating to reserve antibiotics, but also other commonly used antibiotics such as amikacin was not available in Ethiopia and amoxicillin-clavulanate was not available in multiple countries. 2. BARNARDS included areas of low incomes and many antibiotics would be unaffordable. 3. Prices of antibiotics fluctuated between sites that was not relative to income. 4. Some countries provide free healthcare, others offer some free treatment, while in other countries, payment was deferred to patients need. Collectively, these factors will significantly determine the antibiotics that are used for which neonate. For example, the site in Ethiopia only offered ampicillin and gentamicin free of charge to the patient. Patients that cannot afford alternative treatments may not be able to upgrade to more potent antibiotics if no response is seen to the treatment in the neonate. Similarly, families of neonates must pay

for antibiotics in Nigeria and poorer patients will be forced to opt for cheaper, potentially fewer effective options or cannot afford the full antibiotic course. There is a marked paucity around health economics in LMICs and considerably more work is needed to investigate accessibility and affordability of antibiotics in LMICs.

7.8 Antibiotic prescribing practices / Overuse

Antibiotic use is high in many LMICs, with overprescribing or in many countries, antibiotics are available without a prescription (Fink *et al*., 2019; Auta *et al*., 2019), a global problem with reports of antibiotics overprescribed in some HICS. Van Boeckel *et al.* (2014), found a 35% increase in antibiotic consumption between 2000 and 2010, with highest demand for broad-spectrum penicillins and cephalosporins. They also found that India was the highest consumer of antibiotics globally in 2010, in line with high antibiotic resistance profiles found in BARNARDS isolates from India. This is partially due to, as in other countries, the availability of many broad-spectrum antibiotic drugs (cephalosporins, fluoroquinolones, and carbapenems) sold over the counter without official clinical documentation (Van Boeckel *et al*., 2014). The ability to buy carbapenems over the counter needs to be urgently addressed, as carbapenems have broad-spectrum activity against a range of Gram-positive and Gram-negative bacteria and with numerous MDR infections occurring within both African and Asian continents, it is imperative that carbapenems retain their efficacy. However, it would be difficult to enforce these laws. Additionally, this may reduce the number of people that have access to antibiotics, as they may lack transport to get to hospitals. Clinicians in many LMICs will be prescribing carbapenems more readily, as the resistance to other antibiotics is so high, that it may be becoming their go to first line treatment, particularly for critical infections such a sepsis.

A review by Morgan *et al.* (2011) found that non-prescription antibiotics were often used for short durations, sometimes below the recommended dosage, with pharmacists often

dispensing single day courses. Multiple studies in the review found that pharmacists did not enquire about allergies before selling antibiotics and side effects were only discussed in 50% of non-prescription purchases.

Further issues arise with prescription practices of doctors globally and increased stewardship is needed, as many prescribe antibiotics when they are not required due to financial benefits or pressure from the patient. Gani *et al.* (1991) found antibiotics were prescribed in 94% of cases of childhood diarrhoea, when this usually caused by viral infections and requires only rehydration therapy. They found reasons for antibiotic prescribing included knowledge regarding aetiology, rehydration therapy and parental expectations. A study in Iran (Hashemi *et al*., 2013) found that the most common reason for prescriptions of antibiotics was respiratory tract infections, followed by those diagnosed with a common cold. Murphy *et al.* (2012), also found that respiratory ailments were the most common cause for antibiotics prescriptions by GPs in Ireland in addition to European Centre for Disease Prevention and Contol (2014) and appears to be a global trend, though approximately 85% of respiratory tract infections are caused by viruses (Thomas and Bomar, 2022). Studies have found that most doctors felt they slightly overprescribed antibiotics, due mainly to satisfy patients expectations within public health sector, with the additional incentive to retain patients in private care settings, supported by (Lam and Lam, 2003; Barden *et al*., 1998). Additionally, pharmaceutical companies provide incentives to use their antibiotics. Some doctors report their reason for overprescribing antibiotics due to inadequate diagnostic facilities (Chandy *et al*., 2013).

7.9 AMR surveillance

Global AMR surveillance is currently lacking. While structured surveillance systems are in place across Europe, this is not the case for many countries, including many LMICs. From this study, high resistance was found across a range of medically crucial antibiotics. It

is essential to improve AMR surveillance to keep track of the prevalence of resistance across the world, to inform decision making for effective therapy choices and at the governmental level to re-evaluate the currently lacking enforcement of laws regarding antibiotic use. There are recent initiatives, such as the Fleming Fund which aims to provide the necessary equipment and implement / refurbish laboratories for clinical use across a range of LMICs to enable them to capture crucial data on AMR. However, this will take time to implement and get to a point where laboratories are producing consistent, reliable results. It is also important to ensure sustainability, that sites will be able to pay for consumables and staff costs long term.

7.10 Additional issues LMICs LMICs face additional issues regarding AMR. This study and other literature found higher rates of resistance against multiple medically important antibiotics, making neonatal sepsis harder to treat. Furthermore, many healthcare facilities are managing without microbiology facilities, so cannot confirm cases of sepsis and have no way of identifying common pathogens or associated resistance profiles, making treatment selection problematic. Further to this, hospitals may not cover the cost of all treatments and might often need to factor affordability into prescribed medication and may need to recommend sub-optimal treatments. Furthermore, patients may live far from healthcare facilities, causing delayed healthcare seeking. In these instances, patients may have waited until the illness was severe before seeking healthcare and may require more intensive treatment. LMICs have reduced levels of water, sanitation and hygiene (WASH), including reduced running water available and associated reduced levels of hand washing and flushing toilets, in addition to higher exposure to Enterobacteriaceae in the environment from deficient waste management infrastructure and contaminated water. This increases bacterial exposure members of communities, increasing rates of infection (Bartram and Cairncross, 2010). Unregulated agriculture adds to this problem, with run off into water sources and the

environment, alongside unregulated antibiotic usage will likely increase exposure to AMR bacteria. Studies have shown the need for improved WASH facilities for home births in communities in LMICs (Arowosegbe *et al*., 2021).

IPC protocols are insufficient in many healthcare facilities worldwide and implementing good IPC practices comes with its own challenges particularly in LMICs, where healthcare staff and hospital beds are overburdened with limited time and many hospitals lack IPC equipment, as they cannot afford it and some sites lack the expertise to put protocols into place. Besides a lack of funding for items, while visiting BARNARDS sites, staff discussed briefly that there is a possibility of these items, e.g. hand soaps being stolen. Additional measures could compensate for this, such as screwing items to walls but staff at some of these sites are also overburdened with numerous patients and understaffed. The implications of these circumstances are vast, with staff not having time to wash their hands, sterilise instruments appropriately and a lack of postnatal care. Patients were often discharged an hour following childbirth in some hospital sites due to overcrowding and beds are needed by women in labour. Additionally, on site visits, we witnessed many neonates sharing cribs and even resuscitaires due to a lack of space, money, and resources.

7.11 Concepts to reduce the burden of neonatal sepsis

While we cannot 'solve' AMR, there are actions that can be taken to reduce the prevalence of AMR or prevent levels increasing at the current rate. Many of these actions need to be undertaken at government level, but actions from hospital management, doctors and patients are also needed.

Government level

- Governmental bodies need to enforce laws to decrease corruption within the antibiotics markets to reduce the prevalence of substandard drugs. Substandard drugs may increase mortality, as they may not provide a large enough dose to kill the

pathogen, while also potentially increasing AMR, as the bacteria are being treated with a subtherapeutic dose.

- Enforce laws to prevent sales of antibiotics without prescriptions. This would reduce overuse/misuse of antibiotics by the community which would in turn reduce the prevalence of AMR
- Healthcare infrastructure should be reviewed to increase affordability (free or subsidised healthcare paid for by the government)
- Funders to step in and/or governments to fund necessary equipment, training and personnel to facilitate diagnostic microbiology that is sustainable long-term. This is crucial to allow confirm diagnoses, identify pathogens and determine appropriate treatments.
- Government to invest in infrastructure to enable more communities access to running water, to improve sanitation. This, however will be associated with many barriers, but something to consider for the future.
- Train home birth attendants appropriately and educate communities on the importance of having skilled birth assistants present. Or of the pros of giving birth in healthcare facilities with skilled staff, as this could potentially reduce infections in outborn patients.
- Better transport infrastructure or more healthcare facilities in rural areas

Hospital level

- Educate hospital staff on good IPC practices and fund the infrastructure to support this. Increasing education in IPC practices among hospital staff and funding for this to provide hand soap at stations etc. will be invaluable in decreasing sepsis rates and mortality in neonates in some sites in developing countries (Adams-Chapman and Stoll, 2002).

- Add in molecular support to reveal outbreaks and monitor in real-time.

Patient/ community level

- Educate local communities on good sanitation at home to prevent neonatal infection
- Educate communities on the true benefits and disadvantages of having a baby via C-sections before making this decision. We found those born via C-section were at higher risk of developing neonatal sepsis and so this may reduce rate of sepsis slightly.
- Educate expecting mothers/carers on the symptoms of neonatal sepsis, as early intervention will improve outcomes.

Large funding bodies, such as the UN/WHO should also step in to ensure affordability of antibiotic and subsidise these or make them free of charge so that healthcare is available for all.

7.12 Limitations

This study had multiple limitations. Outcomes were not always obtainable as neonates were sometimes lost to follow-up. This is an issue with observational studies, particularly within LMICs, where mothers may live far from clinical sites or have no phone for contacting. Neonatal bodyweight was not reported and estimated from gestational and postnatal age for PK/PD analysis. Regarding MICs, there were no EUCAST breakpoints for some antibiotic/bacteria combinations; we extrapolated breakpoints from similarly related species. FoR data was generated for single antibiotics only as it is problematic to choose concentrations for antibiotic combinations. Isolates selected for analysis of antibiotic therapy given with resistance profiles were based on a subset with available antibiotic data, only those treated with select common antibiotic combinations, potentially biasing the inclusion of neonates from sites with information on antibiotic usage and those that used these treatments more commonly. Differences in antibiotics used per site led to indistinguishable country vs

treatment effects for outcome due to major skews of antibiotics prescribed by certain countries, which will affect outcome in addition to treatment. Clinical data lacked severity of illness prior to treatment, which would have been good to account for when assessing neonatal mortality in combination with treatment and resistance of the pathogen.

The admissions demographic data included many overlapping categories that may have confused participants and was self-reported and therefore subject to potential bias. Significant results within overall analyses may be due to site variation and heterogeneity in responses between sites and the small sample size per site prevented site-specific analyses in addition to preventing analysis of associations according to place of birth. Reporting and collection of GPB was not consistent throughout the enrolment period or across all 12 participating sites and therefore the true burden of GPB neonatal sepsis may be underreported in this study and associations reported for GNB vs GPB may be subject to bias. Fourthly, collecting neonatal outcome up to 60 days was often challenging, and where neonatal data was lost to follow up, the last data point collected was used.

7.13 Conclusion

Rates of neonatal sepsis and mortality are much higher in LMICs than HICs, but this is likely underreported, due partly to insufficient record keeping and reporting systems, high proportions of home births, associated with delayed care seeking, due partly to cultural issues going into hospitals or a lack of funds to get healthcare preventing diagnosis. A lack of microbiology facilities in many LMIC healthcare facilities to confirm diagnosis is another reason why rates may be underreported. This study assessed risk factors for mortality in admissions neonates and we found associations between household hygiene factors highlighting the role of living environment and demographics in neonatal sepsis and mortality, in addition to associations between birth factors and mortality. Multiple outbreaks

were seen during the BARNARDS study, surely contributing to increased rates of neonatal sepsis and mortality, emphasising the importance of instilling proper IPC at sites.

Throughout this thesis, the lack of data from LMICs regarding the burden of neonatal sepsis, common pathogens and resistance profiles has been noted and subsequently empirical therapies are based on data from HICs. Different species from those reported from HICs were found to be common in BARNARDS, as supported by other studies, with lower rates of *Streptococcus* sp., but higher rates of *S. aureus* and GNB. High prevalence of antibiotic resistance was found in GNB isolates against multiple antibiotics, including high resistance against ampicillin and gentamicin, the WHO recommended first-line empirical therapy. Additionally, high levels of resistance against third generation cephalosporins, the WHO recommended second- line therapy for neonatal sepsis. Sites have begun using alternative antibiotics to treat neonatal sepsis and we found a wide range of treatments used throughout BARNARDS. However, due to the prevalence of AMR, treatment alternatives are dwindling, and some options were not available in all sites, or were simply unaffordable for the average patient, with many sites requiring patient funded treatment, or only covering certain, cheaper antibiotics.

Data from this study showed that ceftazidime and amikacin may be a viable alternative empirical therapy for neonatal sepsis in LMICs, due to its enhanced observed survival rate compared to other combinations, reduced FoR, lower cost and broader availability than other alternatives. However, the observed higher survival with neonates treated with ceftazidime could have been for a wide range of reasons, as antibiotic usage was uneven between countries, which could not be accounted for via mixed modelling analyses. Half of the Gram-negative isolates displayed resistance against ceftazidime, and amikacin was reported not to be available in Ethiopia. Meropenem retained low resistance and with no isolates developing resistance in FoR experiments. However, meropenem was shown to be

expensive and carbapenems are reserved as a last resort therapy and therefore should not be utilised as first-line treatment. Other antibiotics showed high resistance or FoR, were expensive or not broadly available, or are last resort antibiotics. An alternative empirical therapy regimen is needed for areas where ampicillin and gentamicin are nearly redundant, which must consider toxicity, cost and availability in addition to how quickly resistance may arise following increased use in addition to current resistance trends.

7.14 Lessons learned

Taking the limitations mentioned above in section 7.9 into account, multiple lessons were learned throughout the duration of the BARNARDS study. I would say as a team carrying out an international research project, the people at the sites you choose to work with can make or break your study. We were fortunate enough to work with excellent collaborators. I would say we also learned the value of meetings with sites to discuss progress in order to catch any issues or misunderstandings early on, this would have prevented outborn neonates without CDS from being enrolled. I wasn't part of the BARNARDS project when it began but joined soon thereafter. It is important to look at the big picture when designing a research project and ensure you are aware of potential routes that you would like the research to take before beginning a project. Bearing this in mind, all sepsis causing pathogens would have been sent to Cardiff University for WGS and we would have obtained a complete picture of sepsis causing pathogens across LMICs and more in depth clinical data regarding outcomes. Furthermore, it would have enabled us to consider collecting data relating to PK/PD modelling, such as weight of the neonate and dosing of antibiotics and to consult with appropriate experts before finalising protocols. The PK/PD findings were reasonably well aligned to predictions from dosing an in vitro MICs for most antibiotics except for AMP-GEN, which importantly reminded me that pathogens and antibiotics will react differently *in vitro* as they may *in vivo* with a plethora of molecules and cell from the immune system

affecting pathogen survival, in addition to potential formation of biofilms and stress responses in bacterial cells.

Despite substantial efforts from research nurses across clinical sites, it was difficult to obtain follow-up data for many neonates. It is therefore important to enrol more neonates than expected for statistically valid complete datasets, in addition to neonates with other missing data. During this project, I also learned the importance of consistent and efficient quality control of data throughout a project to prevent consistent errors and ensure quality data.

We learned a lot about variation of healthcare in LMICs, including facilities available and variation of antibiotic treatment options, making international comparisons of antibiotics more challenging. Lastly, I learned the difficulty of collating quality demographic data and it is important to ensure that questions asked to multiple patients will not be subjective and are phrased to be easily to interpret.

7.15 Further work

Further work would include analysis of genomes for mutations to explain resistance to antibiotics seen without associated ARG. IPC monitoring and enhanced environmental sampling with WGS should be carried out in parallel to monitoring sepsis so that potential routes of transmission can be determined for outbreaks. Cost effective IPC interventions can then be recommended. A more in depth look into antibiotic prescribing and usage will be helpful, as this data was collected retrospectively. It would be noteworthy to obtain data on all changes and reasons for changes, including whether antibiotics were changed based on: lack of availability; cost; in response to microbiology identification or AST results; due to lack of response to the neonate; or other. Survival analyses would be more applicable if higher numbers of neonates were included in analyses, with a mix of antibiotic therapies per site, so that antibiotic effects can be distinguished from site or country effects. Additionally,

further information on severity of illness in the neonate and additional compounding factors should be accounted for.

Further work is required in this field to determine the best empirical therapy option for neonates in LMICs. We carried out frequency of resistance experiments, it would be interesting to build on this, to incorporate more isolates and grow them for a longer time frame in addition to measuring the stability of resistance. Further to this, it would be interesting to sequence isolates before and after phenotypic resistance to examine whether any resistance genes were present before the phenotypic resistance appeared.

In addition to the PK/PD work carried out, it would be interesting to assess blood samples, for creatine levels and antibiotic levels following treatment of a neonate to fully assess the PK/PD of an antibiotic, along with additional information including dosing and neonatal birthweight. We are in the study design phase of BARNARDS II and hope to incorporate the above.

From the data generated in this study and multiple literature articles, it is clear that we need to look for alternatives to ampicillin and gentamicin at least in some LMICs. Clinical trial are eventually needed to fully assess potential alternatives. GARDP have started a new clinical trial of selected antibiotics and further assessments are needed.

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Appendix: Methods

Supplementary Table 1. Questionnaire asked to all enrolled mothers

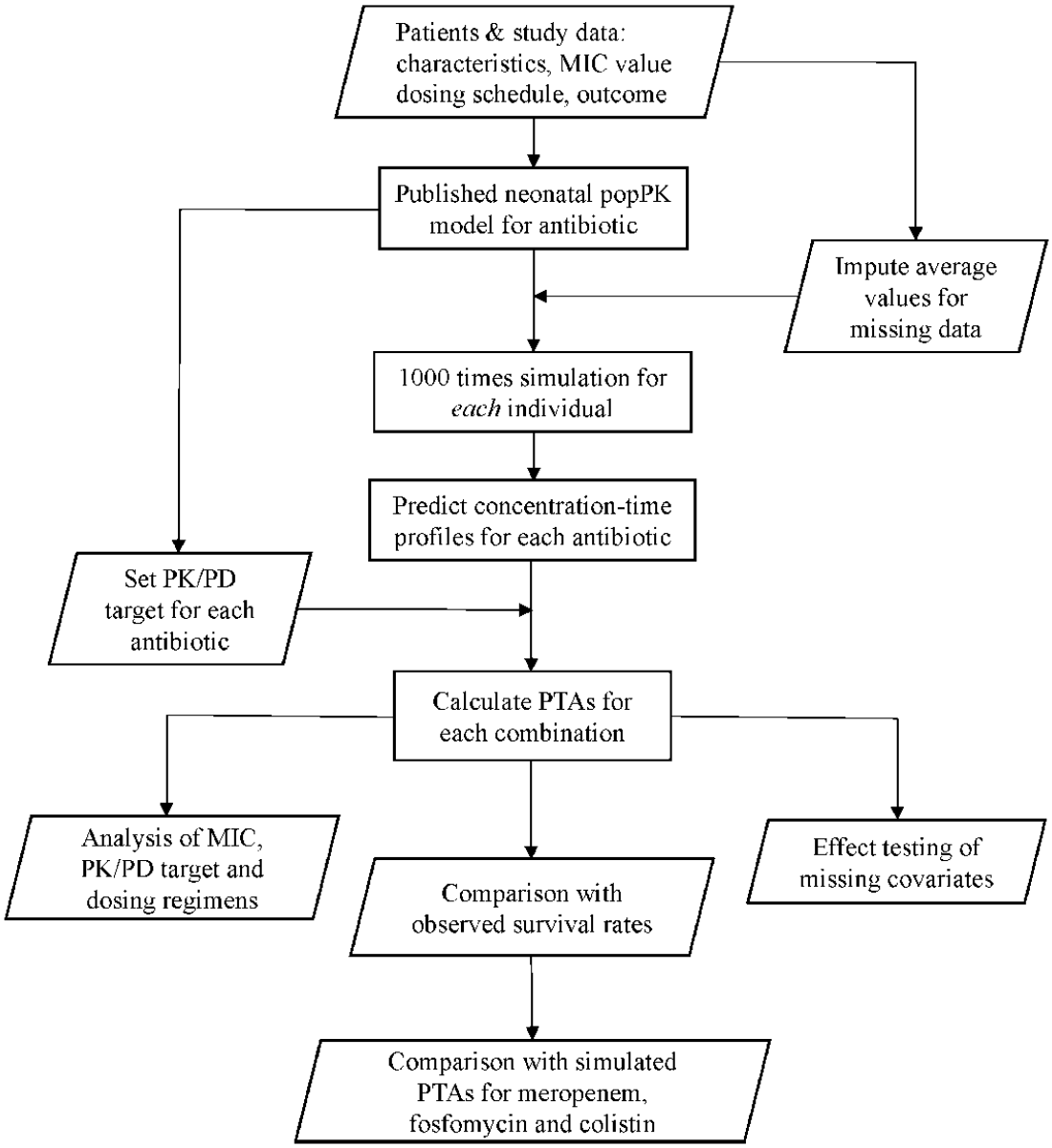
Question	**Responses**
Ward name / number	
Bed number / name	
Type of ward	Accident and emergency (A&E)
	Cardiology
	Critical care
	General surgery
	Gynaecology
	Maternity departments
	Neonatal unit
	Obstetrics and gynaecology units
	Sexual health (genitourinary medicine)
	Other
If other was selected, please specify	
Number of beds in this ward	
Number of bathrooms on this ward	
Where is the Mother situated on the ward?	First 1/3 (closest to door)
	Middle 1/3
	Last 1/3 (furthest from door)
	Side Room
	Other
If other was selected, please specify	
How many beds are in the side room?	
Age of the Mother in years (if not known please write "unknown" and an estimation)	
Date of birth of Mother if known	Date format: **DD/MM/YYYY**
Is this the Mothers first pregnancy?	Yes / No
Number of previous pregnancies	
Number, age and gender of living children	
Has the Mother had any multiple births?	No
	Twins (2)
	Triplets (3)
	Quadruplets (4)
	Quintuplets (5)
	Sextuplets (6)
	Septuplets (7)
	Octuplets (8)
	Nonuplets (9)
	Decuplets (10)
Please detail ages and genders:	
Number of miscarriages (option to bypass)	
Number of abortions (option to bypass)	
Number of stillbirths (option to bypass)	
Number of deceased children (option to bypass)	
Please provide details of deceased children, ages, gender, cause of death.	
In the past three months has the Mother suffered with any of these illnesses / conditions?	Diabetes
	Hypertension or cardiovascular disease
	Immune-compromised (Cancer, HIV, chronic liver diseases, use of steroids)
	TB

Question	Responses
	Malaria
	None
	Other
If other was selected, please specify	
Has the Mother received TB therapy?	Yes / No
In the three months prior to enrolment has the Mother attended a private healthcare clinic?	Yes / No
In the three months prior to enrolment has the Mother visited a traditional healer?	Yes / No
In the 12 months prior to enrolment has the mother travelled outside of the city / province / country?	Yes / No
If yes please specify where?	
In the 12 months prior to enrolment has a household member travelled outside the city / province / country?	Yes / No
Please specify where	
In the 12 months prior to enrolment has the Mother been hospitalised?	Yes / No
Details on reason, duration, hospital	
In the three months prior to enrolment has the Mother used antibiotics (Oral or IV)?	Yes / No
Name of antibiotics used	Amikacin, Amoxicillin, Ampicillin, Aztreonam, Azithromycin, Carbenicillin, Cefaclor, Cefadroxil (cefadroxyl), Cefalexin (cephalexin), Cefaloridine (cephaloradine), Cefamandole, Cefazolin (cephazolin), Cefditoren, Cefepime, Cefixime, Cefotaxime, Cefotetan, Cefoperazone, Cefoxitin, Cefpodoxime, Cefradine (cephradine), Ceftaroline, Ceftazidime, Ceftibuten, Ceftiolene, Ceftizoxime, Ceftobiprole, Ceftriaxone, Cefuroxime, Chloramphenicol, Ciprofloxacin, Clarithromycin, Clindamycin,Cycloserine, Doripenem, Doxycycline, Ertapenem, Erythromycin, Flucloxacillin, Fosfomycin, Gentamicin, Imipenem, Kanamycin, Levofloxacin, Lincomycin, Linezolid, Meropenem, Metronidazole, Minocycline, Moxifloxacin, Nalidixic acid, Neomycin, Nitrofurantoin, Norfloxacin, Ofloxacin, Oxacillin, Oxytetracycline, Penicillin G, Piperacillin, Polymyxin B, Pristinamycin, Quinupristin/dalfopristin, Rifabutin, Rifampin, Streptomycin, Sulfamethoxazole, Telithromycin, Teicoplanin, Tetracycline, Ticarcillin, Tigecycline, Tobramycin, Trimethoprim-Sulfamethoxazole, Vancomycin, Unknown, Other
If other was selected, please specify:	
Overall household income per month (For modelling purposes, we converted this to multiples above and below the local area average household income per month)	20
	$20 - $30
	$30 - $40
	$40 - $50
	$50 - $100
	$100 - $250
	$250 - $500
	$500 - $1000
	$1,000 - $2000
What is the educational status of the Mother?	None
	Can read and write

Question	Responses
	Primary school
	Secondary school
	College / A-Levels
	Undergraduate
	Graduate
	Postgraduate
How would the Mother describe the residential area she lives in	Rural
	Urban
	Semi-rural
	Other
If other was selected, please specify	
What type of residence does the Mother live in?	Apartment
	Separate house
	Shack
	Homeless
	Other
If other was selected, please specify	
Number of bedrooms in home	
Number of people residing in the home	
What is the primary source of drinking water for the household?	Municipal network
	Water vendor (tanker)
	Private well
	Communal taps
	Other
If other was selected, please specify	
Is the drinking water boiled, filtered or untreated?	Boiled
	Filtered
	Neither
What is the primary water source used for?	Domestic water
	Drinking water
	Both
How many hours per day does the household have running water?	Scale: No supply to >12 hours
	Access via communal taps
	Other
If other was selected, please specify	
How many days per week does the household have running water?	Scale: No supply to continuous
Is there a solid waste pile near the Mother's home (proximity of 100m)?	Yes / No
How frequently is the solid waste pile collected?	Once a week or more
	Every 2 weeks
	Every 2 months
	We deal with it ourselves
	Other
If other was selected, please specify	
What sort of toilet does the Mother have within her home?	Sit down with flush
	Squat with flush
	Pit latrine
	Other
If other was selected, please specify	

Question	Responses
Is there stagnant or sewerage water near the Mother's house (proximity of 100m)?	Yes / No
Is the house served by a wastewater network?	Yes / No
Generally, does the Mother have access to soap?	Yes / No / Sometimes
How many times per day does the Mother generally wash her hands?	Scale: <1 to >5
How many days per week does the Mother generally take a shower or bath?	Scale: <1 to everyday
How many hours per day does the household have an electricity supply?	Scale: No supply to continuous
How many days per week does the household have an electricity supply?	Scale: No supply to continuous
Infant DOB	Date format: **DD/MM/YYYY**
Time of birth	HH:MM
Place of birth	Hospital
	Healthcare Centre/Clinic
	Home
	Other
Infant age group at admission	Less than 7 days old
	More than 7 days old
Gestational status	Pre-term
	Term
	Post-date
	Unknown
Gestational age (weeks)	
Premature rupture of membranes	Yes / No
Please provide details if possible	
How many hours after the waters broke until delivery	
Was the baby delivered by Caesarean Section	Yes / No
Type of C-section	Emergency
	Planned
	Unknown
Was the birth assisted in any other way	Yes / No
Please provide details e.g. forcep delivery, episiotomy	
Was this a breech delivery?	Yes / No
Perinatal asphyxia	Yes / No
Infant gender	Female / Male
Any other information in relation to the birth? Gender Outcome / Comments	

Extended PK/PD methodology description



Supplementary Figure 1. An overview of the methodology utilised for pharmacokinetic/pharmacodynamic (PK/PD) modelling. MIC-Minimum inhibitory concentration.

Patient characteristics data

Two subsets of the data were used including either n=290 patients or n=476 patients. For both datasets we included only patients who received one of the four most commonly used antibiotic combinations, which included six antibiotics (ampicillin, amikacin, amoxicillin, ceftazidime, gentamicin and piperacillin). The dataset of n=290 patients only included patients who received a single combination treatment, whereas the larger dataset of n=476 also included patients who received another antibiotic (combination) regimen. Age, gender, patient-specific MIC, antibiotic dosing information, and study-site specific dosing schedules were collected.

Gestational and postnatal age assumptions

Missing gestational ages (GA) and postnatal ages (PNA) were imputed with the average age for preterm and no-preterm infants by sites. GAs for no-preterm infants were assumed to be either 40 weeks (on time) or 42 weeks (late). Postmenstrual ages (PMA) were calculated by PNA adding GA.

Estimation of body weight

Birth weight was estimated by ages and genders from a global reference (Eq. 1) (Mikolajcyk *et al.*, 2011). Ratios of global and local estimates were calculated to predict the region-specific birth weight. Postnatal weight was estimated based on a growth standard for preterm from Lancet (Eq. 2) (Villar *et al.*, 2015) and a WHO standard for no-preterm (Eq. 3-4) with a shifted ratio between the estimated birthweight by Lancet and WHO standard by sites (WHO, 2009). Average birth and postnatal weight were imputed for patients whose gender information were missing.

Estimation for serum creatinine and serum albumin

Serum creatinine (SCR) was also computed using ages which are based on a reference for no-preterm infants (Eq. 5) (Boer *et al.*, 2010). Detected creatinine values were adopted for preterm newborns younger than 1 month (Cuzzolin et al., 2006). For preterm infants older than 1 month, the reference for no-preterm infants was used after adjusting by a ratio of no-preterm and preterm values at day 28. Plasma albumin (ALB) was also estimated by an age-related equation for children (Eq. 6) (Johnson et al., 2006).

$$Birthweight(g) = exp(0.578 + 0.332 \times GA - 0.00354 \times GA^2) \times Ratio (Eq. 1)$$

$$Weight(kg) = exp(2.591 - 0.012 \times PMA^{0.5} - 2201.705 \times PMA^{-2} + 0.091 \times Sex)$$
$$Sex = 1 formale, 0 for female (Eq. 2)$$

$$Formale: weight(kg) = BCPE(x = Age^{0.35}, df(\mu) = 11, df(\sigma) = 7, df(v) = 4, \tau = 2) * Ratio (Eq. 3)$$

$$For female: weight(kg) = BCPE(x = Age^{0.35}, df(\mu) = 11, df(\sigma) = 7, df(v) = 3, \tau = 2) * Ratio (Eq. 4)$$

$$SCR(^{\mu mol}/_{L}) = 10^{(1.75 - 0.07 \times log_2(Age)} \times Ratio (Eq. 5)$$

$$ALB(^{g}/_{L}) = 1.1287 \times ln(Age) + 33.746 (Eq. 6)$$

MIC data handling

For MIC values recorded at the boundaries of their possible values, we assumed the next-lowest or next-highest dilution. In case of missing MIC data we performed random sampling based on the empirical MIC density.

PK/PD simulations to calculate target attainment for commonly used antibiotics

For the six antibiotics studied (ampicillin, amikacin, amoxicillin, ceftazidime, gentamicin and piperacillin) we selected published neonatal population pharmacokinetics (PK) models (Li *et al*., 2013; Fuchs *et al*., 2014; Tang *et al*., 2019; De Cock *et al*., 2012; Tremoulet *et al.*, 2014; Wang *et al*., 2018). Using the population PK models, for each individual we performed 1,000 simulations based on their patient characteristics including MIC and specific dosing schedule used, using the R package RxODE. As PK/PD target we used 50% fT>MIC and Cmax/MIC>8 were used as PK/PD targets for beta-lactam (70% for ceftazidime) (Lodise *et al.*, 2006) and aminoglycosides (Bland *et al*., 2018), respectively. Subsequently, the percentage of PK/PD target attainment (PTA) for each combination by site was computed, determining if at least one of the targets in a combination was reached. PTAs ≥80% was applied as an index of efficacy and 100 times of above simulations were implement to get the distribution of PTAs ≥80%. The final PTAs ≥80%, shown in mean ± SD, for these four combinations were compared with the actual survival rate for each combination in this study. For calculating the PTA per country to assess differences in dosing schedules we used the n=476 patients dataset because here we aimed to identify the impact of differences in dosing schedules used in different countries. For all other PTA calculations we used the single treatment n=290 dataset.

Sensitivity analyses

We performed sensitivity analyses to test the effect of assumptions made regarding missing sex for PTA calculations of all 4 combinations. We also tested the effect of ignoring the effect of co-administration of ibuprofen or dopamine was a covariate for population PK models for gentamicin or amoxicillin. We used a t-test to determine if PTA values were statistically different.

PK/PD simulations for meropenem, fosfomycin and colistin monotherapies

We performed PK/PD simulations for meropenem, fosfomycin and colistin, following the same steps as described for the other antibiotics. For meropenem, a published population PK model in neonates (Smith *et al*., 2011) and a recommended dosage of 10mg/kg every 8h were chosen for simulation (Du *et al*., 2006). 50% fT>MIC were used as the PK/PD target, assuming an approximate protein binding of 2% (Lodise *et al*., 2006; Smith *et al.*, 2011). For fosfomycin and colistin, pharmacokinetics parameter (e.g. clearance and distribution volume)

were based on previous studies (Guggenbichler *et al*., 1978; Molina *et al*., 1977; Nakwan *et al*., 2016). Estimates for inter-individual variability were also included (Nakwan *et al.* 2016; Parker *et al*., 2015. The fraction of colistin methate sodium (CMS) converted to colistin was based on a preclinical study (Landersdorfer *et al*., 2017). The maximum recommended dose regimens were selected for simulation (Traunmuller *et al*., 2011; Nation *et al*., 2016). A 40%fT>MIC with 3% protein binding rate (Lepak *et al.*, 2017; Kirby, 1977) and the average steady state concentration (Css,avg) >2.0mg/L (Ooi *et al*., 2019) were chosen as PK/PD targets for fosfomycin and colistin, respectively.

Supplementary Table 2. Dosing regimens used for simulations in pharmacokinetic/pharmocodynamic modelling.

Meropenem	**Fosfomycin**	**Colistin**
10 mg/kg every 8 h	200 mg/kg every 12h	5 mg/kg per day

Supplementary Table 3. sequence accession numbers for isolates included in virulence factor analysis.

Species	Isolate ID	ENA accession
Escherichia coli	BC-BB312-I	ERS5229805 (SAMEA7472110)
Escherichia coli	BC-BB322-I	ERS5229806 (SAMEA7472111)
Escherichia coli	ESS-BB0379a-I1	ERS5229993 (SAMEA7472298)
Escherichia coli	ESS-BB0140-I1	ERS5229975 (SAMEA7472280)
Escherichia coli	ESS-BB0187-I1	ERS5229979 (SAMEA7472284)
Escherichia coli	NK-BB1367-I	ERS5230076 (SAMEA7472381)
Escherichia coli	NN-BB187-I	ERS5230180 (SAMEA7472485)
Escherichia coli	NN-BB499-I	ERS5230199 (SAMEA7472504)
Escherichia coli	PP-BB2700-I	ERS5230320 (SAMEA7472625)
Escherichia coli	PP-BB2812-I	ERS5230322 (SAMEA7472627)
Escherichia coli	PP-BB5340-I	ERS5230408 (SAMEA7472714)
Escherichia coli	RK-BB62-I	ERS5230567 (SAMEA7472873)
Escherichia coli	RK-BB103-I	ERS5230533 (SAMEA7472839)
Escherichia coli	RK-BB111-I	ERS5230537 (SAMEA7472843)
Escherichia coli	RK-BB1384-I	ERS5230541 (SAMEA7472847)
Escherichia coli	RK-BB1495-I	ERS5230544 (SAMEA7472850)
Escherichia coli	RK-BB2246-I	ERS5230555 (SAMEA7472861)
Escherichia coli	RK-BB91-I	ERS5230584 (SAMEA7472890)
Escherichia coli	RK-BB973-I	ERS5230587 (SAMEA7472893)
Escherichia coli	RU-BB339-I	ERS5230598 (SAMEA7472904)
Escherichia coli	ZAT-BB1448-I1	ERS5230618 (SAMEA7472924)
Escherichia coli	ZAT-BB279-I3	ERS5230636 (SAMEA7472942)
Klebsiella pneumoniae	BC-BB1210-I	ERS5229750 (SAMEA7472055)
Klebsiella pneumoniae	BC-BB1228-I	ERS5229751 (SAMEA7472056)
Klebsiella pneumoniae	BC-BB1283-I	ERS5229755 (SAMEA7472060)
Klebsiella pneumoniae	BC-BB296-I	ERS5229802 (SAMEA7472107)
Klebsiella pneumoniae	BC-BB980-I	ERS5229897 (SAMEA7472202)
Klebsiella pneumoniae	ESI-BB0616-I2	ERS5229913 (SAMEA7472218)
Klebsiella pneumoniae	ESI-BB1044b-I1	ERS5229920 (SAMEA7472225)
Klebsiella pneumoniae	ESI-BB1341a-I1	ERS5229924 (SAMEA7472229)
Klebsiella pneumoniae	ESI-BB1344b-I1	ERS5229926 (SAMEA7472231)
Klebsiella pneumoniae	ESI-BB1384a-I1	ERS5229927 (SAMEA7472232)
Klebsiella pneumoniae	ESO-BB1839b-I1	ERS5229954 (SAMEA7472259)
Klebsiella pneumoniae	ESO-BB2005-I1	ERS5229958 (SAMEA7472263)
Klebsiella pneumoniae	ESS-BB0547a-I1	ERS5230029 (SAMEA7472334)
Klebsiella pneumoniae	ESS-BB0139-I1	ERS5229974 (SAMEA7472279)
Klebsiella pneumoniae	ESI-BB1691-I1	ERS5229940 (SAMEA7472245)
Klebsiella pneumoniae	ESO-BB1839a-I1	ERS5229953 (SAMEA7472258)
Klebsiella pneumoniae	ESS-BB0259-I1	ERS5229982 (SAMEA7472287)
Klebsiella pneumoniae	ESS-BB0304a-I1	ERS5229987 (SAMEA7472292)
Klebsiella pneumoniae	ESS-BB0383-I1	ERS5229995 (SAMEA7472300)
Klebsiella pneumoniae	ESS-BB0405-I1	ERS5229997 (SAMEA7472302)
Klebsiella pneumoniae	ESS-BB0460b-I1	ERS5230015 (SAMEA7472320)

Klebsiella pneumoniae	ESS-BB0463-I2	ERS5230016 (SAMEA7472321)
Klebsiella pneumoniae	ESS-BB0482a-I1	ERS5230018 (SAMEA7472323)
Klebsiella pneumoniae	ESS-BB0490-I1	ERS5230020 (SAMEA7472325)
Klebsiella pneumoniae	ESS-BB0490-I2	ERS5230021 (SAMEA7472326)
Klebsiella pneumoniae	ESS-BB0515a-I1	ERS5230024 (SAMEA7472329)
Klebsiella pneumoniae	ESS-BB0531a-I1	ERS5230027 (SAMEA7472332)
Klebsiella pneumoniae	NK-BB1145-I	ERS5230067 (SAMEA7472372)
Klebsiella pneumoniae	NK-BB1495-I	ERS5230088 (SAMEA7472393)
Klebsiella pneumoniae	NN-BB1542r1-I1	ERS5230132 (SAMEA7472437)
Klebsiella pneumoniae	NN-BB1647-I	ERS5230157 (SAMEA7472462)
Klebsiella pneumoniae	NN-BB169-I	ERS5230165 (SAMEA7472470)
Klebsiella pneumoniae	NN-BB170-I	ERS5230168 (SAMEA7472473)
Klebsiella pneumoniae	NN-BB1721-I	ERS5230172 (SAMEA7472477)
Klebsiella pneumoniae	NN-BB455-I	ERS5230193 (SAMEA7472498)
Klebsiella pneumoniae	NN-BB492r1-I	ERS5230198 (SAMEA7472503)
Klebsiella pneumoniae	NW-BB182ar1-I	ERS5230210 (SAMEA7472515)
Klebsiella pneumoniae	PC-BB31-I	ERS5230217 (SAMEA7472522)
Klebsiella pneumoniae	PC-BB456-I	ERS5230223 (SAMEA7472528)
Klebsiella pneumoniae	PP-BB1935-I	ERS5230279 (SAMEA7472584)
Klebsiella pneumoniae	PP-BB2093-I	ERS5230293 (SAMEA7472598)
Klebsiella pneumoniae	PP-BB2859-I	ERS5230327 (SAMEA7472632)
Klebsiella pneumoniae	PP-BB6586-I	ERS5230453 (SAMEA7472759)
Klebsiella pneumoniae	RK-BB721b-I	ERS5230573 (SAMEA7472879)
Klebsiella pneumoniae	RK-BB1216-I	ERS5230539 (SAMEA7472845)
Klebsiella pneumoniae	RK-BB1813-I	ERS5230548 (SAMEA7472854)
Klebsiella pneumoniae	RK-BB866-I	ERS5230582 (SAMEA7472888)
Klebsiella pneumoniae	RU-BB193-I	ERS5230592 (SAMEA7472898)
Klebsiella pneumoniae	RU-BB284-I	ERS5230596 (SAMEA7472902)
Klebsiella pneumoniae	RU-BB487-I	ERS5230602 (SAMEA7472908)
Klebsiella pneumoniae	ZAT-BB14-I1	ERS5230617 (SAMEA7472923)
Klebsiella pneumoniae	ZAT-BB1262-I4	ERS5230615 (SAMEA7472921)
Klebsiella pneumoniae	ZAT-BB175-I2	ERS5230622 (SAMEA7472928)
Klebsiella pneumoniae	ZAT-BB1830-I1	ERS5230624 (SAMEA7472930)
Klebsiella pneumoniae	ZAT-BB514b-I1	ERS5230641 (SAMEA7472947)

Supplementary Table 3 cont. Sequence accession numbers for isolates included in virulence factor analysis.

Species	**Isolate ID**	**ENA accession**
Staphylococcus aureus	BC-BB1562-I	ERS5229022 (SAMEA7471326)
Staphylococcus aureus	BC-BB991-I	ERS5229026 (SAMEA7471330)
Staphylococcus aureus	ESS-BB0162-I1	ERS5229029 (SAMEA7471333)
Staphylococcus aureus	NK-BB1278-I	ERS5229033 (SAMEA7471337)
Staphylococcus aureus	NK-BB2412-I	ERS5229042 (SAMEA7471346)
Staphylococcus aureus	NN-BB129-I	ERS5229047 (SAMEA7471351)
Staphylococcus aureus	NN-BB1591-I	ERS5229049 (SAMEA7471353)
Staphylococcus aureus	NN-BB1604r1-I	ERS5229050 (SAMEA7471354)
Staphylococcus aureus	NN-BB1727a-I	ERS5229052 (SAMEA7471356)
Staphylococcus aureus	NN-BB1782-I	ERS5229054 (SAMEA7471358)
Staphylococcus aureus	NN-BB651-I	ERS5229055 (SAMEA7471359)
Staphylococcus aureus	PC-BB354b-I1	ERS5229056 (SAMEA7471360)
Staphylococcus aureus	PC-BB356-I	ERS5229057 (SAMEA7471361)
Staphylococcus aureus	PC-BB442-I5	ERS5229058 (SAMEA7471362)
Staphylococcus aureus	PC-BB486-I2	ERS5229059 (SAMEA7471363)
Staphylococcus aureus	PP-BB2079-I	ERS5229063 (SAMEA7471367)
Staphylococcus aureus	PP-BB3938-I	ERS5229071 (SAMEA7471375)
Staphylococcus aureus	PP-BB3956-I	ERS5229072 (SAMEA7471376)
Staphylococcus aureus	PP-BB4507-I	ERS5229073 (SAMEA7471377)
Staphylococcus aureus	PP-BB4614-I	ERS5229075 (SAMEA7471379)
Staphylococcus aureus	PP-BB5936-I	ERS5229084 (SAMEA7471388)
Staphylococcus aureus	PP-BB6944-I	ERS5229093 (SAMEA7471397)
Staphylococcus aureus	PP-BB7632-I	ERS5229095 (SAMEA7471399)
Staphylococcus aureus	PP-BB7955-I	ERS5229096 (SAMEA7471400)
Staphylococcus aureus	PP-BB8010-I	ERS5229097 (SAMEA7471401)
Staphylococcus aureus	PP-BB8048-I	ERS5229099 (SAMEA7471403)
Staphylococcus aureus	PP-BB8061-I	ERS5229100 (SAMEA7471404)
Staphylococcus aureus	RK-BB2000-I	ERS5229102 (SAMEA7471406)
Staphylococcus aureus	ZAT-BB1262-I1	ERS5229103 (SAMEA7471407)
Staphylococcus aureus	ZAT-BB138-I1	ERS5229104 (SAMEA7471408)
Staphylococcus aureus	ZAT-BB2180a-I1	ERS5229109 (SAMEA7471413)
Staphylococcus aureus	ZAT-BB2710-I1	ERS5229113 (SAMEA7471417)
Staphylococcus aureus	ZAT-BB326b-I1	ERS5229115 (SAMEA7471419)

Questionnaire asked to site principal investigators (PIs) regarding antibiotic therapy

All sites have an experienced clinical neonatologist and microbiologist. The questionnaire was completed via consultation between their staff and the local pharmacy department. In some countries such as Nigeria, there is a different between state and federal funding which was also captured in the income levels. Income levels was self-reporting data by the mothers but was ratified by the site PIs who have extensive local knowledge on income levels etc.

Name of person completing form ……
Date……

Job role……..

Name of hospital……

Country …….

Region……

1.0 What is the estimated monthly prevalence of clinical diagnosis for neonatal sepsis?

……
……..

2.0 Do you have the necessary equipment to perform blood cultures? (Y/N – Indicate if this is on site or off site facilities if yes)

……
……..

2.1 If yes, what is the estimated monthly prevalence of positive blood cultures for neonatal sepsis?

……
……..

3.0 Please complete the table below:

	Do you have access to the following antibiotics for treatment of neonatal sepsis? (Y/N)	What are the dosing recommendations? (IV, mg/Kg, Interval (Hours)	Estimated cost per dose? ($)
Example	Y	IV, 7.5, 12	5
Ampicillin			
Gentamicin			
Ceftazidime			
Piperacillin/Tazobactam			
Amikacin			
Amoxicillin			
Fosfomycin			
Tigecycline			
Colistin			

4.0 What is the primary empirical treatment for neonatal sepsis at the facility?

……
……...

5.0 What is the common recommended second line of treatment for neonatal sepsis?

……..

6.0 Which causative pathogens for neonatal sepsis are of most concern at the facility? (Delete as appropriate)
Klebsiella pneumoniae/Staphylococcus aureas/ Escherichia coli/

7.0 What is the estimated average monthly neonatal morbidity from sepsis?

…….

8.0 What is the estimated average total cost for stay, treatment and administration for a neonate with suspected clinical sepsis? ($ per 24 hours?)

……..

9.0 How much of the total average cost is invoiced to the patient at the facility? ($ per 24 hours)

……

10.0 Are the cost of antibiotics included in the figure above? (Y/N)

…….

11.0 Are the patients at the facility charged prescription fees? (If yes- please state amount $)

……

12.0 What is the average weekly income for the immediate area the facility serves?

…….

13.0 Is the facility public/private/part private (delete as appropriate)

Thank you for taking the time to complete this survey.

Sensitivity analyses

Supplementary Table 4. Mortality% associated with different antibiotic therapies (n=476), comparison with untraceable neonates removed from analysis. Mortality increased slightly when untraceable neonates were removed, due to the lower denominator, but similar p values between treatments were found for both analyses.

Antibiotic combination	All neonates		Untraceable neonates removed	
	Total number	Mortality %	Total number	Mortality%
AMP-GEN	111	16.2	96	18.8
AMC-AMK	78	24.4	78	24.4
CTZ-AMK	172	9.3	138	11.6
PIP-TAZ-AMK	115	27.8	90	35.6

X^2 all: N=476, X^2=18.825, df=3, p<0.001
X^2 untraceable neonates removed: N=402, X^2=19.573, df=3, p<0.001

Supplementary Table 5. Mortality% associated with different therapies for empirical dataset only, comparison with untraceable neonates removed from analysis. Mortality increased slightly when untraceable neonates were removed, due to the lower denominator, but similar p values between treatments were found for both analyses.

Antibiotic combination	All neonates		Untraceable neonates removed	
	Total number	Mortality %	Total number	Mortality%
AMP-GEN	78	10.3	72	11.1
AMC-AMK	27	29.6	27	29.6
CTZ-AMK	109	8.3	90	10.0
PIP-TAZ-AMK	76	22.4	58	29.3

X^2 all: N=290, X^2=13.354, df=3, p=0.004
X^2 untraceable neonates removed: N=247, X^2=14.174, df=3, p=0.003

Supplementary Table 6. Chi-square (X^2) statistical results for minimum inhibitory concentration (MIC) vs outcome empirical therapy compared to repeated analysis with untraceable neonates removed. Df=degrees of freedom. Similar p values were found for both analyses.

Antibiotic combination	All neonates				Untraceable neonates removed			
	Number	X^2	Df	P value	Number	X^2	Df	P-value
AMP-GEN	76	0.804	2	0.669	70	0.718	2	0.698
AMC-AMK	NA -No untraceable neonates							
CTZ-AMK	107	2.818	3	0.421	89	3.162	3	0.367
PIP/TAZ-AMK	76	5.465	3	0.145	58	6.391	3	0.094

Supplementary Table 7. Mann-Whitney U test results for association of pathogenicity index and outcome, compared to repeated analysis with untraceable neonates removed. Similar p-values were found for both analyses.

Dataset	All			Untraceable neonates removed		
	Number	Mann Whitney U test statistic	P value (exact)	Number	Mann Whitney U test statistic	P-value (exact)
E. coli	22	33.000	0.837	20	30.000	0.892
K. pneumoniae	55	178.500	0.517	46	189.000	0.549
S. aureus	33	113.000	0.954	28	91.000	1.000

Supplementary Table 8. Mann-Whitney U test results for association of virulence factors and outcome, compared to repeated analysis with untraceable neonates removed. Similar p values were found for both analyses.

Dataset	All			Untraceable neonates removed		
	Number	Mann Whitney U test statistic	P value	Number	Mann Whitney U test statistic	P-value
E. coli	22	12.500	0.042	20	9.500	0.029
K. pneumoniae	55	188.000	0.663	46	173.000	0.870
S. aureus	33	128.000	0.630	28	108.000	0.408

Results

Intrinsic resistance

Overall, colistin resistance was present in 29.11% of isolatesfrom all countries involved in BARNARDS displaying except for Ethiopia. Bangladesh had the highest rate of colistin resistance followed by Nigeria and Pakistan with a higher rate of colistin resistance in Asia than in Africa. However, these analyses included bacteria, such as *Serratia* spp. which made up a large proportion of resistant isolates, which are intrinsically resistant to colistin. Resistance profiles were reanalysed discounting any bacteria that were found to be intrinsically resistant to the antibiotics tested (Supplementary Table 9, Supplementary Figure 2).

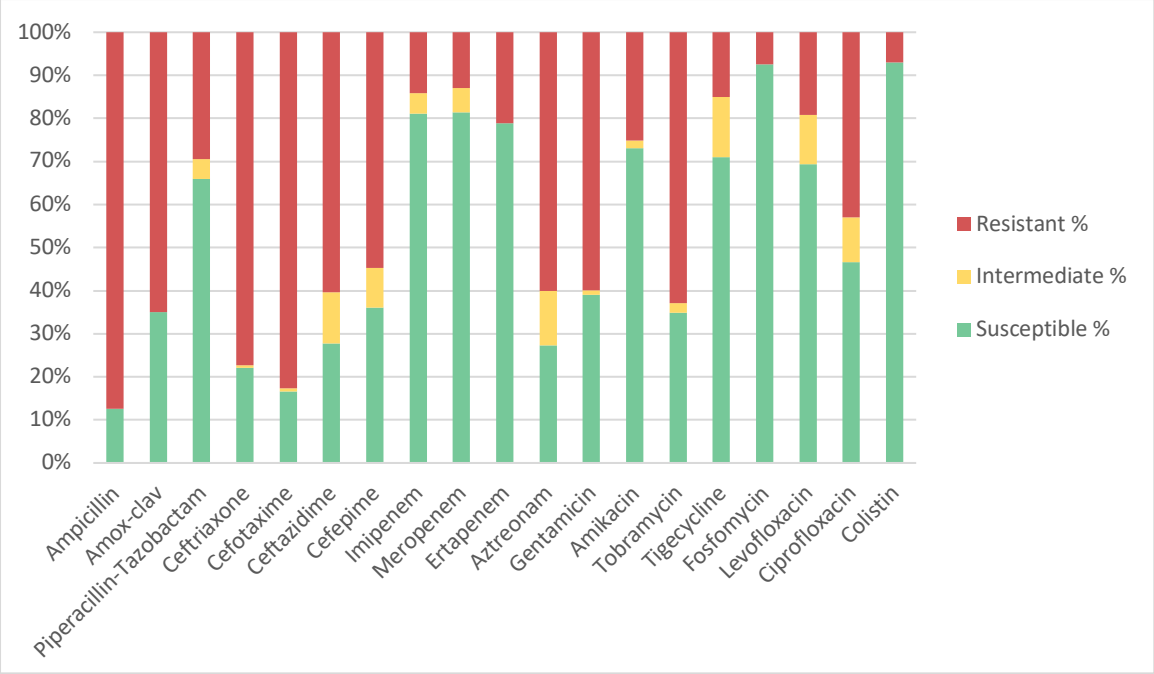
Supplementary Table 9. Bacteria analysed in BARNARDS that are considered intrinsically resistant to each antibiotic tested and number overall of each species included in BARNARDS sepsis isolates. Information sourced from [1]EUCAST, Intrinsic Resistance and Unusual Phenotypes version 3.2, February 2020.

Antibiotic	Intrinsically resistant species	Number overall
Ampicillin	*Enterobacter* cloacae complex	78
	Citrobacter spp.[1]	5
	Klebsiella spp.	395
	Pseudomonas spp.	32
	Ralstonia mannitolytica	17
	Acinetobacter spp.	47
	Raoultella ornithinolytica	1
	Serratia marcescens	149
	Aeromonas sp.	3
	Achromobacter xylosoxidans	1
	Burkholderia cepacia complex	59
	Stenotrophomonas maltophilia	3
Amoxicillin-clavulanate	*Acinetobacter* spp.	47
	Enterobacter clocae complex	78
	Aeromonas sp.	3
	Citrobacter freundii	3
	Pseudomonas spp.	32
	Serratia marcescens	149
	Burkholderia cepacia complex	59
	Stenotrophomonas maltophilia	3
Piperacillin-tazobactam	*Burkholderia cepacia* complex	59
	Stenotrophomonas maltophilia	3
Ceftriaxone Cefotaxime	*Acinetobacter* spp.	47
	Achromobacter xylosoxidans	1
	Burkholderia cepacia complex	59
	Pseudomonas sp.	32
	Ralstonia mannitolytica	17

Imipenem	*Stenotrophomonas maltophilia*	3
Meropenem	*Stenotrophomonas maltophilia*	3
Ertapenem	*Acinetobacter* spp. *Achromobacter xylosoxidans* *Burkholderia cepacia* complex *Pseudomonas* sp. *Ralstonia mannitolytica* *Stenotrophomonas maltophilia*	47 1 59 32 17 3
Aztreonam	*Acinetobacter* spp. *Burkholderia cepacia* complex *Stenotrophomonas maltophilia*	47 59 3
Gentamicin	*Burkholderia cepacia* complex *Stenotrophomonas maltophilia*	59 3
Amikacin	*Burkholderia cepacia* complex *Stenotrophomonas maltophilia*	59 3
Tigecycline	*Pseudomonas* sp. *Ralstonia mannitolytica*	32 17
Fosfomycin	*Acinetobacter* spp. *Burkholderia cepacia* complex *Stenotrophomonas maltophilia*	47 59 3
Ciprofloxacin	*Burkholderia cepacia* complex	59
Colistin	*Morganella morganii* *Proteus mirabilis* *Serratia marcescens* *Burkholderia cepacia* complex	1 3 149 59
Ceftazidime Cefepime Tobramycin Levofloxacin	NA	-
Minocycline	NA (no resistant breakpoint)	-

[1]*Pseudomonas* spp. were included as intrinsically resistant when EUCAST guidelines stated *Pseudomonas aeruginosa*

[2]*Rastonia mannitolytica* was also included as intrinsically resistant when EUCAST stated *P. aeruginosa* was intrinsically resistant.

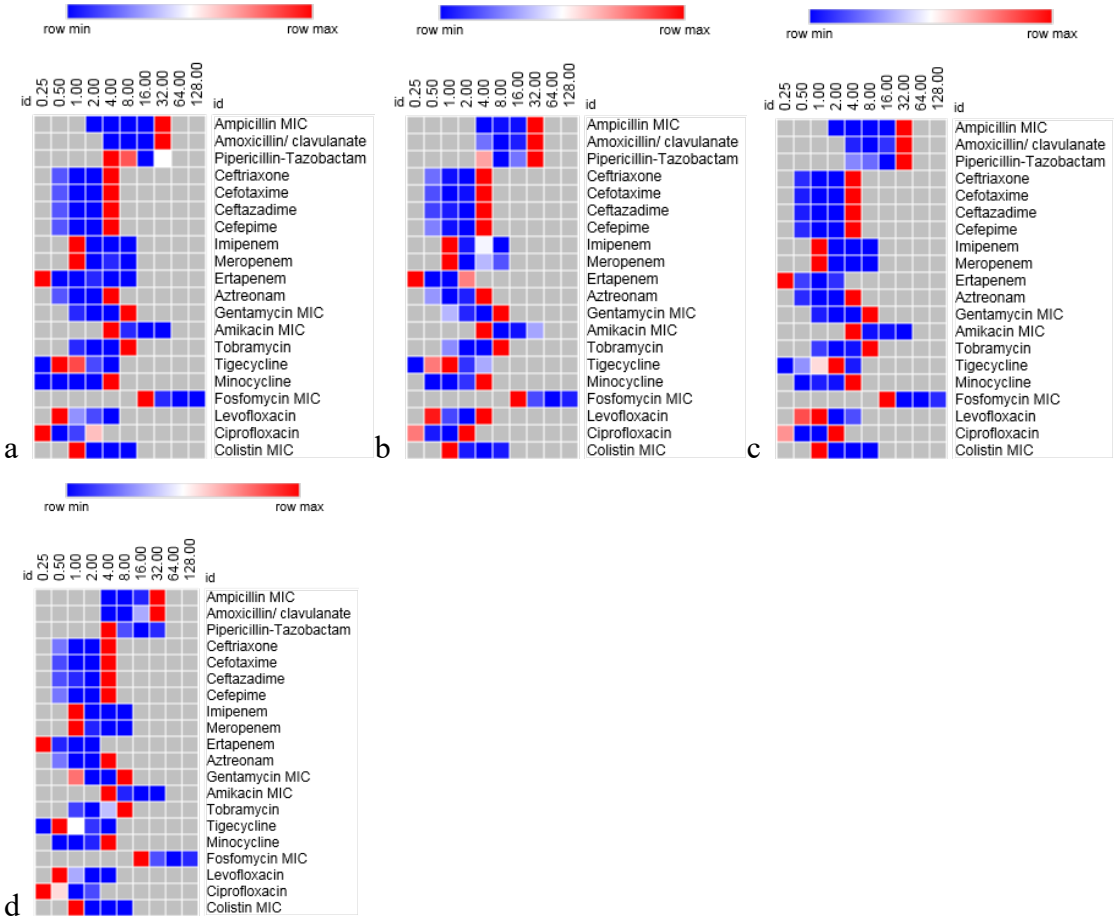


Supplementary Figure 2. Resistance profiles re-analysed excluding all bacterial isolates with intrinsic resistance according to EUCAST v9.0. Red sections show isolates exhibiting resistance, yellow those requiring increased exposure and green susceptible isolates.

When resistance against colistin was reanalysed excluding intrinsically resistant bacteria, resistance was lower at 7.07%. *Serratia marcescens* (n=149) was commonly found in cases of sepsis, particularly within Bangladesh (n=120), where an outbreak of *Serratia marcescens* occurred during the timeframe of the study. A high rate of resistance against ampicillin remained when the large number of intrinsically resistant isolates were removed (n=185/206, 89.32%). Despite the occurrence of intrinsically resistant bacteria, results prior to and after this section display resistance inclusive of intrinsic and acquired resistance, to showcase numbers of isolates antibiotics will be ineffective against, but we have explored the impact of intrinsic resistance here.

K. pneumoniae Africa MICs

MICs were similar between countries in Africa, although there was some variation, particularly regarding resistance to carbapenems. Piperacillin-tazobactam MICs were lower in South Africa, ciprofloxacin MICs were higher in Nigeria and Ethiopia. Carbapenem MICs were higher for Nigeria, as was amikacin.

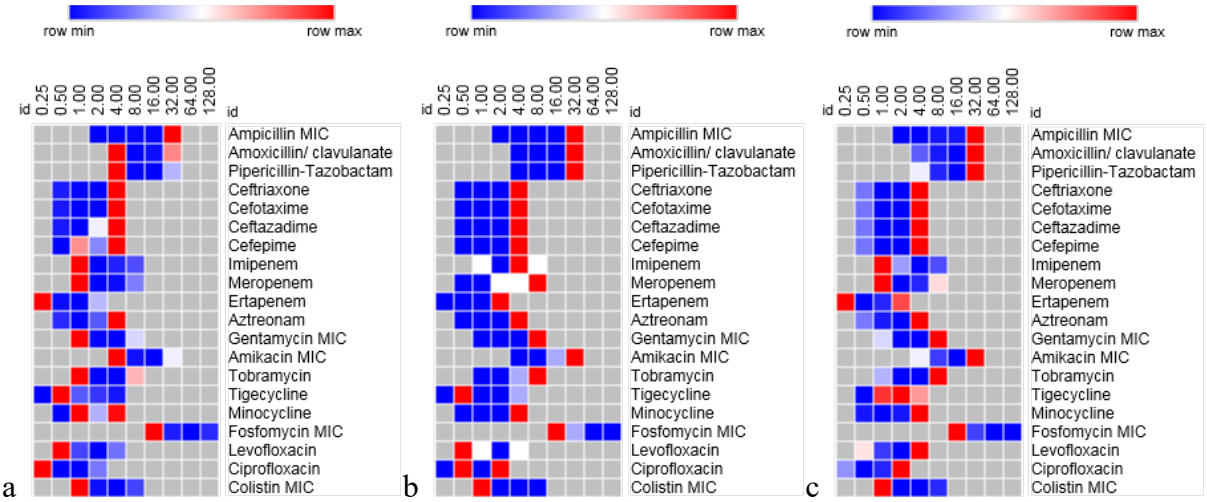


Supplementary Figure 3. Minimum inhibitory concentration (MIC) heatmaps for K. pneumoniae isolates per countries in Africa. a) Rwanda; b) Nigeria; c) Ethiopia; d) South Africa. Red displays highest numbers of isolates that had a certain MIC and dark blue the lowest, as shown by the key.

Klebsiella pneumoniae isolates from sites in Rwanda, South Africa and Ethiopia showed complete resistance to ampicillin, with Nigeria showing nearly complete resistance to ampicillin. Carbapenem resistance was found to be reasonably low in *K. pneumoniae* isolates from three countries in Africa, but high for isolates from Nigeria, which had resistance levels of approximately 40% against the carbapenems. No resistance against colistin was seen in Rwanda, South Africa or Ethiopia, with only 4.4% resistance occurring in isolates from Nigeria. High resistance was seen against extended spectrum cephalosporins with all sites showcasing over 85% resistance to these.

K. pneumoniae South Asia MICs

From sites in South Asia, there were 67 *K. pneumoniae* isolates, 61 of which were MDR. MICs for amoxicillin-clavulanate, piperacillin-tazobactam, cefepime, levofloxacin and ciprofloxacin were lower in Pakistan. MICs for the three carbapenems were highest in India. Tigecycline MICs were highest in Bangladesh. (Supplementary Figure 4).

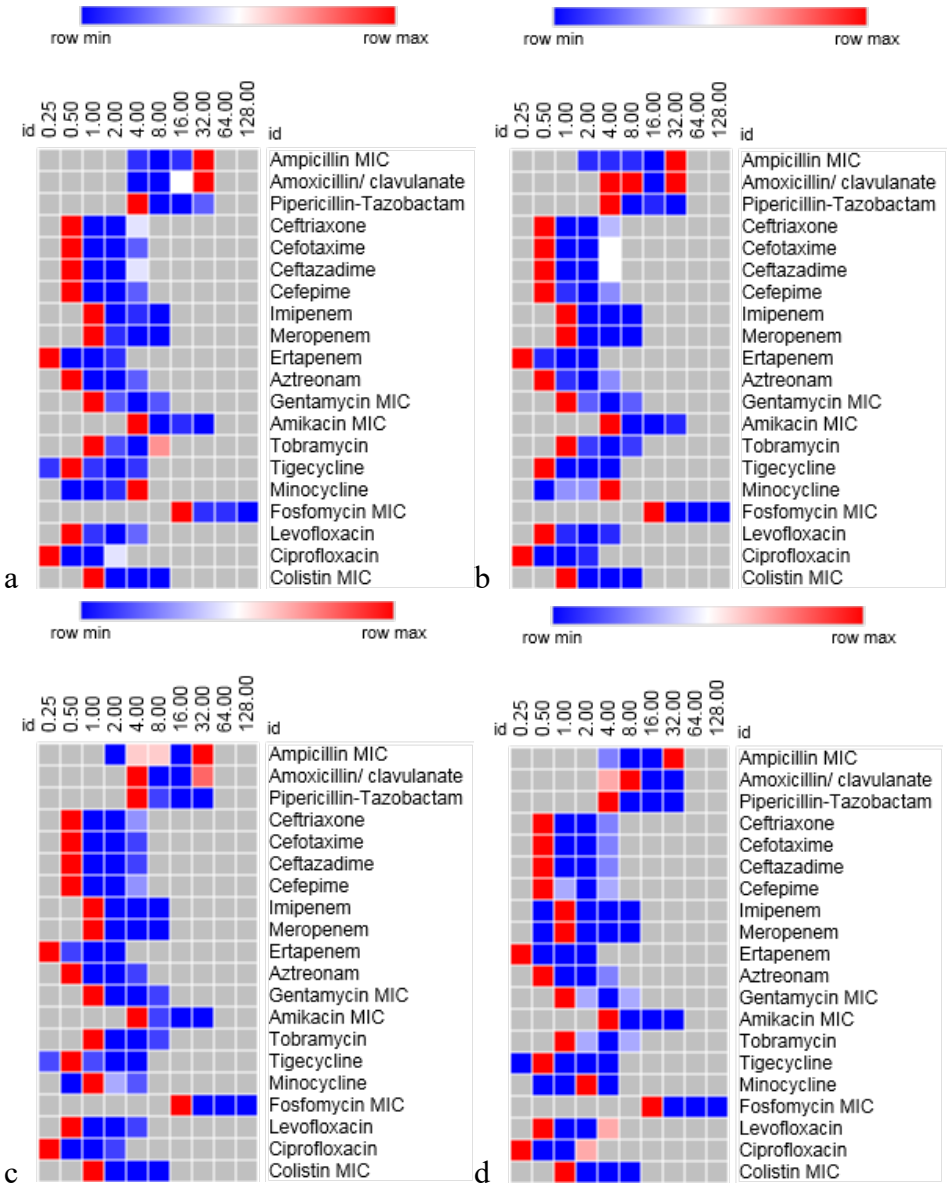


Supplementary Figure 4Minimum inhibitory concentration (MIC) heatmaps for Klebsiella pneumoniae isolates from South Asia a) Pakistan; b) India; c) Bangladesh. Red displays highest numbers of isolates that had a certain MIC and dark blue the lowest, as shown by the key.

More variation was seen between countries in South Asia compared to those within Africa regarding resistance profiles for *Klebsiella* sp. isolates. As with isolates from Africa, complete or high resistance to ampicillin was found (98.51%). High resistance was seen against the carbapenems tested in *Klebsiella pneumoniae* isolates from Asia (32.84% for imipenem and meropenem and 65.67% resistance against ertapenem), and was particularly high in India, with 75% resistance against imipenem and meropenem and complete resistance against Ertapenem. Resistance was also high against cephalosporins tested (92.54% ceftriaxone, 94.03% cefotaxime, 88.06% ceftazidime, 85.07% cefepime). Colistin resistance was found to be 5.97% in *K. pneumoniae* isolates from Asia, with two isolates from BC and two from PP. *K. pneumoniae* isolates from India showed a concerning total resistance against a range of antibiotics including all penicillins (and B-lactamase inhibitor combinations); 3G cephalosporins; and ertapenem.

E. coli MICs Africa

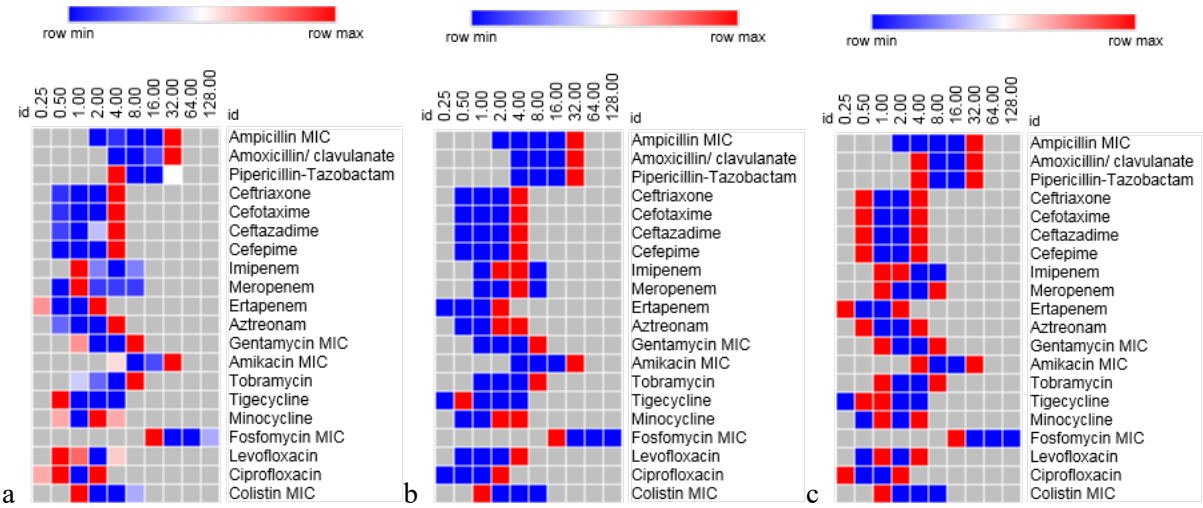
Most *E. coli* isolates analysed were from sites in Africa (n=56/74), with ten isolates from ESS, 15 from NK, seven from NN, one from NW, 15 from RK, two from RU and six from ZAT. Resistance to ampicillin was reduced to 76.79%, which was still witnessed in all sites in Africa, but lowest in Ethiopia (40.00%). A lower level of resistance to third generation cephalosporins was found in isolates from Africa (28.57% ceftriaxone, 23.21% cefotaxime, 21.43% ceftazidime) and cefepime (12.50%), resistance present at low levels in all sites. No resistance was found against imipenem or meropenem from sites in Africa, and only 1.79% resistance against ertapenem, with only one resistant isolate from RK. Additionally, only one isolate from NK was resistant to amikacin (1.79%) (Supplementary Figure 5).



Supplementary Figure 5. . Minimum inhibitory concentration (MIC) heatmaps for E. coli isolates from South Asia. a) Rwanda; b) Nigeria; c) Ethiopia; d) South Africa. Red displays highest numbers of isolates that had a certain MIC and dark blue the lowest, as shown by the key.

E. coli MICs South Asia

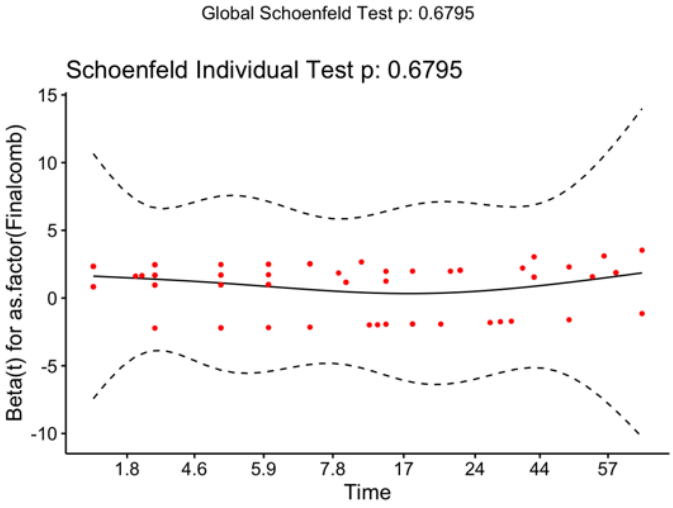
Fewer *E. coli* isolates were found to cause sepsis in South Asia, with 18 isolates analysed, including three isolates from BC, two from IN, three from PC and ten from PP. *E. coli* isolates from sites in South Asia showed some level of resistance to all antibiotics tested, except for fosfomycin and colistin, with no isolates demonstrating resistance (Supplementary Figure 6).



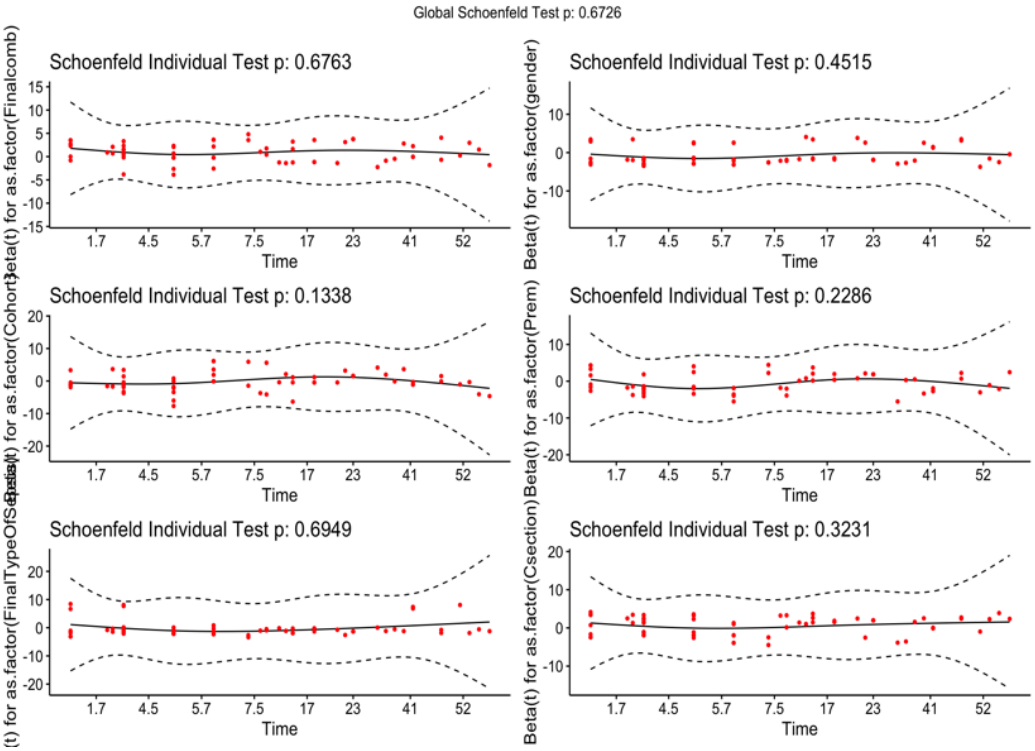
Supplementary Figure 6. Minimum inhibitory concentration (MIC) heatmaps from E. coli isolates from sites in South Asia: a) Pakistan; b) India; c) Bangladesh. Red displays highest numbers of isolates that had a certain MIC and dark blue the lowest, as shown by the key.

Over half of the *E. coli* isolates tested showed resistance against ampicillin (94.44%), amoxicillin-clavulanate (61.11%), ceftriaxone (83.33%), cefotaxime (88.89%), ceftazidime (72.22%), cefepime (66.67%), aztreonam (61.11%) and levofloxacin (66.67%).

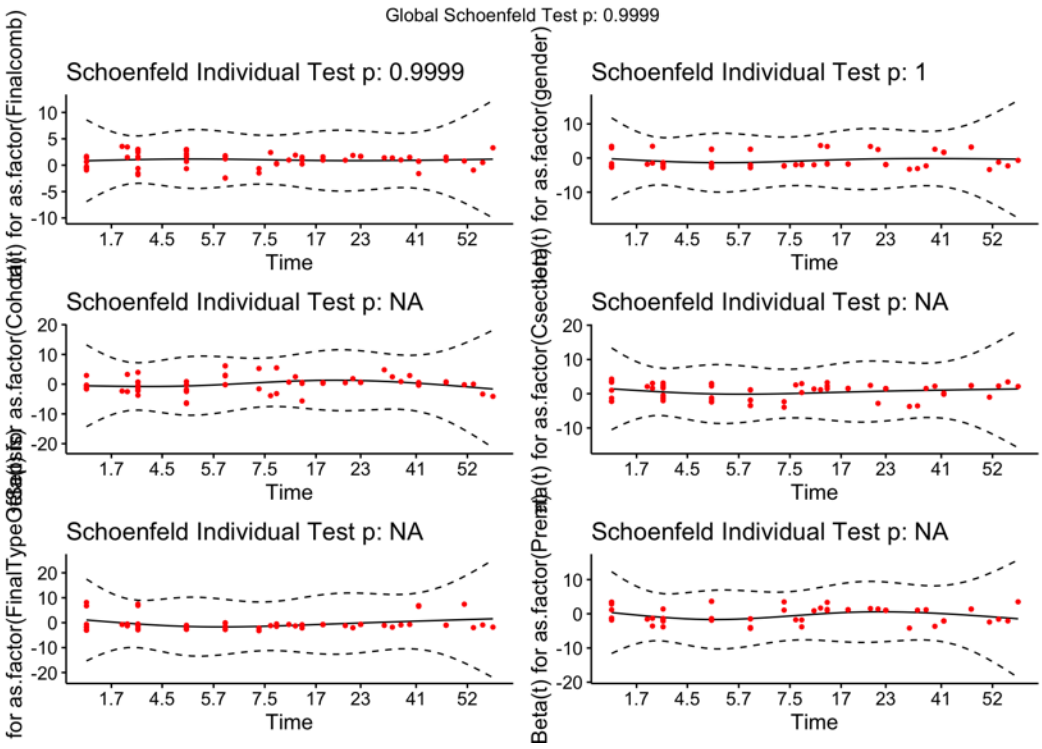
Pakistan *E. coli* isolates (n=13) displayed broad resistance, with >90% resistance against ampicillin (92.31%) and third generation cephalosporins (92.31% ceftriaxone and cefotaxime, 69.23% ceftazidime). Only two *E. coli* isolates from India were analysed, although they both demonstrated complete resistance against most antibiotics with the exceptions of imipenem, meropenem, aztreonam, tigecycline, fosfomycin and colistin. Only three isolates were analysed from Bangladesh, demonstrating at least one resistant isolate against most antibiotics except for meropenem, fosfomycin and colistin and complete resistance against ampicillin. However, resistance profiles for *E. coli* from these two countries should not be relied upon, due to the small number of isolates, therefore there are not enough results to extrapolate to a wider scale.



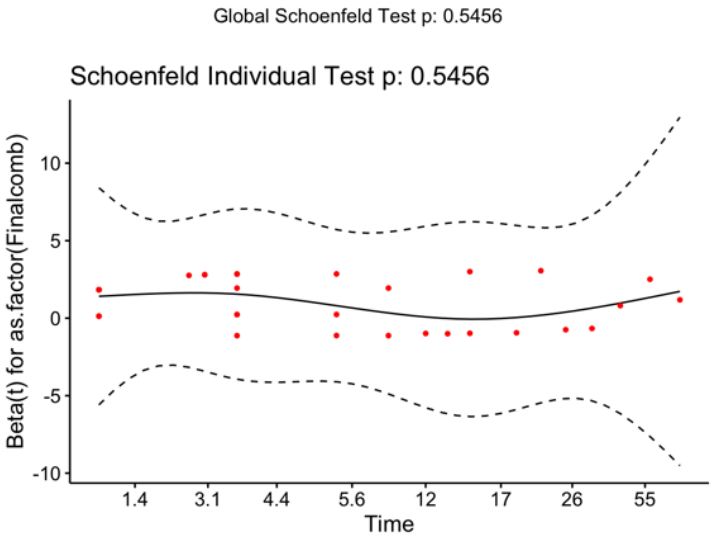
Supplementary Figure 7. Schoenfeld residual plot for unadjusted cox regression for overall subset of 476 neonates for hazard with the final combination coefficient. The 95% confidence interval is shown as a dotted line.



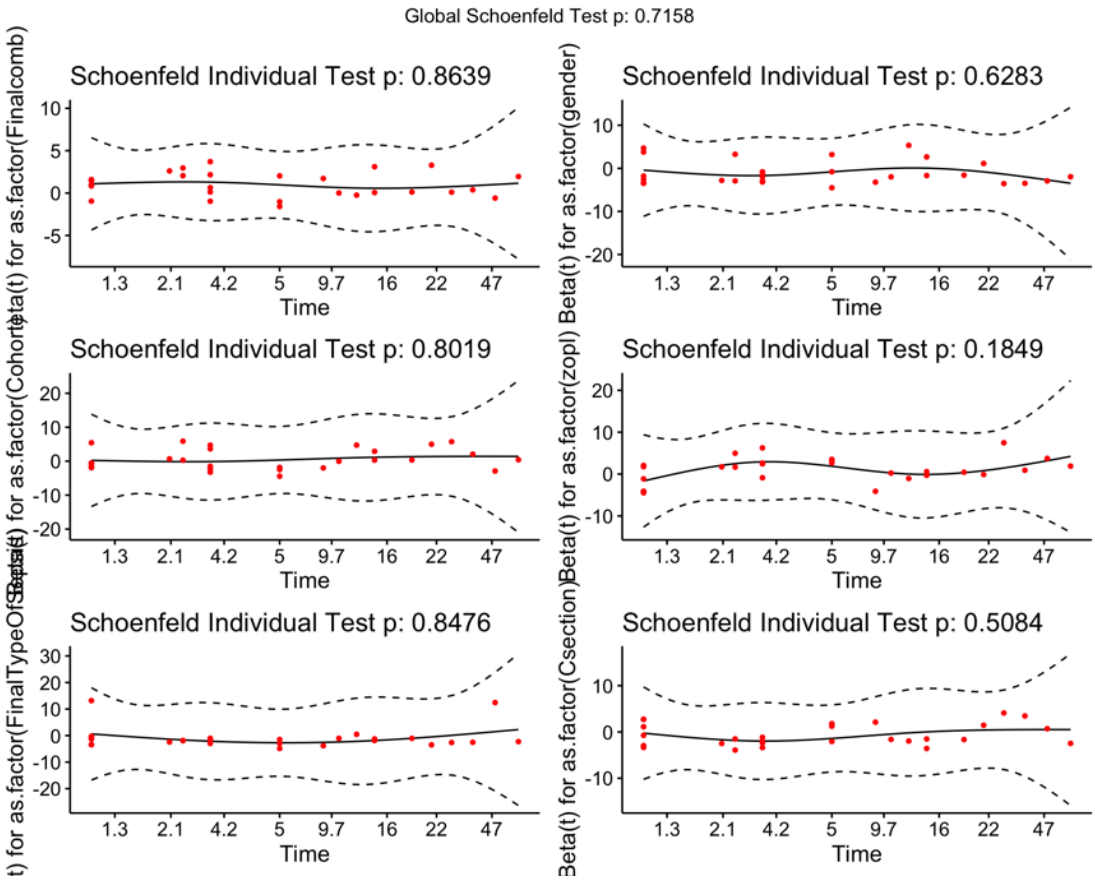
Supplementary Figure 8. Schoenfeld residual plots for adjusted Cox proportional hazard regression models carried out on the overall subset, n=476. Type of sepsis as early onset or late onset sepsis (EOS/LOS) was stratified within the adjusted model to ensure that Cox proportional hazard assumptions were met.



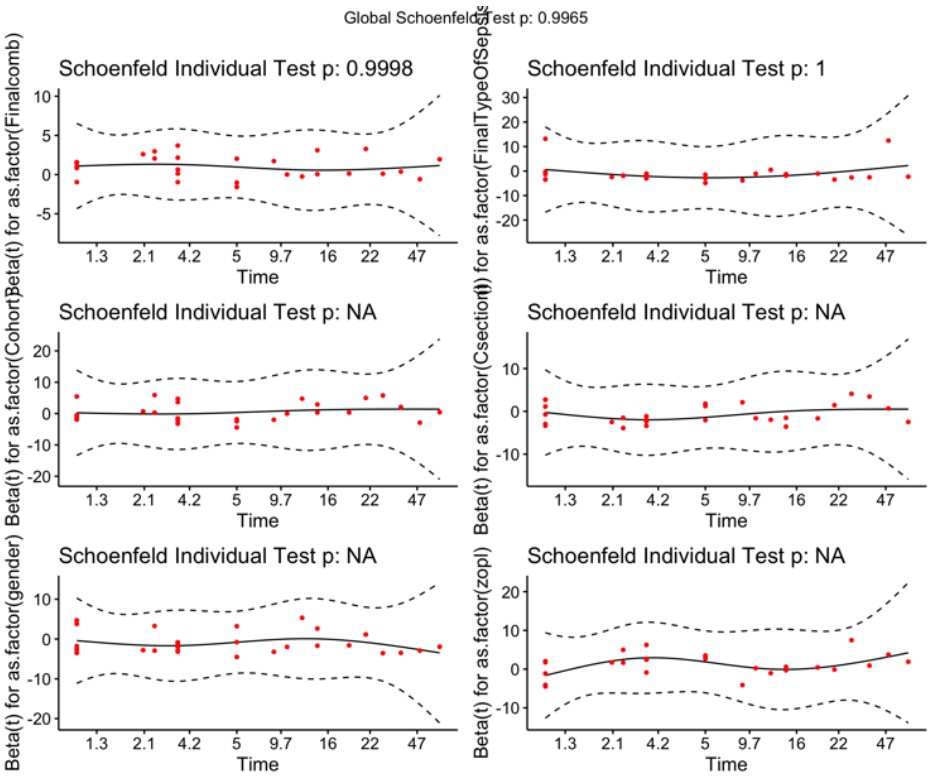
Supplementary Figure 9. Mixed-effect model: Proportional Hazards assumptions displayed NA for multiple variables, showing that the mixed-effect model with country incorporated as a random effect did not fit the data well, due to the dispersion of the data between countries.



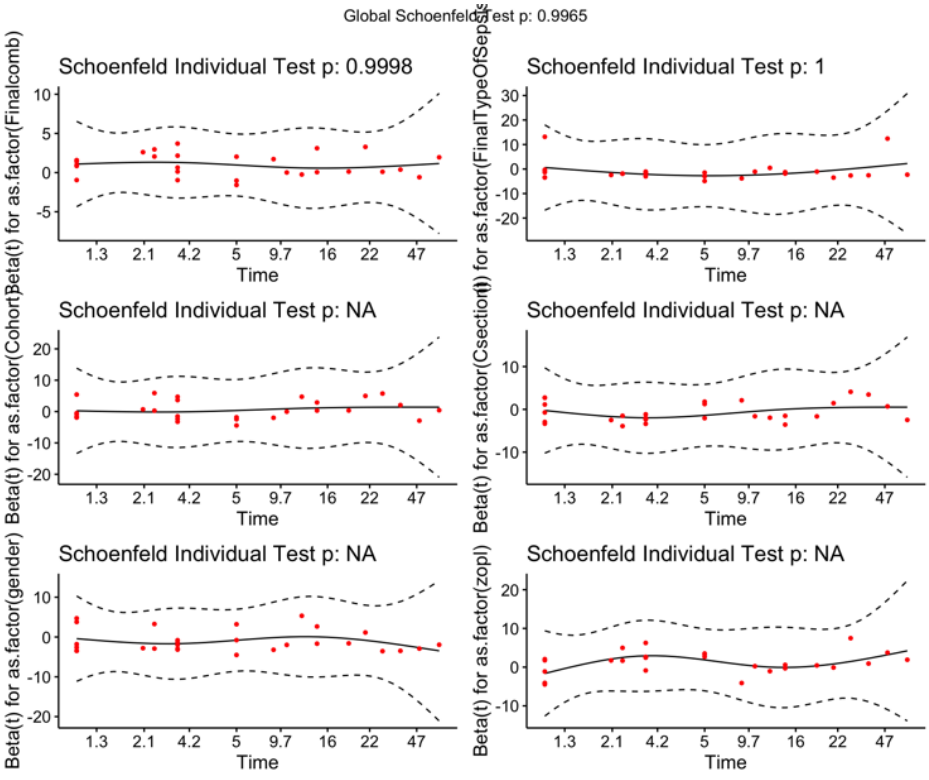
Supplementary Figure 10. Schoenfeld residual plots for unadjusted Cox regression carried out on the empirical treatment only subset of neonates (n=290).



Supplementary Figure 11. Schoenfeld residual plots for adjusted Cox proportional hazard regression models carried out on n=290. Type of sepsis early onset/late onset (EOS/LOS) was stratified within the adjusted model to ensure that Cox proportional hazard assumptions were met.



Supplementary Figure 12. Mixed-effect model Schoenfeld residual plots displayed NA for multiple variables in the mixed-effect model with country incorporated as a random effect, showing that this model did not fit the data well, due to the skewed dispersion of the antibiotics used between countries.



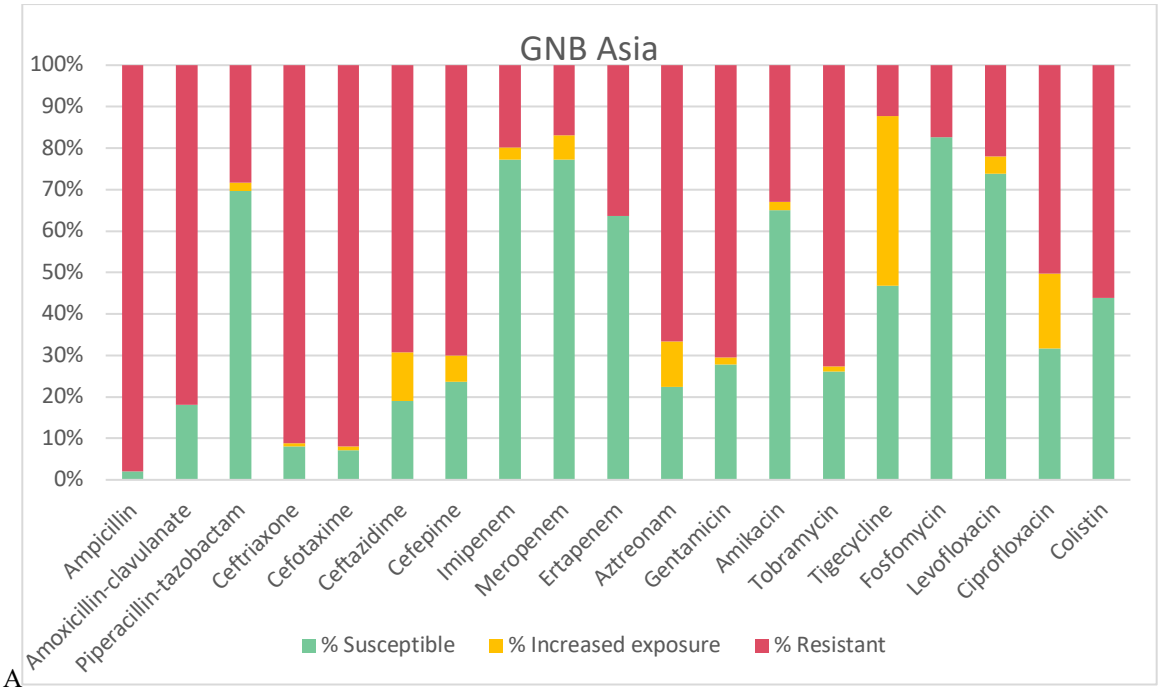
Supplementary Figure 13. Mixed-effect model Schoenfeld residual plots displayed NA for multiple variables in the mixed-effect model with country incorporated as a random effect, showing that this model did not fit the data well, due to the skewed dispersion of the antibiotics used between countries.

Supplementary Table 4. MIC_{50} and MIC_{90} results from minimum inhibitory concentration (MIC) testing for Gram-negative isolates included in this study (n=401). Highest concentration tested on all isolates input.

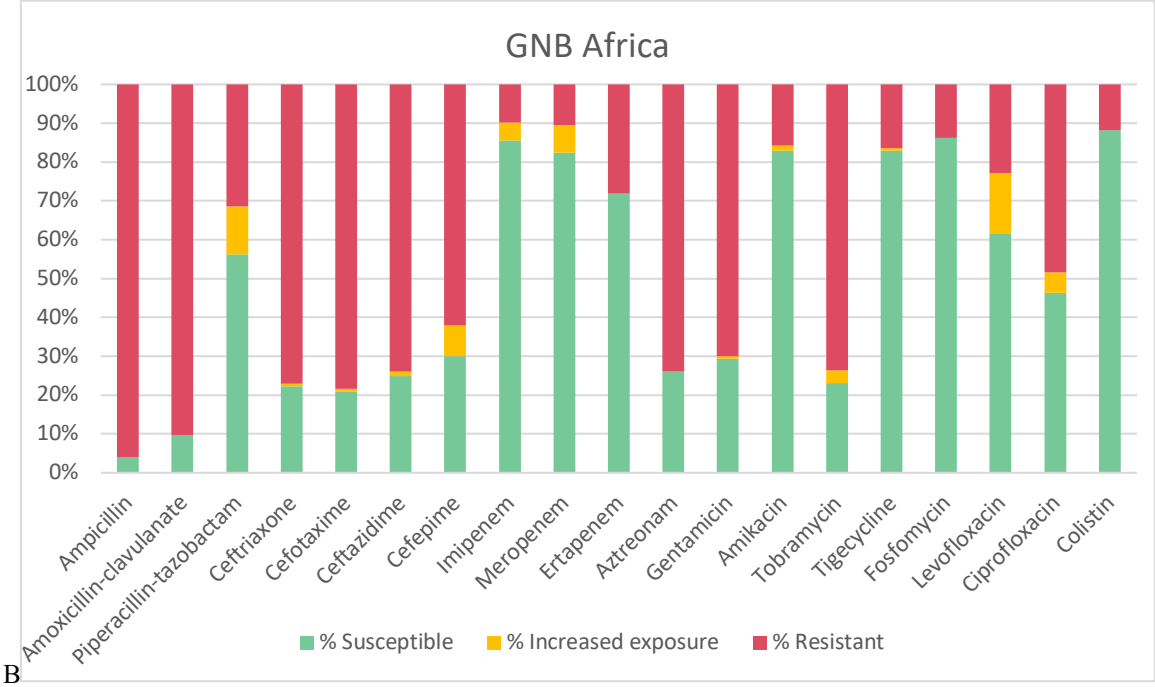
Antibiotic	**MIC_{50} (µg/mL)**	**MIC_{90} (µg/mL)**
Ampicillin	>32	>32
Amoxicillin-clavulanate	>32	>32
Piperacillin-tazobactam	4	>32
Ceftriaxone	>4	>4
Cefotaxime	>4	>4
Ceftazidime	>4	>4
Cefepime	>4	>4
Imipenem	1	>8
Meropenem	1	>8
Ertapenem	0.25	>2
Aztreonam*	>4	>4
Gentamicin	>8	>8
Amikacin	4	>32
Tobramycin	>8	>8
Tigecycline	1	2
Minocycline	4	>4
Fosfomycin	16	64
Levofloxacin	0.5	>4
Ciprofloxacin	0.5	>2
Colistin	1	>8

Supplementary Table 5. MIC_{50} and MIC_{90} results from minimum inhibitory concentration (MIC) testing for Gram-positive isolates tested that were included in this study (n=56). Highest concentration tested on all isolates input.

Antibiotic	**MIC_{50} (µg/mL)**	**MIC_{90} (µg/mL)**
Ampicillin	4	64
Oxacillin	2	>8
Flucloxacin	1	>8
Levofloxacin	0.5	>4
Ciprofloxacin	0.5	>4
Gentamicin	0.5	>4
Amikacin	4	8
Tobramycin	0.5	>4
Tigecycline	0.25	0.5
Minocycline	0.25	1
Rifampicin	0.03	0.03
Vancomycin	1	2
Azithromycin	4	>8
Linezolid	2	4

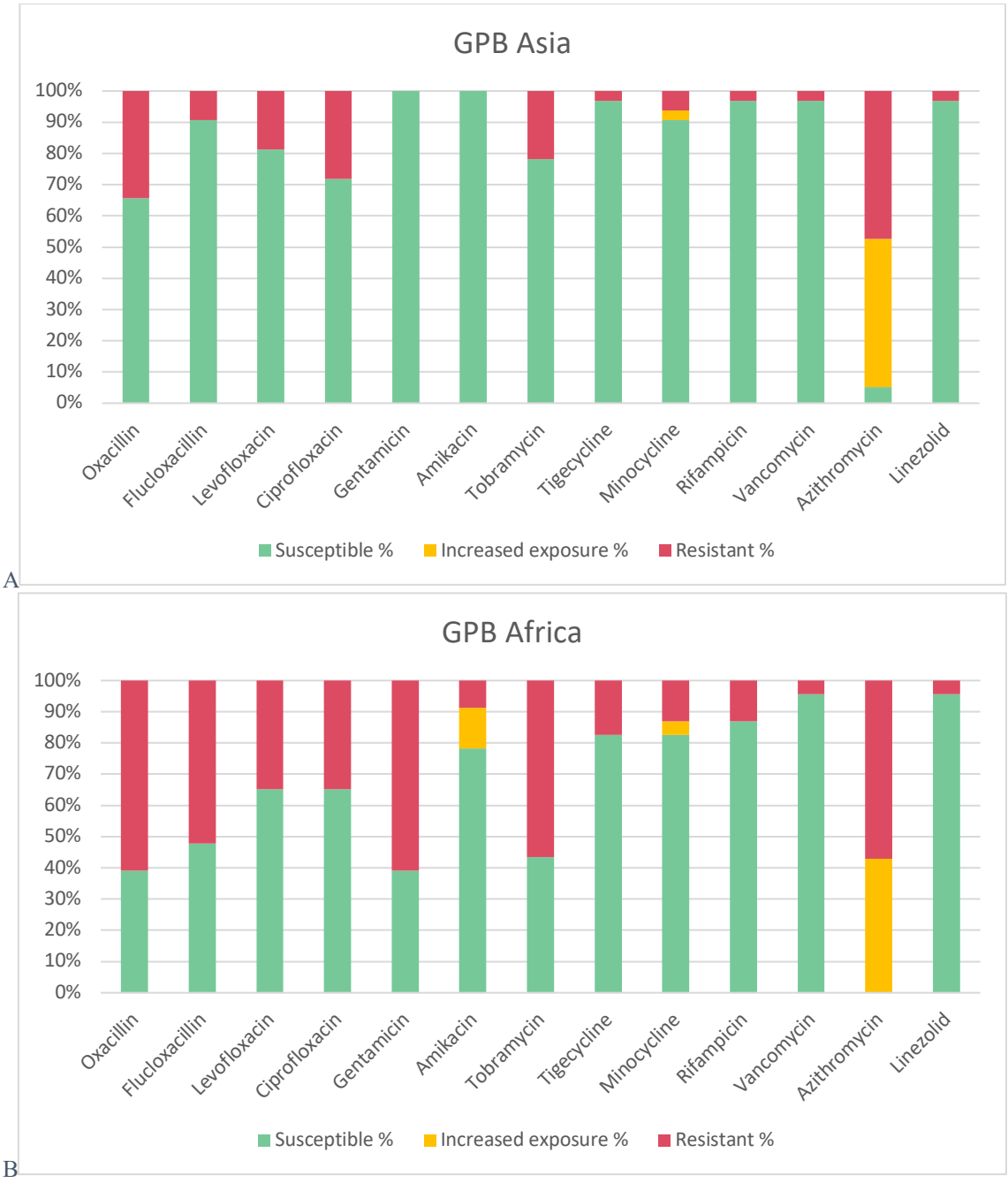


A



B

Supplementary Figure 14. Antibiotic resistance profiles according to EUCAST v9.0 (2019)[1] breakpoints for Gram-negative (GNB) isolates within this subset from sites in A. Asia (n=237) and B. Africa (n=153). Similar resistance profiles can be seen per continent for most antibiotics. However, differences are seen for tigecycline, as resistance for this antibiotic was determined via ECOFF values and therefore reliant on species diversity in a continent. Colistin also displayed a higher prevalence of resistance in Asia. However, this was due to higher frequency of intrinsically resistant species, including S. marcescens.

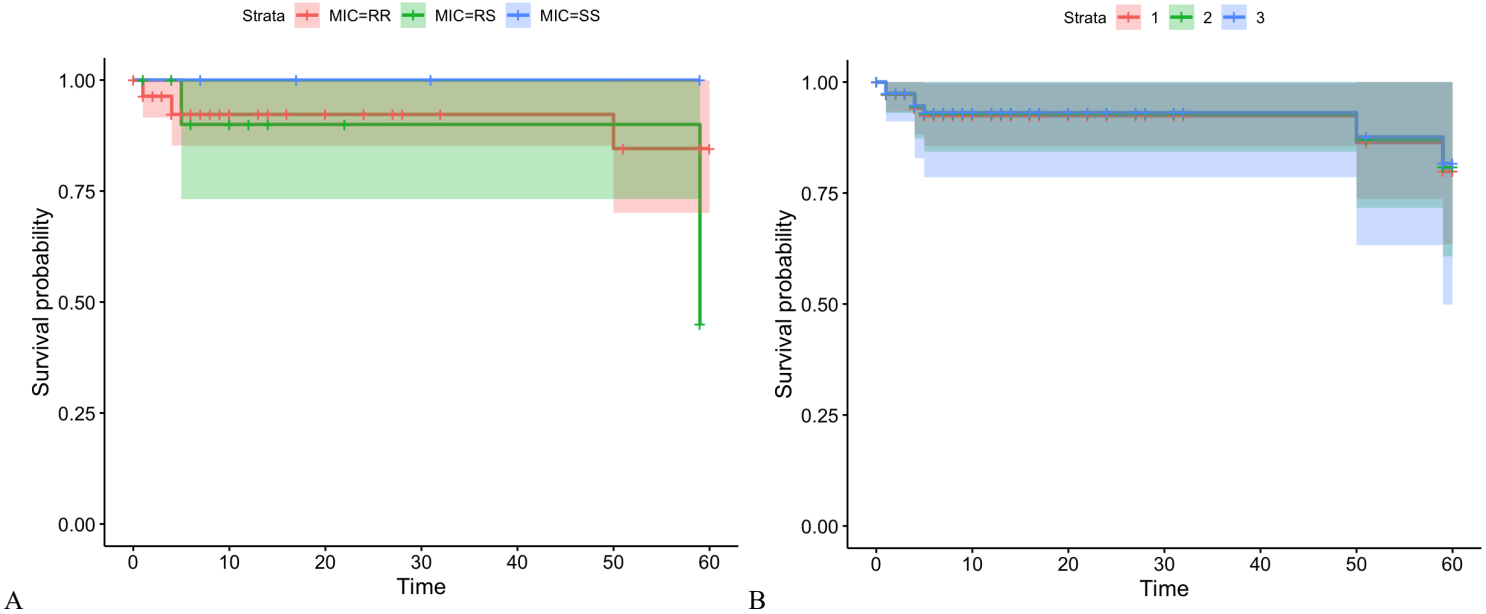


Supplementary Figure 15. Antibiotic resistance profiles, according to EUCAST v9.0 (2019) breakpoints for Gram-positive bacteria (GPB) testedwithin this sub-set from sites in A. Asia (n=32) and B. Africa (n=23). Overall, lower resistance profiles were demonstrated, with exception of high Azithromycin resistance in both continents. Higher levels of resistance were seen in isolates in from clinical sites in Africa for most antibiotics tested.

MIC vs outcome survival curves for differing resistance profiles (RR; RS; SS) for each treatment combination with outcome for neonates treated only with one empirical therapy, n=290.

Supplementary Table 6. Cox regression proportional hazards results for empirical dataset for neonates treated with only ampicillin and gentamicin, n=76 (MIC values not available for 2 isolates) for unadjusted and adjusted models as per clinical information provided in Table 5.6. No confidences intervals (CIs) were available for the mixed effect model. Associated graphs provided below.

	Unadjusted				Adjusted for clinical factors				Mixed effects model, country variation			
	HR	95% CI		P-value	HR	95% CI		P-value	HR	95% CI		P-value
		Lower	Upper			Lower	Upper					
RR												
RS	1.762	0.341	9.11	0.499	0.648	0.027	15.483	0.789	0.648	-	-	0.790
SS	<0.001	0.000	Inf	0.999	<0.001	0.000	Inf	0.999	<0.001	-	-	1.000

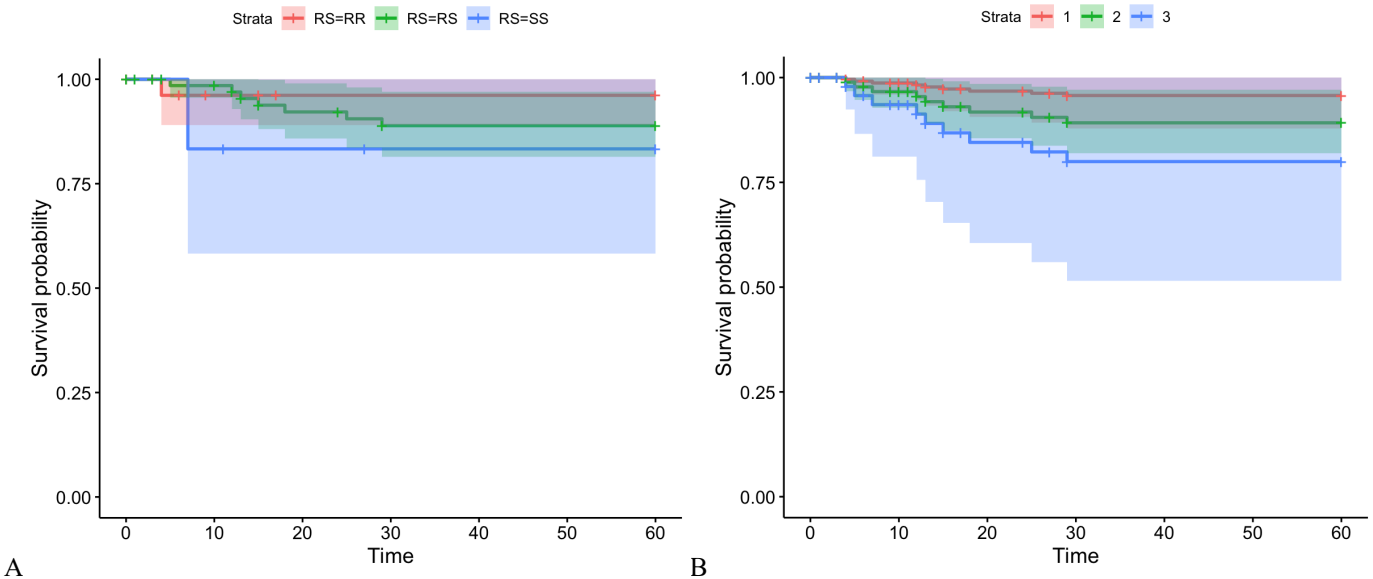


A B

Supplementary Figure 16. Cox regression proportional hazards results displayed as graphs for neonates treated only with ampicillin and gentamicin, n=76 per MIC of sepsis causing pathogen, provided for A) unadjusted and B) adjusted models as per clinical information provided in Supplementary Table 11. For adjusted graph, clinical information was set at: gender: male, cohort: inborn, Type of sepsis: early onset sepsis;, sepsis pathogen type: Gram-negative bacteria, C-section: no, premature: no. Strata 1=RR (resistant to both antibiotics received); 2=RS (resistant to 1 antibiotic received); 3=SS (susceptible to both antibiotics received). Survival curves were made in R Studio, with the survival and survminer packages.

Supplementary Table 7. Cox regression proportional hazards results for empirical dataset for neonates treated with ceftazidime and amikacin, n=107 (minimum inhibition concentration values not available for 2 isolates) for unadjusted and adjusted models as per clinical information provided in Table 5.6. No confidences intervals were available for the mixed effect model. Associated graphs provided below. A mixed effect model and adjusted for country was not carried out as all isolates were from Bangladesh. HR=Hazards ratio

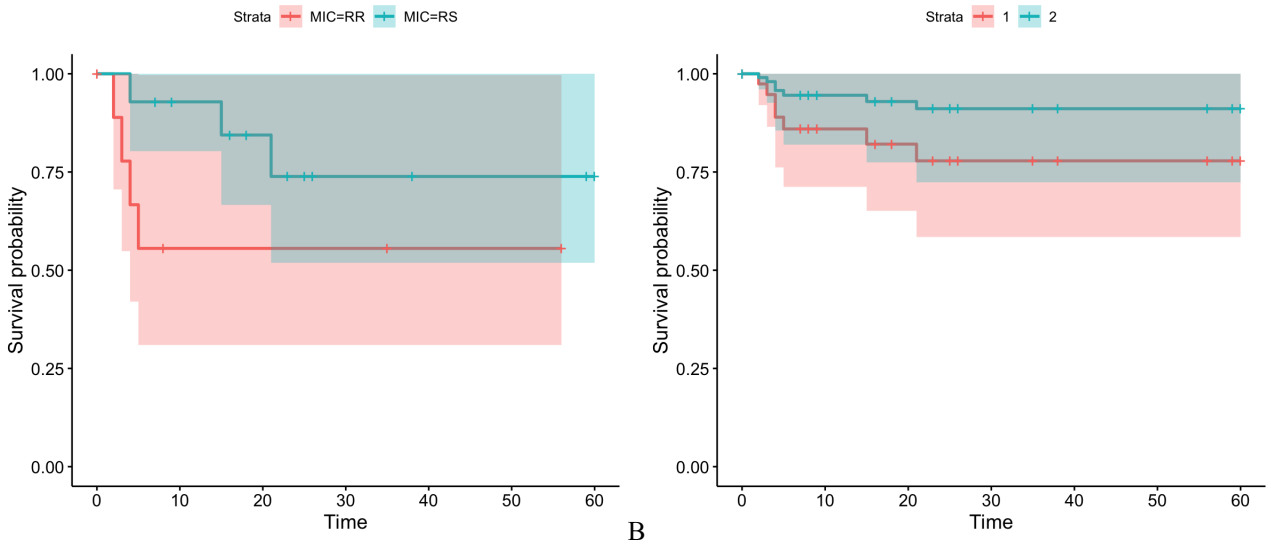
	Unadjusted				Adjusted for clinical factors				Mixed effects model, country variation			
	HR	95% CI		P-value	HR	95% CI		P-value	HR	95% CI		P-value
		Lower	Upper			Lower	Upper					
RR												
RS	1.069	0.321	21.22	0.369	5.649	0.526	60.660	0.153	5.649	-	-	0.150
SS	5.113	0.319	81.94	0.249	<0.001	0.000	Inf.	0.999	<0.001	-	-	1.000



Supplementary Figure 17. Cox regression proportional hazards survival curves for neonates treated only with ampicillin and gentamicin, per minimum inhibitory concentration of sepsis causing pathogen, provided for A) unadjusted and B) adjusted models as per clinical information provided in Supplementary Table 11. For adjusted graph, clinical information was set at: gender: male, cohort: inborn, Type of sepsis: early onset, sepsis pathogen type: Gram-negative, C-section: no, premature: no. Strata 1=RR (resistant to both antibiotics received); 2=RS (resistant to one antibiotic received); 3=SS (susceptible to both antibiotics received). Survival curves were made in R Studio, with the survival and survminer packages.

Supplementary Table 8. Cox regression proportional hazards results for empirical dataset for neonates treated with amoxicillin-clavulanate and amikacin, n=24 (values not available for 3 isolates) for unadjusted and adjusted models as per clinical information provided in Table 5.6. A mixed effect model and adjusted for country was not carried out as all isolates were from Nigeria. No isolates susceptible to both antibitoics were able to be included in this dataset, therefore only comparisons between those resistant to both or one antibiotics could be made. HR=Hazards ratio. Associated graphs provided below.

	Unadjusted				Adjusted for clinical factors			
	HR	95% CI		P-value	HR	95% CI		P-value
		Lower	Upper			Lower	Upper	
RR								
RS	0.372	0.083	1.669	0.196	0.285	0.008	9.998	0.489

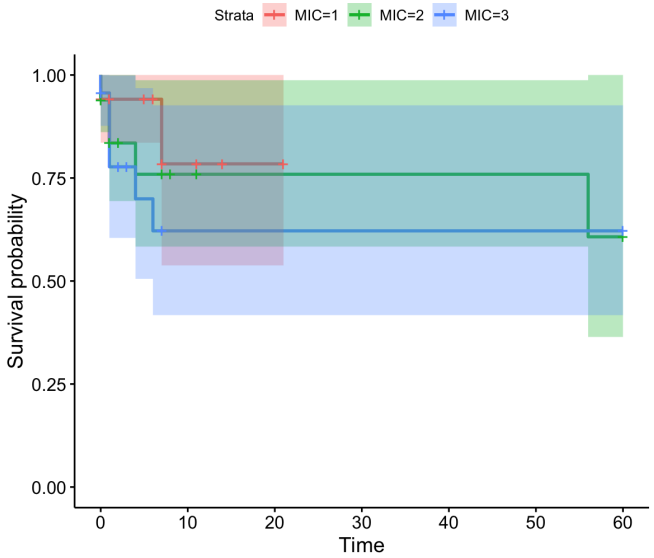


Supplementary Figure 18. Cox regression proportional hazards results displayed as graphs for neonates treated only with amoxicillin-clavulanate and amikacin, per minimum inhibitory concentration of sepsis causing pathogen, provided for A) unadjusted and B) adjusted models as per clinical information provided in Supplementary Table 11. For the adjusted graph, clinical information was set at: gender: male, cohort: inborn, Type of sepsis: early onset, sepsis pathogen type: Gram-negative, C-section: no, premature: no. Strata 1=RR(resistant to both antibiotics received); 2=RS (resistant to one antibiotic received) (No isolates susceptible to both antibiotics were found in this dataset). Survival curves were made in R Studio, with the survival and survminer packages.

Supplementary Table 9. Cox regression proportional hazards results for empirical dataset for neonates treated with piperacillin-tazobactam and amikacin, n=76 (values not available for 3 isolates) for unadjusted and adjusted models as per clinical information provided in Table 5.6. High confidence intervals

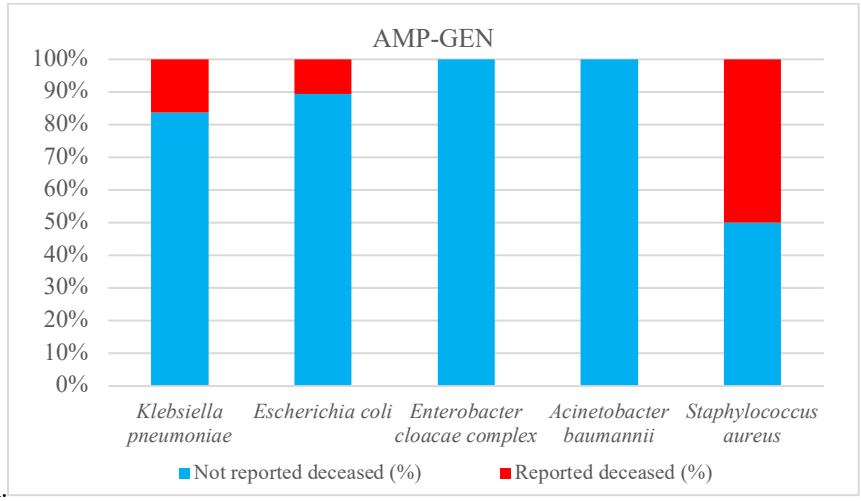
were witnessed for the adjusted analysis, partly due to 47 observations deleted from analysis with missing data, all RS isolates of which were removed. Associated graphs provided below. A mixed effect model and adjusted for country was not carried out as all isolates were from Pakistan. EOS=Early-onset sepsis; LOS=Late onset sepsis; GNB= Gram-negative bacteria; GPB=Gram-positive bacteria as the infecting pathogen. RR=resistant to both antibiotics received; RS=resistant to one antibiotic received; SS= susceptible to both antibiotics. HR=hazard ratio.

	Unadjusted				Adjusted for clinical factors			
	HR	95% CI		P-value	HR	95% CI		P-value
		Lower	Upper			Lower	Upper	
RR								
RS	1.582	0.316	7.914	0.576	31.37	0.911	1079.92	0.056
SS	1.915	0.382	9.605	0.430	17.40	0.721	419.93	0.079

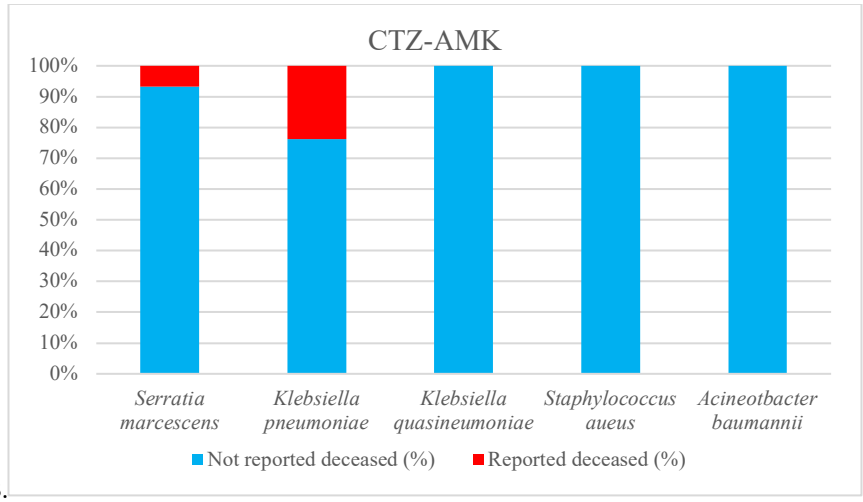


Supplementary Figure 19. Cox regression proportional hazards results displayed as graphs for neonates treated only with piperacillin-tazobactam and amikacin, per MIC of sepsis causing pathogen, provided for unadjusted cox regression analysis. MIC1=RR, resistant to both antibiotics received, MIC2=RS resistant to one antibiotic received, MIC3=SS SS= susceptible to both antibiotics. Red line displaying RR isolates stops at 21 days, as this was the last observation for neonates with RR pathogens. No adjusted graph is displayed due to high confidence interval, this was not easily visualised. Survival curves were made in R Studio, with the survival and survminer package.

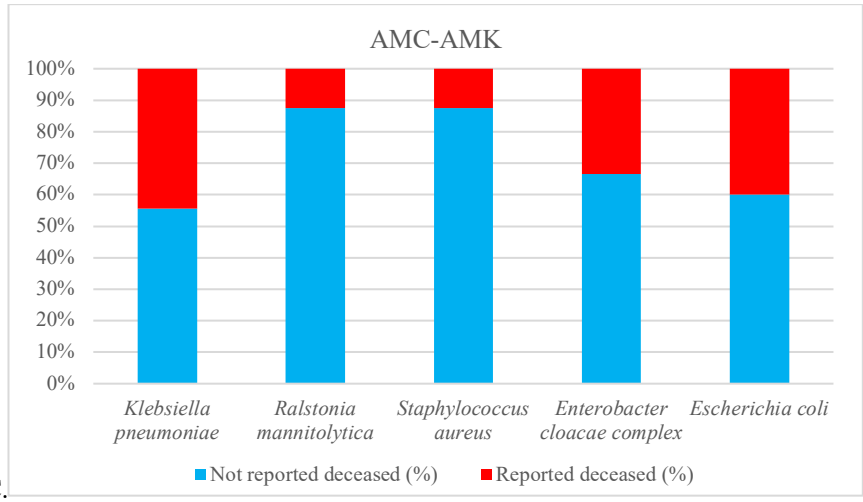
A.


AMP-GEN
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Klebsiella pneumoniae
Escherichia coli
Enterobacter cloacae complex
Acinetobacter baumannii
Staphylococcus aureus
Not reported deceased (%)
Reported deceased (%)

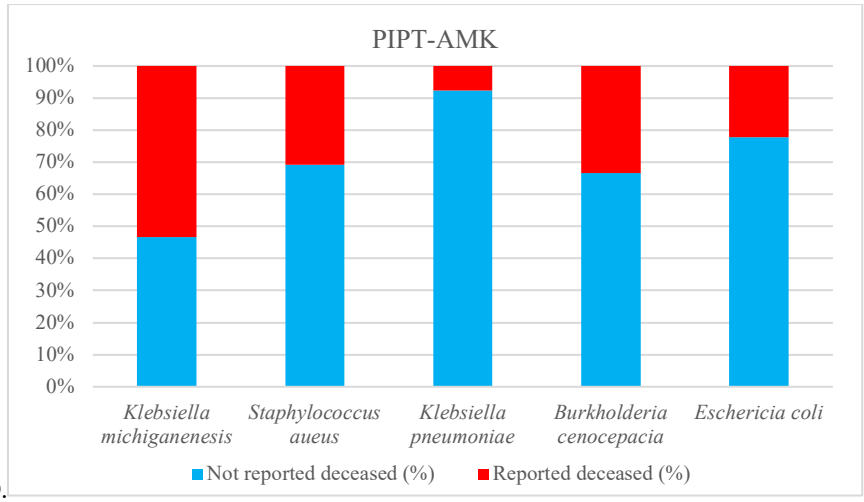
B.


CTZ-AMK
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Serratia marcescens
Klebsiella pneumoniae
Klebsiella quasineumoniae
Staphylococcus aueus
Acineotbacter baumannii
Not reported deceased (%)
Reported deceased (%)

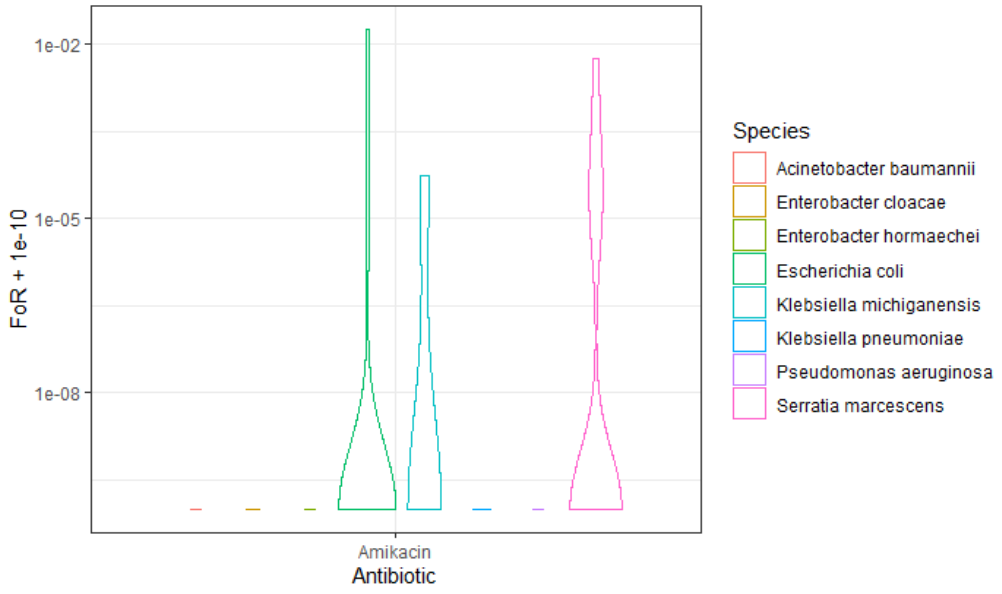
C.


AMC-AMK
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Klebsiella pneumoniae
Ralstonia mannitolytica
Staphylococcus aureus
Enterobacter cloacae complex
Escherichia coli
Not reported deceased (%)
Reported deceased (%)

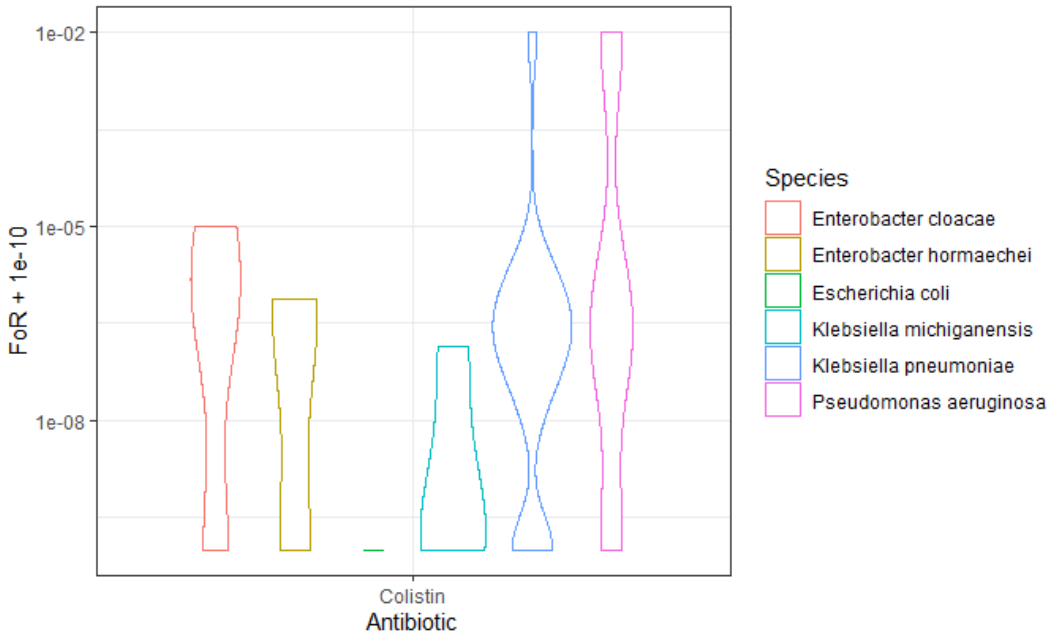
D.



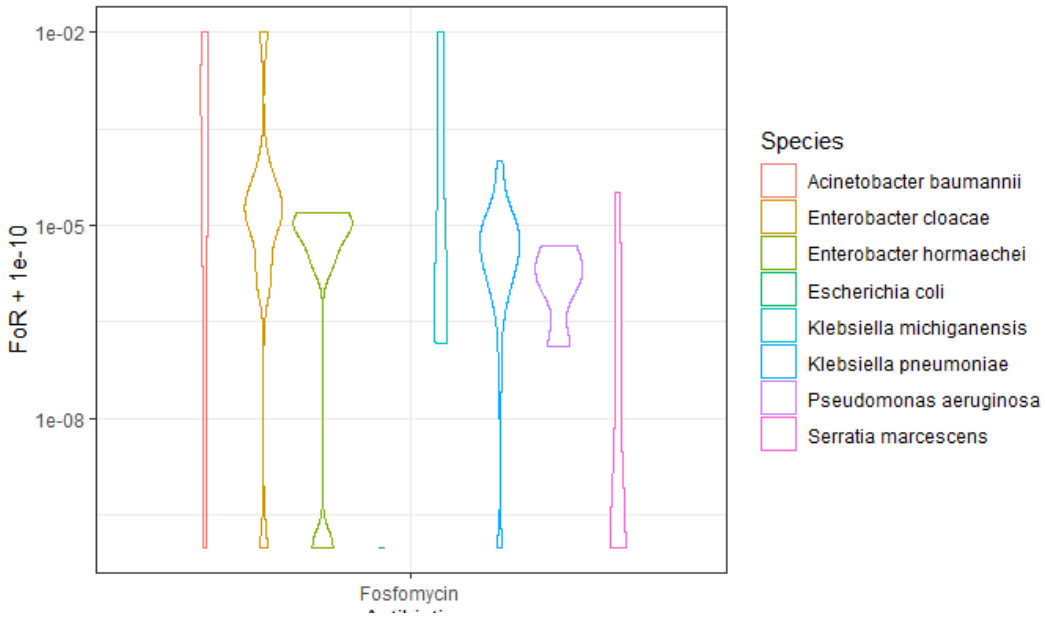
Supplementary Figure 20. Outcomes considered per species found to be sepsis causing pathogen for each antibiotic combination therapy. A.AMP-GEN (ampicillin and gentamicin); B.CTZ-AMK (ceftazidime and amikacin); C.AMC-AMK (amoxicillin-clavulanate and amikacin); D.PIP/TAZ-AMK (piperacillin-tazobactam and amikacin). Klebsiella spp. in A and B refer to other Klebsiella species excluding K. pneumoniae, which is portrayed separately due to higher numbers. Worst outcomes were found for P. aeruginosa, S. aureus and S. maltophilia when treated with piperacillin-tazobactam and amikacin, with all neonates reported as deceased. Cases of infections with Acinetobacter sp., Achromobacter sp. and Citrobacter sp. had the best outcomes across all treatment combinations where reported.


1e-02
1e-05
1e-08
FoR + 1e-10
Amikacin
Antibiotic
Species
Acinetobacter baumannii
Enterobacter cloacae
Enterobacter hormaechei
Escherichia coli
Klebsiella michiganensis
Klebsiella pneumoniae
Pseudomonas aeruginosa
Serratia marcescens

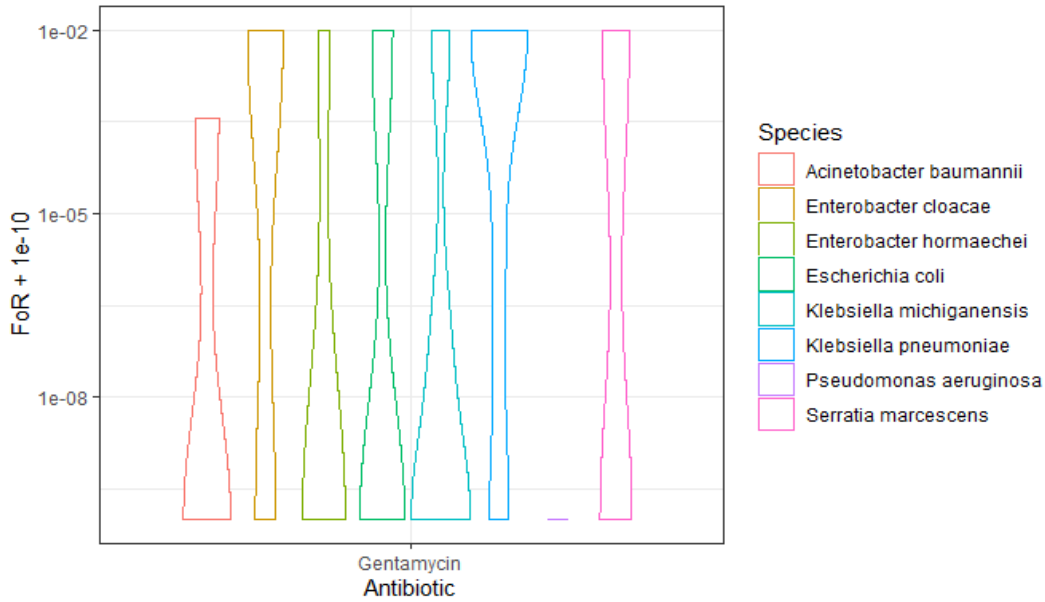
A


1e-02
1e-05
1e-08
FoR + 1e-10
Colistin
Antibiotic
Species
Enterobacter cloacae
Enterobacter hormaechei
Escherichia coli
Klebsiella michiganensis
Klebsiella pneumoniae
Pseudomonas aeruginosa

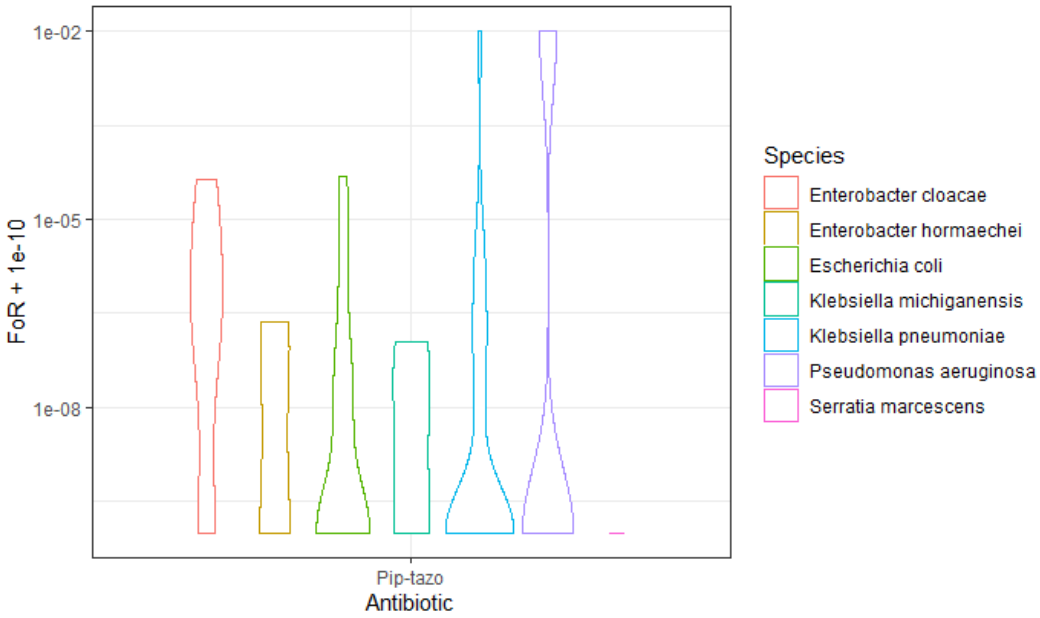
B


1e-02
1e-05
1e-08
FoR + 1e-10
Fosfomycin
Species
Acinetobacter baumannii
Enterobacter cloacae
Enterobacter hormaechei
Escherichia coli
Klebsiella michiganensis
Klebsiella pneumoniae
Pseudomonas aeruginosa
Serratia marcescens

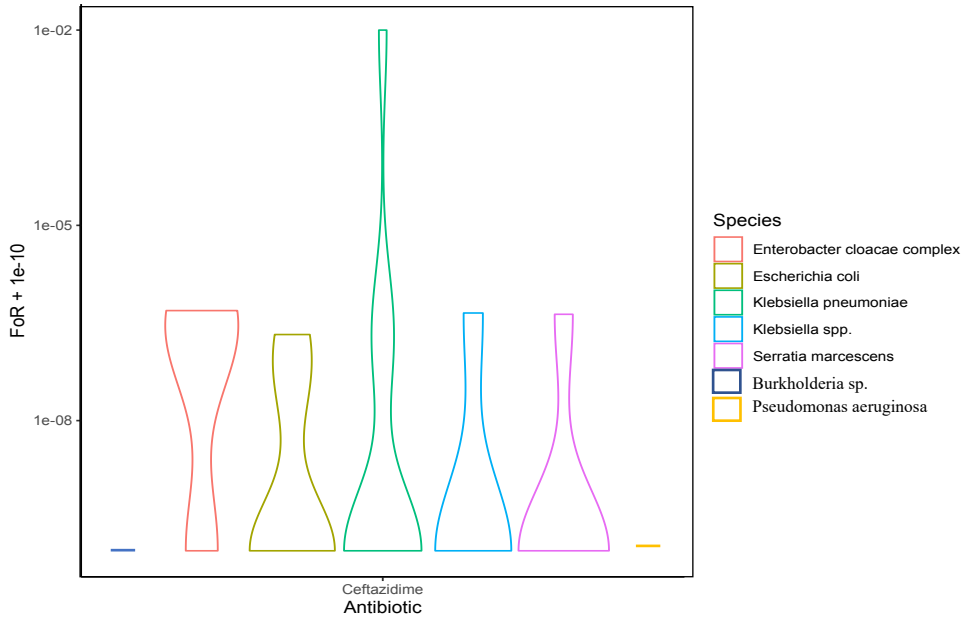
C


1e-02
1e-05
1e-08
FoR + 1e-10
Gentamycin
Antibiotic
Species
Acinetobacter baumannii
Enterobacter cloacae
Enterobacter hormaechei
Escherichia coli
Klebsiella michiganensis
Klebsiella pneumoniae
Pseudomonas aeruginosa
Serratia marcescens

D



E



F

Supplementary Figure 21. Frequency of resistance (FoR) for all Gram-negative species tested against A. Amikacin; B. Colistin; C. Fosfomycin; D. Gentamicin; E. Piperacillin-tazobactam; F. Ceftazidime. Results have been log transformed with a standard of $1x10^{-10}$ added to enable incorporation of zero values. This standard was chosen, as the lowest rate of FoR found was $1x10^{-9}$.

Supplementary Table 10. Frequency table. Frequencies (counts and percentages) for each variable for all neonates with CDS in admissions cohort.

Variable	Categories	Total count	Total %
Mothers first pregnancy?	No	2309	63.6
	Yes	1316	36.2
	Missing	7	0.2
Previous pregnancies	1	819	22.5
	2	515	14.2
	3	298	8.2
	4	201	5.5
	5	135	3.7
	6	103	2.8
	7	90	2.5
	8	41	1.1
	9	43	1.2
	10	21	0.6
	11	43	1.2
	Missing	1323	36.4
Miscarriages	0	1636	45
	1	347	9.6
	2	112	3.1
	3+	66	1.9
	Missing	1471	40.5
Abortions	0	2004	55.2
	1	116	3.2
	2	27	0.7
	3+	7	0.1
	Missing	1478	40.7
Previous stillbirth	0	1927	53.1
	1	179	4.9
	2	24	0.7
	3+	11	0.4
	Missing	1491	41.1
Deceased children	0	1723	47.4
	1	314	8.6
	2	72	2
	3+	49	1.3
	Missing	1474	40.6
Previous multiple birth	No	2255	62.1
	Yes	52	1.4
	Missing	1325	36.5
Current pregnancy multiple	No	3296	96.3
	Yes	136	3.7

Diabetes	No	3595	99.0
	Yes	37	1.0
Hypertension	No	3388	93.3
	Yes	244	6.7
Immune disorders	No	3585	98.7
	Yes	47	1.3
Malaria	No	3050	85.0
	Yes	582	16.0
Infection	No	3478	95.8
	Yes	154	4.2
Typhoid	No	3619	99.6
	Yes	13	0.4
Other illness	No	959	26.4
	Yes	2673	73.6
Mother attended private healthcare in past three months	No	2106	58.0
	Yes	1517	41.8
	Missing	9	0.2
Has the mother Visited traditional healer in the past 3 months	No	2737	75.4
	Yes	891	24.5
	Missing	4	0.1
Has the Mother travelled outside of the city/area in last 12 months	No	3094	85.2
	Yes	492	13.5
	Missing	46	1.3
Member of household travelled outside of the city /area in last 12 months	No	2633	72.5
	Yes	753	20.7
	Missing	246	6.7
Has the mother been Hospitalised in the last 12 months	No	3341	92.0
	Yes	230	6.3
	Missing	61	1.6
Has the mother used antibiotics in the last 3 months	No	2832	78.0
	Yes	558	15.4
	Missing	242	6.7
Beta Lactams	Yes	386	10.6
Aminoglycosides	Yes	6	0.2
Fluoroquinolones	Yes	6	0.2
Macrolides	Yes	12	0.3
Tetracyclines	Yes	0	0.0
Sulfonamide	Yes	2	0.1

Lincosamides	Yes	2	0.1
Chloramphenicol	Yes	0	0.0
Metronidazole	Yes	365	10.0
Polymyxins	Yes	0	0.0
Glycopeptides	Yes	1	0.0
Rifampicins	Yes	1	0.0
Streptogramins	Yes	0	0.0
Cycloserine	Yes	0	0.0
Any other antibiotic?	Yes	37	1
Overall household income per month	>2 times below average	1050	28.9
	Up to 2 times below average	731	20.1
	Average	2064	35.1
	Up to 2 times above average	320	8.8
	> 2 times above average	232	6.4
	Missing	25	0.7
What is the educational status of the Mother	None	397	10.9
	Limited	855	23.5
	Secondary	2064	56.8
	University	310	8.5
	Missing	6	0.2
How would the Mother describe the residential area she lives in	Rural	1302	35.8
	Urban	1914	52.7
	Semi-rural	411	11.3
	Other	2	0.1
	Missing	3	0.1
Number of bedrooms in entire residence	0	114	3.1
	1	719	19.8
	2	1379	38
	3	721	20.4
	4+	679	18.7
Number of people residing there	1-3	1122	30.9
	4-6	1494	41.1
	7+	1016	28
Primary source of drinking water for the household	Municipal network	797	21.9

	Water vendor (tanker or sachet)	193	5.3
	Private well	701	19.3
	Communal tap	726	20
	Sachet or bottled water	462	12.7
	Ground water	740	20.4
	Missing	13	0.4
Treatment of drinking water	Boiled	411	11.3
	Filtered	330	9.1
	Neither	2887	79.5
	Missing	4	0.1
How many days per week does the household have running water	No household supply	2654	73.1
	<=1 day	39	1.1
	2-3 days	137	3.8
	4-6 days	437	12
	7 days	361	9.9
	Missing	4	0.1
Solid waste pile near the Mother's home (proximity of 100m)	No	2405	66.2
	Yes	1224	33.7
	Missing	3	0.1
How frequently is the solid waste pile collected	We deal with it ourselves	805	22.2
	Once a week or more	307	8.5
	Less than once a week	89	2.5
	Not disposed	17	0.5
	Other	6	0.2
	NA	2408	66.3
What sort of toilet does the Mother have within her home	No toilet	82	2.3
	sit down with flush	667	18.4
	Squat with flush	978	26.9
	Pit latine	1892	52.1
	Both sit down and squat	7	0.2

	Other	3	0.1
	Missing	3	0.1
Is there stagnant or sewerage water near the Mother's house (proximity 100m)	No	3099	85.3
	Yes	530	14.6
	Missing	3	0.1
Is the house served by a wastewater network	No	1863	51.3
	Yes	1765	48.6
	Missing	4	0.1
Does the mother generally have access to soap	No	68	1.9
	Yes	3518	96.9
	Sometimes	43	1.2
	Missing	3	0.1
How many times per day does the mother generally wash her hands	Occasionally	2305	63.5
	Frequently	1324	36.5
	Missing	3	0.1
How many days per week does the Mother generally take a shower or bath	Less than daily	1554	42.8
	Daily	751	20.7
	Missing	1327	36.5
How many days per week does the household have an electricity supply	No supply	444	12.2
	Less than half the week	499	13.7
	More than half the week (with interrupted service)	1845	50.8
	Continuous supply	840	23.1
	Missing	4	0.1
Gender	Male	1935	53.3
	Female	1159	31.9
	Missing	538	14.8
Place of birth	Home	1020	28.1
	Hospital	1939	53.4
	Healthcare Centre/Clinic	618	17
	Other	27	0.7
	Missing	28	0.8
Term of baby at delivery	On time	2861	78.8
	Premature	489	13.5
	Late	242	6.7

	Missing	40	1.1
Premature rupture of membrane (PROM)	No	3459	95.2
	Yes	171	4.7
	Missing	2	0.1
Was it 24 hours or more between waters breaking and delivery	No	3481	95.8
	Yes	151	4.2
Was the baby delivered by caesarean section	No	2659	73.2
	Yes	832	22.9
	Missing	141	3.9
Emergency or planned Caesarean section	Emergency	591	16.3
	Planned	214	5.9
	NA	2827	77.8
Did the birth have assisted delivery	No	1878	51.7
	Yes	1613	44.4
	Missing	141	3.9
Breech birth	No	3219	88.6
	Yes	99	2.7
	Missing	314	8.6
Perinatal asphyxia	No	3000	82.6
	Yes	607	16.7
	Missing	25	0.7
Outcome	Alive	2467	67.9
	Deceased	294	8.1
	Untraceable	871	24.0

Supplementary Table 11. Species of bacteria not multidrug resistant in the admissions cohort

Row Labels	**Count of Species ID**
Escherichia coli	21
Klebsiella michiganensis	19
Klebsiella pneumoniae	13
Enterobacter cloacae	7
Salmonella enterica	4
Serratia marcescens	3
Klebsiella quasipneumoniae	2
Pseudomonas stutzeri	2
Enterobacter cloacae complex	2
Enterobacter hormaechei	2
Klebsiella variicola	1
Enterobacter asburiae	1
Morganella morganii	1
Burkholderia cenocepacia	1
Proteus mirabilis	1
Burkholderia sp.	1
Raoultella ornithinolytica	1
Serratia nematodiphila	1
Citrobacter freundii	1
Acinetobacter baumannii	1
Aeromonas sp.	1
Acinetobacter schindleri	1
Grand Total	**87**

Supplementary Table 12. Bacterial species categorised as multidrug resistant within the admissions cohort.

Row Labels	**Count of Species ID**
Serratia marcescens	84
Klebsiella pneumoniae	62
Acinetobacter baumannii	10
Klebsiella michiganensis	8
Pseudomonas aeruginosa	8
Burkholderia cenocepacia	7
Klebsiella quasipneumoniae	7
Escherichia coli	7
Enterobacter cloacae	7
Ralstonia mannitolilytica	5
Pseudomonas alcaligenes	3
Burkholderia sp.	2
Citrobacter freundii	2
Acinetobacter bereziniae	2
Acinetobacter nosocomialis	1
Franconibacter pulveris	1
Acinetobacter schindleri	1
Staphylococcus aureus	1
Pseudomonas stutzeri	1
Burkholderia gladioli	1
Klebsiella variicola	1
Burkholderia cepacia	1
Achromobacter sp.	1
Enterobacter hormaechei	1
Grand Total	**224**

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