UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Molecular Epidemiology of Antibiotic Resistant ESKAPE and *Escherichia coli* Pathogens Isolated from Bloodstream Infections in Public Sector Hospitals in uMgungundlovu District, KwaZulu-Natal, South Africa

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BAKOENA ASHTON HETSA

BSc Hons Microbiology (North-West University), MSc Biology (NWU)

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BAKOENA ASHTON HETSA

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A thesis submitted to the School of Laboratory Medicine and Medical Sciences, College of Health Science, University of KwaZulu-Natal, for the degree of Doctor of Philosophy in Medicine (Medical Microbiology).

This is a thesis in which the chapters are written as a set of discrete research publications, with an overall introduction and final summary.

This is to certify that the content of this thesis is the original research work of Mr Bakoena Ashton Hetsa, carried out under our supervision at the Antimicrobial Research Unit (ARU), Discipline of Pharmaceutical Sciences, School of Health Sciences, and School of Laboratory Medicine and Medical Sciences, College of Health Sciences, Westville campus, University of KwaZulu-Natal (UKZN), Durban, South Africa.

As the candidate's supervisor, I have approved this thesis for examination.

Supervisor:

Supervisor: Signed: -- [redacted] Name: Prof. Sabiha. Y. Essack Date: 29/12/2024

Co-Supervisor: Signed: --- [redacted] Name: Dr. Joshua Mbanga Date: 20/12/2024

Co-Supervisor: Signed: --- [redacted] ---- Name: Dr. Akebe L.K. Abia Date: 29/12/2024

Co- Supervisor: Signed: ------ [redacted] ----- Name: Dr. Daniel Amoako Date: 29/12/2024

DECLARATION

I, Bakoena Ashton Hetsa, declare that

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DEDICATION

This work is dedicated to my Lord Jesus Christ who has granted me strength and courage to complete. Also, I dedicate this work to my late mother and father who selflessly worked hard to give me an education.

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LIST OF MANUSCRIPTS INCLUDED IN THIS THESIS

1. **Bakoena A. Hetsa,** Jonathan Asante, Joshua Mbanga, Akebe L. K Abia, Daniel G. Amoako, and Sabiha Y. Essack. Genomic Characterization of Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Implicated in Bloodstream Infections, KwaZulu-Natal, South Africa: A Pilot Study. [published in Antibiotics 2024, 13, 796. https://doi.org/10.3390/antibiotics13090796].

2. **Bakoena A. Hetsa,** Jonathan Asante, Joshua Mbanga, Akebe L. K. Abia, Daniel G. Amoako, and Sabiha Y. Essack. Genomic Analysis of Virulent, Multidrug Resistant *Klebsiella pneumoniae* and *Klebsiella oxytoca* from Bloodstream Infections in South Africa. [published in Microbial Pathogenesis 2025, 200, 107272. https://doi.org/10.1016/j.micpath.2024.107272]

3. **Bakoena Ashton Hetsa**, Jonathan Asante, Akebe L.K. Abia, Daniel G. Amoako, and Sabiha Y. Essack. Multidrug-Resistant ESKAPEEc Pathogens from Bloodstream Infections in South Africa: A Cross-Sectional Study Assessing Resistance to WHO AWaRe Antibiotics. [published in Health Science Reports, 2025; 8:e70897. https://doi.org/10.1002/hsr2.70897].

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LIST OF ABBREVIATIONS AND ACRONYMS

ABR	Antibiotic resistance
ACME	Arginine catabolic mobile element
AMR	Antimicrobial resistance
ARU	Antimicrobial Research Unit
AST	Antimicrobial susceptibility testing
ATCC	American Type Culture Collection
BSI	Bloodstream infection
BPPL	Bacterial Priority Pathogens List
CARD	Comprehensive Antibiotic Resistance Database
CGE	Centre for Genomic Epidemiology
CLSI	Clinical and Laboratory Standards Institute
CPE	Carbapenemase-producing *Enterobacterales*
CRAB	Carbapenem-resistant *Acinetobacter baumannii*
CRISPR	Clustered regularly interspaced short palindromic repeat
CTX-M	Cefotaxime-Munichen
DNA	Deoxyribonucleic acid
DRG	Disinfectant resistance gene
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ESBL	Extended-spectrum β-lactamase
GES	Guiana extended-spectrum
GLASS	Global Antimicrobial Resistance Surveillance System
HA	Hospital-acquired
HA-MRSA	Hospital acquired-methicillin resistant *Staphylococcus aureus*
HMRG	Heavy metal resistance gene
ICU	Intensive care unit

IMP	Imipenem metallo-β-lactamase
IS	Insertion sequence
KPC	*Klebsiella pneumoniae carbapenemase*
KZN	KwaZulu-Natal
LMIC	Low- and middle-income country
MARI	Multiple antibiotic resistance index
MBL	Metallo β-lactamase
MDR	Multidrug resistant
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLS_B	Macrolide-Lincosamide-Streptogramin B
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NDM	New Delhi metallo β-lactamase
NHLS	National Health Laboratory Services
OXA	Oxacillinase
PAI	Pathogenicity island
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PVL	Panton-Valentine leukocidin
qPCR	Quantitative polymerase chain reaction
RAST	Rapid annotation using subsystem technology
REP-PCR	Repetitive palindromic-polymerase chain reaction
RNA	Ribonucleic acid
SHV	Sulphydryl variable

SNP	Single nucleotide polymorphism
ST	Sequence type
TEM	Temoniera
VIM	Verona integron metallo β-lactamase
VISA	Vancomycin-intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant Enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WGS	Whole-genome sequencing
WHO	World Health Organization

ABSTRACT

Enterococcus faecium, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp*. (ESKAPE) pathogens and *Escherichia coli* (ESKAPEEc) are major contributors to bloodstream infections (BSIs) and pose significant treatment challenges. This study describes the molecular epidemiology and genomic profiles of ESKAPEEc isolates from BSIs in two public hospitals in the uMgungundlovu District, South Africa.

Blood samples (n = 195) were collected from adult and paediatric patients with suspected BSIs between November 2017 and December 2018. Isolates were identified using VITEK 2 system, and confirmed by polymerase chain reaction (PCR). Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method and interpreted according to EUCAST/CLSI guidelines. Whole-genome sequencing (WGS) and bioinformatics tools were used to determine the resistome, virulome, mobilome, clonality and phylogenies of selected *K. pneumoniae*, *K. oxytoca*, and *S. aureus* isolates.

Out of 195 presumptive isolates, 159 were confirmed as ESKAPEEc. *K. pneumoniae* (28.9%) and *S. aureus* (28.3%) were the most predominant pathogens. In total, 151 (94.9%) of isolates were multi-drug resistant (MDR). *K. pneumoniae* and *K. oxytoca* carried β-lactamase genes including the $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1B}}$, bla_{SHV}, and bla_{OXY} genes. The $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ genes were associated with Tn3 transposons and insertion sequences (ISs) (IS*Ec9*, IS*91*). Virulence genes detected in *Klebsiella* spp. were associated with ISs (IS*1*, IS*3*, IS*630*) and integrase. Six sequenced *S. aureus* isolates harboured ARGs including *blaZ*, *aac(6')-aph(2''), ant(9)-Ia*, *ant(6)-Ia*, and *fosB* among others. Most ARGs were associated with ISs (IS*6*, IS*1182*) and recombinase. Virulence genes identified in *S. aureus* isolates were mostly associated with ISs, recombinases and integrases. Phylogenetic analysis of selected *K.*

pneumoniae and *S. aureus* isolates revealed clear grouping patterns based on Multilocus Sequence Typing (MLST) and geographic origin.

WGS and bioinformatics analysis revealed that *Klebsiella* strains and *S. aureus* have a rich repertoire of ARGs, virulence genes, and MGEs in several permutations and combinations, indicating a complex situation of mobilized antibiotic resistance and pathogenic characteristics in clonal and multi-clonal strains responsible for BSIs in this healthcare context.

CHAPTER 1 – INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction

Antimicrobial resistance (AMR) remains a subject of global interest related to public health, especially as the use of antibiotics continues to rise in both clinical and veterinary practice. The extensive use of antibiotics is recognised as the primary cause of the development of antibiotic resistance due to sustained selection pressure on bacteria (Kraemer et al., 2019). Bacteria can survive the effects of antibiotics in various ways, including gene mutations in intrinsic chromosomal genes or through the acquisition of antimicrobial resistance genes (ARGs) from other microorganisms (Sekyere and Asante, 2018). Thus, infections caused by pathogenic bacteria have become increasingly difficult to treat due to various antibiotic resistance mechanisms deployed by bacteria to evade the effects of antibiotics (Sekyere and Asante, 2018). AMR poses a threat to both public health and the economy; it is estimated that by 2030, the indirect impact of drug-resistant infections will cost the world economy between US$1 – 3.4 trillion, resulting from premature deaths, disability, disease, and ineffective labour (WHO, 2020).

The ESKAPE pathogens, including *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp along with *Escherichia coli* (ESKAPEEc) are an important group of opportunistic pathogens of humans and animals and a leading cause of invasive diseases globally. ESKAPEEc infections have been detected in hospital and community settings with varying morbidity and mortality rates in all age groups globally. Among invasive infections, bloodstream infections (BSIs) are estimated to affect about 31.5 million people, leading to 5.3 million deaths yearly (Kolesnichenko et al., 2021). ESKAPEEc pathogens have been implicated in BSIs, associated

with major healthcare costs and limited treatment options (De Angelis et al., 2018). Despite advances in healthcare, a long-term surveillance study undertaken in a large Italian teaching hospital that described the trends in ESKAPEEc BSI and AMR over 9 years (2007– 2015) revealed that ESKAPEEc contributed 50–70% of all causative pathogens implicated in BSIs. However, understanding and assessing their prevalence and significance given AMR patterns and human health were still incomplete because the shift in the distribution and AMR patterns of ESKAPEEc was ongoing (De Angelis et al., 2018).

Generally, broad-spectrum antibiotics are the backbone of treatment for BSIs. The initial empiric antibiotic treatment of BSIs aims to cover all probable pathogens until identification and antimicrobial susceptibility testing (AST) of the causative micro-organisms is available. However, treatment of BSIs caused by antibiotic-resistant bacteria (ARB) is often associated with ineffective empiric antibiotic therapy due to organism-drug mismatch, resulting in high mortality, morbidity, hospital stay, and costs. The key concern for potential organism-drug mismatch drives the extensive use of broad-spectrum antibiotic therapy in patients, particularly in patients who are critically ill. Thus, empirical therapy with broad-spectrum antibiotics is often practised in BSI management (Munford, 2006). However, this practise has resulted in high selective pressure favouring the emergence and spread of multi-drug resistant (MDR) bacteria (Obeng-Nkrumah et al., 2016; Parajuli et al., 2017). Thus, selecting appropriate antibiotics for empiric treatment of BSIs is increasingly challenging.

This study describes the genomic profiles of ESKAPEEc pathogens from blood cultures obtained from public hospitals, including regional and tertiary hospitals within the KwaZulu-Natal Province in South Africa, using WGS and bioinformatics analysis. Molecular testing methods employed in this study can significantly improve both hospital and patient

management of BSIs through accurate identification of ARGs to opotimize antibiotic therapy, potentially shortening hospital stays, reducing readmissions, and lowering healthcare costs.

2.0 Literature review

This section provides a brief overview of the literature on antibiotic resistance, the epidemiology of resistance genes, the molecular mechanisms of resistance in ESKAPEEc bacteria, their role in BSIs, and the use of WGS and bioinformatics analysis in AMR research.

2.1 AMR in ESKAPEEc isolated from hospital environments and clinical samples

The AMR crisis facing hospitals globally is driven by the ESKAPE pathogens (Gram positives: *Enterococcus faecium, Staphylococcus aureus*; and Gram negatives: *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp.) and *Escherichia coli* (*E. coli*) (ESKAPEEc) which are responsible for the majority of infections in hospital patients that are difficult to manage with antimicrobial therapy. Notably, ESKAPE pathogens are environmental or commensal bacteria that cause opportunistic infections in hospitalised or immunocompromised patients. There are documented reports on detecting resistant pathogens, including ESKAPE, in common areas in the hospital environments (Chng et al., 2020; Rodrigues et al., 2020). A Brazilian study investigated the epidemiology of bacterial contamination on surfaces and equipment in a hospital and investigated their antimicrobial susceptibility and MDR in two public hospitals. The ARB were isolated on selective media and subjected to biochemical tests before antibiotic susceptibility testing. Bacterial contamination was detected in 94.7% of the 208 sampled surfaces and equipment in the hospital, with the predominance of *S. aureus*. MRSA, and 4th generation cephalosporins and quinolone- resistant

P. aeruginosa were identified in the study with higher rates of MDR, particularly in the adult intensive care unit (Rodrigues et al., 2020). A study by Chng et al. (2020) used deep shotgun metagenomics to characterize microbiomes, pathogens and antibiotic resistance gene (ARG) cassettes in a tertiary-care hospital in Singapore. The study detected clinically important ARGs conferring resistance to a wide range of antibiotics such as carbapenems (OXA-23), methicillin (*mecA*), broad-spectrum β-lactams (*cme-1*), gentamicin (*aac3-IIa*), colistin (*mcr1*), tetracycline (*tetC*), fosfomycin (*fosA, fosA2*) and macrolides (*mphE*), in different hospital sites (Chng et al., 2020).

Drug-resistant infections, particularly those caused by MDR organisms in healthcare settings, pose a severe threat to clinicians and patients. ESKAPEEc are commonly associated with high levels of antibiotic resistance (Marturano and Lowery, 2019). A Hungarian study, by Benkő et al. (2020) isolated ESKAPE pathogens from different clinical samples including blood, urine, wound swabs, and respiratory specimens, collected in intensive care units (ICUs) and medical and surgical units. Bacterial isolates were cultured on selective media and identified using the VITEK 2 Compact ID/AST and Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). AST was done using the Kirby-Bauer method. The study discovered that 72.2% of isolates recovered from clinical specimens were ESKAPE pathogens. The study further recorded an overall MDR rate of 23.8% and the highest MDR rate among the ESKAPE pathogens was observed for *A. baumannii* (67.74%). A study was undertaken to investigate the molecular characterization of AMR genes in ESKAPEEc pathogens from clinical samples in Chonburi, Thailand. Isolates were cultured on selective media and identified using biochemical tests. The detection of ARGs was done using PCR and sequencing. Isolates were positive for various ARGs against carbapenems [*bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{OXA-48-like}}$, *bla*$_{\text{OXA-23,}}$ *bla*$_{\text{OXA-58}}$ *bla*$_{\text{IMP}}$ and *bla*$_{\text{VIM}}$], colistin [*mcr-1* and *mcr-3*], third and

fourth generation cephalosporins genes [*bla*$_{\text{CTX-15}}$, *bla*$_{\text{CTX-M-55}}$, *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTXM-24}}$, *bla*$_{\text{CTX-M-27}}$, and *bla*$_{\text{OXA-1}}$], methicillin [*mec*A], mupirocin [*mupA*], quaternary ammonium compounds [*qacA* and *qacB*], and vancomycin [*vanA*] (Ruekit et al., 2022).

Resistance to various classes of antibiotics such as β-lactams sulfonamides, aminoglycosides, fluoroquinolones, macrolides, glycopeptides, and trimethoprim has been frequently detected in ESKAPEEc pathogens (Kritsotakis et al. 2022; Pandey et al. 2021). A retrospective cohort study in Crete, Greece, investigated the epidemiology of ESKAPEEc-associated bacteraemia and AMR distribution. ESKAPEEc isolates were identified to species level by phenotypic tests, and antibiotic susceptibility was determined with the MicroScan autoSCAN-4 System (Beckman Coulter, Leriva SA, Greece) (Kritsotakis et al. 2022). Isolates were phenotypically resistant to antibiotics tested, with *A. baumannii* displaying high levels (>80% to 100%) of non-susceptibility to all agents tested except colistin (33%). Antibiotic susceptibility results revealed many MDR phenotypes among *A. baumannii* (97%), *Enterobacter spp.* (91%) and *K. pneumoniae* (60%) (Kritsotakis et al. 2022). In the study done in Nepal by Pandey et al. (2021), phenotypic antibiotic assays were used to detect susceptibility profiles and extended-spectrum β-lactamase (ESBL) production. The AST results in the study done in Nepal by Pandey et al. (2021), revealed that MDR rates among Gram-positive isolates, including *E. faecium* (80%) and *S. aureus* (67.5%) were high. ESBL and metallo-beta-lactamase (MBL) production was confirmed by the double disk synergy test (DDST), combination disk test (CDT) or E-test. Results from the study showed the occurrence of ESBL- and MBLproducers among *K. pneumoniae*, *A. baumannii,* and *P. aeruginosa* isolates. They then used a phenotypic assay to detect vancomycin-resistant enterococci (VRE) before detecting the *vanA* and *vanB* genes using conventional polymerase chain reaction (PCR). *vanA* (20%) was the only VRE gene identified (Pandey et al., 2021). Another study in Lebanon, done by Salloum et al. (2020), used

the disk diffusion method to investigate the patterns of resistance and incidence of MDR among Gram-negative and Gram-positive bacteria. The results of the study showed high rates of MDR among *E. coli* (54.4%) and *K. pneumoniae* (41.7%), while 89.1% of *A. baumannii* was extremely drug-resistant (XDR) (Salloum et al., 2020).

Studies from Africa include an investigation in Kenya, where the distribution of ESKAPEEc isolates across the different hospital levels was studied (Odoyo et al., 2023). Swabs in neutralizing buffer (NB) were used to sample 617 selected high-touch areas in various hospital departments. Culture-based methods were used to isolate ESKAPEEc pathogens, and the VITEK-2 system was used to determine antibiotic resistance. Results of the study showed that all the sampled high-touch areas were contaminated with MDR ESKAPEEc pathogens, with high levels observed in beddings, new-born incubators, baby cots, and sinks in surgical and maternity wards. Their susceptibility test results revealed that most *A. baumannii, Enterobacter* spp., *K. pneumoniae*, and *E. coli* isolates displayed resistance to antibiotics, including cephalosporins, and meropenem. In contrast, MRSA, *E. faecalis* and *E. faecium* isolates had high levels of resistance to erythromycin, levofloxacin, and tetracycline (Odoyo et al., 2023). A study from Tanzania analysed the prevalence and resistance patterns of ESKAPEEc pathogens isolated in pus swabs from hospitalized patients. Isolates were subjected to initial identification using conventional methods and biochemical tests. Disk diffusion was used to determine AST profiles, and a high level of resistance against 3rd generation cephalosporins, aminoglycosides, and sulfonamides was recorded in *A. baumannii, K. pneumoniae, E. coli,* and *P. aeruginosa* isolates. The study also recorded high rates of resistance to carbapenems (67%) and fluoroquinolones (83.3%) among *A. baumannii* isolates (Masoud et al., 2022). A prospective observational study in Malawi investigated the incidence of antibiotic-resistant Gram-negative and Gram-positive bacteria from various clinical specimens. The culture-based method, Gram stain, biochemical test and analytical profile index (API) 20E and 20NE systems

(BioMerieux, Durham, US) were used to isolate and confirm the identity of isolates. The disk diffusion assay was used to determine antibiotic resistance. The study identified ESBL-producing isolates, MDR ESKAPEEc, and other ARB in various hospital departments, emphasizing the need for strict infection prevention and control programs to curb spread (Choonara et al., 2022).

In South Africa, ESKAPEEc pathogens have been reported in studies done on patients with bacteraemia from the National Institute for Communicable Diseases (NICD) (Ismail et al., 2019; Ismail and Perovic, 2018). Ismail et al. (2019) investigated the spectrum of ESKAPE and *E. coli* isolated from blood cultures from the public and private health sectors. The study used the disk-diffusion method to determine isolates' susceptibility profiles and recorded a significant decrease in susceptibility to ciprofloxacin, carbapenems, and aminoglycosides from both health sectors. Both studies recorded the non-susceptibility of fluoroquinolones, third- and fourth-generation cephalosporins, and carbapenems, indicating that these drugs may not be relied upon in a few years to come (Ismail et al., 2019; Ismail and Perovic, 2018). A five-year retrospective study by Ramsamy et al. (2018) investigated ESKAPE pathogens isolated from blood, respiratory, urine, catheter, and sputum. The study used the VITEK 2 system to determine pathogen identification and antibiotic susceptibility. The AST results revealed an increase in resistance of Gram-negative ESKAPE pathogens to amoxicillin-clavulanate and ceftriaxone, with resistance to ceftriaxone increasing from 54.6% in 2011 to 65.5% in 2015. The study recorded that ESBL-producing *K. pneumoniae* increased from 54.9% in 2011 to 65.5% in 2015. Increased resistance to ciprofloxacin from 35% in 2011 to 42% in 2015 was also reported among *K. pneumoniae*. Of note was the significant increase in carbapenem resistance from 5% between 2011 and 2014 to 16% in 2015, an observation that is a cause for

concern as treating infections caused by ESBL-producing bacteria relies on carbapenems (Ramsamy et al. 2018).

Several studies in Africa have reported phenotypic resistance rates among ESKAPEEc pathogens from various clinical samples, including blood, but did not integrate genomics. The ESKAPEEc pathogens harbor several virulence factors, ARGs, and MGEs such as plasmids transposons, integrons, and insertion sequences, which may enhance their transmission, and their pathogenicity (Venkateswaran et al. 2023). Despite the growing recognition of the prevalence of MDR ESKAPEEc in African BSIs, significant gaps exist in understanding the underlying molecular mechanisms contributing to their virulence and resistance. Many studies examine pathogens within the ESKAPEEc group in isolation or from non-human or environmental sources. Given the surge in the incidence of BSIs caused by ESKAPEEc pathogens and the clear gap in linking clinical outcomes to genomic data that mediate resistance, virulence and their transferribility across pathogens, there is an urgent need to collectively analyse ESKAPEEc pathogens and integrate their resistome, virulome and mobilome to understand the underlying molecular mechanisms associated with virulence and resistance, and transferribility.

2.2 The burden of AMR and Multi-drug resistance of ESKAPEEc pathogens

ESKAPE pathogens are recognised as leading causative agents of nosocomial infections in seriously ill and immunocompromised patients, and are associated with antibiotic resistance (Santajit and Indrawattana, 2016). ESKAPE pathogens have developed various resistance mechanisms through mutation and acquisition of mobile genetic elements (MGEs) (Beatson and Walker, 2014). Resistance to clinically important antibiotics leaves clinicians with limited

options in treating infectious diseases, especially those caused by multidrug-resistant ESKAPEEc.

ESKAPE pathogens are known for their propensity to escape the effects of drugs through various resistance mechanisms (Santajit and Indrawattana, 2016). These MDR ESKAPEEc bacteria cause difficult-to-treat infections. Consequently, the WHO has listed MDR Gram-negative and Gram-negative ESKAPEEc organisms in the high-priority pathogens list, emphasising the need for research and drug development of novel therapies (WHO, 2024). The Gram-positive pathogens included in the WHO priority list comprise MRSA, vancomycin-resistant *S. aureus* (VRSA), vancomycin-intermediate *S. aureus* (VISA), and VRE. Gram-negative pathogens include carbapenem-resistant *Enterobacterales* (CRE) and ESBL-producing *Enterobacterales*. Of the CRE, the *K. pneumoniae* (CRKP), carbapenem-resistant *A. baumannii* (CRAB), carbapenem-resistant *P. aeruginosa* (CRPA), and carbapenem-resistant *E. coli* (CREC) are key (WHO, 2024).

2.3 Mechanisms of antibiotic resistance

AMR is caused by various mechanisms such as antibiotic inactivation or alteration, modification of antibiotic's binding sites, enzymatic inactivating of the antibiotic, target site protection, efflux pump activation to eliminate the antibiotic from the bacterial cell, and changes in the outer membrane permeability through porin alteration or loss to hinder effective antibiotic entry (Reygaert, 2018). Antibiotic resistance in bacteria can either be intrinsically (innate) expressed or acquired. Intrinsic resistance refers to the bacteria's ability to naturally resist the effects of certain antibiotics through functional and structural characteristics within the genome. Acquired resistance occurs through acquiring foreign genes either through

conjugation (plasmids), transformation (naked DNA) and transduction (bacteriophages) (Jain et al., 2024).

Enzymatic inactivation falls into two groups, which either deactivate the antibiotic's active site or alter the drug's structural components to prevent interaction with the bacterial target site (Gauba and Rahman, 2023). Enzymatic inactivation is observed in β-lactams, where β-lactamases can bind to and inactivate them. When β-lactams are hydrolysed, they form open rings that are ineffective in binding to their target, penicillin-binding proteins (PBPs) (Cag et al., 2016). Chloramphenicol resistance is mediated by chloramphenicol acetyltransferase (CAT), which alters the drug by adding chemical groups to the drug molecule, decreasing antibiotics' affinity to their target molecules. The CAT changes the antibiotic chloramphenicol to an inactive mono- or diacetate form (Zienkiewicz et al., 2017). Resistance to aminoglycosides is mediated by the production of aminoglycoside-modifying enzymes (AMEs), which can acetylate, phosphorylate, or adenylylate the antibiotic. AMEs inactivate aminoglycosides by covalently attaching acetyl groups to the hydroxyl groups of the aminoglycoside molecule, rendering it inactive (Davies, 2010)

Bacteria modify the antibiotic target site, making the drug molecules unable to bind to their intended target, thus evading the effects of antibiotics (Cag et al., 2016). This mechanism enhances resistance to β-lactams, fluoroquinolone and macrolide-lincosamide-streptogramin B (MLSB) antibiotics. Resistance to β-lactam antibiotics is facilitated by altering the penicillin-binding protein (PBPs,) which renders most β-lactam antibiotics ineffective. For instance, resistance to methicillin in MRSA is encoded by the *mecA* gene, which facilitates the modification of PBPs (Lakhundi and Zhang, 2018). Altering the drug target D-Ala–D-Ala terminus of peptidoglycan favours bacterial cell wall synthesis despite the presence of

antibiotics such as glycopeptides (vancomycin) (Arias and Murray, 2012), which has been recorded in *S. aureus* and enterococci.

Fluoroquinolone resistance may occur due to modifications in DNA gyrase (*gyrA*) and (*gyrB*) (Chien et al., 2016), while the efficacy of macrolide antibiotics can be affected through ribosomal target site modification through methylation of 23s rRNA (Asante et al., 2019). Bacteria can avert antibiotic build-up at the target sites by either reducing drug entry into cells, increasing drug expulsion from the cell or by using both mechanisms in parallel (Devi et al., 2024). The internal concentration of antibiotics in bacteria is controlled by membrane-associated mechanisms of resistance, including porin defects and increased levels of efflux pumps. Efflux pumps are classified into six major families, namely resistance-nodulation division (RND), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), small multidrug resistance (SMR), ATP-binding cassette (ABC), and proteobacterial antimicrobial compound efflux (PACE) (Du et al., 2018). RND efflux pumps are more prevalent among Gram-negative, and are associated with resistance to a broad range of antibiotics (De Oliveira et al., 2020). The RND family transporters consist of unique proteins, namely a periplasmatic adaptor protein (encoded by MexA, MexX, MexC, MexE), a resistant-nodulation-cell division transporter (RNDt), (encoded by MexB, MexY, MexD, and MexF), and an outer membrane factor (OMF), (encoded by OprM, OprJ, or OprN) (Daury et al., 2016). For instance, in *P. aeruginosa*, about twelve RND family efflux pumps have been identified (Dreier et al., 2015). The expression of genes encoding multidrug efflux pumps (Mex) including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM have been reported (Li et al., 2015). In particular, expression of MexAB-OprM in *P. aeruginosa* strains is linked with resistance to different antibiotic classes, including carbapenem-resistance (Beig et al., 2020)

2.4 *Enterococcus species* (*E. faecium*)

Enterococci are ubiquitous Gram-positive, non-spore-forming, and facultative anaerobes found in various hospital environments, known to be common residents of human and animal gastrointestinal tracts (García-Solache and Rice, 2019). *Enterococcus* spp. possess features that enable them to grow and survive under harsh environmental conditions (Sparo et al., 2018). Although enterococci are commensal organisms of the human gastrointestinal tract, they can cause a range of infections, including urinary tract infections, intra-abdominal, pelvic or soft tissue infections, and bacteraemia, particularly in the immunocompromised (Billington et al., 2014; Quiñones et al. 2018). Among *Enterococcus* spp. *E. faecalis* and *E. faecium* are commonly implicated in most enterococcal infections and have been identified in patients with bacteraemia (Lee et al., 2020). Enterococcal infection is commonly associated with device-associated healthcare infection. Their clinical relevance is related to their ability to behave as a pathogen when it reaches the bloodstream through the urinary tract or gut translocation, and colonising indwelling devices (Sparo et al., 2018).

AMR in *Enterococci* is attributed to genomic mutations and the acquisition of mobile genetic elements (MGEs) such as transposons and plasmids. *Enterococcus* spp. are intrinsically resistant to a wide range of antibiotics, including cephalosporins and trimethoprimsulfamethoxazole, and exhibit low-level resistance to β-lactams and aminoglycosides macrolides, tetracyclines, streptogramins, and glycopeptides (Boccella et al., 2021; GarcíaSolache and Rice 2019; Hollenbeck and Rice 2012). Among these, strains that display high-level aminoglycoside-resistance (HLAR) and VRE pose particular treatment difficulties (Yangzom and Singh 2019). A study done in East Sikkim, India, sought to identify *Enterococcus* spp. and determine their antimicrobial resistance patterns, including VRE and

HLAR (Yangzom and Singh 2019). The authors used standard culture and biochemical methods to identify *Enterococcus* spp.; antibiograms were determined by AST. The study revealed high resistance to ciprofloxacin, ampicillin, HLAR, and VRE among *E. faecium* and *E. faecalis*.

VRE infections are treated with antibiotics such as linezolid and daptomycin, but acquired resistance to these antibiotics has also been reported (Guzman Prieto et al., 2016). *E. faecium* resistance to various antibiotic classes, including last-resort antibiotics, has been increasing, further reducing therapeutic options for enterococcal infections (Kristich et al., 2014). Moreover, *E. faecium* has the propensity to acquire and exchange AMR determinants through mutation or horizontal transfer of ARGs located on mobile genetic elements (MGEs) (Sanderson et al., 2022). Vancomycin-resistant enterococci are common causes of BSIs with high morbidity and mortality rates. VRE display resistance to glycopeptide antibiotics, mediated by the production of D-AlaD-Lac or D-Ala-D-Ser, encoding for low-glycopeptide affinity. Vancomycin resistance is mediated by various vancomycin resistance operons termed VanA/B/D/E/G/L/M/N, which mediate vancomycin resistance on varying levels. The *vanA* and *vanB* are most commonly reported genes in *E. faecium*, both transferable through MGEs (He et al., 2020). The retrospective study by López-Luis et al. (2021) described the clinical and epidemiological characteristics of enterococcal BSIs and factors associated with VRE. Species identification and antibiotic susceptibility testing were done using an automated VITEK 2 system, and the *vanA* and *vanB* genes were detected using conventional PCR after being confirmed by culture on selective media containing 8 mg/mL vancomycin. Results revealed that *E. faecium* (94%) isolates were *vanA* gene positive, but no *vanB* gene was identified. Another study was a population-based prospective longitudinal study by Correa-Martínez et al. (2022) in North-Rhine-Westphalia (NRW), Germany, where they investigated the molecular

epidemiology of VRE isolated from patients with VRE bloodstream infections. They used conventional PCR for identification of isolates, followed by sequencing. They used qPCR assay to detect ARGs conferring resistance to glycopeptide antibiotics. They identified all VRE isolates as *E. faecium*, with the majority harbouring *vanB* (n = 539, 71.4%), while *vanA* (n = 216, 28.6%) was detected in a few isolates. MLST analysis revealed that isolates belonged to ST117, ST80, ST203, ST192, ST17. A previous South African study described the clinical and molecular characteristics of VRE causing BSIs at a tertiary-level paediatric hospital in Cape Town (Lochan et al., 2016). Presumptive isolates were cultured on selective media containing 30 µg vancomycin; identification and antibiotic susceptibility was done by VITEK 2 system before confirmation with the Hain Genotype *Enterococcus* line probe assay. Conventional PCR was used to detect *vanA, vanB* and *vanC*. MLST was done by PCR followed by sequencing (Lochan et al., 2016). The results showed that all VRE isolates haboured *vanA* and *vanB*. MLST analysis revealed that isolates belonged to ST817, ST80, ST203, and ST18. In Australia, a study by Xie et al. (2020) described the epidemiology of VRE BSIs in patients with solid tumours or hematological malignancies. Identification of enterococci and antibiotic susceptibility to teicoplanin and vancomycin was determined by the VITEK 2 system. They used PCR to detect the presence of *vanA*, but *vanB* detection was based on phenotypic resistance to teicoplanin on the VITEK 2 system. Out of 96 isolates analysed, all (100%) were identified as *E. faecium*, 99% were positive for *vanB*, while only one *vanA*-positive isolate was recorded.

Lee et al. (2020) characterised *E. faecium* isolates from hospitalized patients with BSIs in Australia. The authors conducted WGS on 1,025 isolates. They detected 26 ARGs conferring resistance to aminoglycosides (*aadE, aac (6')-aph(2''), ant(6)-Ia, aph(2'')-Ie, aph(3')-III*), chloramphenicol (*cat* (pC221), tetracycline (*tet(S), tet(L), tet(M), tet(U)*), macrolide (*erm(A),*

erm(B), erm(T), msrC), glycopeptide (*vanA, vanB*), lincosamide (*lnu(B)*), trimethoprim (*dfrG*), and streptogramin B (*msrC*). The isolates were found to harbour virulence genes including those encoding *bsh* (bile salt hydrolase), *clpP* (protease), *ecbA* (collagen type-V binding microbial surface component recognizing adhesive matrix molecule [MSCRAMM], acm (collagen binding MSCRAMM), *bopD* (biofilm formation), *fss3* (fibrinogen-binding MSCRAMM), *psaA* and *sgrA* (adhesion and biofilm formation), *cpsF* (cleavage and polyadenylation specific factor). Isolates belonged to STs including ST17, ST18, ST78, ST80, ST192, ST203, ST262, ST555, ST796, ST1421 and ST1424.

A study that was done in Germany by Eichel et al. (2020) investigated the clonal relationship of vancomycin-resistant *E. faecium* (VREfm) bacteraemia using WGS. The study results revealed the presence of ARGs encoding resistance to glycopeptides (*vanA, vanB*), tetracycline (*tet(L), tet(M)*), trimethoprim (*dfrF, dfrG*), macrolides (*msr(C), erm(B), erm(T)*) aminoglycosides (*ant(6)-Ia, sat4, aph(2'')-Ia and aph(3')-IIIa, spc*) a gene conferring highlevel gentamicin resistance (*aph(2'')-Ia*). The study also detected the presence of virulence genes associated with biofilm formation (*ecbA*) and adhesion genes (*fss3, scm*), *acm*, (adhesion and biofilm (sgrA) in VRE isolates. The majority of isolates belonged to ST117 (74%). Other STs identified include ST203, ST80, ST17, and ST192.

2.5 *S. aureus*

Staphylococcus aureus is a Gram-positive coccal bacterium inhabiting healthy individuals' nostrils and skin and is also found in other mammals. Approximately 20-30% of the human population is mostly colonized by *S. aureus*, and they are known as asymptomatic carriers. *S. aureus* is a common opportunistic pathogen that causes different community and hospital-

acquired infections, ranging from severe skin infections, pneumonia, endocarditis, and bloodstream infections (BSIs) (Benkő et al. 2020; Pandey et al. 2021).

S. aureus has an exceptional ability to acquire and develop resistance to antibiotics, particularly β-lactams (Turner et al., 2019); in particular, resistance to penicillin is mediated by the *blaZ* gene (Guo et al., 2020). *S. aureus* resistant to methicillin is clinically important, termed MRSA and poses a major public health threat because of multidrug resistance to different antibiotic classes that limit treatment options (Turner et al., 2019). MRSA resistance is mediated by the *mecA* gene that encodes penicillin-binding protein 2a (PBP2a), found on an MGE known as the staphylococcal cassette chromosome *mec* (*SCCmec*) (Hadyeh et al., 2019). MRSA strains have high resistance rates against β-lactam antibiotics, including penicillins, cephalosporins, and carbapenems. The ability of *S. aureus* strains to cause an infection is predicated by resistance to antibiotics and the production of a battery of virulence factors that aid in adherence, haemolysis, toxin production, and biofilm formation (Foster, 2019; Foster et al., 2014). Virulence factors and multiple resistance genes can be transmitted by HGT (Warnes et al., 2012) on diverse MGEs, amongst which plasmids are reported as the primary sources for dissemination (Diekema et al., 2019).

MRSA causes the most difficult-to-treat infections, including BSIs due to multidrug resistance. Vancomycin is an option recommended for treating infections caused by MRSA. However, the emergence of VRSA, and VISA and their global spread have been reported (Shariati et al., 2020). There is growing interest in determining the occurrence of MRSA and associated ARGs in BSIs to curb the spread of MDR MRSA strains. An Iranian study by Zamani et al. (2023) investigated the antibiotic resistance pattern, toxin profile, and molecular characteristics of *S. aureus* isolates obtained from patients with bacteraemia. Bacterial isolates were cultured on

blood agar and mannitol salt agar and characterised by conventional methods. The *mecA* gene and virulence genes, and coagulase typing were detected by PCR. The AST results from the study revealed the highest resistance rate for ampicillin (92.5%), gentamicin (69.2%), amikacin (68.3%), and erythromycin (61.7%). Fifty-five of the 120 isolates were methicillin-resistant *S. aureus* and positive for *mecA* gene. PCR revealed the existence of virulence genes toxic shock syndrome toxin-1 (*tst*), panton-valentine leukocidin (*pvl*), and exfoliative toxins (*eta*, *etb*) encoding genes. Another study that characterized MRSA and MSSA isolates from patients admitted to tertiary hospitals was done in Chlef, Algeria, by Namoune et al. (2023), where standard microbiological methods were used for bacterial identification and confirmed using PCR. The researchers used PCR for genotyping and WGS to detect ARGs. WGS revealed the presence of ARGs including *mecA* (41%), *blaZ* (41%), *ermC* (47%), *fosB* (17.6%), *fusB* (23.5%), *tetK* (29.4%), parC (17.6%), *aad(6)* (35.3%), *aph(3')-IIIa* (35.3%) and mutations conferring resistance to fusidic acid (*fusA*), fosfomycin (*glpT* and *murA*) and fluoroquinolones (*parC*). The study results revealed two dominant MRSA clones, MRSA clone ST80-*spa*-t044-SCCmec-IVc(2b)-PVL+ and ST1-t127 clone, which were found in both MRSA and MSSA isolates.

A prospective observational study characterized *S. aureus* bacteraemia in 58 hospitals in Argentina, Bolivia, Brazil, Paraguay and Uruguay between April and October 2019 using WGS. Identification of *S. aureus* isolates was done using conventional methods and confirmed by MALDI-TOF MS. AST was done using the disc diffusion method. Out of 404 isolates analysed, the *mecA* gene was detected in 165 isolates, while 239 isolates were defined as MSSA because of the absence of the *mecA* gene. Other ARGs detected in their study included *fosB*, *ermT*. The results revealed three MRSA genotypes, including CC30-MRSA-IVc-*t019-lukS/F*PV+, CC5-MRSA-IV-*t002-lukS/F*-PV- and CC8-MRSA-IVc-*t008-lukS/F*-PV+-

COMER+ were the most prevalent in the MRSA (Gregorio et al., 2023). A Japanese study by Sato et al. (2023) analysed MRSA strains isolated from patients with BSIs in a Japanese university hospital using PCR and WGS. Conventional PCR was used to detect virulence genes such as PVL (*Luks-PV*) and toxic shock syndrome toxin-1 (TSST-1); multiplex PCR was used for SCC*mec* typing. Results of the study showed that the *mecA* gene was detected in all the MRSA strains, and the presence of virulence genes, including *tst-1* (41, 30.4%), *lukS-PV* (4, 2.96%). The next generation sequencing results revealed the existence of ARGs conferring resistance to β-lactams (*blaZ*), erythromycin (*erm(A), erm(C)*), tetracycline (*tet(M*), fosfomycin (*fosB4, fosD*), aminoglycosides (*aac(6')-aph(2'')*), and virulence genes including enterotoxins (*sec, seg, sei, sel, sem, sen, seo, seu, tst*), pore-forming toxins (*hlgA, hlgB, hlgC, lukD, lukE, lukF-PV, lukS-PV*), and others including staphylococcal complement inhibitor (SCIN) (encoded by the *scn* gene) staphylokinase (encoded by *sak*), and arginine catabolic mobile element (ACME). The MRSA strains were grouped into clonal complex CC5 (34 strains), CC8 (27 strains) and CC1 (27 strains), belonging to sequence types ST5, ST8, ST1, ST2613, ST764, ST630.

Another study in Barcelona, Spain, investigated the molecular epidemiology, genomic diversity, and clinical characteristics of MRSA-BSIs in a tertiary care hospital over 30 years, from 1990–2019 (Antonio et al., 2022). They genotyped MRSA strains using pulse-field gel electrophoresis (PFGE), and characterized mechanisms of resistance using WGS. Results showed five different genotypes including CC8-ST247-Ia n=162, CC5-ST228-I n=72, CC5-IV belonged to ST125 and ST146 n=279, CC8-ST8-IV n=120, CC22-ST22-IVh n=42. WGS results revealed various resistance mechanisms to ciprofloxacin associated with substitutions in the quinolone resistance-determining regions (QRDRs) of DNA gyrase (*gyrA*), and topoisomerase IV (*grlA*). ARGs conferring resistance to aminoglycosides (*aac(6')-Ie-*

aph(2'')Ia, *ant(4')-Ia*), macrolides and lincosamides (*mph(C), msr(A), erm(A), erm(B)*), rifampicin (associated with mutations in (*rpoB*), tetracyclines (*tet(M), tet(K)*), chloramphenicol (*catA*), cotrimoxazole (*dfrC, drfG, dfrK*), fosfomycin (*fosB, alterations in uhpT*), mupirocin (*mupA*), fusidic acid (*fusC)* were detected. Alteration in PBP2a (N146K or E239K) was associated with resistance to ceftaroline, while changes in *mprF* gene (T345I and L826F) led to daptomycin resistance (Antonio et al., 2022). A study done by Smith et al. (2021) characterised MRSA and methicillin-susceptible *S. aureus* (MSSA) isolates from paediatric and adult patients with BSIs in New Hampshire, USA. WGS revealed the existence of 20 ARGs consisting of genes encoding resistance to β-lactams (*blaZ, mecA*), aminoglycosides (*aadD, ant(9)-Ia, aph(3')IIIa*), erythromycin (*erm(A),* and macrolides (*msrA, mphC*) (Smith et al., 2021). The results also revealed the presence of virulence determinants encoding leucotoxin (*hlgABC*), pantonvalentine leukocidin (PVL) (*lukF* and *lukS*), aureolysin (*aur*), staphylokinase (*sak*), staphylococcal complement inhibitor (*scn*), enterotoxins (*seg, sei, sem, sen, seo, sek, sed, sej, ser* and *seu*), and toxic-shock syndrome toxin-1 (TSST-1).

2.6 *K. pneumoniae*

K. pneumoniae is a non-motile, non-fastidious, Gram-negative, usually capsule-forming bacterium belonging to the *Enterobacterales* family. *K. pneumoniae* recognized as a significant opportunistic pathogen responsible for invasive nosocomial infections has an outstanding ability to develop resistance to commonly used antibiotics. The treatment of infections caused by *K. pneumoniae* is restricted by the emergence of multidrug resistance resulting from the production of ESBLs and carbapenemase, acquisition of AMR genes and mutations in core genes, and overexpression of efflux pumps (Tooke et al., 2019; Wyres and Holt, 2018). *K. pneumoniae* infections are associated with two major strains classified into two major

pathotypes: classic *K. pneumoniae* (cKP) and hypervirulent *K. pneumoniae* (hvKP). The latter is more virulent and can bear virulent plasmids and associated resistance, such as carbapenem resistance (Lan et al. 2021; Zhang et al. 2022). Compared to cKP, hvKP strains characteristically display high capsule polysaccharide production, forming a hypermucoviscous phenotype, siderophores (enterobactin, salmochelin, yersiniabactin and aerobactin), and virulence plasmids which enable them to cause highly invasive infections (Lan et al., 2021). Antibiotic resistance and virulence genes in bacterial isolates may lead to life-threatening infections that are difficult to treat or prolong illness. A Chinese study that described the molecular epidemiology of *K. pneumoniae* in BSI patients at a tertiary hospital identified 17 ST11-K64 isolates harboring the $bla_{\text{KPC-2}}$ gene; most coharbored *rmpA2*, *iucA*, *iutA*, and *Kvar_1549* genes (Wu et al., 2021). Sundaresan et al. (2022) recently identified ESBL and carbapenemase-producing isolates co-expressing virulence genes, including *ybtS, iutA,* and *rmpA, magA*.

The major burden of AMR in *K. pneumoniae* is the worldwide spread of certain successful AMR clones, named high-risk clones. Several studies have reported various high-risk clones, including those belonging to ST11, ST15, ST101, ST147, ST258, and ST307 (Peirano et al. 2020). Reports from South Africa have shown that the prevalence of the ST307 clone is increasing and is made up of carbapenemase-producing *K. pneumoniae* isolates. A study in KwaZulu-Natal, South Africa, characterized 14 carbapenemase-producing *Enterobacterales* (CPE) strains. Results indicated that all isolates belonged to ST307 and harbored the $bla_{\text{OXA-181}}$ carbapenemase in different combinations with other β-lactamases. Isolates from the study contained several ARGs and MGEs, including integrons, plasmids and virulence genes (Madni et al., 2021). A study done in South Africa by Lowe et al. (2019) characterized *K. pneumoniae* ST307 strains with OXA-48-like enzymes. Detection of ARGs was done using conventional

PCR and WGS. They reported that the *K. pneumoniae* ST307 contained an IncX3 plasmid with *bla*$_{\text{OXA-181}}$ (ST307_X3-OXA-181). The ARGs detected in the study included *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{OXA-181,}}$ *bla*$_{\text{NDM}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{VIM}}$, with the dominance of *bla*$_{\text{OXA-48}}$–like (n = 471) (Lowe et al., 2019).

K. pneumoniae is a leading cause of serious human infections, including respiratory tract infections, urinary tract infections (UTIs) and BSIs (Martin and Bachman, 2018). A study in West Bengal, India, on hypervirulent *K. pneumoniae* (hvKP) causing neonatal BSIs that used a PCR and sequencing approach revealed the existence of 107 ARGs conferring resistance to β-lactams (*bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{OXA}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{AmpC}}$, *bla*$_{\text{NDM-1}}$), aminoglycosides (*armA*, *aac(6')-Ib*, *rmtC*, *rmtB*) and fluoroquinolones (*qnrB1*, *qnrS1*, *oqxA*, *oqxB*, *aac(6')-Ib-cr*) in hvKPs. For analysis of plasmids, WGS and microbial identification and characterization through read analysis (MICRA) is an automatic pipeline that uses iterative mapping against reference genomes to identify genes and variations (Caboche et al., 2017). WGS and MICRA revealed the presence of conjugative plasmids carrying the *bla*$_{\text{NDM}}$ genes conferring resistance to carbapenems in two isolates (Mukherjee et al., 2023). A study in Denmark sought to characterise ESBL/AmpC and carbapenemase-producing *K. pneumoniae* isolates from blood cultures. The combination disk method using Neo-Sensitabs$^{\text{TM}}$ (Rosco, Taastrup, Denmark) was used to identify ESBL and AmpC phenotypes (Hansen et al., 2020). The study identified, ESBL resistance genes (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-154}}$, *bla*$_{\text{SHV-27}}$, *bla*$_{\text{SHV-12}}$), carbapenemases (*bla*$_{\text{OXA48}}$, *bla*$_{\text{OXA-232}}$), and AmpC β-lactamases (DHA-1) using WGS. Also detected were the fluoroquinolone resistance genes (*oqxA, oqxB, qnrB1, qnrB4, qnrB6, qnrS1*) and genes conferring resistance to aminoglycosides (*aac(3)-IIa, aph(6')-Id, aph(3')-Ib, aadA2, aph(3')-Ia, aadA5, aadA16, strA, strB*). Interestingly, the study found that 85% percent of the isolates contained one or several plasmid-mediated aminoglycoside resistance genes (Hansen et al.,

2020). Another study in China investigated the microbiological characteristics and epidemiology of *K. pneumoniae* bloodstream isolates based on ompK36 genotyping. The VITEK2 system was used for identification and antimicrobial susceptibility testing of *K. pneumoniae*. Isolates were assayed for carbapenemase resistance genes, ompK36, plasmid-mediated virulence genes, biofilm formation using PCR-based genotyping, sequencing, and microtitre plate assay, and multi-locus sequence typing (MLST) was done using PCR and sequencing to determine the sequence types (ST) based on seven house-keeping genes (Du et al., 2019). The capsular type K1, K54, K20, K57 and K2 K5, and sequence types ST23, ST65, ST11, ST147, ST25 and ST133 were identified. Yang et al. (2024) investigated the resistomes, virulence determinants, and the phylogenetic relationship between BSI-KP in Hong Kong, China. *K. pneumoniae* isolates were cultured on Columbia blood agar containing 5% sheep blood (Becton Dickinson, USA) and identified using MALDI-TOF MS (Bruker, Germany). A total of 51 different ARGs and virulence genes were identified using WGS. Another study done in Massachusetts, United States (USA) used WGS to investigate the clinical characteristics, bacterial diversity, and ARGs. Identification of isolates was done using MALDI-TOF MS. They used WGS to detect ARGs, including $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{KPC-2}}$, $bla_{\text{KPC-3}}$, $bla_{\text{OXA-48}}$, and $bla_{\text{NDM-5}}$. They identified a set of virulence genes consisting of aerobactin, yersiniabactin, and *rmpADC/rmpA*_2 genes associated with hypermucoidity (Roach et al., 2024).

2.7 *A. baumannii*

Acinetobacter baumannii is a Gram-negative, non-fermentative bacterium that is not only ubiquitous in the environment but also an opportunistic pathogens. *A. baumannii* has become a leading nosocomial pathogen responsible for infections, including catheter-associated urinary tract infections, ventilator-associated pneumonia, skin and soft tissue infections, and BSIs

(Ayobami et al., 2020). Moreover, *A. baumannii* has become a global threat in the healthcare setting because of its propensity to acquire MDR, XDR, PDR phenotypes and tolerance to desiccation (Cavallo et al., 2023). Increasing reports of *A. baumannii* strains resistant to fluoroquinolones, aminoglycosides, carbapenems, and colistin are alarming (Cavallo et al., 2023). In addition to being MDR pathogens, *A. baumannii* harbours virulence factors, including secretion systems, immunity interaction, or adhesion to the host cells, which enhance virulence and pathogenicity in *A. baumannii* (Kumar et al. 2021; Tiku 2022).

Several studies have used various molecular assays to detect *A. baumannii* in blood cultures. A study in Shaanxi, China, by Gu et al. (2021) investigated molecular characteristics of *A. baumannii* BSIs and non-bloodstream *A. baumannii* infections. The researchers used MALDI-TOF for species identification and WGS to determine ARGs and virulence genes. The agar diffusion method was used to determine the antibiotic resistance. They identified ARGs, including $bla_{\text{OXA-23}}$, $bla_{\text{OXA-146}}$, $bla_{\text{OXA-167}}$, $bla_{\text{OXA-72}}$, and $bla_{\text{NDM-1}}$, and virulent genes such as *bauA* (siderophore receptor gene) and *bap* (biofilm-associated gene) were identified. The sequence types identified included ST195, ST208, and ST195. In central Italy, a study analysed antibiotic-resistant and virulent *A. baumannii* strains isolated from COVID-19 patients affected by sepsis (Cherubini et al., 2022). The study used broth microdilution to determine antibiotic susceptibility and investigated ARGs, MGEs, virulence factors, and STs using WGS. The results showed that all isolates belonged to ST2, and the presence of MGEs including IS3, IS4, IS5, IS6 (IS26, IS6100), IS30, IS91 and IS256. ARGs consisted of genes encoding resistance to β-lactam, tetracycline, aminoglycosides, macrolides, fluoroquinolone, sulphonamides, and chloramphenicol. Major findings in the study were that the Tn6207 transposon in all analysed strains was associated with the tetracycline efflux pump and its regulator gene (*tetB*) and

aminoglycoside resistance genes *strB* and *strA*, implying that these ARGs are transposon-mediated.

A study done at ROK University Hospital, Korea, found that *A. baumannii* isolates displayed the highest resistance rates to ciprofloxacin and imipenem (100%), piperacillin (99.4%), cefepime (99.4%), ampicillin/sulbactam (97.6%), and gentamicin (90%). The PCR assays identified $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-5}}$ genes encoding resistance to carbapenems in all carbapenem-resistant *A. baumannii* (CRAB) isolates, and virulence genes, including those associated with biofilm formation (Park et al., 2023). The MLST analysis revealed ST191, ST195, ST208, ST357, ST369, ST451, ST469, ST491, ST784, ST1599, and ST1653, of which ST191 (28.8%) was the most abundant. In South Africa, a prospective cross-sectional study in Gauteng, South Africa, analyzed the molecular characteristics of *A. baumannii* isolated from blood cultures in patients at a tertiary level hospital. Bacterial species identification was done using the VITEK 2 system and MALDI-TOF MS. The *mcr-1* to *mcr-5* genes were identified using PCR. ARGs were detected using conventional PCR and WGS. WGS was used on colistin-resistant isolates to detect sequence types (STs), ARGs, and virulent factors. The study results revealed that most ST1 and ST2 isolates displayed resistance to colistin and carried multiple ARGs encoding resistance to β-lactams/carbapenem ($bla_{\text{NDM-1}}$, $bla_{\text{OXA-23}}$, $bla_{\text{PER-7}}$), aminoglycosides (*aacC1, aadA, aphA1, strA, strB*), polymyxin (*lpsB*), macrolides (*msrE, mphE*) tetracycline (*tetB*), trimethoprim (*dfrA1*), sulfonamides (*sul1, sul2*), rifampicin (*arr-2*), disinfecting agents and antiseptics (*qacE*), chloramphenicol (*cmlA5*) (Lowe et al., 2022). Another interesting finding of the study was that the *ISAba1* was associated with $bla_{\text{OXA–23}}$ (n = 6) and ISAba125 was found upstream of bla_{NDM-1} gene, implying that these ISs are responsible for the mobilisation of carbapenem resistance genes.

2.8 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, facultative anaerobic, rod-shaped bacterium found in the digestive tracts of humans. It is a leading opportunistic pathogen that frequently causes nosocomial infections such as urinary tract infections and bloodstream infections (Ababneh et al. 2021; Magalhães et al. 2020). BSIs caused by *P. aeruginosa* isolates are typically difficult to treat due to intrinsic antimicrobial resistance and the ability to acquire resistance to multiple classes of antimicrobial agents (Reynolds and Kollef, 2021). Antibiotics, including cephalosporins, monobactams, carbapenems, fluoroquinolones, aminoglycosides, β-lactam/β-lactamase inhibitors, have been used to treat *P. aeruginosa* infections (Ibrahim et al., 2020). However, *P. aeruginosa* strains display resistance to several antimicrobial agents through various resistance mechanisms, including the production of β-lactamases, upregulation of multidrug efflux pumps and cell wall mutations (Glen and Lamont, 2021). A Chinese study that investigated the incidence and antimicrobial resistance profiles of *P. aeruginosa* from BSIs revealed that isolates phenotypically displayed low levels of resistance to aminoglycosides, cephalosporins, fluoroquinolones, and polymyxin B (Xiao et al., 2023). However, a study at King Abdullah University Hospital (KAUH) in Jordan reported higher resistance to imipenem, piperacillin-tazobactam, cefepime, ciprofloxacin and gentamicin (Ababneh et al., 2021). In addition, *P. aeruginosa* possesses a battery of virulence factors that contribute to its pathogenicity. A study investigated the antibiotic sensitivity and virulence genes of *P. aeruginosa* isolates obtained from clinical specimens, including blood, urine, broncho-alveolar lavage, and wound swabs (Bogiel et al., 2022). The study isolates were phenotypically resistant to ticarcillin/clavulanate, piperacillin, imipenem and piperacillin/tazobactam. The virulence genes were investigated using conventional PCR. All the 71 isolates assayed in the study carried *lasB, plC, nan2, aprA, phzM, exoU exoS, pilA,* and *pilB* genes.

Spottiswoode et al. (2023) undertook a study in the United States to investigate recurrent *P. aeruginosa* bloodstream infections in a severely immunocompromised patient. Species identification was done using the MALDI-TOF MS, and minimum inhibitory concentrations (MICs) were determined using the Trek Sensititre (Thermo Scientific, Oakland, Ohio, USA) automated broth microdilution system. Mutations in the *ampD* and *ampE* genes were identified using WGS and were associated with β-lactam resistance and cefepime treatment failure reported in the study. *ampD* represses the expression of the *ampC* β-lactamase gene. Base pair deletion in the *oprD* gene was associated with increased resistance to ceftazidime. The study results showed that the co-occurrence of ARGs conferring MDR in *P. aeruginosa* BSIs impedes treatment. A study was undertaken in Iran to investigate the association of the *mcr-1* gene with the emergence of MBL/AmpC in *P. aeruginosa* strains. Bacterial isolation and confirmation were done using standard microbiology and biochemical techniques on selective media. The ARGs and MLST genes were identified using PCR. Ten of 69 isolates were positive for the *mcr-1*, while β-lactamase genes identified included bla_{NDM} (n = 11, 15.94%) bla_{FOX} gene (n = 31, 42.94%), bla_{ACC} (n = 27, 39.13%), bla_{SPM} (n = 19, 27.53%), bla_{VIM} (n = 27, 39.13%) and bla_{TEM} (n = 20, 28.98%). Of the 69 isolates, 43 different STs were identified, of which ST235 and ST3340 were associated with MDR phenotypes (Tahmasebi et al., 2020). A study in Larissa, Greece, characterized nine $bla_{\text{NDM-1}}$-positive *P. aeruginosa* isolates from various clinical specimens, including blood. The identification and susceptibility testing of the microorganisms were done using the Vitek-2 system. WGS was used to identify ARGs and virulence genes. WGS analysis revealed 22 ARGs, including ARGS against β-lactams ($bla_{\text{NDM-1}}$, bla_{PAO}, $bla_{\text{OXA-10}}$, $bla_{\text{OXA-488}}$), aminoglycosides (*aph(3')-Ib*, *aph(6)-Id*, *aac(3)-Id*, *aac(6')-Ib3*, *aac(6)-II*, *aadA10*, *rmtF*), quinolones (*crpP*, *aac(6')-Ib-cr*, *qnrVC1*), folate (*sul1*, *sul2*, *dfrB5*), chloramphenicol (*catB7*, *floR*), macrolides (*msrE*), fosfomycin (*fosA*), and quaternary

ammonium compounds (*qacE*) with strains possessing several different virulence genes (Tsilipounidaki et al., 2023).

2.9 *Enterobacter* species

Enterobacter species are fermentative, Gram-negative, rod-shaped bacteria in the *Enterobacterales* family (Santajit and Indrawattana, 2016). They exist as normal flora of the gastrointestinal microbiota and respiratory tract of humans and animals (Pati et al., 2018). However, *Enterobacter* spp. are recognised as opportunistic nosocomial pathogens responsible for infections including bacteraemia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, ophthalmic infections (Rizi et al., 2019). *Enterobacter* spp. have been reported in nosocomial outbreaks in susceptible patients, especially in neonatal intensive care units (NICUs) (Ferry et al., 2020) and adult ICUs (Rahal et al., 2021). Currently, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterobacter bugandensis*, and *E. asburiae* are the most frequent members of this species isolated from clinical samples. Several *Enterobacter* spp., such as *Enterobacter cloacae* complex (ECC) are considered clinically significant species and have been identified in BSIs (Manandhar et al., 2022).

Treatment of infections with *Enterobacter* spp. is increasingly difficult because of the production of AmpC β-lactamase, which confers intrinsic resistance to third-generation cephalosporins (Ellington et al., 2019), as well as resistance to other antibiotics, including aminopenicillins, amoxicillin-clavulanate, cefoxitin and penicillin (Chavda et al., 2016). Resistance to antibiotics such as carbapenems, aminoglycosides, chloramphenicol, and ciprofloxacin has also been reported (Manandhar et al., 2022).

A retrospective study in Kathmandu, Nepal, characterized *Enterobacter cloacae* from BSIs in terms of their ARGs and phylogenetic relationships. The study showed that isolates, were resistant to cefotaxime, gentamicin, chloramphenicol, co-trimoxazole, ciprofloxacin, and carbapenems. Further, a diverse set of ARGs conferring resistance to β-lactams [$bla_{\text{CTX-M-1}}$, bla_{ACT}, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1D}}$], quinolones [*qnrB*], trimethoprim [*dfr*A5] streptomycin [*strA/B*], aminoglycosides [*aac(3)-IIa*, *aac-aad*], chloramphenicol [*catA*], tetracyclines [*tet(A)*], sulfonamides [*sulII*] and fosfomycin [*fosA2*] were evident. No resistance to carbapenems, macrolides and colistin was observed (Manandhar et al., 2022). The management of infections caused by *Enterobacter* spp., resistant to carbapenems, has been achieved through colistin. However, the emergence of colistin resistance has been reported since the detection of plasmid-mediated gene (*mcr-1*) conferring resistance to colistin (Wang and Feng, 2020). A Chinese study by Lin et al. (2020) identified an isolate co-harbouring *mcr-9* and $bla_{\text{NDM-1}}$ from a patient with BSI. Resistome analysis revealed 24 other ARGs mediating resistance to aminoglycosides [*aac(6')-IIc*, *aadA5*, *aph(3'')-Ib*, *aph (3')-Ia*, *aph(6)-Id*, *armA*], β-lactams [$bla_{\text{ACT-16}}$, $bla_{\text{DHA-1}}$, $bla_{\text{OXA-1}}$, $bla_{\text{SHV-12}}$], chloramphenicol [catA2, catB3], fosfomycin [*fosA*], macrolides (*msr(E)*, *mph(E)*, *ere(A)*], quinolones [*aac(6')-Ib-cr*, *oqxB*, *oqxA*, *qnrB4*], sulphonamides [s*ul1*], and trimethoprim [*dfrA1*, *dfrA19*] (Lin et al., 2020). In addition to ARGs, isolates harbored yersinia high-pathogenicity island (HPI), which harbored yersiniabactin biosynthesis operon *ybtSXQPAUTE, irp1/ 2*, and *fyuA*, and a battery of virulent genes.

2.10 *E. coli*

E. coli is a Gram-negative bacterium belonging to the *Enterobacterales* family. It lives as a normal commensal of the human intestine, but can opportunistically cause life-threatening

infections, including bacteraemia (Diekema et al., 2019). *E. coli* has become a common cause of BSIs. Previously, two studies from Italy revealed that the most commonly isolated pathogen from patients' blood was *E. coli* (32.8%) (De Angelis et al., 2018) and (34%) (De Socio et al. 2019). *E. coli* has become more resistant to antibiotics, including broad-spectrum penicillins, third-generation cephalosporins, fluoroquinolones, aminoglycosides, and polymyxins, rendering treatment difficult. Carbapenem-resistant *E. coli* has resulted in the revival of polymyxins as a last-resort antibiotic treatment option (Quan et al., 2017). However, the emergence of polymyxin resistance has been reported in Gram-negative pathogens, leaving limited treatment options. Genotypic determinants for resistance, such as *mcr-1*, bla_{NDM}, $bla_{\text{OXA-48}}$, and bla_{IMP} have been identified among MDR *E. coli* (Mediavilla et al., 2016). The co-existence of the colistin resistance gene (*mcr*) with ARGs encoding resistance to multiple antibiotics, such as carbapenems and extended-spectrum β-lactams, raises concerns about potentially pan-drug-resistant strains. Several pandemic strains responsible for BSI belong to clonal lineages of MDR *E. coli*, including sequence types (ST) ST131, ST73, ST95, ST69, and ST38 (Manges et al., 2019).

In the study by Ragupathi et al. (2020), WGS was used to detect ARGs, encoding resistance to β-lactams, carbapenems, fluoroquinolones, tetracycline, aminoglycosides and colistin. The aminoglycoside ARGs (*aadA*5 and *aac(6')lb-cr*, *aadA*2 and *rmtB*) were the most abundant, followed by the β-lactam ARGs ($bla_{\text{CTX-M-15}}$, $bla_{\text{NDM-5}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1B}}$). The study reported the increasing frequency of antimicrobial resistance in clinical *E. coli* isolates, which were associated with $bla_{\text{CTX-M}}$, bla_{NDM}, and *mcr* genes. Son et al. (2021) investigated resistance patterns of *E. coli* strains isolated from patients with BSI in Vietnam. The species identification and antimicrobial susceptibility testing were determined by the VITEK 2 system. Isolates were assayed for ESBL ARGs using multiplex PCR and double disk diffusion methods. Several

resistance genes were identified, including *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ (β-lactams), *bla*$_{\text{NDM}}$, and *bla*$_{\text{VIM}}$ (carbapenem).

E. coli infections are exacerbated by the presence of virulence factors, depending on the pathotype involved. The *E. coli* pathotypes associated with extraintestinal pathogenic *E. coli* (ExPEC) strains are frequently associated with BSIs and encode virulence factors that aid in colonizing the digestive tract and urinary tract. ExPEC contains genes encoding virulence factors such as adhesins to adhere to host cells, capsular polysaccharides and lipopolysaccharides (LPS), toxins that enable invasion, iron acquisition systems, and serum resistance proteins (Desvaux et al., 2020).

A comprehensive understanding of the epidemiology of ESKAPEEc and the nature and extent of antibiotic resistance is vital. Combining phenotypic and molecular techniques like WGS is recommended as a basis for antibiotic resistance surveillance to develop public health guidelines (WHO, 2020). Therefore, this study used a combination of phenotypic and genomic-based methods to describe the molecular epidemiology of drug-resistant ESKAPEEc implicated in BSIs in the uMgungundlovu district, KwaZulu-Natal (KZN), South Africa, focusing on resistome, virulome, mobilome, clonality, and phylogenies.

3.0 Aim and Objectives

3.1 Aim

To describe the molecular epidemiology, resistome, virulome, mobilome, clonality, and phylogenies of antibiotic-resistant ESKAPE and *E. coli* pathogens isolated from blood cultures in uMgungundlovu District, KwaZulu-Natal (KZN), South Africa.

3.2 Specific objectives

- To ascertain the incidence of ESKAPEEc from hospitals in the uMgungundlovu District of the KwaZulu-Natal Province from blood cultures routinely processed by the central microbiology laboratory using culture techniques.

- To identify ESKAPEEc obtained from blood cultures using VITEK-2 system followed by real-time PCR using primer-specific genes.

- To ascertain the antibiotic resistance patterns of ESKAPE and *E. coli* by Kirby-Bauer disk diffusion according to the European Committee on Antimicrobial Susceptibility (EUCAST) and/or Clinical and Laboratory Standards Institute (CLSI) guidelines as appropriate.

- To delineate resistome and virulome using WGS and bioinformatics tools to provide a comprehensive understanding of the mechanisms of resistance and virulence in selected *K. pneumoniae, K. oxytoca* and *S. aureus* isolates, including but not limited to ResFinder, Comprehensive Antibiotic Resistance Database (CARD), VirulenceFinder, virulence factor database (VFDB), and BacWGSTdb.

- To identify and characterise mobile genetic elements to comprehend how clinical *K. pneumoniae, K. oxytoca* and *S. aureus* spread ARGs and virulence factors, using WGS and bioinformatics tools including Mobile, ElementFinder, PlasmidFinder, ISFinder. PHASTER, and SCC*mec*Finder.

- To assess the phylogenetic relationships in order to understand the evolutionary history and relatedness among *K. pneumoniae*, *K. oxytoca* and *S. aureus* from blood cultures using WGS and bioinformatics tools, including Bacterial and Viral Bioinformatics Resource Center (BV-BRC), iTOL and FigTree.

4.0 Synopsis of Methodology

4.1 Ethical Approval

Ethical approval for the study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal under the following reference number BCA444/16. All bacterial isolates were obtained as part of routine clinical management, and no patient-identifiable information was collected. As such, the requirement for individual informed consent was waived by the ethics committee. Permission was obtained from the Head of the Provincial Department of Health and the relevant Health District Manager of the Province of KwaZulu-Natal to conduct the study in the Province and District, respectively. Permission was sought from the Chief Executive Officers/Medical Managers of the public sector health facilities and the Head of Medical Microbiology in the uMgungundlovu NHLS to conduct research in their healthcare facilities and laboratories, respectively, and Memoranda of Agreement/Understanding were entered into between the University, the healthcare facility and NHLS.

4.2 General Methodology

The study describes the molecular epidemiology, and phenotypic and genotypic characteristics of ESKAPEEc isolates implicated in blood stream infections from hospitals in the uMgungundlovu District of the KwaZulu-Natal Province, South Africa. The study was part of a larger surveillance study that used the Global Antimicrobial Resistance and Use Surveillance System (GLASS) guidelines. Presumptive ESKAPEEc isolates (from blood cultures) were obtained from routine blood cultures processed by the central microbiology laboratory that receives samples from hospitals in the uMgungundlovu district. Isolates were collected over 18 months, from October 2018 to February 2020 and stored at the Antimicrobial Research Unit, University of KwaZulu-Natal. The inclusion criteria were patients with suspected bloodstream infections from whom blood samples were collected for microbiological analysis and were positive for ESKAPEEc pathogens. Bacterial identification was done using the VITEK 2 system (BioMérieux, MarcyL'Etoile, France), followed by molecular confirmation of isolates using qPCR-specific primer sequences. PCR of the *mecA* gene was used to confirm methicillin-resistant *S. aureus*. The antibiotic susceptibility profiles of ESKAPEEc pathogens against twenty antibiotics and enterococci against sixteen antibiotics were determined using the disk diffusion assay according to the CLSI/EUCAST guidelines (CLSI, 2020; EUCAST, 2020). The resistome, virulome, mobilome, clonality and phylogenomic relationships of ESKAPE and *E. coli* clones were determined using WGS and bioinformatics tools. Comparative genomics of ESKAPE and *E. coli* was done to assess dominant endemic clones spreading in the hospital setting.

4. 3 Outline of the Thesis

This PhD study is presented in the form of journal articles in press, submitted manuscripts, and consists of the following five chapters:

Chapter 1: An introduction and literature review on antibiotic resistance in hospital environments and BSIs. The MDR ESKAPEEc, such as MRSA, VRE, and ESBL-resistant pathogens listed in the WHO priority list and their inclusion in antibiotic resistance surveillance studies, is reviewed.

Chapter 2: Manuscript 1. Multidrug-Resistant ESKAPEEc Pathogens from Bloodstream Infections in South Africa: A Cross-Sectional Study Assessing Resistance to WHO AWaRe Antibiotics. [Hetsa, B.A., Asante, J., Amoako, D.G., Abia, A.L., Mbanga, J. and Essack, S.Y., 2025. Multidrug-Resistant ESKAPEEc Pathogens from Bloodstream Infections in South Africa: A Cross-Sectional Study Assessing Resistance to WHO AWaRe Antibiotics. *Health Science Reports*, *8*(6), p.e70897]. The manuscript describes the prevalence and antibiotic resistance patterns of ESKAPE and *E. coli* pathogens in the uMgungundlovu district in the KZN Province, South Africa.

Chapter 3: Manuscript 2. Genomic Analysis of Virulent, Multidrug-Resistant *Klebsiella pneumoniae* and *Klebsiella oxytoca* from Bloodstream Infections, South Africa. [Accepted in Microbial Pathogenesis] [Hetsa, B.A., Asante, J., Mbanga, J., Amoako, D.G., Abia, A.L., Ismail, A. and Essack, S.Y., 2025. Genomic analysis of virulent, multidrug-resistant *Klebsiella pneumoniae* and *Klebsiella oxytoca* from bloodstream infections, South Africa. *Microbial Pathogenesis*, *200*, p.107272]. This manuscript describes the antibiotic resistome, mobilome, virulome and phylogenetic analysis of *K. pneumoniae* in the KwaZulu-Natal Province, South Africa, using WGS.

Chapter 4: Manuscript 3: Genomic Characterisation of Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Implicated in Blood-stream Infections, KwaZulu-Natal,

South Africa: A Pilot Study**.** [Hetsa, B.A.; Asante, J.; Mbanga, J.; Ismail, A.; Abia, A.L.K.; Amoako, D.G.; Essack, S.Y. Genomic Characterization of Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Implicated in Bloodstream Infections, KwaZulu-Natal, South Africa: A Pilot Study. *Antibiotics* **2024**, *13*, 796. https://doi.org/10.3390/antibiotics13090796]. The study describes the antibiotic resistome, mobilome, virulome, and phylogenic analysis of clinical methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates in the Kwa-Zulu Natal Province, South Africa, using WGS and bioinformatics tools.

Chapter 5: **Conclusion:** This chapter gives a summary and significance of the work. The limitations and recommendations for future work are also discussed.

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CHAPTER 2- MULTIDRUG-RESISTANT ESKAPEEC PATHOGENS FROM BLOODSTREAM INFECTIONS IN SOUTH AFRICA: A CROSS-SECTIONAL STUDY ASSESSING RESISTANCE TO WHO AWARE ANTIBIOTICS

Authors contribution

Bakoena Ashton Hetsa, as the principal investigator, co-conceptualized the study, undertook the laboratory work and drafted the manuscript.

- **Dr Jonathan Asante** and undertook critical revision of the manuscript.

- **Dr Joshua Mbanga**, as co-supervisor undertook critical revision of the manuscript.

- **Dr Akebe L. K. Abia**, as co-supervisor, supervised the laboratory work, vetted the results and undertook critical revision of the manuscript.

- **Dr Daniel G. Amoako**, as co-supervisor, supervised the laboratory work, vetted the results and undertook critical revision of the manuscript.

- **Professor Sabiha Y. Essack**, as the principal supervisor, co-conceptualized the study, guided the literature review and ethical approval application, facilitated data collection and analysis, and undertook critical revision of the manuscript.

Objective(s) met: This paper addresses objectives 1, 2 and 3

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Multidrug-Resistant ESKAPEEc Pathogens From Bloodstream Infections in South Africa: A Cross-Sectional Study Assessing Resistance to WHO AWaRe Antibiotics

Bakoena A. Hetsa[1] | Jonathan Asante[1,2] | Daniel G. Amoako[1,3] | Akebe L. K. Abia[1] | Joshua Mbanga[1,4] | Sabiha Y. Essack[1]

[1]Antimicrobial Research Unit, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa | [2]School of Pharmacy and Pharmaceutical Sciences, University of Cape Coast, Ghana | [3]Department of Pathobiology, University of Guelph, Ontario, Canada | [4]Department of Applied Biology & Biochemistry, National University of Science and Technology, Bulawayo, Zimbabwe

Correspondence: Bakoena A. Hetsa (hetszab@gmail.com)

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ABSTRACT

Background and Aims: Multidrug-resistant (MDR) pathogens, particularly members of the ESKAPE group and *Escherichia coli* (collectively referred to as ESKAPEEc), are major contributors to bloodstream infections (BSIs) and pose significant treatment challenges. This study aimed to characterize the antimicrobial resistance (AMR) profiles of ESKAPEEc isolates from BSIs in public hospitals in the uMgungundlovu District, South Africa, and to assess their resistance to World Health Organization (WHO) Access, Watch, and Reserve (AWaRe) antibiotics.

Methods: Between November 2017 and December 2018, blood samples ($n = 195$) were collected from adult and paediatric patients with suspected BSIs. Isolates were identified using the VITEK 2 system and confirmed by polymerase chain reaction (PCR). Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method and interpreted according to EUCAST/CLSI guidelines. The multiple antibiotic resistance index (MARI) was calculated. One-way analysis of variance (ANOVA) was used to assess associations between MARI and clinical variables, including ward type and facility level.

Results: Out of 195 presumptive isolates, 159 were confirmed as ESKAPEEc. The most frequently identified pathogens were *Klebsiella pneumoniae* (28.9%) and *Staphylococcus aureus* (28.3%). High resistance rates were observed across WHO Access and Watch antibiotics, including ampicillin (76% in *E. coli*), gentamicin (67.4% in *K. pneumoniae*), and ciprofloxacin (≥ 60% in most species). Carbapenem resistance in *Acinetobacter baumannii* reached 90%. Overall, 94.9% of isolates were MDR, and 93.1% had MARI ≥ 0.2. Significant differences in MARI values were observed across ward groups and facility levels, with the highest values recorded in intensive care units (mean = 0.67, 95% CI: 0.62–0.72) and tertiary hospitals (mean = 0.64, 95% CI: 0.60–0.68), compared to regional hospitals (mean = 0.52, 95% CI: 0.47–0.57).

Conclusion: The findings reveal a high burden of MDR ESKAPEEc in BSIs and widespread resistance to WHO Watch antibiotics. Targeted antimicrobial stewardship and the implementation of microbiology-guided therapy are urgently needed to optimize patient outcomes and curb the spread of resistance.

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1 | Introduction

Enterococcus faecium, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* spp. are called ESKAPE pathogens that escape the action of antibiotics due to their increasing multidrug resistance [1, 2]. Together with third-generation cephalosporin and carbapenem-resistant *Escherichia coli*, these bacteria are identified by the World Health Organization as bacterial priority pathogens for the research and development of new antibiotics [3]. The ESKAPE and *E. coli* pathogens (ESKAPEEc) are increasingly implicated in most nosocomial infections, including bloodstream infections (BSIs) and sepsis [4]. These infections are commonly reported among neonates, the elderly, and immunocompromised patients [5].

BSIs are invasive infections characterized by high mortality rates and healthcare costs [6]. Several studies on BSIs have reported different causative organisms with varying occurrences of Gram-negative [7] and Gram-positive [8, 9] pathogens. A 20-year surveillance study by Tian et al. [10] in China found that *E. coli*, *S. aureus*, and *K. pneumoniae* were the most predominantly identified pathogens causing BSIs. Their results revealed that *K. pneumoniae* and *E. coli* displayed resistance to antibiotics, including third-generation cephalosporins and fluoroquinolones [10].

In South Africa, carbapenem-resistant *Enterobacterales* (CRE) have been reported in a retrospective survey done in patients with bacteraemia from 2015 to 2018 through the Group for Enteric, Respiratory and Meningeal Disease Surveillance in South Africa (GERMS-SA) surveillance platform. Perovic et al. [11] revealed that more than 75% of CRE isolates were *K. pneumoniae* and carried $bla_{OXA\text{-}48}$ and bla_{NDM} genes conferring resistance to carbapenems. Moreover, a more recent study that compared the BSI incidence, pathogens, and antimicrobial resistance profiles in two large neonatal units in Botswana and South Africa showed that *K. pneumoniae* and *S. aureus* were dominant pathogens and displayed high levels of resistance to antibiotics [12]. Another South African study on ESKAPEEc isolated from patients with bacteraemia in private and public sectors/hospitals identified *K. pneumoniae* as the leading pathogen. Isolates displayed varying non-susceptibility to ciprofloxacin, cephalosporins, carbapenems, ciprofloxacin, amikacin, gentamicin, and tigecycline [13].

The surveillance and characterization of antibiotic-resistant pathogens, including ESKAPEEc, is essential in managing the rise of AMR. We assessed the antibiotic resistance profiles of ESKAPEEc implicated in BSIs among adults and children in two public hospitals in the uMgungundlovu district in KwaZulu-Natal, South Africa.

2 | Materials and Methods

2.1 | Ethical Approval

Ethical approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Reference Number: BCA444/16). All bacterial isolates were obtained as part of routine clinical diagnostics, and no patient-identifiable information was collected. As such, the requirement for individual informed consent was waived by the ethics committee.

2.2 | Study Setting and Sample Collection

Blood cultures were collected from November 2017 to December 2018 at one regional and one tertiary public sector hospital in the uMgungundlovu District. The regional hospital comprised 824 beds, while the tertiary hospital is a 530-bed facility. A total of 195 presumptive ESKAPEEc isolates implicated in BSIs constituted the sample. Patients' demographic data (age, sex, ward type, and specimen source) were collected. Patients' names were excluded to maintain anonymity.

2.3 | Bacterial Isolation and Identification

Preliminary identification of isolates was done using the VITEK 2 system (BioMérieux, Marcy-L'Etoile, France). Pure isolates were stored in tryptic soy broth (Basingstoke, Hampshire, England) broth supplemented with 20% glycerol at −80°C until further analysis.

2.4 | DNA Extraction and Molecular Confirmation of ESKAPE and *E. coli* Isolates

DNA was extracted using the boiling method as previously described [14]. The purity and concentration of extracted DNA were determined by gel electrophoresis. Molecular confirmation of ESKAPEEc isolates was done using species-specific oligonucleotide primer sequences and PCR conditions listed in Supporting Information S1: Table 2. PCR was performed in a 10 μL reaction volume with 3 μL DNA template, 5 μL Luna® Universal qPCR master mix (Biolabs, New England, South Africa), 0.5 mL (1.25 μM) of each primer, and 1 mL of nuclease-free water. *E. coli* ATCC 25922, *S. aureus* ATCC 29213, *K. pneumoniae* ATCC 35657, *E. aerogenes* ATCC 13048, *Enterococcus* spp. ATCC 29212, *P. aeruginosa* ATCC 35032, and *A. baumannii* ATCC 19606 were used as positive controls, while nuclease-free water was used as negative control.

2.5 | Molecular Identification of Methicillin-Resistant *S. aureus* (MRSA)

Multiplex PCR was conducted targeting *S. aureus* specific thermonuclease (*nuc)* gene, and the methicillin-resistance-encoding *mecA* gene using primers and PCR conditions shown in Table S2. All reactions were performed in a total volume of 10 uL, which consisted of 5 μL 2× Luna Universal qPCR Master Mix (New England Biolabs, South Africa) 0.5 μL (1.25 mM) of the forward and reverse primers, 1 μL of nuclease-free water, and 3 μL of template DNA. The PCR conditions were as previously described [15], with slight modifications of the hot start activation (94°C for 5 min), followed by 35 cycles of denaturation (94°C for 30 s), and annealing/extension at (62°C for 30 s). A melt curve was prepared by ramping up the melting temperature from 60°C to 95°C at a rate of 0.15°C/s in continuous

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mode following a pre-melt step at 95°C for 15 s on the 1st step. The methicillin-resistant *S. aureus* ATCC 43300, was used as a positive control, and nuclease-free water was used as the negative control. All reactions were carried out in an Applied Biosystems QuantStudio 5 Real-time PCR system (Thermo Fisher Scientific, Waltman, MA).

2.6 | Antimicrobial Susceptibility Testing (AST)

The antibiotic susceptibility profiles were determined by the Kirby–Bauer disk diffusion method, and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [16]. Breakpoints for ampicillin, azithromycin, tetracycline, and nalidixic acid were from the Clinical and Laboratory Standards Institute (CLSI) breakpoints [17]. Antibiotic resistance profiles were classified using the WHO AWaRe arrangement of Access (first line), Watch (restricted), Reserve (last resort) antibiotics (WHO AWaRe, 2023). For *Enterobacterales* the following 20 antibiotics were used: amikacin (AMK, 30 μg), ampicillin (AMP, 10 μg), azithromycin (AZM, 15 μg), amoxicillin-clavulanic acid (AMC, 30 μg), cefepime (FEP, 10 μg), cefotaxime (CTX, 30), cefoxitin (FOX, 30 μg), ceftazidime (CAZ, 30 μg), ceftriaxone (CRO, 30 μg), cephalexin (LEX, 30 μg), ciprofloxacin (CIP, 5 μg), chloramphenicol (CHL, 30 μg), gentamicin (GEN, 10 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), nalidixic acid (NAL, 30 μg), piperacillin-tazobactam (TZP, 110 μg), tetracycline (TET, 30 μg), tigecycline (TGC, 15 μg), and trimethoprim-sulfamethoxazole (SXT, 25 μg) (Oxoid, Basingstoke, UK), were used. All antibiotic discs used in this study were purchased from Oxoid (Oxoid Ltd., Basingstoke, UK) (Supporting Information S1: Table 1). *E. coli* ATCC 25922 was used for quality control.

For *E. faecium*, 16 commercial antibiotic disks were tested, which included ampicillin (AMP, 10 μg), chloramphenicol (CHL, 30 μg), ciprofloxacin (CIP, 5 μg), erythromycin (ERY, 15 μg), imipenem (IPM, 10 μg), gentamycin (GEN, 120 μg), levofloxacin (LVX, 5 μg), linezolid (LZD, 30 μg), tetracycline (TET, 30 μg), nitrofurantoin (NIT, 300 μg), quinupristin-dalfopristin (Q-D, 15 μg), streptomycin (STR, 300 μg), teicoplanin (TEC, 30 μg), tigecycline (TGC, 15 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), and vancomycin (VAN, 30 μg), disks (Oxoid, Basingstoke, UK), (interpreted using EUCAST breakpoint (EUCAST, 2019). Breakpoints for chloramphenicol (CHL 30 μg), tetracycline (TET, 30 μg), and erythromycin (ERY, 15 μg) were interpreted according to CLSI [17]. *E. faecalis* ATCC 29212 was used for quality control.

For *S. aureus* the following 20 antibiotics were used: penicillin G (PEN 10 μg), ampicillin (AMP 10 μg), amikacin (AMK, 30 μg), cefoxitin (FOX, 30 μg), chloramphenicol (CHL, 30 μg), clindamycin (CLI, 2 μg), ciprofloxacin (CIP, 5 μg), erythromycin (ERY, 15 μg), gentamicin (GEN, 10 μg), levofloxacin (LVX, 5 μg), linezolid (LZD 30 μg), moxifloxacin (MXF, 5 μg), quinupristin-dalfopristin (Q-D, 15 μg), rifampicin (RIF, 5 μg), tetracycline (TET, 30 μg), tigecycline (TGC, 15 μg), trimethoprim-sulfamethoxazole (SXT, 25 μg), (interpreted using EUCAST breakpoints), and doxycycline (DOX, 30 μg), nitrofurantoin (NIT, 300 μg), teicoplanin (TEC, 30 μg) interpreted using CLSI breakpoints. All antibiotics were purchased from Oxoid (Oxoid, Basingstoke, UK). The minimum inhibitory concentrations (MICs) for vancomycin (VAN) were determined through the broth microdilution method using the CLSI guidelines, due to the absence of breakpoints for the disc diffusion method [17]. A methicillin-sensitive strain, *S. aureus* ATCC 29213, was used as a positive control.

2.7 | Multidrug-Resistance and Multiple Antimicrobial Resistance Index (MARI) Analysis

Multidrug resistance was defined as resistance to at least one agent in three distinct antibiotic classes. Multiple antibiotic resistance phenotypes were displayed by all isolates in the study, regardless of antibiotic classes. The MAR index was evaluated using the formula $MAR=x/y$, where x is the number of antibiotics the isolate was resistant to, and y is the total number of antibiotics tested against the isolate [18]. The MAR index was used as an indicator of health risk assessment to ascertain whether isolates originate from excessive or low antibiotic use environments.

2.8 | Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 27.0 (IBM Corp., Armonk, NY, USA) and Python 3.11 with the statsmodels and seaborn libraries for data visualization. Descriptive statistics were used to summarize demographic characteristics and antimicrobial resistance data. Continuous variables were presented as means with standard deviations (SD) or medians with interquartile ranges (IQR), where appropriate. Categorical variables were reported as frequencies and percentages.

The multiple antibiotic resistance index (MARI) was calculated for each isolate by dividing the number of antibiotics to which the isolate was resistant by the total number of antibiotics tested. One-way analysis of variance (ANOVA) was used to compare MARI values across two key categorical variables: facility level (regional vs. tertiary hospitals) and clinical setting, classified into Ward Group categories (Intensive Care Units, General Inpatient Wards, Surgical Wards, Paediatric Units, Emergency/Trauma Units, Specialist Wards, and Outpatient/Clinic-Based Services).

The assumptions of normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. Where these assumptions were met, ANOVA was conducted to identify overall group differences. Tukey's Honestly Significant Difference (HSD) test was used post hoc to identify specific group pairwise differences. Effect sizes were reported using partial eta squared (η^2) to quantify the proportion of variance in MARI explained by each factor. Confidence intervals (95% CI) for group means were calculated to assess the precision of the estimates. All statistical tests were two-sided, and a significance level (α) of 0.05 was used to determine statistical significance. No imputation was performed for missing data. All analyses were pre-specified in the study protocol, and no exploratory subgroup analyses were conducted. Statistical

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terminology, abbreviations, and test types were clearly defined in the results section to ensure interpretability and consistency.

3 | Results

3.1 | Isolation, Molecular Identification, and Distribution of Isolates

The VITEK 2 system was utilized for bacterial identification. Of the 185 presumptive isolates, 159 (85.9%) were confirmed as members of the ESKAPEEc group using the VITEK 2 system. Species-level identification was further validated using qPCR with species-specific oligonucleotide primers listed in Supporting Information S1: Table 1. No discrepancies were observed between phenotypic and molecular identification across all isolates. Among the ESKAPEEc pathogens, *K. pneumoniae* was the most frequently isolated species ($n = 46$, 28.9%), followed closely by *S. aureus* ($n = 45$, 28.3%), and *E. coli* ($n = 25$, 15.7%) (Figure 1A). Twenty-six (26) isolates were identified as non-ESKAPEEc organisms, including *Enterococcus faecalis* ($n = 13$, 50.0%), *Serratia marcescens* ($n = 11$, 42.3%), and *Proteus mirabilis* ($n = 2$, 7.6%).

Regarding facility level distribution, the majority of isolates were obtained from regional hospitals ($n = 128$, 80.5%), while tertiary hospitals contributed 31 isolates (19.5%) (Figure 1B). Stratification by broader clinical settings (Ward Group) revealed that the highest number of isolates originated from Intensive Care Units ($n = 43$), followed by Outpatient/Clinic-Based Services ($n = 31$), and Paediatric Units ($n = 24$) (Figure 1C). Across clinical departments, the Intensive Care Unit (ICU) accounted for the largest share of isolates ($n = 33$, 20.7%), followed by surgical and paediatric wards (Figure 1D). Within the ICU, *K. pneumoniae* was the predominant pathogen (15/33, 45.5%), while *S. aureus* was most frequently recovered from the paediatric outpatient department ($n = 12$). The remaining species were variably distributed across other ward types levels (Figure 1D; Supporting Information S1: Table 2).

Seventy-nine of the 159 patients (49.7%) were males, and 71 (44.7%) were females, while the remaining 9 (5.6%) were unidentified. The participants' age ranged from 0 months to 77 years. The mean age was 19.35 years. Also, 3 (6.7%), of the 45 *S. aureus* isolates were confirmed as MRSA by PCR detection of *mec*A gene.

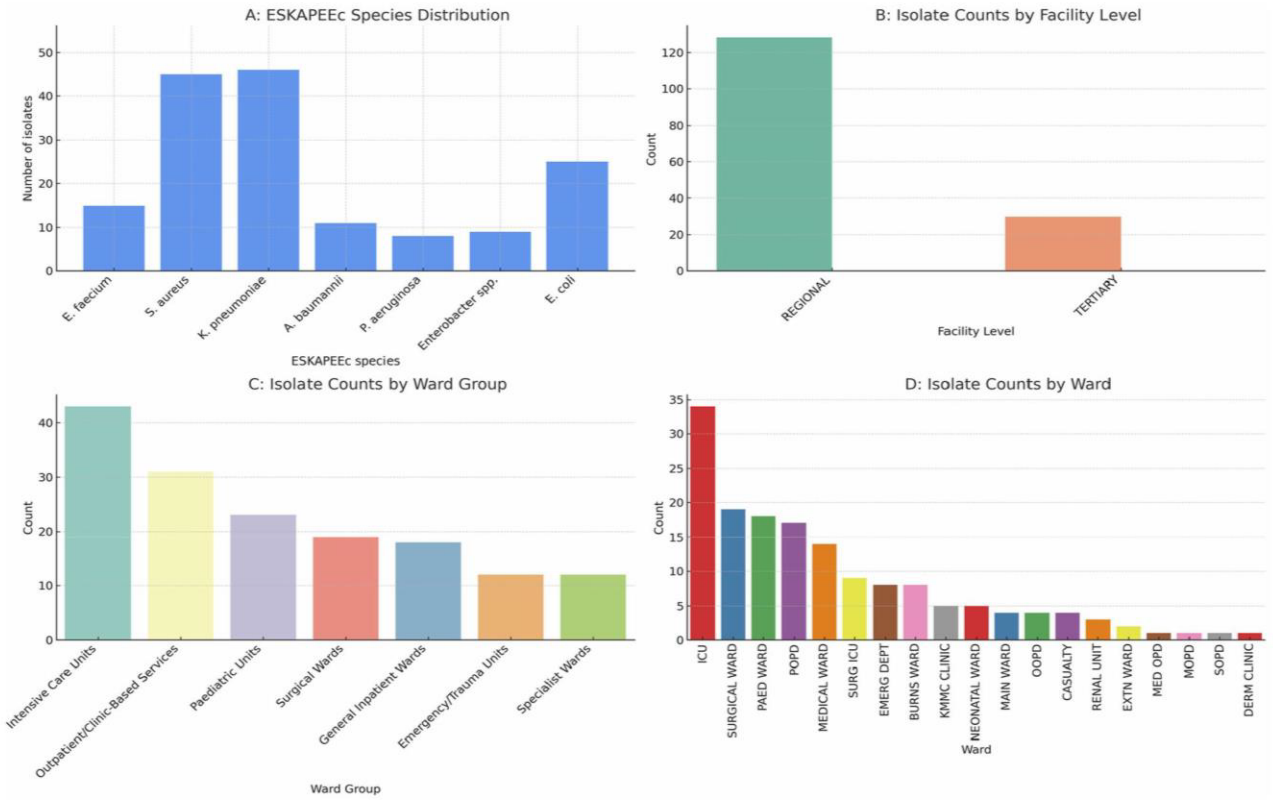

FIGURE 1 | Distribution of ESKAPEEc Isolates by Species, Facility Level, Ward Group, and Ward Type. (A) Frequency distribution of ESKAPEEc species isolated from bloodstream infections ($n = 159$). *Klebsiella pneumonia* and *Staphylococcus aureus* were the most common species. (B) Isolate counts stratified by healthcare facility level, showing a predominance of isolates from regional hospitals compared to tertiary hospitals. (C) Isolate distribution across broader clinical categories (WardGroup), with Intensive Care Units (ICUs) and Outpatient/Clinic-Based Services accounting for the highest numbers. (D) Detailed breakdown of isolate counts by specific hospital wards, with ICU, surgical, and pediatric wards contributing the highest proportions.

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3.2 | Prevalence of Antibiotic-Resistance Profiles of ESKAPEEc Isolates

The antibiotic susceptibility profiles of confirmed ESKAPEEc isolates are summarized in Table 1. *K. pneumoniae* exhibited the highest resistance to Access group antibiotics, notably cephalexin (78.3%), amoxicillin-clavulanic acid (69.6%), and trimethoprim-sulfamethoxazole (67.4%), with the lowest resistance to amikacin (39.1%). Resistance to Watch group antibiotics ranged from 30.4% to 69.0%, with highest resistance against cefotaxime, ceftazidime, and ciprofloxacin (each 67.4%–69.0%), and the lowest against imipenem (30.4%) and meropenem (32.6%). All *K. pneumoniae* isolates were susceptible to tigecycline (91.3%) and were not tested for ampicillin due to intrinsic resistance [19]. *Escherichia coli* showed high resistance to ampicillin (76.0%) and amoxicillin-clavulanic acid (68.0%) in the Access group, and 68.0% resistance to Watch antibiotics such as cefepime and ceftazidime. Lowest resistance was recorded for imipenem and meropenem (both 12.0%).

E. cloacae displayed high resistance to ampicillin (77.8%) and cefoxitin (66.7%), while resistance was lowest for gentamicin and nalidixic acid (11.1%). Isolates were highly susceptible to tigecycline (88.9%). *A. baumannii* showed extensive resistance to several Access antibiotics including ampicillin (72.7%) and chloramphenicol (63.6%), and to Watch antibiotics such as ceftazidime, ceftriaxone, and ciprofloxacin (each 72.7%). Lowest resistance was observed for amikacin (36.4%), azithromycin (45.5%), imipenem (45.5%), and meropenem (45.5%). Tigecycline remained highly effective (90.9% susceptibility). *P. aeruginosa* isolates were highly resistant to Access antibiotics such as ampicillin (75.0%) and amoxicillin-clavulanic acid (62.5%), but showed lower resistance to chloramphenicol and gentamicin (both 12.5%). In the Watch group, resistance was highest to cefoxitin and cefotaxime (62.5%) and lowest for piperacillin-tazobactam (12.5%). Tigecycline was not tested due to known ineffectiveness against *P. aeruginosa* (Table 1).

The antibiotic susceptibility profiles of all confirmed *S. aureus* and *E. faecium* isolates are presented in Table 2. *S. aureus* exhibited high resistance to Access group antibiotics, particularly penicillin G (93.3%) and tetracycline (60.0%), with the lowest resistance observed for chloramphenicol (17.8%). Among Watch group antibiotics, notable resistance was seen against rifampicin (62.2%), moxifloxacin (60.0%), and ciprofloxacin (57.8%). In contrast, isolates were highly susceptible to Reserve antibiotics including linezolid (95.6%), tigecycline (93.3%), and quinupristin-dalfopristin (93.3%). *E. faecium* isolates also showed substantial resistance to Access antibiotics, including ampicillin (80.0%), tetracycline (66.7%), and trimethoprim-sulfamethoxazole (66.7%). Resistance in the Watch category was recorded against erythromycin and streptomycin (both 66.7%), with the lowest resistance observed for imipenem (33.3%). Reserve agents such as vancomycin (93.3%), linezolid (96.0%), tigecycline (93.3%), and quinupristin-dalfopristin (93.3%) remained highly effective. The activity of trimethoprim and SXT against enterococci is uncertain; hence, the wild-type population is categorized as intermediate [20]. Consequently, there were no susceptible isolates (Table 2).

Overall, high levels of resistance were observed to Access and Watch group antibiotics across the pathogens (Figure 2). *K. pneumoniae* showed the highest resistance to both Access (64.6%) and Watch (56.7%) antibiotics. *A. baumannii* followed closely with 58.0% and 60.3% resistance to Access and Watch groups, respectively. *E. faecium*, *E. coli*, and *S. aureus* also demonstrated notable resistance rates, exceeding 40% for at least one category. In contrast, resistance to Reserve antibiotics remained relatively low across all species, with the highest being *E. cloacae* (11.1%) and *A. baumannii* (9.1%). *P. aeruginosa* displayed no resistance to Reserve agents, though it maintained moderate resistance to Access and Watch categories (Figure 2). These findings emphasize the widespread resistance to first-line and second-line treatments.

3.2.1 | Multidrug Resistance and Multiple Antibiotic Resistance (MAR) Index (MARI)

The overall MARI across all isolates ranged between 0.05 and 1.0 (mean = 0.56) (Supporting Information S1: Table S3). In total 148 (93.1%) had MARI > 0.2 isolates indicating that isolates were from environments with high antibiotic use. Multidrug resistance was recorded in 151 (94.9%) isolates (Supporting Information S1: Tables S4–S6). The isolates displayed varying resistance patterns that were grouped into 131 different antibiograms (Supporting Information S1: Table S7).

3.2.2 | Statistical Analysis

The multiple antibiotic resistance index (MARI) was used as a proxy for health risk assessment, providing insight into whether isolates originated from environments with high or low antibiotic pressure. To examine differences in MARI values across clinical settings, a one-way analysis of variance (ANOVA) was conducted for two grouping variables: ward group and facility level. The assumptions of normality and homogeneity of variances were evaluated before analysis using the Shapiro–Wilk and Levene's tests, respectively. Both assumptions were satisfied, with Levene's test indicating equal variances across ward groups ($F = 1.001$, $p = 0.427$) and facility levels ($F = 3.303$, $p = 0.071$) (Supporting Information S1: Table S8A).

A statistically significant difference in MARI values was observed among ward group categories ($F(6, 129) = 2.895$, $p = 0.011$), with a moderate effect size ($\eta^2 = 0.103$), suggesting that 10.3% of the variation in resistance levels was attributable to ward type (Supporting Information S1: Table S8B). The highest mean MARI values were recorded in Specialist Wards, Surgical Wards, and Intensive Care Units (Figure 3A). Post hoc analysis using Tukey's HSD revealed a significant pairwise difference between Intensive Care Units and Outpatient/Clinic-Based Services ($p = 0.031$), while other pairwise comparisons were not statistically significant (Supporting Information S1: Table S8C). In addition, a significant difference was observed between regional and tertiary hospitals ($F(1, 156) = 17.520$, $p < 0.001$), with an effect size of $\eta^2 = 0.101$ (Supporting Information S1: Table S8B). Tertiary hospitals had higher average MARI scores than regional facilities, likely reflecting greater antimicrobial selection pressure and patient acuity (Figure 3B).

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TABLE 1 | Antimicrobial susceptibility of confirmed *Enterobacterales*, and non-fermenting Gram-negative isolates from blood samples.

	K. pneumoniae ($n = 46$)			*E. coli* ($n = 25$)			*E. cloacae* ($n = 9$)			*A. baumanii* ($n = 11$)			*P. aeruginosa* ($n = 8$)		
Antibiotics (concentration)	***R*** **(%)**	***I*** **(%)**	***S*** **(%)**	***R*** **(%)**	***I*** **(%)**	***S*** **(%)**	***R*** **(%)**	***I*** **(%)**	***S*** **(%)**	***R*** **(%)**	***I*** **(%)**	***S*** **(%)**	***R*** **(%)**	***I*** **(%)**	***S*** **(%)**
Access antibiotics															
Ampicillin (10 μg)	NT	NT	NT	19 (76.0)	0	6 (24.0)	7 (77.8)	0	2 (22.2)	8 (72.7)	0	3 (27.3)	6 (75.0)	0	2 (25.0)
Amoxicillin-clavulanic acid (30 μg)	32 (69.6)	0	14 (30.4)	17 (68.0)	0	8 (32.0)	6 (66.7)	0	3 33.3)	7 (63.6)	0	4 (36.4)	5 (62.5)	0	3 (37.5)
Cephalexin (30 μg)	36 (78.3)	0	10 (21.7)	16 (64.0)	0	9 (36.0)	6 (66.7)	0	3 (33.3)	[a]7 (63.6)	0	4 (36.4)	5 (62.5)	0	3 (37.5)
Amikacin (30 μg)	18 (39.1)	18 (39.1)	10 (21.7)	11 (44.0)	0	14 (56.0)	0	3 (33.3)	6 (66.7)	4 (36.4)	0	7 (63.6)	1 (12.5)	0	7 (87.5)
Chloramphenicol (30 μg)	30 (65.2)	0	16 (34.8)	10 (40.0)	0	15 (60.0)	2 (22.2)	0	7 (77.8)	7 (63.6)	0	4 (36.4)	4 (50.0)	0	50.0
Gentamycin (10 μg)	31 (67.4)	0	15 (32.6)	14 (56.0)	6 (24.0)	5 (20.0)	1 (11.1)	4 (44.4)	4 (44.4)	5 (45.5)	0	6 (54.5)	1 (12.5)	0	7 (87.5)
Tetracycline (30 μg)[a]	30 (65.2)	7 (15.2)	9 (19.6)	16 (64.0)	0	9 (36.0)	3 (33.3)	0	6 (66.7)	7 (63.6)	0	4 (36.4)	5 (62.5)	0	3 (37.5)
Trimethoprim-sulfamethoxazole (25 μg)	31 (67.4)	0	15 (32.6)	14 (56.0)	0	11 (44.0)	4 (44.4)	0	5 (55.6)	6 (54.5)	0	5 (45.5)	NT	NT	NT
Watch antibiotics															
Azithromycin (15 μg)[a]	18 (39.0)	0	61.0	16 (64.0)	0	9 (36.0)	2 (22.2)	0	7 (77.8)	4 (45.5)	0	6 (54.5)	2 (25.0)	0	6 (75.0)
Cefepime[a] (10 μg)	26 (57.0)	0	20 (43.0)	14 (56.0)	0	11 (44.0)	3 (33.3)	2 (22.2)	4 (44.4)	[a]6 (54.5)	0	5 (45.5)	3 (37.5)	0	5 (62.5)
Cefotaxime[a] (30 μg)	32 (69.6)	0	14 (30.4)	17 (68.0)	3 (12.0)	5 (20.0)	5 (55.6)	1 (11.1)	3 (33.3)	[a]7 (63.6)	0	4 (36.4)	5 (62.5)	0	3 (37.5)
Ceftazidime[a] (30 μg)	31 (67.4)	0	15 (32.6)	17 (68.0)	0	8 (32.0)	4 (44.4)	3 (33.3)	2 (22.2)	[a]8 (72.7)	0	3 (27.3)	4 (50.0)	0	4 (50.0)
Ceftriaxone[a] (30 μg)	31 (67.4)	0	15 (32.6)	17 (68.0)	1 (4.0)	7 (28.0)	5 (55.6)	0	4 (44.4)	[a]8 (72.7)	0	3 (27.3)	3 (37.5)	0	5 (62.5)
Cefoxitin[a] (30 μg)	30 (65.2)	0	16 (34.8)	12 (48.0)	0	13 (52.0)	6 (66.7)	0	3 (33.3)	[a]8 (72.7)	0	3 (27.3)	5 (62.5)	0	3 (37.5)
Ciprofloxacin (5 μg)	31 (67.4)	0	15 (32.6)	15 (60.0)	0	10 (40.0)	5 (55.6)	2 (22.2)	2 (22.2)	8 (72.7)	0	3 (27.3)	3 (37.5)	0	5 (62.5)
Imipenem (10 μg)	14 (30.4)	0	32 (69.6)	3 (12.0)	2 (8.0)	20 (80.0)	3 (33.3)	0	6 (66.7)	5 (45.5)	0	6 (54.5)	2 (25.0)	0	6 (75.0)
Meropenem (10 μg)	15 (32.6)	0	31 (67.4)	3 (12.0)	2 (8.0)	20 (80.0)	3 (33.3)	0	6 (66.7)	5 (45.5)	0	6 (54.5)	2 (25.0)	0	6 (75.0)
Piperacillin-tazobactam (110 μg)	30 (65.2)	0	16 (34.8)	10 (40.0)	6 (24.0)	9 (36.0)	2 (22.2)	3 (33.3)	4 (44.4)	7 (63.6)	0	4 (36.4)	1 (12.5)	0	7 (87.5)
Nalidixic acid (30 μg)[a]	29 (63.0)	6 (13.0)	11 (24.0)	14 (56.0)	0	11 (44.0)	1 (11.1)	4 (44.4)	4 (44.4)	6 (54.5)	0	5 (45.5)	5 (62.5)	0	3 (37.5)
Reserve antibiotics															
Tigecycline (15 μg)	4 (8.7)	0	42 (91.3)	2 (8.0)	0	23 (92.0)	1 (11. 1)	0	8 (88.9)	1 (9.1)	—	10 (90.9)	0	0	8 (100.0)

Note: NT; AMP is not tested for *K. pneumoniae* because of intrinsic AMP resistance, and TGC is not tested for *P. aeruginosa*.
Abbreviations: I, intermediate susceptibility; R, resistant; S, susceptible.
[a]CLSI guidelines used [17]. SXT was not tested for *P. aeruginosa* because it is intrinsically resistant to SXT.

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TABLE 2 | Antimicrobial susceptibility of confirmed *S. aureus* and *E. faecium* isolates.

Antibiotics (concentration)	*S. aureus* ($n = 45$)			*E. faecium* ($n = 15$)		
	R (%)	*I* (%)	*S* (%)	*R* (%)	*I* (%)	*S* (%)
Access antibiotics						
Penicillin G (10 μg)	42 (93.3)	0	3 (6.7)	NT	NT	NT
Ampicillin (10 μg)	19 (42.2)	0	26 (57.8)	12 (80.0)	0	3 (20.0)
Amikacin (30 μg)	19 (42.2)	9 (20.0)	17 (37.8)	NT	NT	NT
Chloramphenicol (30 μg)	8 (17.8)	—	37 (82.2)	4 (26.7)	3 (20.0)	8 (53.3)
Clindamycin (2 μg)	11 (24.4)	2 (4.4)	32 (71.2)	NT	NT	NT
Doxycycline (30 μg)	13 (29.0)	5 (11.1)	27 (59.9)	NT	NT	NT
Gentamycin (10 μg)	20 (44.4)	—	25 (55.6)	[c]53.3	—	—
Tetracycline[b] (30 μg)	27 (60.0)	—	18 (40.0)	[b]10 (66.7)	3 (20.0)	2 (13.3)
Trimethoprim-sulfamethoxazole (25 μg)	23 (51.1)	2 (4.4)	20 (44.5)	10 (66.7)	5 (33.3)	0
Nitrofurantoin[a] (300 μg)	12 (26.7)	5 (11.1)	28 (62.2)	5 (33.3)	3 (20.0)	7 (46.7)
Average	43.1%			54.5		
Watch antibiotics						
Cefoxitin (30 μg)	17 (37.8)	—	28 (62.2)	NT	NT	NT
Ciprofloxacin (5 μg)	26 (57.8)	—	19 (42.2)	8 (53.3)	0	7 (46.7)
Erythromycin[a] (15 μg)	19 (42.2)	—	26 (57.8)	10 (66.7)	5 (33.3)	0
Imipenem[b] (10 μg)	NT	NT	NT	5 (33.3)	2 (13.3)	8 (53.3)
Levofloxacin (5 μg)	20 (44.4)	—	25 (55.6)	8 (53.3)	0	7 (46.7)
Moxifloxacin (5 μg)	27 (60.0)	—	18 (40.0)	NT	NT	NT
Rifampicin (5 μg)	28 (62.2)	11 (24.4)	6 (13.3)	NT	NT	NT
Streptomycin[c] (300 μg)	NT	NT	NT	10 (66.7)	—	—
Teicoplanin[b] (30 μg)	[a]18 (40.0)	3 (6.7)	24 (53.3)	[b]6 (40.0)	—	9 (60.0)
Vancomycin (30 μg)[b]	0	0	45 (100.0)	1 (6.7)	0	14 (93.3)
Average	43.1%			42.9%		
Reserve antibiotics						
Linezolid (30 μg)	2 (4.4)	—	43 (95.6)	1 (6.7)	—	14 (93.3)
Tigecycline (15 μg)	3 (6.7)	—	42 (93.3)	1 (6.7)	—	14 (93.3)
Quinupristin-dalfopristin (15 μg)[b]	3 (6.7)	0	42 (93.3)	2 (13.3)	—	13 (86.7)
Average	5.9%			4.9%		

Note: —, no breakpoints available.
Abbreviations: I = intermediate susceptibility; NT=Not tested PEN G, FOX, CLI, RIF, DOX, AMK; R = resistant; S = susceptible.
[a]CLSI guidelines used [17].
[b]Based on *Enterococcus faecium* only; SXT concentration (25 μg).
[c]Only HLR considered. HLR, high-level resistance GEN (120 μg).

Ninety-five percent confidence intervals (95% CI) were calculated around the group means to assess the precision of the estimates. Full descriptive statistics for both ward groups and facility levels are provided in Supporting Information S1: Tables S9A and S9B, respectively. Visual comparisons of MARI distribution and mean values are presented in Figure 3 (boxplots with significance markers) and Figure 4 (point plots with 95% confidence intervals and annotated sample sizes).

4 | Discussion

This study investigated the prevalence, distribution, and antibiotic resistance profiles of ESKAPEEc pathogens causing BSIs in KwaZulu-Natal, South Africa. Our results confirmed ESKAPE and *E. coli* as common causative bacteria in BSIs in children and adults and highlighted their diverse antibiotic susceptibility profiles.

One hundred and fifty-nine (159, 85.9%) isolates were identified as ESKAPE and *E. coli*. *K. pneumoniae* (28.9%) and *S. aureus* (28.3%) were the leading isolated pathogens, followed by *E. coli* (15.7%). The dominance of *K. pneumoniae* and *S. aureus* in the current study corroborates the findings of Ismail and Perovic [13], who studied antimicrobial susceptibility profiles of ESKAPE pathogens isolated from patients with bacteraemia in South Africa from 2016 to 2018. The occurrence of *E. coli* (15.7%) in this study was slightly lower than 19% and 36% from

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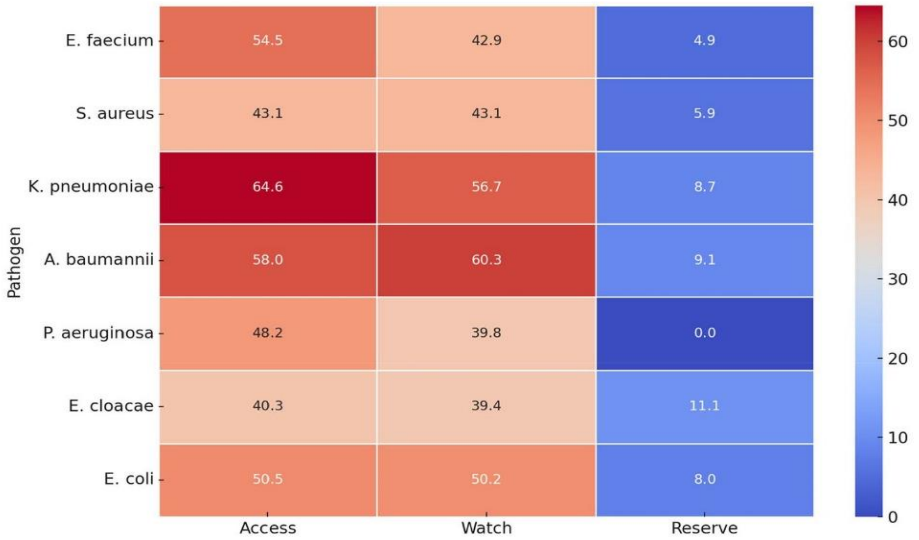

FIGURE 2 | Heatmap showing the percentage of resistance to WHO AWaRe (Access, Watch, Reserve) antibiotics among ESKAPEE pathogens isolated from bloodstream infections. Resistance levels are color-coded, with higher percentages indicated by warmer tones. *Klebsiella pneumoniae* and *Acinetobacter baumannii* exhibited the highest resistance to both Access and Watch category antibiotics, while *Enterobacter cloacae* had the highest resistance to Reserve antibiotics.

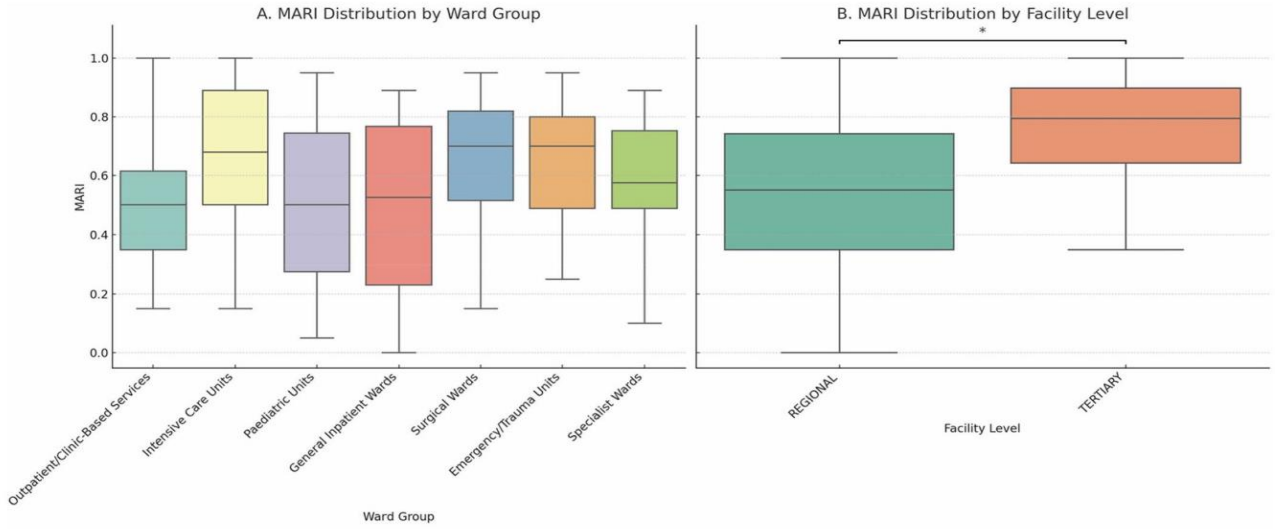

FIGURE 3 | Distribution of Multiple Antibiotic Resistance Index (MARI) values by clinical setting. (A) Boxplot showing the distribution of MARI values across seven clinical ward groupings. The highest median MARI scores were observed in Intensive Care Units, Surgical Wards, and Specialist Wards, while Outpatient/Clinic-Based Services had the lowest. (B) Boxplot comparing MARI values between facility levels. Tertiary hospitals showed significantly higher MARI values compared to regional hospitals ($p < 0.001$). The asterisk denotes a statistically significant difference.

the public and private hospitals reported in another South African study that analyzed antimicrobial susceptibility patterns of ESKAPE organisms isolated from patients with bacteraemia between 2016 and 2017. Their results showed that *E. coli* was the most common organism, followed by *K. pneumoniae* [21].

The overall antibiotic susceptibility profiles showed that more than two-thirds of the *Enterobacterales* and *non-Enterobacterales* isolates ($\geq 50\%$) were resistant to antibiotics in the Access category (ampicillin, amoxicillin-clavulanic acid, gentamycin) and Watch category (cephalosporins, piperacillin-tazobactam, ciprofloxacin), similar to that reported by Ismail and Perovic [13]. Choonara et al. [22] also observed resistance to cephalosporins, ciprofloxacin, gentamycin, and trimethoprim-sulfamethoxazole in a study that was done in Malawi, which investigated the antimicrobial susceptibility profiles of ESKAPEEc isolates from clinical samples, including blood. However, their analysis differs

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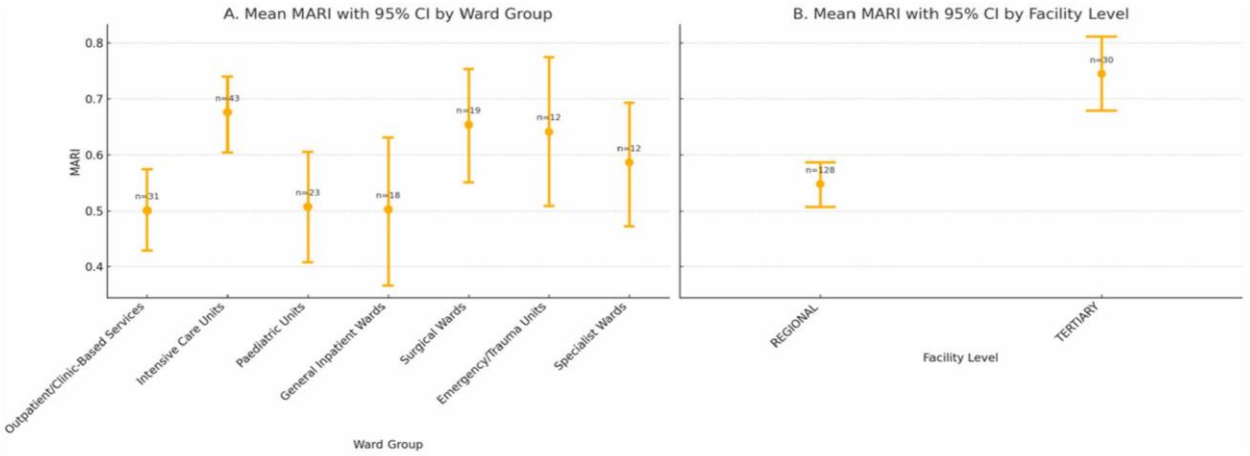

FIGURE 4 | Mean Multiple Antibiotic Resistance Index (MARI) with 95% confidence intervals by clinical setting. (A) Mean MARI values with 95% confidence intervals across seven ward group categories. Intensive Care Units, Surgical Wards, and Specialist Wards exhibited the highest mean MARI values. Sample sizes for each group are annotated above the error bars. (B) Mean MARI comparison between regional and tertiary hospitals, showing significantly higher resistance levels in tertiary facilities. Error bars represent 95% confidence intervals, with sample size (*n*) labeled above each mean point.

from ours because their sample size was larger and included urine, blood, and pus specimens. Their study also identified *P. mirabilis* and *Salmonella* spp., which were included for further analysis. The majority (≥ 82%) of isolates in this study displayed high susceptibility to antibiotics in the reserve category, such as tigecycline. The high susceptibilities recorded against tigecycline could be due to the reserved use of tigecycline, mainly for the treatment of difficult-to-treat bacterial infections caused by MDR bacteria [23]. Therefore, high susceptibility to tigecycline suggests that they may still be relied upon in treating infections caused by *Enterobacterales* and *non-Enterobacterales*, although resistance has been documented [24].

In this study, 90% of *A. baumannii* and 27.2% of *K. pneumoniae* isolates were resistant to imipenem and meropenem (Table 1). The high resistance to carbapenems observed in this study (90%) among *A. baumannii* isolates is similar to that reported by Lowe et al. [25] where more than 80% resistance to meropenem and imipenem was recorded. This is an important finding as treatment options for carbapenem-resistant *Enterobacterales* (CRE) infections are generally limited to the use of Reserve antibiotics such as tigecycline and colistin, even though resistance against these antibiotics too is progressively increasing [26]. In this study, we observed relatively low levels of tigecycline resistance in *K. pneumoniae* (8.7%), *E. coli* (8.0%), *E. cloacae* (11.1%), and *A. baumannii* (9.1%) (Table 1). However, resistance to tigecycline should be closely monitored as it is reserved for the treatment of infections caused by MDR pathogens. The *E. cloacae* displayed high susceptibility to tigecycline, indicating that it may still be relied upon for effective treatment of infections caused by *E. cloacae* isolates.

In total, 44 (97.8%) *S. aureus*, displayed multidrug resistance, with isolates displaying high resistance to penicillin (93.3%), rifampicin (62.3%), tetracycline (60%), moxifloxacin (60.0%), and ciprofloxacin (57.8%). This contrasts with a South African study investigating patients with *S. aureus* bacteremia (SAB), which reported high susceptibility to antibiotics tested except for penicillin [27]. Resistance to antibiotics in the Watch category, like rifampicin (62.2%), moxifloxacin (60.0%), ciprofloxacin (57.8%), is of particular concern and indicates a potential emerging resistance. The 28.9% MRSA prevalence reported in this study is similar to 27% reported in a South African study that investigated molecular epidemiology and virulence characteristics of *S. aureus* isolates from bacteraemic patients at Tygerberg Hospital, Cape Town [28]. However, *S. aureus* isolates in this study exhibited high levels of sensitivity to Access antibiotics including chloramphenicol (82.2%), clindamycin (75.6%), Watch antibiotics such as vancomycin (100%), and reserve antibiotics such as tigecycline (93.3%), quinupristin-dalfopristin (93.3%), and linezolid (88.9%). Moodley and Perovic [29], also observed high susceptibilities to vancomycin, linezolid, chloramphenicol, and clindamycin which is comparable with the findings of this study. The high susceptibility to linezolid and vancomycin indicates that they may still be relied upon in treating infections caused by *S. aureus*.

The *E. faecium* (100%) isolates in this study were MDR and displayed resistance to ampicillin (80.0%), erythromycin (67%), streptomycin (66.7%), tetracycline (67%), imipenem (53.3%), and ciprofloxacin (53.3%). However, the *E. faecium* isolates displayed high susceptibility against antibiotics in Watch category, such as vancomycin (93.3%), and teicoplanin (60%). Furthermore, *E. faecium* isolates displayed high susceptibility against reserve antibiotics such as quinupristin-dalfopristin (93.3%), tigecycline (93.3%), and linezolid (80%), implying that the drugs can still be relied upon for the treatment of infections caused by MDR pathogens. Similarly, high susceptibility of *E. faecium* isolates was observed against linezolid, vancomycin, and teicoplanin in another South African study that determined the prevalence and AMR patterns of enterococci, including *E. faecium* isolated from blood cultures at the South African public hospitals from January 2016 to December 2020 [30]. Most (94.9%) isolates displayed a multidrug resistance phenotype,

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with 151 resistance patterns (antibiograms) (Supporting Information S1: Table S3). The assortment of 157 antibiograms observed in MDR isolates indicates a wide diversity of resistant phenotypes in different bacteria. The varying resistance patterns observed in all isolates, including *A. baumannii* isolates, may limit treatment options [31]. Most of our study isolates were primarily isolated from the paediatric outpatient department (POPD), intensive care unit (ICU), medical wards, surgical wards, burn units, and the orthopedic outpatient department (OOPD), with most isolates from ICU. The distribution of pathogens in this study concurs with a review conducted by Pons and Ruiz [32], who stated that ESKAPEEc pathogens are frequently isolated in ICUs, with high levels of resistance to antibiotics commonly used in ICUs. Most patients in this study (60.4%) were less than a year old. This is significant since neonates and infants are at high risk of infection because of their underdeveloped immune systems, making them vulnerable to infections, especially in the ICU [33]. Neonatal sepsis is commonly associated with antibiotic resistance and is recognised as a global concern because of limited access to effective antimicrobials, warranting a search for new antimicrobials [34–36].

The MARIs observed in this study were largely high, with more than 148 (93.1%) of isolates having MARI of ≥ 0.20 (Figure 1), indicating high selective pressure and that isolates originate from a high-risk source where antibiotics are regularly used [37]. *A. baumannii* (45.5%), and *K. pneumoniae* (32.6%) isolates were the leading pathogens isolated from ICU participants. Most isolates from ICU participants showed multidrug resistance phenotype, with isolates displaying high resistance to commonly used antibiotics in the Access category, such as ampicillin, amoxicillin clavulanic acid, and those in the Watch category, including piperacillin-tazobactam, cephalosporins, and carbapenems (Table 1). Similar high resistance patterns have been observed against these antibiotics in South Africa [25], and India [38]. The MARI values for both *A. baumannii* and *K. pneumoniae* isolates from ICU were higher than ≥ 0.4 indicating isolates that these isolates were from environments where antibiotics are frequently used, as would be expected in ICU environments [39]. The observed variation in MARI values across clinical settings underscores the significant influence of healthcare environments on antimicrobial resistance (AMR) patterns. Although the overall difference in MARI across ward groups was statistically significant, post hoc analysis indicated that the only meaningful pairwise difference occurred between Intensive Care Units (ICUs) and Outpatient/Clinic-Based Services. This observation aligns with existing evidence that ICUs are high-risk zones for AMR due to the routine use of broad-spectrum antibiotics, frequent empirical treatment, invasive procedures, and prolonged patient stays, all of which contribute to intensified antimicrobial selection pressure [40, 41]. In contrast, outpatient services are characterized by shorter patient interactions and lower acuity cases, resulting in comparatively reduced resistance levels [42]. The significantly higher MARI values observed in tertiary hospitals compared to regional facilities further highlight the elevated risk of resistance emergence in referral-level institutions. Tertiary hospitals often manage more complex cases and serve as referral centers for critically ill patients, leading to increased antibiotic exposure and a higher prevalence of multidrug-resistant organisms [43]. These findings emphasize the need for context-specific antimicrobial stewardship (AMS) interventions, particularly targeting high-risk environments such as ICUs and tertiary healthcare settings.

Overall, the alarming rates of antimicrobial resistance (AMR) identified in this study reflect a complex interplay of microbiological and systemic healthcare factors within the public sector in KwaZulu-Natal. These findings are particularly concerning in the context of empiric therapy for bloodstream infections, where timely and effective treatment is critical. The detection of phenotypic resistance to carbapenems among *K. pneumoniae* and *A. baumannii* isolates suggests the presence of carbapenemase-producing organisms, likely mediated by resistance genes such as bla_{NDM}, $bla_{OXA\text{-}23}$, or $bla_{OXA\text{-}48}$, which have been increasingly reported in South African tertiary hospitals [11, 25, 44]. Similarly, the identification of the *mecA* gene in *Staphylococcus aureus* highlights the burden of methicillin-resistant strains (MRSA), further restricting treatment options and increasing reliance on last-line agents such as linezolid and vancomycin [45]. These resistance mechanisms exacerbate clinical outcomes, contributing to prolonged hospital stays, increased healthcare costs, and heightened mortality risk.

Several factors within the local healthcare setting likely contribute to the high prevalence of resistance observed. Unregulated antibiotic prescribing, especially for agents such as third-generation cephalosporins and amoxicillin-clavulanate, remains a major driver of resistance, as previously documented in the region [46]. Compounding this issue is the limited implementation of robust antimicrobial stewardship (AMS) programs. Many public hospitals face shortages of specialized personnel, including clinical microbiologists and infectious disease practitioners, which hinders effective oversight and enforcement. A recent AMS situational analysis across KwaZulu-Natal public sector facilities confirmed critical gaps in infrastructure and stewardship practices [47]. Additional contributors may include widespread empirical antibiotic use, restricted access to rapid diagnostics, and prolonged ICU admissions, all of which increase the risk of horizontal gene transfer in high-risk hospital wards. These findings underscore the need for urgent, coordinated action. Strengthening antimicrobial stewardship programs across healthcare institutions should be prioritized through strategies such as formulary restrictions for Watch and Reserve antibiotics, regular audit-and-feedback mechanisms, and strict adherence to national treatment guidelines [47]. Investment in diagnostic microbiology, including molecular methods to detect key resistance genes, will facilitate timely, targeted therapy. Moreover, integrating IPC (Infection Prevention and Control) measures with real-time AMR surveillance will be essential for minimizing nosocomial transmission and guiding evidence-based interventions [48].

The limitations of this study include the absence of epidemiological data such as prior antibiotic exposure, treatment history, and clinical parameters, which restricted our ability to assess risk factors for antimicrobial resistance. Additionally, the study was limited to culture-confirmed bloodstream infection (BSI) cases recorded in hospital databases. As a result, patients with less severe infections or those managed in outpatient settings

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may have been excluded, potentially limiting the generalizability of the findings to the broader patient population. Future studies should aim to incorporate larger, more comprehensive data sets, including clinical and treatment information, to enable more risk factor analysis and improve the external validity of resistance surveillance.

5 | Conclusion

This study demonstrates a high prevalence of multidrug resistance (MDR) among ESKAPEEc pathogens isolated from bloodstream infections, along with considerable variability in their resistance profiles. Notably, widespread resistance to WHO Watch group antibiotics was observed, potentially necessitating increased reliance on Reserve category agents for effective treatment. These findings underscore the urgent need for strengthened antimicrobial stewardship and routine AMR surveillance, particularly within public hospital settings, to inform treatment strategies and curb the spread of resistant pathogens.

Author Contributions

Bakoena A. Hetsa: conceptualization, investigation, writing – original draft, methodology, formal analysis, writing – review and editing, software. **Jonathan Asante:** writing – review and editing, formal analysis, validation, methodology, conceptualization, software. **Daniel G. Amoako:** conceptualization, validation, writing – review and editing, methodology, project administration, formal analysis, supervision, resources. **Akebe L. K. Abia:** conceptualization, writing – review and editing, validation, methodology, supervision, resources, project administration. **Joshua Mbanga:** conceptualization, writing – review and editing, validation, supervision, methodology. **Sabiha Y. Essack:** conceptualization, funding acquisition, writing – review and editing, validation, methodology, supervision, resources, project administration.

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Conflicts of Interest

S.Y.E. is a chairperson of the Global Respiratory Partnership and a member of the Global Hygiene Council, both supported by unrestricted educational grants from Reckitt (Pty.) Ltd. UK. The other authors declare no conflicts of interest.

Data Availability Statement

The generated data used to support the findings of this study are included in the main article and its supporting materials.

Transparency Statement

The lead author Bakoena A. Hetsa affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

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Table S1: Oligonucleotide primers and cycling conditions for the detection of housekeeping genes

Organism	Gene	Primer sequence (5′-3′)	PCR Program	Amplicon size (bp)	Reference
E. faecium	*sodA*	F-TACTGACAAACCATTCATGATG R-AACTTCGTCACCAACGCGAAC	UNG 50s 98 °C, 50s 95 °C, 1min 55 °C, 1min 72°C	112	(Furlaneto-Maia et al., 2014) (Jackson et al., 2004)
E. cloacae	*hsp60*	F-GGTAGAAGAAGGCGTGGTTGC R-ATGCATTCGGTGGTGATCATCAG	5min 95°C, 30s 57°C, 1min 72°C	341	(Bakhshi et al., 2019)
S. aureus	*nuc*	F-ATGAACAACGTTCTGAAATTCTCTGCT R- CTTGCGGCTGGCTTTTTCCAG	5min 95°C, 30s 57°C, 1min 72°C	270	(Javid et al., 2018)
S. aureus	*mecA*	F- ACAGGTGAATTATTAGCACTTGTAAG R- ATTGCTGTTAATATTTTTTGAGTTGAA	5min 94°C, 55°C, 45s, 45s 72°C	174	(Asante et al., 2019)
S. aureus	*mecA*	F-′AACAGGTGAATTATTAGCACTTGTAAG3′ R-5′ATTGCTGTTAATATTTTTTGAGTTGAA3′	UNG 5min 94 °C, 30s 94 °C, 30s, 62 °C, 45s, 72°C		(Amoako et al., 2019)
K. pneumoniae	*khe*	F-CATCTGCCACACCTTTCTCA R-CCGGGATTGAGCGGGTAATA	UNG 50s 98 °C, 10s 94 °C , 30s 55.8 400 °C, 1min 72°C		(Hartman et al., 2009)
A. baumannii	*sp4* F	F-CACGCCGTAAGAGTGCATTA R-AACGGAGCTTGTCAGGGTTA	5min 95°C, 30s 60°C, 30s 72°C	490	(Higgins et al., 2007)
P. aeruginosa	*oprl* F	F-ATGAACAACGTTCTGAAATTCTCTGCT R-CTTGCGGCTGGCTTTTTCCAG	5min 95°C, 30s 57°C, 1min 72°C	249	(Gholami et al., 2016)
E. coli	*uida*	F-AAAACGGCAAGAAAAAGCAG R-ACGCGTGGTTAACAGTCTTGCG	UNG 50s 98 °C, 10s 95 °C, 30s 62 °C, 1min 72°C	162	(Godambe et al., 2017)

Table S2: Demographic characteristic characteristics of patients

Isolate ID	Species	Sample	Location	Department	Gender	Age
K1	*K. pneumoniae*	Blood	Regional	Intensive care unit	Female	6
K2	*K. pneumoniae*	Blood	Regional	KMMC CLINIC	U	3
K3	*K. pneumoniae*	Blood	Regional	Intensive care unit	F	<1 year
K4	*K. pneumoniae*	Blood	Regional	Intensive care unit	F	6
K5	*K. pneumoniae*	Blood	Regional	Medical ward	F	<1 year
K6	*K. pneumoniae*	Blood	Regional	Surgical ward	M	40
K7	*K. pneumoniae*	Blood	Tertiary	Casualty	F	70
K8	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	F	<1 year
K9	*K. pneumoniae*	Blood	Regional	Burns ward	M	65
K10	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	M	<1 year
K12	*K. pneumoniae*	Blood	Regional	Intensive care unit	F	<1 year
K13	*K. pneumoniae*	Blood	Tertiary	Renal unit	F	37
K14	*K. pneumoniae*	Blood	Regional	Medical ward	M	<1 year
K15	*K. pneumoniae*	Blood	Regional	Surgical ward	M	20
K21	*K. pneumoniae*	Blood	Regional	KMMC CLINIC	M	<1 year
K22	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	M	42
K23	*K. pneumoniae*	Blood	Regional	Surgical ward	F	18
K24	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	M	<1 year
K26	*K. pneumoniae*	Blood	Regional	Surgical ward	M	28
K28	*K. pneumoniae*	Blood	Tertiary	Renal unit	M	55
K29	*K. pneumoniae*	Blood	Regional	Surgical ward	M	28
K30	*K. pneumoniae*	Blood	Regional	Paediatric ward	M	<1 year
K31	*K. pneumoniae*	Blood	Regional	EMERG DEPT	F	59
K32	*K. pneumoniae*	Blood	Regional	EMERG DEPT	F	<1 year
K33	*K. pneumoniae*	Blood	Regional	Paediatric ward	F	<1 year
K34	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	F	<1 year
K35	*K. pneumoniae*	Blood	Regional	Paediatric ward	M	<1 year
K36	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	F	48
K37	*K. pneumoniae*	Blood	Regional	Paediatric ward	M	<1 year
K38	*K. pneumoniae*	Blood	Regional	Surgical intensive care unit	F	64
K39	*K. pneumoniae*	Blood	Regional	Surgical intensive care unit	M	34
K40	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	M	<1 year
K41	*K. pneumoniae*	Blood	Tertiary	Renal unit	F	36
K42	*K. pneumoniae*	Blood	Regional	Paediatric ward	M	<1 year
K43	*K. pneumoniae*	Blood	Regional	Paediatric ward	M	<1 year
K44	*K. pneumoniae*	Blood	Regional	Paediatric ward	F	4
K45	*K. pneumoniae*	Blood	Regional	Medical ward	F	<1 year
K46	*K. pneumoniae*	Blood	Regional	Paediatric ward	M	<1 year
K47	*K. pneumoniae*	Blood	Regional	Intensive care unit	F	40
K48	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	U	<1 year
K49	*K. pneumoniae*	Blood	Regional	Medical ward	M	<1 year
K50	*K. pneumoniae*	Blood	Regional	Intensive care unit	F	<1 year

K51	*K. pneumoniae*	Blood	Regional	Burns ward	M	30
K52	*K. pneumoniae*	Blood	Regional	Medical ward	F	<1 year
K53	*K. pneumoniae*	Blood	Tertiary	Intensive care unit	U	<1 year

E1	*E. coli*	Blood	Regional	Medical ward	M	22
E2	*E. coli*	Blood	Regional	Medical ward	U	19
E3	*E. coli*	Blood	Tertiary	Casualty	F	48
E4	*E. coli*	Blood	Regional	Medical ward	F	56
E5	*E. coli*	Blood	Regional	Intensive care unit	U	17
E6	*E. coli*	Blood	Regional	Intensive care unit	M	44
E7	*E. coli*	Blood	Regional	Emergency department	F	42
E8	*E. coli*	Blood	Regional	Medical ward	F	75
E10	*E. coli*	Blood	Regional	Surgical intensive care unit	M	27
E21	*E. coli*	Blood	Tertiary	Surgical ward	F	25
E13	*E. coli*	Blood	Regional	Emergency department	M	67
E16	*E. coli*	Blood	Tertiary	Casualty	M	40
E17	*E. coli*	Blood	Regional	Surgical intensive care unit	M	61
E19	*E. coli*	Blood	Regional	Surgical intensive care unit	M	40
E26	*E. coli*	Blood	Regional	Surgical ward	F	77
E30	*E. coli*	Blood	Regional	Surgical ward	M	28
E12	*E. coli*	Blood	Tertiary	intensive care unit	M	<1 year
E20	*E. coli*	Blood	Tertiary	intensive care unit	M	<1 year
E28	*E. coli*	Blood	Regional	Paediatric ward	M	<1 year
E27	*E. coli*	Blood	Regional	Intensive care unit	F	<1 year
E24	*E. coli*	Blood	Regional	Intensive care unit	F	<1 year
E11	*E. coli*	Blood	Regional	Paediatric ward	F	<1 year
E14	*E. coli*	Blood	Regional	Paediatric outpatient	M	<1 year
E18	*E. coli*	Blood	Regional	Paediatric ward	F	2
E25	*E. coli*	Blood	Regional	Intensive care unit	M	<1 year
EC3	*E. cloacae*	Blood	Regional	Surgical intensive care unit	M	29
EC10	*E. cloacae*	Blood	Regional	Surgical intensive care unit	F	40
EC9	*E. cloacae*	Blood	Tertiary	Casualty	M	63
EA1	*E. cloacae*	Blood	Regional	intensive care unit	M	53
EC7	*E. cloacae*	Blood	Tertiary	Surgical intensive care unit	M	59
EC6	*E. cloacae*	Blood	Tertiary	Dermatology clinic	F	23
EC8	*E. cloacae*	Blood	Regional	Paediatric ward	F	<1 year
EC5	*E. cloacae*	Blood	Regional	Paediatric ward	M	<1 year
EC2	*E. cloacae*	Blood	Regional	Paediatric ward	F	<1 year
A5	*A. baumanii*	Blood	Regional	Surgical ward	M	32
A2	*A. baumanii*	Blood	Tertiary	Surgical ward	F	26
A10	*A. baumanii*	Blood	Regional	Medical ward	F	50
A12	*A. baumanii*	Blood	Tertiary	Intensive care unit	F	31
A3	*A. baumanii*	Blood	Regional	Surgical ward	F	<1 year
A6	*A. baumanii*	Blood	Tertiary	intensive care unit	U	<1 year
A8	*A. baumanii*	Blood	Tertiary	intensive care unit	F	<1 year

A11	*A. baumanii*	Blood	Tertiary	intensive care unit	F	<1 year
A9	*A. baumanii*	Blood	Regional	Paediatric outpatient	U	<1 year
A1	*A. baumanii*	Blood	Tertiary	Paediatric outpatient	M	<1 year
A4	*A. baumanii*	Blood	Regional	intensive care unit	M	<1 year
P5	*P. aeruginosa*	Blood	Regional	Burns ward	M	17
P6	*P. aeruginosa*	Blood	Regional	Burns ward	U	50

P2	*P. aeruginosa*	Blood	Regional	Surgical ward	M	47
P7	*P. aeruginosa*	Blood	Regional	Burns ward	M	17
P1	*P. aeruginosa*	Blood	Regional	Surgical ward	M	<1 year
P3	*P. aeruginosa*	Blood	Regional	Paediatric outpatient	M	<1 year
P8	*P. aeruginosa*	Blood	Regional	Intensive care unit	M	<1 year
P4	*P. aeruginosa*	Blood	Regional	Burns ward	M	2
S2	*S. aureus*	Blood	Regional	Medical ward	F	68
S3	*S. aureus*	Blood	Regional	Emergency department	F	17
S11	*S. aureus*	Blood	Regional	Surgical ward	F	17
S21	*S. aureus*	Blood	Regional	Emergency department	M	46
S22	*S. aureus*	Blood	Regional	Medical ward	F	74
S24	*S. aureus*	Blood	Regional	Surgical ward	M	33
S25	*S. aureus*	Blood	Regional	Surgical ward	M	58
S33	*S. aureus*	Blood	Regional	Medical ward	F	54
S38	*S. aureus*	Blood	Regional	Medical ward	F	76
S39	*S. aureus*	Blood	Regional	Surgical ward	M	71
S41	*S. aureus*	Blood	Regional	Medical ward	M	73
S42	*S. aureus*	Blood	Regional	Orthopedic out-patient department	M	37
S43	*S. aureus*	Blood	Regional	Medical ward	F	67
S49	*S. aureus*	Blood	Regional	Emergency department	F	56
S18	*S. aureus*	Blood	Regional	Extension ward	M	<1 year
S30	*S. aureus*	Blood	Regional	Burns ward	F	<1 year
S31	*S. aureus*	Blood	Regional	Burns ward	F	3
S13	*S. aureus*	Blood	Regional	Paediatric ward	M	<1 year
S46	*S. aureus*	Blood	Regional	Extension ward	M	7
S6	*S. aureus*	Blood	Tertiary	Intensive care unit	M	<1 year
S7	*S. aureus*	Blood	Regional	Orthopedic out-patient department	F	6
S12	*S. aureus*	Blood	Regional	Paediatric ward	F	<1 year
S15	*S. aureus*	Blood	Regional	Paediatric outpatient department	F	<1 year
S19	*S. aureus*	Blood	Regional	Paediatric outpatient department	M	<1 year
S28	*S. aureus*	Blood	Regional	Paediatric outpatient department	U	<1 year
S29	*S. aureus*	Blood	Regional	Paediatric ward	M	<1 year
S32	*S. aureus*	Blood	Regional	Intensive care unit	M	<1 year
S34	*S. aureus*	Blood	Regional	Paediatric outpatient department	M	<1 year
S40	*S. aureus*	Blood	Regional	Paediatric outpatient department	M	6
S44	*S. aureus*	Blood	Regional	Intensive care unit	F	<1 year
S45	*S. aureus*	Blood	Regional	Orthopedic out-patient department	F	6
S48	*S. aureus*	Blood	Tertiary	Intensive care unit	U	<1 year

S1	*S. aureus*	Blood	Regional	Paediatric outpatient department	F	<1 year
S4	*S. aureus*	Blood	Regional	KMMC clinic	F	<1 year
S5	*S. aureus*	Blood	Regional	KMMC clinic	F	<1 year
S8	*S. aureus*	Blood	Regional	Emergency department	M	11
S9	*S. aureus*	Blood	Regional	Paediatric outpatient department	F	<1 year
S10	*S. aureus*	Blood	Regional	Paediatric outpatient department	M	<1 year
S13	*S. aureus*	Blood	Regional	Paediatric outpatient department	F	<1 year
S14	*S. aureus*	Blood	Regional	Orthopedic out-patient department	M	10
S16	*S. aureus*	Blood	Regional	Paediatric outpatient department	F	<1 year
S17	*S. aureus*	Blood	Regional	Paediatric outpatient department	M	<1 year
S20	*S. aureus*	Blood	Regional	Surgical ward	M	<1 year
S26	*S. aureus*	Blood	Regional	Paediatric outpatient department	M	<1 year
S37	*S. aureus*	Blood	Regional	Paediatric ward	M	<1 year
EF10	*E. faecium*	Blood	Regional	Paediatric outpatient department	F	<1 year
EF6	*E. faecium*	Blood	Regional	Intensive care unit	F	<1 year
EF5	*E. faecium*	Blood	Regional	Medical ward	F	<1 year
EF7	*E. faecium*	Blood	Tertiary	Medical ward	F	57
EF8	*E. faecium*	Blood	Regional	Paediatric ward	F	<1 year
EF9	*E. faecium*	Blood	Regional	Medical ward	M	<1 year
EF12	*E. faecium*	Blood	Regional	Paediatric outpatient department	M	2
EF13	*E. faecium*	Blood	Regional	Paediatric outpatient department	F	<1 year
EF15	*E. faecium*	Blood	Regional	Paediatric outpatient department	M	<1 year
EF19	*E. faecium*	Blood	Regional	KMM CLINIC	M	<1 year
EF21	*E. faecium*	Blood	Regional	Medical ward	M	<1 year
EF23	*E. faecium*	Blood	Regional	Intensive care unit	M	<1 year
EF24	*E. faecium*	Blood	Tertiary	Surgical ward	F	43
EF25	*E. faecium*	Blood	Regional	KMMC CLINIC	M	<1 year
EF26	*E. faecium*	Blood	Regional	Paediatric ward	M	<1 year

Table S3: Multiple antibiotic resistance index (MARI) of ESKAPEEc isolates

MAR index	No of isolates (%)						
	E. faecium	***S. aureus***	***K. pneumoniae***	***A. baumannii***	***P. aeruginosa***	***E. cloacae***	***E. coli***
0.05	0 (0)	0 (0)	2 (4.3)	0 (0)	0 (0)	0 (0)	0 (0)
0.10	0 (0)	2 (4.4)	0 (0)	0 (0)	0 (0)	0 (0.0)	0 (0)
0.15	3 (20.0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (11.1)	0 (0)
0.20	0 (0)	6 (13.3)	0 (0)	1 (9.1)	0 (0)	1 (11.1)	1 (4.0)
0.25	0 (0)	3 (6.7)	1 (2.2)	0 (0)	2 (25.0)	2 (22.2)	3 (12.0)
0.30	2 (13.3)	7 (15.6)	2 (4.3)	0 (0)	1 (12.5)	0 (0.0)	1 (4.0)
0.35	1(6.7)	3 (6.7)	5 (10.9)	1 (9.1)	3 (37.5)	0 (0.0)	0 (0)
0.40	1(6.7)	4 (8.9)	1 (2.2)	1 (9.1)	0 (0)	1 (11.1)	2 (8.0)
0.45	0 (0)	5 (11.1)	3 (6.5)	1 (9.1)	0 (0)	2 (22.2)	2 (8.0)
0.50	2 (13.3)	5 (11.1)	2 (4.3)	1 (9.1)	0 (0)	1 (11.1)	3 (12.0)
0.55	0 (0)	1 (6.7)	8 (17.4)	1 (9.1)	2 (25.0)	0 (0)	3 (12.0)
0.60	2 (13.3)	4 (8.9)	9 (19.6)	1 (9.1)	0 (0)	0 (0)	3 (12.0)
0.65	0 (0)	3 (6.7)	2 (4.3)	1 (9.1)	0 (0)	0 (0)	3 (12.0)
0.70	0 (0)	0 (0)	3 (6.5)	0 (0)	0 (0)	0 (0)	1 (4.0)
0.75	0 (0)	2 (4.4)	4 (8.7)	0 (0)	0 (0)	2 (11.1)	2 (8.0)
0.80	0 (0)	0 (0)	4 (8.7)	1 (9.1)	0 (0)	0 (0)	0 (0)
0.85	0 (0)	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	1 (4.0)
0.90	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
0.95	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
1.0	0 (0)	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	0 (0)

Table S4: Detailed phenotypic profiles of Gram-negative isolates

Isolate ID	Species	Ward type	Antibiotic resistance profile																		
			AMP	AMC	TZP	CTX	CAZ	CRO	FEP	LEX	FOX	IMP	MEM	NAL	CIP	GEN	AMK	TGC	CHL	SXT	AZM	TET
E1	*E. coli*	Med ward	S	S	I	S	I	I	I	R	R	I	I	I	S	I	I	S	S	R	S	S
E2	*E. coli*	Med ward	R	R	S	I	R	R	R	R	S	S	S	S	S	I	I	S	S	R	S	S
E3	*E. coli*	Casualty	R	R	S	R	R	R	R	R	S	I	S	R	R	R	R	I	S	R	R	R
E4	*E. coli*	Med ward	R	R	I	R	R	R	R	R	S	I	I	R	R	R	R	I	S	R	R	R
E5	*E. coli*	ICU	R	R	R	R	I	R	I	S	S	I	S	R	R	I	R	I	R	R	S	R
E6	*E. coli*	ICU	R	R	R	R	R	R	R	R	S	I	S	I	S	S	R	S	S	R	S	R
E7	*E. coli*	Emerg dpt	R	R	S	S	S	R	I	R	R	R	R	R	R	R	R	I	R	R	S	R
E8	*E. coli*	Med ward	R	R	I	R	R	R	R	R	S	S	S	R	R	R	S	I	S	R	S	1
E10	*E. coli*	SICU	R	R	I	I	I	R	I	R	R	R	R	R	R	S	S	S	R	R	R	R
E21	*E. coli*	Surg ward	R	R	S	R	R	R	R	R	S	I	S	R	R	R	I	S	R	R	R	R
E13	*E. coli*	Emerg dpt	R	R	R	R	R	R	R	R	1	I	S	R	R	R	R	I	S	R	R	R
E16	*E. coli*	Casualty	R	R	I	R	R	R	R	R	S	I	S	R	R	R	R	S	S	R	R	R
E17	*E. coli*	SICU	R	R	S	R	R	R	R	R	S	I	S	R	R	R	R	S	S	R	R	R
E19	*E. coli*	SICU	R	R	S	S	S	I	I	S	S	S	S	R	R	R	I	S	1	R	R	R
E26	*E. coli*	Surg ward	R	R	R	R	R	R	R	R	1	S	S	R	R	R	I	I	S	R	R	R
E30	*E. coli*	Surg ward	R	R	R	R	R	R	R	R	1	S	S	R	R	R	I	I	S	R	R	R
E12	*E. coli*	ICU	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
E20	*E. coli*	ICU	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	I	R	R	R	R
E28	*E. coli*	Paed ward	R	R	R	R	R	R	R	R	R	S	S	I	R	R	R	R	S	1	S	S
E27	*E. coli*	ICU	R	R	R	R	R	R	R	R	R	S	S	I	R	R	I	R	S	1	S	S
E24	*E. coli*	ICU	R	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	S	1	S	S
E11	*E. coli*	Paed ward	R	R	S	I	I	S	I	S	R	S	S	I	R	I	I	I	S	R	R	R
E14	*E. coli*	POPD	R	R	S	R	S	R	I	R	S	I	S	R	R	S	S	I	S	R	R	R
E18	*E. coli*	Paed ward	R	R	S	R	R	R	R	R	R	I	S	R	R	R	I	I	R	R	R	R
E25	*E. coli*	ICU	R	R	I	S	S	S	I	S	S	S	S	I	S	R	S	I	S	R	R	I
EC3	*E. cloacae*	SICU	R	R	R	R	R	R	R	R	R	S	S	R	R	R	I	I	R	R	S	R
EC10	*E. cloacae*	SICU	R	R	R	R	R	R	R	R	R	S	S	I	I	S	I	I	S	R	S	S
EC9	*E. cloacae*	Casualty	R	R	I	R	R	R	R	R	R	S	S	I	I	S	S	I	S	R	S	S
EA1	*E. cloacae*	ICU	R	S	S	S	S	R	I	S	S	S	S	I	R	S	I	I	S	S	S	S
EC7	*E. cloacae*	SICU	R	R	I	R	R	I	I	R	R	S	S	S	R	S	I	R	S	S	S	S
EC6	*E. cloacae*	Dermatology	R	R	S	R	S	1	R	R	R	S	S	S	R	S	I	I	S	S	R	S

EC8	*E. cloacae*	Paed ward	R	R	I	I	I	R	I	R	R	S	S	S	S	S	S	I	R	S	S	S
EC5	*E. cloacae*	Paed ward	R	R	S	S	I	S	I	S	S	S	S	S	S	S	S	S	S	R	R	R
EC2	*E. cloacae*	Paed ward	R	R	S	S	S	I	I	R	R	S	S	I	R	S	S	I	S	S	S	S
K1	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	I	S	I	R	R	I	R	R	R	S	R
K2	*K. pneumoniae*	KMMC CLINIC	NT	R	R	R	R	R	R	R	R	I	S	R	R	R	R	R	R	R	R	R
K3	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	S	R
K4	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	I	S	R	R	R	R	R	R	R	S	R
K5	*K. pneumoniae*	Med ward	NT	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	S	I
K6	*K. pneumoniae*	Surg ward	NT	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
K7	*K. pneumoniae*	Casualty	NT	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
K8	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	S	R	R	R
K9	*K. pneumoniae*	Burns ward	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	I	I	R	R	R	R
K10	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	S	R
K12	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	I	R	R	R	S	R
K13	*K. pneumoniae*	Renal unit	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	I	R	R	R	S	I
K14	*K. pneumoniae*	Med ward	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	I	R	R	R	S	R
K15	*K. pneumoniae*	Surg ward	NT	R	R	I	R	R	R	R	R	S	S	I	S	R	I	R	R	S	R	R
K21	*K. pneumoniae*	KMMC CLINIC	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	S	I	S	R	S	S
K22	*K. pneumoniae*	ICU	NT	R	R	S	R	R	R	S	R	S	R	I	R	S	S	R	S	R	S	R
K23	*K. pneumoniae*	Surg ward	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	S	R
K24	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	S	R	S	R
K26	*K. pneumoniae*	Surg ward	NT	R	R	S	I	S	R	S	R	S	S	I	R	S	I	R	S	S	S	S
K28	*K. pneumoniae*	Renal unit	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	S	I	R	R	S	S
K29	*K. pneumoniae*	Surg ward	NT	R	R	R	R	R	R	R	R	S	1	R	R	R	R	R	R	R	R	R
K30	*K. pneumoniae*	Paed ward	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	S	R	S	S
K31	*K. pneumoniae*	Emrg dept	NT	R	R	R	R	R	R	R	R	S	R	R	R	R	I	R	R	R	R	R
K32	*K. pneumoniae*	Emrg dept	NT	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R
K33	*K. pneumoniae*	Paed ward	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R	R	R
K34	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	S	R	R	R	R	S	R	S	R	S	R
K35	*K. pneumoniae*	Paed ward	NT	R	R	R	R	R	R	R	R	S	R	R	R	R	I	I	S	R	S	R
K36	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R
K37	*K. pneumoniae*	Paed ward	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	S	R	S	R	S	R
K38	*K. pneumoniae*	SICU	NT	R	R	I	S	R	R	R	R	S	R	R	R	R	I	R	S	S	S	I
K39	*K. pneumoniae*	SICU	NT	R	R	R	R	R	R	R	S	S	S	R	R	R	I	I	R	R	S	R
K40	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	S	S	S	R	R	R	I	I	R	R	S	R
K41	*K. pneumoniae*	Renal unit	NT	R	R	R	R	R	R	R	S	S	S	I	I	R	S	I	S	S	S	S

K42	*K. pneumoniae*	Paed ward	NT	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R
K43	*K. pneumoniae*	Paed ward	NT	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	I
K44	*K. pneumoniae*	Paed ward	NT	R	R	R	R	R	R	R	R	R	R	R	R	I	I	R	R	R	I
K45	*K. pneumoniae*	Med ward	NT	S	S	S	S	1	I	S	S	S	S	S	S	S	S	S	S	S	S
K46	*K. pneumoniae*	Paed ward	NT	R	R	R	R	R	S	R	S	S	S	R	R	R	R	I	S	1	S	S
K47	*K. pneumoniae*	ICU	NT	S	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S	S
K48	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	I	I	R	R	R	R
K49	*K. pneumoniae*	Med ward	NT	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	R	I
K50	*K. pneumoniae*	ICU	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	S	R	S	1	S	S
K51	*K. pneumoniae*	Burns ward	NT	R	R	R	R	R	R	R	R	S	S	R	R	R	I	R	S	R	R	R
K52	*K. pneumoniae*	Med ward	NT	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I
K53	*K. pneumoniae*	ICU	NT	R	I	R	R	R	R	R	S	S	S	R	R	R	S	I	R	R	S	R
A5	*A. baumanii*	Surg ward	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
A2	*A. baumanii*	Surg ward	R	R	R	I	R	R	R	R	I	I	I	I	S	R	R	S	R	R	S	S
A10	*A. baumanii*	Med ward	R	R	R	I	R	R	R	R	R	R	R	R	R	R	I	I	R	R	S	R
A12	*A. baumanii*	ICU	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R
A3	*A. baumanii*	Surg ward	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
A6	*A. baumanii*	ICU	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
A8	*A. baumanii*	ICU	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
A11	*A. baumanii*	ICU	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
A9	*A. baumanii*	POPD	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	S	R
A1	*A. baumanii*	POPD	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	R	S	R
A4	*A. baumanii*	ICU	R	R	R	I	I	R	R	R	I	I	I	I	S	S	R	I	R	I	S	S
P5	*P. aeruginosa*	Burns ward	S	R	S	S	I	S	R	R	S	S	S	R	R	S	S	R	R	R	S	S
P6	*P. aeruginosa*	Burns ward	R	R	R	S	I	S	R	R	S	S	S	R	S	S	S	R	R	R	S	R
P2	*P. aeruginosa*	Surg ward	R	R	R	I	R	R	R	R	S	S	S	R	R	S	S	R	R	R	S	R
P7	*P. aeruginosa*	Burns ward	R	R	R	R	I	R	R	R	I	S	S	R	S	R	S	R	I	R	S	R
P1	*P. aeruginosa*	Surg ward	R	R	R	S	R	R	R	R	R	S	I	R	R	S	S	R	R	R	R	R
P3	*P. aeruginosa*	POPD	R	R	R	S	I	S	R	R	S	S	S	R	S	S	S	R	R	R	R	R
P8	*P. aeruginosa*	ICU	R	R	R	S	S	S	R	R	S	S	S	R	S	S	S	R	R	R	S	R
P4	*P. aeruginosa*	Burns ward	R	R	R	S	R	S	R	R	S	S	S	R	S	S	S	R	R	R	S	R

Abbreviations: AMP, ampicillin; AMC, amoxicillin–clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; LEX, cephalexin; FOX, cefoxitin; TZP, piperacillin–tazobactam; IPM, imipenem; MEM, meropenem; NAL, nalidixic acid; CIP, ciprofloxacin; GEN, gentamicin; AMK, amikacin; TET, tetracycline; TGC, tigecycline; CHL, chloramphenicol, SXT, trimethoprim–sulfamethoxazole, and AZM, azithromycin. R, resistant; I, intermediate susceptibility; S, susceptible; ICU, Intensive Care Unit; Med ward, medical ward; POPD, paediatric outpatient department; Paed ward; Paediatric ward; Surg ward, Surgical ward;

Table S5: Detailed phenotypic profiles of *S. aureus* isolates

Isolate ID	Species	Ward type	PEN	AMP	FOX	CIP	MXF	LEV	GEN	AMK	TGC	CHL	NIT	SXT	ERY	LZD	TEC	TET	DO	RIF	CLI	VAN
S2	*S. aureus*	Med ward	R	S	S	R	S	S	S	I	R	S	S	S	I	R	I	R	R	I	I	S
S3	*S. aureus*	Emerg dept	R	S	S	S	S	S	S	S	R	R	R	I	I	R	I	I	I	S	I	S
S11	*S. aureus*	Surg ward	R	R	R	R	R	R	R	I	R	S	S	R	1	S	I	R	R	R	R	S
S21	*S. aureus*	Emerg dept	R	S	S	R	R	R	R	I	R	S	S	R	I	S	I	R	R	R	S	S
S22	*S. aureus*	Med ward	R	S	S	S	S	S	S	S	R	S	S	S	I	S	I	I	I	I	I	S
S24	*S. aureus*	Surg ward	R	R	R	S	R	S	S	S	R	R	R	R	R	R	R	R	R	R	R	S
S25	*S. aureus*	Surg ward	R	S	S	R	S	R	R	I	S	S	S	R	I	S	I	R	R	I	S	S
S33	*S. aureus*	Med ward	R	S	S	S	S	S	R	I	R	S	S	S	I	S	S	I	R	R	S	S
S38	*S. aureus*	Med ward	R	S	S	S	S	S	S	S	R	S	S	S	I	S	S	S	S	I	S	S
S39	*S. aureus*	Surg ward	R	R	R	R	S	S	R	S	S	S	S	S	S	S	S	R	R	I	S	S
S41	*S. aureus*	Med ward	R	S	S	R	R	R	R	S	S	S	S	R	R	R	R	I	R	R	R	S
S42	*S. aureus*	OOPD	R	R	R	R	S	R	R	I	R	S	S	I	R	S	I	S	R	I	S	S
S43	*S. aureus*	Med ward	R	R	R	R	R	R	R	S	R	S	S	R	R	S	S	I	I	I	S	S
S49	*S. aureus*	Emerg dept	R	S	S	R	R	R	S	I	R	S	S	S	I	S	I	I	I	S	I	S
S18	*S. aureus*	Extn ward	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R	S	S
S30	*S. aureus*	Burns ward	R	S	S	R	S	S	S	S	S	S	S	S	S	S	I	I	S	S	S	S
S31	*S. aureus*	Burns ward	R	R	R	R	R	I	R	R	S	S	I	R	R	S	R	R	R	I	S	S
S13	*S. aureus*	Paed ward	R	S	S	S	S	R	S	S	S	S	I	R	R	R	I	R	S	R	S	S
S46	*S. aureus*	Extn ward	R	R	R	S	S	S	I	S	S	S	I	S	S	S	I	I	S	S	S	S
S6	*S. aureus*	ICU	R	S	S	R	R	I	R	S	R	S	R	R	R	I	R	R	S	I	S	S
S7	*S. aureus*	OOPD	R	R	R	R	R	R	I	R	R	S	R	R	R	I	R	S	S	I	S	S
S12	*S. aureus*	Paed ward	R	R	S	R	R	R	R	I	S	S	R	R	R	I	I	R	S	I	S	S
S15	*S. aureus*	POPD	R	R	S	R	R	1	S	R	S	S	R	S	S	I	R	R	S	R	S	S
S19	*S. aureus*	POPD	R	R	S	R	R	I	R	R	S	S	S	S	S	I	S	I	R	I	S	S
S28	*S. aureus*	POPD	S	S	S	S	R	S	I	R	S	S	R	S	S	S	I	R	R	I	S	S
S29	*S. aureus*	Paed ward	R	R	R	R	R	S	R	S	S	S	S	R	S	I	I	I	R	I	S	S

S32	*S. aureus*	ICU	R	R	R	S	R	S	I	R	S	S	S	R	S	S	R	I	R	R	S	S
S34	*S. aureus*	POPD	R	S	S	R	S	S	S	I	S	S	S	S	S	S	S	I	S	R	S	S
S40	*S. aureus*	POPD	R	S	S	S	R	S	S	R	S	S	S	S	S	S	S	I	R	S	S	S
S44	*S. aureus*	ICU	R	R	R	R	R	R	S	R	S	S	R	R	R	R	R	R	R	R	S	S
S45	*S. aureus*	OOPD	R	S	R	R	R	S	I	R	S	S	I	R	S	I	R	R	R	R	S	S
S48	*S. aureus*	ICU	R	R	R	R	R	S	R	R	S	S	S	R	S	I	S	I	S	S	S	S
S1	*S. aureus*	POPD	R	S	S	R	R	S	R	R	R	S	R	S	S	R	R	R	S	R	S	S
S4	*S. aureus*	KMMC clinic	R	R	R	S	S	1	R	R	R	S	S	R	R	R	R	R	S	R	S	S
S5	*S. aureus*	KMMC clinic	R	S	S	R	R	R	S	R	R	S	S	R	R	I	R	I	R	I	S	S
S8	*S. aureus*	Emerg dept	R	S	S	R	R	R	R	I	S	S	R	R	R	I	R	I	S	R	S	S
S9	*S. aureus*	POPD	S	S	S	S	S	R	R	R	R	S	S	R	R	R	R	I	S	R	S	S
S10	*S. aureus*	POPD	R	S	S	R	S	I	R	R	R	S	S	R	S	R	I	R	R	I	S	S
S13	*S. aureus*	POPD	R	R	R	R	R	R	S	I	R	S	I	R	R	R	R	R	R	I	S	S
S14	*S. aureus*	OOPD	R	S	S	S	R	R	I	R	S	S	S	R	S	I	I	I	S	I	S	S
S16	*S. aureus*	POPD	R	S	S	R	R	I	I	I	S	S	S	R	S	I	I	R	S	I	S	S
S17	*S. aureus*	POPD	R	S	S	S	S	I	S	R	R	S	S	R	S	I	I	S	S	I	S	S
S20	*S. aureus*	Surg ward	S	S	S	S	S	I	I	R	S	S	S	S	R	I	I	R	S	I	S	S
S26	*S. aureus*	POPD	R	R	R	R	R	R	I	S	R	R	R	R	R	R	R	R	R	R	S	S
S37	*S. aureus*	Paed ward	R	I	I	R	S	S	I	S	S	S	S	R	S	S	I	I	S	R	S	S

Abbreviations: PEN= penicillin G; AMP= ampicillin; FOX= cefoxitin; CIP= ciprofloxacin; MXF= moxifloxacin; LEV= levofloxacin; GEN= gentamicin; AMK= amikacin; TGC= tigecycline; CHL= chloramphenicol; NIT= nitrofurantoin; SXT= sulphamethoxazole/trimethoprim; ERY= erythromycin; LZD= linezolid; TEC= teicoplanin; TET= tetracycline; DOX= doxycycline; RIF= rifampicin; CLI= clindamycin; VAN= vancomycin; R, resistant; Emerg dept, Emergency department; Ext ward, extension ward; ICU, Intensive care unit; Med ward, medical ward; Surg ward, surgical ward; OPD, Outpatient Department; OOPD; Orthopedic out-patient department, Paed ward; Paediatric ward, POPD; Paediatric Outpatient Department.

Table S6: Detailed phenotypic profiles of *E. faecium* isolates

Isolate ID	Species	Ward type	Antibiotics															
			AMP	IMP	CIP	LEV	GEN	STR	ERY	TGC	CHL	NIT	SXT	LZD	TEC	VAN	TET	QD
EF10	*E. faecium*	POPD	S	I	S	S	S	S	R	R	S	R	R	R	R	R	R	R
EF6	*E. faecium*	ICU	R	R	R	R	R	R	I	S	I	S	R	R	S	S	I	R
EF5	*E. faecium*	Med ward	R	R	R	R	R	S	R	I	S	S	R	S	S	S	R	R
EF7	*E. faecium*	Med ward	R	R	R	R	R	S	I	S	S	R	R	S	S	S	1	S
EF8	*E. faecium*	Paed ward	R	R	R	R	R	R	I	I	S	S	R	S	S	S	I	I
EF9	*E. faecium*	Med ward	S	S	S	S	S	S	R	I	S	S	R	S	S	S	S	R
EF12	*E. faecium*	POPD	S	S	S	S	S	R	I	I	R	S	R	R	S	S	R	R
EF13	*E. faecium*	POPD	S	R	R	R	R	R	R	S	I	S	R	S	S	S	S	I
EF15	*E. faecium*	POPD	R	R	R	R	R	R	R	I	I	S	R	S	S	S	R	R
EF19	*E. faecium*	KMMC clinic	S	I	S	S	S	S	R	I	R	R	I	S	S	S	R	R
EF21	*E. faecium*	Med ward	S	S	S	S	S	S	I	I	R	S	R	S	S	S	R	R
EF23	*E. faecium*	ICU	R	R	R	S	S	S	R	S	R	R	I	S	S	S	R	R
EF24	*E. faecium*	Surg ward	R	R	R	R	R	R	R	S	S	I	R	S	S	S	R	R
EF25	*E. faecium*	KMMC clinic	R	R	R	R	R	R	R	I	S	S	R	S	S	S	I	R
EF26	*E. faecium*	Paed ward	S	S	S	S	S	S	R	I	S	S	I	S	S	S	R	R

PEN = penicillin G; AMP= ampicillin; AMC= amoxicillin-clavulanic acid; LEX= cephalexin; CTX= cefotaxime; CAZ=cefotaxime; FEP= cefepime; CRO= ceftriaxone; FOX = cefoxitin; TZP= piperacillin-tazobactam; IMP= imipenem; MEM= meropenem; CIP = ciprofloxacin; MXF = moxifloxacin; AZM = azithromycin; ERY = erythromycin; GEN = gentamicin; AMK = amikacin; CHL = chloramphenicol; TET = tetracycline; DOX = doxycycline; TEC = teicoplanin; TGC = tigecycline; LZD = linezolid; CLI = clindamycin; NAL= nalidixic acid; RIF = rifampicin; SXT = sulfamethoxazole/trimethoprim; NIT = nitrofurantoin. I, intermediate susceptibility; Q-D, quinupristin-dalfopristin; R, resistant; S, susceptible; VAN, vancomycin OOPD; Orthopedic out-patient department; Paed ward, Paediatric ward

Table S7: Multiple antibiotic resistant phenotypes displayed by ESKAPE and *E. coli* isolates

Species	Resistance Phenotype	No. observed
E. faecium	AMP-IMP-CIP-GEN-ERY-SXT-TET-STR-LEV-QD	2
	ERY-TGC-NIT-SXT-LZD-TEC-TET-VAN-QD	1
	AMP-IMP-CIP-GEN-ERY-SXT-TET-LEV- QD	1
	AMP-IMP-CIP-GEN-ERY-SXT-STR-LEV- QD	1
	AMP-IMP-CIP-GEN-SXT-LZD-STR-LEV-QD	1
	IMP-CIP-GEN-ERY-SXT-STR-LEV	1
	AMP-IMP-CIP-GEN-STR-LEV	1
	CHL-SXT-LZD-TET-STR-QD	1
	ERY-CHL-NIT-TET-QD	1
	CHL-SXT-TET-QD	1
	ERY-SXT-QD	1
S. aureus	PEN-G-AMP-FOX-CIP-MXF-LEV-GEN-AMK-TGC-NIT-SXT-ERY-LZD-TEC-TET-DOX-RIF	1
	PEN-G-CIP-AMP-FOX-MXF-LEV-TGC-CHL-NIT-SXT-ERY-LZD-TEC-TET-DOX-RIF	1
	PENG-AMP-FOX-CIP-MXF-LEV-AMK-NIT-SXT-ERY-LZD-TEC-TET-DOX-RIF	1
	PEN-G-AMP-FOX-MXF-TGC-CHL-NIT-SXT-ERY-LZD-TEC-TET-DOX-RIF-CLI	1
	PEN-G-AMP-FOX-CIP-MXF-LEV-GEN-TGC-SXT-ERY-TET-DOX-RIF-CLI	1
	PENG-AMP-FOX-LEV-GEN-AMK-TGC-SXT-ERY-LZD-TEC-TET-RIF	1
	PENG-AMP-FOX-CIP-MXF-GEN-AMK-SXT-ERY-TEC-TET-DOX	1
	PEN G-AMP-FOX-CIP-MXF-LEV-TGC-SXT-ERY-LZD-TEC-TET-DOX	1
	PENG-CIP-MXF-LEV-GEN-SXT-ERY-LZD-TEC-DOX-RIF-CLI	1
	PENG-AMP-FOX-CIP-MXF-LEV-AMK-TGC-NIT-SXT-ERY-TEC	1
	PENG-AMP-CIP-MXF-LEV-GEN-NIT-SXT-ERY-TET	1
	PENG-AMP-CIP-MXF-LEV-AMK-NIT-TEC-TET-RIF	1
	PENG-AMP-FOX-CIP-MXF-AMK-SXT-TEC-TET-DOX-RIF	1
	PENG-CIP-MXF-GEN-AMK-TGC-NIT-LZD-TEC-TET-RIF	1
	PENG-AMP-FOX-CIP-GEN-AMK-TGC-SXT-LZD-TET-DOX	1
	PENG-CIP-MXF-LEV-GEN-TGC-SXT-TET-DOX-RIF	1

	PENG-AMP-FOX-CIP-MXF-LEV-GEN-TGC-SXT-ERY	1
	PENG-CIP-MXF-GEN-TGC-NIT-SXT-ERY-TEC-TET	1
	PENG-CIP-MXF-LEV-AMK-TGC-SXT-ERY-TEC-DOX	1
	PENG-CIP-MXF-LEV-GEN-NIT-SXT-ERY-TEC-RIF	1
	PENG-AMP-FOX-CIP-LEV-GEN-TGC-ERY-DOX	1
	LEV-GEN-AMK-TGC-SXT-ERY-LZD-TEC-RIF	1
	PENG-AMP-FOX-MXF-AMK-SXT-TEC-DOX-RIF	1
	PENG-AMP-FOX-CIP-MXF-GEN-SXT-DOX	1
	PENG-AMP-FOX-CIP-MXF-GEN-AMK-SXT	1
	PENG-CIP-LEV-GEN-SXT-TET-DOX	1
	PENG-AMP-FOX-CIP-GEN-TET-DOX	1
	PENG-CIP-TGC-LZD-TET-DOX	1
	PENG-LEV-SXT-ERY-LZD-TET-RIF	1
	PENG-AMP-CIP-MXF-GEN-AMK-DOX	1
	PENG-CIP-MXF-LEV-TGC	1
	MXF-AMK-NIT-TET-DOX	1
	PENG-TGC-CHL-NIT-LZD	1
	PENG-GEN-TGC-DOX-RIF	1
	PENG-MXF-LEV-AMK-SXT	1
	PENG-CIP-MXF-SXT-TET	1
	PENG-AMK-TGC-SXT	1
	PENG-MXF-AMK-DOX	1
	PENG-CIP-SXT-RIF	1
	PENG-CIP-RIF	1
	AMK-ERY-TET	1
K. pneumoniae	AMC-CTX-TZP -CAZ-CRO-FEP-LEX-FOX -IMP-MEM-NAL-CIP-GEN-AMK-TGC-CHL-SXT-AZM-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX -MEM-NAL-CIP-GEN-AMK-TGC-CHL-SXT-AZM-TET	2
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX -IMP-MEM-NAL-CIP-GEN-AMK-TGC-CHL-SXT-AZM	2
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-TGC-CHL-SXT-AZM-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-AMK-TGC-CHL-SXT-AZM-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-MEM-NAL-CIP-GEN-AMK-TGC-CHL-SXT-TET	1

	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-AMK-CHL-SXT-AZM	1
	AMC-TZP -CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-AMK-TGC-CHL-SXT-AZM-TET	1
	AMC-TZP -CTX-CAZ-CRO-FEP-LEX-FOX-MEM-NAL-CIP-GEN-TGC-CHL-SXT-AZM-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-AMK-TGC-CHL-SXT-TET	3
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-NAL-CIP-GEN-AMK-TGC-CHL-SXT	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-TGC-SXT-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-AMK-TGC-SXT-AZM-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-AMK-TGC-CHL-SXT-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-CIP-GEN-AMK-TGC-CHL-SXT	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-CHL-SXT-AZM	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-CHL-SXT-AZM-TET	2
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-TGC-SXT-AZM-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-MEM-NAL-CIP-GEN-TGC-SXT-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-CHL-SXT-TET	1
	AMC-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-TGC-CHL-SXT-TET	1
	AMC-TZP -CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-TGC-CHL-SXT	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-AMK-TGC-SXT	1
	AMC-TZP -CTX-CAZ-CRO-FEP-LEX-FOX-MEM-NAL-CIP-GEN-SXT-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-CIP-GEN-TGC-CHL-SXT-TET	1
	AMC-TZP -CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-CHL-SXT	1
	AMC-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-TGC-SXT-TET	1
	AMC-CTX-CAZ-CRO-FEP-LEX-NAL-CIP-GEN-CHL-SXT-TET	3
	AMC-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-TGC-SXT	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX -NAL-CIP-GEN-SXT	1
	AMC-CAZ-CRO-FEP-LEX-FOX-GEN-TGC-CHL-AZM-TET	1
	AMC-TZP-CTX-CAZ-CRO-LEX-NAL-CIP-GEN-AMK-SXT	1
	AMC-TZP-CAZ-CRO-FEP-FOX -MEM-CIP-TGC-SXT-TET	1
	AMC-CRO-FEP-LEX-FOX-MEM-NAL-CIP-GEN-TGC	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX -GEN	1
	AMC-TZP-FEP-FOX -CIP-TGC	1
A. baumanii	AMP-TZP -CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-AMK-CHL-SXT-AZM-TET	3

	AMP -AMC-TZP -CTX-CAZ-CRO-LEX-FOX-IMP-MEM-NAL-CIP-GEN-AMK-TGC-CHL-AZM-TET	1
	AMP-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-AMK-CHL-SXT-AZM-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-TGC-CHL-SXT-TET	1
	AMP-AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-AMK-SXT-TET	1
	AMP-AMC-TZP-CTX-CRO-FEP-LEX-IMP-MEM-NAL-CIP-GEN-TGC-CHL-SXT-TET	1
	AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-TGC-CHL-SXT-TET	1
	AMP-AMC-FEP-FOX-GEN-AMK-SXT	1
	AMC-CTX-LEX-FOX-AMK-CHL	1
P. aeruginosa	AMP-AMC-TZP-CTX-CRO-FEP-LEX-FOX-NAL-CIP-TGC-CHL-SXT-AZM-TET	1
	AMP-AMC-CTX-LEX-FOX-NAL-TGC-CHL-SXT-TET	3
	AMP-AMC-CTX-CRO-FEP-LEX-FOX-NAL-CIP-TGC-CHL-SXT-TET	1
	AMP-AMC-CTXCAZ-FEP-LEX-FOX-NAL-GEN-TGC-SXT-TET	1
	AMP-AMC-CTX-LEX-FOX-NAL-TGC-CHL-SXT-AZM-TET	1
	AMC-LEX-FOX-NAL-CIP-TGC-CHL-SXT	1
***Enterobacter* spp.**	AMP-AMC-TZP-CTX-CAZ-CRO-FEP- LEX-FOX-NAL-CIP-GEN-CHL-SXT- TET	1
	AMP-AMC-TZP-CTX-CAZ-CRO-LEX-FOX-SXT	1
	AMP-AMC-CTX-CAZ-CRO-FEP-LEX- FOX-SXT	1
	AMP-AMC-CTX-CRO-FEP-LEX-FOX-CIP-AZM	1
	AMP-AMC-CTX-CAZ-LEX-FOX-CIP-TGC	1
	AMP-AMC-CRO-LEX-FOX-CHL	1
	AMP-AMC-SXT-AZM-TET	1
	AMP-AMC-LEX-FOX-CIP	1
	AMP-CRO-CIP	1
E. coli	AMP-AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-IMP-MEM-NAL-CIP-GEN-AMK-TGC-CHL-SXT-AZM-TET	1
	AMP-AMC-CTX-CAZ-CRO-FEP-LEX-NAL-CIP-GEN-AMK-SXT-AZM-TET	4
	AMP-AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-SXT-AZM-TET	2
	AMP-AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-AMK-SXT-AZM-TET	1
	AMP-AMC-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-CHL-SXT-AZM-TET	1
	AMP-AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-NAL-CIP-GEN-AMK-TGC-SXT	1
	AMP-AMC-CTX-CAZ-CRO-FEP-LEX-NAL-CIP-GEN-AMK-CHL-SXT-AZM-TET	1
	AMP-AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-CIP-GEN-AMK-TGC-SXT	1
	AMP-AMC-CTX-CAZ-CRO-FEP-LEX-NAL-CIP-GEN-CHL-SXT-AZM-TET	1

	AMP-AMC-TZP-CTX-CAZ-CRO-FEP-LEX-FOX-CIP-GEN-TGC-SXT	1
	AMP-AMC-CRO-LEX-FOX-IMP-MEM-NAL-CIP-CHL-SXT-AZM-TET	1
	AMP-AMC-CRO-LEX-FOX-IMP-MEM-CIP-GEN-AMK-CHL-SXT-TET	1
	AMP-AMC-CTX-CAZ-CRO-FEP-LEX-NAL-CIP-GEN-SXT-TET	1
	AMP-AMC-TZP-CTX-CRO-NAL-CIP-AMK-CHL-SXT-TET	1
	AMP-AMC-CTX-CRO-LEX-NAL-CIP-SXT-AZM-TET	1
	AMP-AMC-TZP-CTX-CAZ-CRO-FEP-LEX -SXT	1
	AMP-AMC-NAL-CIP-CHL-SXT-AZM-TET	1
	AMP-AMC-FOX-CIP-SXT-AZM-TET	1
	AMP-AMC-GEN-SXT-AZM	1

PEN = penicillin G; AMP= ampicillin; AMC= amoxicillin-clavulanic acid; LEX= cephalexin; CTX= cefotaxime; CAZ=cefotaxime; FEP= cefepime; CRO= ceftriaxone; FOX = cefoxitin; TZP= piperacillin-tazobactam; IMP= imipenem; MEM= meropenem; CIP = ciprofloxacin; MXF = moxifloxacin; AZM = azithromycin; ERY = erythromycin; GEN = gentamicin; AMK = amikacin; CHL = chloramphenicol; TET = tetracycline; DOX = doxycycline; TEC = teicoplanin; TGC = tigecycline; LZD = linezolid; CLI = clindamycin; NAL= nalidixic acid; RIF = rifampicin; SXT = sulfamethoxazole/trimethoprim; NIT = nitrofurantoin.

Table S8A: Results of Shapiro–Wilk and Levene's tests for normality and homogeneity of variances

Comparison	Test	Statistic	P-value / Comment
WardGroup	Levene's Test	1.001	0.427 Assumption met
Facility Level	Levene's Test	3.303	0.071 Assumption met

* Shapiro–Wilk tests indicated acceptable normality across groups for both Ward Group and Facility Level.

Table S8B: One-way ANOVA summary statistics for MARI values across ward groups and facility levels.

Comparison	F-value	P-value	η^2 (Effect Size)	Post hoc
WardGroup	2.895	0.011	0.103	Tukey HSD: significant pairwise differences
Facility Level	17.520	< 0.001	0.101	Not applicable (2-group ANOVA)

Table S8C: Tukey's HSD post hoc pairwise comparisons of MARI values among ward groups.

Group 1	**Group 2**	**Mean Difference**	**P-Value**	**Lower CI**	**Upper CI**	**Significant**
Emergency/Trauma Units	General Inpatient Wards	-0.1394	0.6924	-0.4024	0.1236	False
Emergency/Trauma Units	Intensive Care Units	0.0341	0.9994	-0.1963	0.2645	False
Emergency/Trauma Units	Outpatient/Clinic-Based Services	-0.1413	0.5767	-0.3813	0.0986	False
Emergency/Trauma Units	Paediatric Units	-0.1347	0.6813	-0.386	0.1166	False
Emergency/Trauma Units	Specialist Wards	-0.055	0.9975	-0.3431	0.2331	False

Emergency/Trauma Units	Surgical Wards	0.012	1.0	-0.2482	0.2722	False
General Inpatient Wards	Intensive Care Units	0.1736	0.1278	-0.0245	0.3717	False
General Inpatient Wards	Outpatient/Clinic-Based Services	-0.0019	1.0	-0.211	0.2072	False
General Inpatient Wards	Paediatric Units	0.0047	1.0	-0.2174	0.2268	False
General Inpatient Wards	Specialist Wards	0.0844	0.9617	-0.1786	0.3474	False
General Inpatient Wards	Surgical Wards	0.1515	0.4509	-0.0807	0.3836	False
Intensive Care Units	Outpatient/Clinic-Based Services	-0.1755	0.0313	-0.3418	-0.0092	True
Intensive Care Units	Paediatric Units	-0.1689	0.0891	-0.3512	0.0135	False
Intensive Care Units	Specialist Wards	-0.0891	0.9091	-0.3195	0.1413	False
Intensive Care Units	Surgical Wards	-0.0221	0.9999	-0.2165	0.1723	False
Outpatient/Clinic-Based Services	Paediatric Units	0.0066	1.0	-0.1876	0.2008	False
Outpatient/Clinic-Based Services	Specialist Wards	0.0863	0.9344	-0.1536	0.3263	False
Outpatient/Clinic-Based Services	Surgical Wards	0.1534	0.2864	-0.0523	0.359	False
Paediatric Units	Specialist Wards	0.0797	0.9639	-0.1716	0.331	False
Paediatric Units	Surgical Wards	0.1467	0.4163	-0.0721	0.3655	False
Specialist Wards	Surgical Wards	0.067	0.9875	-0.1932	0.3272	False

Table S9A: **Descriptive statistics of MARI values by ward group, including mean, standard deviation, sample size, and 95% confidence intervals.**

WardGroup	Mean MARI	Sample Size	Standard Deviation	Standard Error	CI Lower (95%)	CI Upper (95%)
Emergency/Trauma Units	0.6416	12	0.2438	0.0703	0.5036	0.7796
General Inpatient Wards	0.5022	18	0.2953	0.0696	0.3657	0.6386
Intensive Care Units	0.6758	43	0.2156	0.0328	0.6113	0.7402
Outpatient/Clinic-Based Services	0.5003	31	0.2143	0.0384	0.4248	0.5757
Paediatric Units	0.5069	23	0.2568	0.0535	0.4020	0.6119
Specialist Wards	0.5866	12	0.2208	0.0637	0.4616	0.7116
Surgical Wards	0.6536	19	0.2314	0.0530	0.5496	0.7577

Table S9B: **Descriptive statistics of MARI values by facility level (regional vs tertiary), including mean, standard deviation, sample size, and 95% confidence intervals.**

Level	Mean MARI	Sample Size	Standard Deviation	Standard Error	CI Lower (95%)	CI Upper (95%)
REGIONAL	0.5475	128	0.2420	0.0213	0.5055	0.5894
TERTIARY	0.7450	30	0.1855	0.0338	0.6785	0.8114

CHAPTER 3- GENOMIC ANALYSIS OF VIRULENT, MULTIDRUG RESISTANT *KLEBSIELLA PNEUMONIAE* AND *KLEBSIELLA OXYTOCA* FROM BLOODSTREAM INFECTIONS, SOUTH AFRICA

Author contributions

Bakoena Ashton Hetsa, as the principal investigator, co-conceptualized the study, undertook the laboratory work and drafted the manuscript.

- **Dr Jonathan Asante** undertook a critical revision of the manuscript.

- **Dr Joshua Mbanga,** as co-supervisor, supervised the work, and undertook critical revision of the manuscript.

- **Dr Akebe L. K. Abia**, as co-supervisor, supervised the laboratory work, and critically revised the manuscript.

- **Dr Daniel G. Amoako**, as co-supervisor, supervised the laboratory work, vetted the results and undertook critical revision of the manuscript.

- **Professor Sabiha Y. Essack**, as the principal supervisor, co-conceptualized the study, guided the literature review and ethical approval application, facilitated data collection and analysis, vetted the results and critically revised the manuscript.

Objective(s) met: This paper addresses objectives 4, 5 and 6.

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Genomic analysis of virulent, multidrug resistant *Klebsiella pneumoniae* and *Klebsiella oxytoca* from bloodstream infections, South Africa

Bakoena A. Hetsa [a,*], Jonathan Asante [a,b], Joshua Mbanga [a,c], Daniel G. Amoako [a,d], Akebe L.K. Abia [a], Arshad Ismail [e,f], Sabiha Y. Essack [a,g]

[a] Antimicrobial Research Unit, College of Health Sciences, University of KwaZulu-Natal, Durban, 4000, South Africa
[b] School of Pharmacy and Pharmaceutical Sciences, University of Cape Coast, PMB, Cape Coast, Ghana
[c] Department of Applied Biology & Biochemistry, National University of Science and Technology, Corner Cecil Avenue & Gwanda Road, Bulawayo 263, Zimbabwe
[d] Department of Pathobiology, University of Guelph, Guelph, ON, N1G 2W1, Canada
[e] Department of Biochemistry and Microbiology, Faculty of Science, Engineering and Agriculture, University of Venda, Thohoyandou, 0950, South Africa
[f] Sequencing Core Facility, National Institute for Communicable Diseases, Division of the of the National Health Laboratory Service, Johannesburg, 2193, South Africa
[g] School of Pharmacy, University of Jordan, Amman, 11942, Jordan

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ABSTRACT

The study investigated the resistome, virulome and mobilome of multidrug resistant (MDR) *Klebsiella pneumoniae* and *Klebsiella oxytoca* clinical isolates.

Methods: A total of 46 suspected *Klebsiella* species (*spp.*) were collected from blood cultures within the uMgungundlovu District in the KwaZulu-Natal Province. Antibiotic susceptibility was determined against a panel of 19 antibiotics using the disk diffusion test. A subset of 14 MDR *K. pneumoniae* (n = 10) and *K. oxytoca* (n = 4) isolates were selected based on their antibiograms and subjected to whole genome sequencing (WGS). The sequence types (STs), resistome, virulome, mobilome, capsule loci (KLs) were analysed using relevant WGS and bioinformatics tools.

Results: Of the 10 *K. pneumoniae* sequence types (ST) identified, the most common were ST25 (n = 3), ST101 (n = 3), and 4 *K. oxytoca* belonged to ST450 (n = 3). The two high-risk *K. pneumoniae* clones ST15, and ST17 were identified. O and K capsule types were identified, with predominance of KL2, KL17, KL29, O1/O2v2, O1/O2v1, and OL104 respectively. The majority of isolates displayed multidrug resistance predominantly carrying β-lactamase genes, including $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, $bla_{SHV,}$ and $bla_{OXA\text{-}1}$, and bla_{OXY} including the carbapenemase $bla_{OXA\text{-}181}$ in two (14.3 %) study isolates. Other resistance genes included: *aac(6′)-lb-cr*, *aac(3)*, *aac*, *aph*, *aad*, *dfr*, *tet(A)*, and *tet(D)*, *mph(A)*, *sul1*, *sul2*, *oqx*, *qnr*, *acrR*, *ramR*, *parC*, *gyrA*, *arr-3*, *cat*, *fosA*, *qacE* genes conferring resistance to aminoglycosides, trimethoprim, tetracycline, macrolide, sulfonamides, fluoroquinolones, rifampicin phenicols, fosfomycin, and quaternary ammonium compound disinfectant. Virulence factors related to hypervirulence: encoding aerobactin (*iuc*, *iutA*), salmochelin (*iro*), yersiniabactin (*ybt*), enterobactin (*ent*), type 1 and 3 (*mrk* and *fim*), and capsule synthesis (*rcsA* and *rcsB*) were identified. IncF, IncR, and Col plasmid replicon types and class I integrons were detected, with IncFIB(K) predominance. The $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ genes were bracketed by Tn3 transposons, ISEc9, recombinase and IS91 insertion sequences. **Conclusions**: The convergence of multidrug resistance and hypervirulence genes in *Klebsiella* strains is a potential clinical concern. Carbapenemase, ESBL screening and genomic surveillance are urgently required in hospital environments.

1. Introduction

Klebsiella spp. are Gram-negative, anaerobic, rod-shaped, bacteria belonging to the *Enterobacterales* family. Bacterial species belonging to the genus *Klebsiella*, most notably *Klebsiella pneumoniae* and *Klebsiella oxytoca*, are important nosocomial pathogens. These *Klebsiella* spp. are responsible for a plethora of nosocomial infections such as respiratory tract infections, urinary tract infections (UTIs), and bloodstream infections (BSIs) [1]. The extensive use β-lactam antibiotics for treatment of multidrug-resistant (MDR) bacterial infections has led to the

* Corresponding author.
E-mail address: bakoenah@yahoo.com (B.A. Hetsa).

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production of extended-spectrum β-lactamases (ESBLs) and carbapenemase-producing Enterobacterales (CPE) [2]. The emergence of carbapenem-resistant *Enterobacterales* (CRE), and ESBL-producing *Klebsiella* strains pose significant medical concern associated with treatment failure, morbidity and mortality [3].

In South Africa, high rates of resistance to β-lactams particularly mediated by carbapenemases and ESBLs have been reported in clinical isolates of *K. pneumoniae* and *K. oxytoca* [5,6]. Broad-spectrum β-lactam resistance in *Klebsiella* spp. is attributable to the acquisition of ESBL genes such as bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ types, with predominance of $bla_{\text{CTX-M-15}}$ [7]. OXY β-lactamases are chromosomal class A β-lactamases found in *K. oxytoca*, mediating resistance to penicillin's, and cephalosporins [9]. Carbapenem resistance in *Klebsiella* spp. is predominantly mediated by the production of carbapenem-hydrolysing β-lactamase enzymes, the carbapenemases. More recently, Magobo et al. identified *K. pneumoniae* with $bla_{OXA\text{-}181}$, and $bla_{NDM\text{-}1}$ genes isolated from bloodstream infections in a hospital in Tembisa, South Africa. All the *K. pneumoniae* isolates identified were resistant to ertapenem and meropenem, and 94 % of the isolates carried carbapenemase genes [4].

In addition to antimicrobial resistance (AMR), highly virulent *Klebsiella* spp. carry a battery of virulence genes that encode aerobactin (*iuc, iut*), salmochelin (*iro*), yersiniabactin (*ybt, fyuA, irp1/2*), enterobactin (ent) involved in iron acquisition and pathogenicity factors such as capsular polysaccharide (CPS) (K antigen) (K1 or K2 capsules; O1 or O2 LPS), as well as regulators of mucoid phenotype (*rmpA* and *rmpA2*) [10]. There are also other pathogenicity factors, such as *fim* and *mrk* genes encoding type 1 and type 3 fimbriae, and lipopolysaccharides (LPS) biosynthesis, and type VI secretion systems (T6SS) The *fim* and *mrk* genes and type IV secretion systems (T6SS) are virulent factors required for host cell adherence and biofilm formation [11,12], which makes eradication of infections extremely difficult [13].

Convergence of AMR genes and virulence factors in pathogenic *Klebsiella* spp., is a key feature associated with increased pathogenesis [14]. The burden of infections caused by *Klebsiella* spp., is associated with global spread of MDR strains known as high-risk clones. Among the best characterised *K. pneumoniae* high risk clones are those belonging to clonal group ST11, ST512, ST14 and ST15 [15]. *K. oxytoca* STs, and specifically ST2, ST9, ST85, ST43, ST92, and ST145 are commonly identified in clinical isolates of *K. oxytoca* displaying antibiotic resistance [16].

In this study, we describe genomic characteristics of MDR and ESBL-producing *K. pneumoniae* and *K. oxytoca* isolates isolated from blood culture in hospitals within the KwaZulu-Natal province in South Africa, in terms of the virulome, resistome, mobilome, genetic environment and phylogenomics.

2. Materials and methods

Ethical approval

Ethical approval for the study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal under the following reference number BCA444/16.

2.1. Bacterial collection, and identification

A total of 46 suspected *Klebsiella* species were collected from blood cultures obtained from two hospitals within the uMgungundlovu District in the KwaZulu-Natal Province as part of a larger surveillance study that used the global antimicrobial resistance and use surveillance system (GLASS) guidelines. All the isolates had been identified by the National Health Laboratory Services (NHLS) using the automated VITEK 2 system (BioMérieux, MarcyL'Etoile, France) The WGS study isolates comprised of a subset of 14 isolates of which 10 were *K. pneumoniae* and four were *K. oxytoca* (n = 4). The selection of isolates was based on their antibiograms and multidrug resistance (MDR).

2.1.1. Antibiotic susceptibility testing (AST)

Antibiotic susceptibilities of the isolates were carried out using the disk diffusion method against a panel of 19 antibiotics, on Mueller–Hinton agar as recommended by the Clinical and Laboratory Standards Institute (CLSI) [17], or European Committee on Antimicrobial Susceptibility Testing (EUCAST) [18]. The antibiotics tested and interpreted according to CLSI guidelines (CLSI, 2017) included: amoxicillin–clavulanic acid (AMC, 30 μg), cefotaxime (CTX, 30 μg), ceftazidime (CAZ, 30 μg), ceftriaxone (CRO, 30 μg), cefepime (FEP, 10 μg), cephalexin (LEX, 30 μg), cefoxitin (FOX, 30 μg), piperacillin–tazobactam (TZP, 110 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), nalidixic acid (NAL, 30 μg), ciprofloxacin (CIP, 5 μg), gentamicin (GEN, 10 μg), amikacin (AMK, 30 μg), tetracycline (TET, 30 μg), chloramphenicol (CHL, 30 μg), trimethoprim–sulfamethoxazole (SXT, 25 μg) and azithromycin (AZM, 15 μg). Only tigecycline (15 μg) was interpreted according to the EUCAST breakpoints (EUCAST, 2017), as the CLSI guidelines has no breakpoints. The antibiotic disks were obtained from Oxoid (Oxoid Ltd., Basingstoke, UK) (Table 1). *Escherichia coli* ATCC25922 was used as a quality control strain. MDR was defined as resistance to three or more antibiotic classes [19].

2.2. DNA extraction and whole-genome sequencing

The genomic DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, St. Louis, MO, USA) following the instructions of the manufacturer. The quantity and quality of the extracted gDNA was analysed using a Nanodrop 8000 (Thermo Fisher Scientific Waltham, MA, USA). Multiplexed paired-end libraries (2 × 300 bp) were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, United States), and sequences were determined on an Illumina MiSeq platform with 100 × coverage at the National Institute of Communicable Diseases (NICD) Sequencing Core Facility, South Africa.

2.3. Genomic analyses and annotation

Raw sequencing reads were quality trimmed and *de-novo* assembled into contigs using the CLC Genomics Workbench version 10 (CLC, Bio-QIAGEN, Aarhus, Denmark) [20]. The *de-novo* assembled reads were uploaded in GenBank and annotated using the NCBI prokaryotic genome annotation pipeline. The assembled genomes were deposited at GenBank under the BioProject **PRJNA669537**. The resistome, virulome, and mobilome (plasmids, integrons, transposons, insertion sequences, and prophages) were predicted using various online tools, including ResFinder 4.1 (https://cge.cbs.dtu.dk/services/ResFinder-4.1), and the comprehensive antibiotic resistance database (https://card.mcmaster.ca/analyze/rgi accessed on October 1, 2023). Virulence determinants were identified with default settings using the virulence factor database (VFDB: http://www.mgc.ac.cn/VFs/main.htm accessed on November 15, 2023), and VirulenceFinder 2.0 https://cge.food.dtu.dk/services/VirulenceFinder/

accessed on November 15, 2023). PlasmidFinder database 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/accessed on October 19, 2023), ISFinder (https://isfinder.biotoul.fr/), MGE (https://cge.food.dtu.dk/services/MobileElementFinder/), INTEGRAL database http://integrall.bio.ua.pt/accessed on October 20, 2023) and PHASTER (https://phaster.ca/accessed on October 1, 2023). Multilocus sequence typing (MLST) was predicted using the online database (https://cge.cbs.dtu.dk/services/MLST/accessed on October 10, 2023). Capsular typing was done using Kaptive online tool (https://kaptive-web.erc.monash.edu/) to determine the serotypes (K-type and O-type and wzi allelic) [21,22]. The genetic environment of ARGs, virulence genes and MGEs were visualized by Geneious Prime 2021 (https://www.genious.com accessed on December 5, 2023). Whole-genome sequences of *K. pneumoniae* and *K. oxytoca* isolates curated at the bacterial and viral bioinformatics resource center (BV-BRC) online platform (https://www.

Table 1
Antibiotic susceptibility profiles of *K. pneumoniae* and *K. oxytoca* implicated in BSIs.

Isolate ID	Strain	Antibiotics																	
		AMC 30 µg	CTX 30 µg	CAZ 30 µg	CRO 30 µg	FEP 10 µg	LEX 30 µg	FOX 30 µg	TZP 110 µg	IMP 10 µg	MEM 10 µg	NAL 30 µg	CIP 5 µg	GEN 10 µg	AMK 30 µg	TGC 15 µg	TET 30 µg	CHL 30 µg	SXT 25 µg	AZM 15 µg
K. pneumoniae																				
K8	*K. pneumoniae*	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	R	S	R	R
E24	*K. pneumoniae*	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	S	S	R	S
E28	*K. pneumoniae*	R	R	R	R	R	R	R	R	R	R	I	R	R	R	S	S	S	I	S
K13	*K. pneumoniae*	R	R	R	R	R	R	R	R	S	S	R	R	R	I	S	I	R	R	S
K33	*K. pneumoniae*	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	R	R	R	R
E14	*K. pneumoniae*	R	R	R	R	R	R	R	R	S	R	R	R	R	R	S	R	R	R	R
K31	*K. pneumoniae*	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	S	S	R	S
K32	*K. pneumoniae*	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R
K35	*K. pneumoniae*	R	R	R	R	R	R	R	R	S	R	R	R	R	I	S	R	S	R	S
K37	*K. pneumoniae*	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S	I	R	R	R
K. oxytoca																				
K51	*K. oxytoca*	I	R	I	I	I	I	I	I	S	S	I	I	I	I	S	I	S	I	R
EC2	*K. oxytoca*	I	R	I	I	S	I	I	S	S	S	I	I	R	I	S	S	S	S	R
EC7	*K. oxytoca*	I	I	I	I	I	I	I	I	S	S	S	I	S	I	S	S	S	S	S
A11	*K. oxytoca*	I	R	R	R	I	R	I	R	I	I	R	R	R	R	S	R	I	I	R

Amoxicillin-clavulanic acid (AMC), Cefoxatime (CTX), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Cephalexin (LEX), Cefoxitin (FOX), Piperacillin/Tazobactam (TZP), Imipenem (IPM), Meropenem (MEM), Nalidixic acid (NAL), Ciprofloxacin (CIP), Gentamycin (GEN), Amikacin (AMK), Tigecycline (TGC), Tetracycline (TET), Chloramphenicol (CHL), Trimethoprim/Sulfamethoxazole (SXT), Azithromycin (AZM), and *Resistant (I), susceptible (S), and intermediate (I).

bv-brc.org/accessed on December 10, 2023) from Africa were downloaded and used together with our study's isolates for phylogeny analysis. A phylogenomic tree of *K. pneumoniae* and *K. oxytoca* was built using BV-BRC's Phylogenetic Tree Building tool, using the nucleotide and amino acid sequences from 1000 shared genes (https://www.bv-brc.org/). The generated phylogenetic trees were visualized, annotated, and edited using iTOL (https://itol.embl.de/accessed on December 10, 2023) and Figtree (http://tree.bio.ed.ac.uk/software/figtree/accessed on December 10, 2023).

3. Results

3.1. Demographic and patient details

The 14 isolates investigated in this study were obtained from patients who visited a regional hospital (n = 10, 71.4 %) and a tertiary hospital (n = 4, 28.6 %). All the bloodstream infections in this study were monomicrobial. Most isolates were obtained from the medical ward (n = 4, 28.3 %), and intensive care unit (n = 4, 28.3 %). One each of the remaining isolates were obtained from the burn ward, surgical ward, renal unit, paediatric ward, paediatric outpatient department, and emergency department. Seven patients were females, and seven patients were males. The age distribution of patients ranged from 0 to 59 years old, and the mean age was 19.7 years (Table S1). The demographic details of the source participants of the 14 isolates that were selected for WGS are shown in Supplementary Table 1.

3.2. Antibiotic susceptibility of isolates

The 14 isolates showed similar phenotypic resistance patterns with most displaying resistance to all β-lactams, including cephamycin (FOX), cephalosporins, (CRO, CAZ, and FEP). Resistance to β-lactamase inhibitors (TZP), aminoglycosides, (GEN, and AMK), and fluoroquinolone (CIP) was also observed (Table 1). However, isolates were highly susceptible to IMP (78.6 %), MEM (64.3 %), and CHL (57.1 %) (Table 1). All isolates were MDR, and the resistance phenotype was confirmed by the ARGs detected by WGS (Table 3).

3.3. Genome characteristics

The genomic features of the sequences, in terms of size, coverage, L50, N50, CRISPR arrays, GC content, number of RNAs, and coding sequences are shown in Table S2.

3.4. Virulome analysis

All isolates carried similar battery of virulence genes (Table 2). The genes for the regulator of mucoid phenotype (*rcsA/B, phoP*), siderophores including yersiniabactin (*ybtAEPQSTUX*, *irp1/2*, *fyuA*), aerobactin (*iutA*), enterobactin (*entABCDEFS*, *fepABCDG*, *fes*), salmochelin (*iroBDEN*)) were present in all isolates (Table 2). Therefore, all isolates were putatively designated as hypervirulent strains. Hyper-mucoviscous regulators encoding (*rmp*A and *rmp*A2) genes were not found in tested isolates. The type I fimbriae locus *fimABCDEFHIJ*, and type 3 fimbriae locus *mkrABCDFHIJ* were present in all isolates. The two yersiniabactin genes, *fyuA*, and *irp2* genes were associated with the integrative and conjugative element (ICEKp1) in two *K. pneumoniae* isolates (Table 4).

3.5. Resistome analysis

The resistome confirmed the resistance phenotypes in most isolates (Table 3). However, a few discordances were detected for azithromycin where is the presence of *mphA* gene (n = 1) did not translate into phenotypic azithromycin resistance on AST. Also, two isolates that were phenotypically resistant to azithromycin, did not carry the corresponding ARGs (Table 3). Resistome analysis showed that 13/14 (99 %)

Table 2
Virulence factors in *K. pneumoniae* and *K. oxytoca* isolates.

Isolate ID	Species	Virulence genes				
		Regulation of mucoid phenotype A	Aerobactin	Ent siderophore	Salmochelin	Yersiniabactin
K. pneumoniae						
K8	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
E24	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entF, entS entA, entB, entC, entD, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
E28	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
K13	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
K33	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
E14	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
K31	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
K32	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
K35	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
K37	*K. pneumoniae*	*rcsA, rcsB*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
K. oxytoca						
K51	*K. oxytoca*	*rcsA, rcsB, phoP*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
EC2	*K. oxytoca*	*rcsA, rcsB, phoP*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
EC7	*K. oxytoca*	*rcsA, rcsB, phoP*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*
A11	*K. oxytoca*	*rcsA, rcsB, phoP*	*iucA, iucB, iucC, iucD, iutA*	*entA, entB, entC, entD, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes*	*iroB, iroC, iroD, iroE, iroN*	*fyuA, irp1, irp2, ybtA, ybtE, ybtP, ybtQ, ybtS, ybtT, ybtU, ybtX*

of isolates harboured β-lactamase encoding genes. Five different families were detected, viz., $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{OXA}, bla_{SHV}, and bla_{OXY} in all *K. pneumoniae* isolates (Table 3). The most common β-lactamase genes found in *K. pneumoniae* isolates were $bla_{CTX\text{-}M\text{-}15}$ (10/10), we found 9 variants of bla_{SHV} [$bla_{SHV\text{-}106}$ (4/10), $bla_{SHV\text{-}11}$ (4/10), $bla_{SHV\text{-}1}$ (3/10), $bla_{SHV\text{-}81}$ (2/10), $bla_{SHV\text{-}28}$ (2/10), $bla_{SHV\text{-}187}$ (1/10), $bla_{SHV\text{-}96}$ (1/10), $bla_{SHV\text{-}94}$ (1/10), $bla_{SHV\text{-}172}$ (1/10)], followed by bla_{TEM} [$bla_{TEM\text{-}1B}$ (9/10), $bla_{TEM\text{-}1C}$ (1/10), $bla_{TEM\text{-}79}$ (1/10)], and bla_{OXA} [$bla_{OXA\text{-}1}$ (8/10), $bla_{OXA\text{-}9}$ (2/10)] (Table 3). *K. oxytoca* isolates, contained the $bla_{OXY\text{-}2\text{-}5}$ (4/4), $bla_{OXY\text{-}2\text{-}4}$ (3/4) gene (Table 3). A single carbapenemase gene $bla_{OXA\text{-}181}$ was identified in two *K. pneumoniae* isolates. No carbapenemases from classes A, B, and C were detected. Additional genes included those conferring resistance to aminoglycosides [*aac(3)-IIa, aph(3′)-Ia, aph(6)-Id, aph(3″)-Ib, aac(6′)-Ib-cr, aac(3)-IIe, aadA16, baeR*], trimethoprim [*dfrA1, dfrA14, dfrA27*], tetracycline [*tet(A), tet(D)*] macrolide [*mph(A)*], sulfonamides]*sul*1, *sul*2], fosfomycin [*fosA, fosA5, fosA6*], rifampicin [*arr*-3], phenicols [*catA2, catII, catB3*], fluoroquinolones [*qnrB1, qnrB6, oqxB, oqxA, gyrA, parC, emrR*] and quaternary ammonium compound disinfectant [*qac*E] (Table 3). Mutations in chromosomal genes associated with quinolone/ciprofloxacin (*gyrA* and/or *parC*) and tigecycline (*acrR*, *ramR*) resistance were found in some *K. pneumoniae* isolates (Table S5).

The study isolates displayed significant agreement between resistance phenotypes and genotypes detected with ARGs affirming the phenotype, except in a few instances. There was discordance between azithromycin-resistant phenotype and the *mphA* genotype was observed (K33 and E14) were phenotypically resistant to azithromycin, there was no corresponding ARGs detected, while strain E24 was phenotypically susceptible to azithromycin, but carried the *mphA* gene mediating resistance to azithromycin.

3.6. MLST, capsular serotypes (KLO and wzi) typing

A variety of STs was detected among the *Klebsiella* spp. For *K. pneumoniae* six different STs were detected with ST25 (n = 3), ST101 (n = 3), and singletons of ST17 (n = 1), ST152 (n = 1), ST985 (n = 1), and ST15 (n = 1), while ST450 (n = 3) was detected in *K. oxytoca* isolates, one *K. oxytoca* isolate was not assigned to any ST (Table 3). *K. oxytoca* isolates had the same capsular serotype KL29 (capsule locus type associated with wzi386 allele) with an OL104 O locus (Table 3). The *K. pneumoniae* isolates of the same ST, (ST25, and ST101) possessed the same capsular serotypes (K and O loci) and were strain specific (Table 3).

3.7. Mobilome (plasmids, ISs, intact prophages, and integrons) and the genetic environment

The PlasmidFinder revealed twelve different plasmid replicons, with most isolates (57.1 %) having more than one plasmid in different combinations (Table 3). The most frequently detected plasmid replicon was IncFIB(K) in 10/14 (71.4 %). Additionally, IncFIB(K) plasmid replicon was found in three isolates belonging to ST25 sequence types of *K. pneumoniae* (Table 3). Also, the IncFIB(pKPHS1) replicon was found in two *K. oxytoca* isolates only (Table 3). ISfinder analysis detected 32 insertion sequences (ISs), of which IS3 and IS6 were the most prevalent, and Tn3 family transposases in different permutations and combinations (Table 3).

Using synteny analysis confirmed the locations of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in our study isolates. These elements were located on plasmids, except for the SHV gene including $bla_{SHV\text{-}11}$, $bla_{SHV\text{-}1}$, and $bla_{SHV\text{-}106}$, which were encoded on the chromosome (Table S4). The ARGs were mainly associated with insertion sequences, transposons, and class 1 integrons (Table 4). The $bla_{CTX\text{-}M\text{-}15}$ gene was often associated with Tn3 transposase, insertion

Table 3
Kaptive typing, Resistome characteristics in *K. pneumoniae* and *K. oxytoca* isolates.

Isolate ID (MLST)	WGS *in-silico* typing			Antibiotic resistance genes				
	K type	Allelic type	KLO typing	ESBL	β-lactamases	Aminoglycosides	Fluoroquinolones/ Quinolones	Other resistance genes
K. pneumoniae								
K8 (ST25)	KL2	wzc2, wzi72	O1/ O2v2	+	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *bla*$_{SHV-81}$, *bla*$_{SHV-11}$	*aac(3)-IIa, aph(3′)-Ia, aph(6)-Id, aph(3″)-Ib, aac(6′)-Ib-cr, aac (3)-IIe*	*qnrB1, aac(6′)-Ib-cr*	*mph(A), tet(A), dfrA14, sul2, opmA, ompK37*
E24 (ST25)	KL2	wzc2, wzi72	O1/ O2v2	+	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *bla*$_{SHV-11}$	*aac(3)-IIa, aph(3′)-Ia, aph(6)-Id, aph(3″)-Ib, aac(6′)-Ib-cr, aac (3)-IIe*	*qnrB6, aac(6′)-Ib-cr*	*sul2, acrR, dfrA14, mph(A), catB3, uhpT, marR, ompA*
E28 (ST25)	KL2	wzc2, wzi72	O1/ O2v2	+	*bla*$_{OXA-1}$, *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-181}$, *bla*$_{SHV-81}$, *bla*$_{SHV-11}$	*aac(3)-IIa, aac(6′)-Ib-cr, aph (6)-Id, aph(3″)-Ib, aac(3)-IIe*	*aac(6′)-Ib-cr*	*sul2, dfrA14, kpnG, kpnE, eptB, ompA*
K13 (ST101)	KL17	wzc18, wzi137	O1/ O2v1	+	*bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1C}$, *bla*$_{OXA-1}$, *bla*$_{OXA-9}$, *bla*$_{SHV-106}$, *bla*$_{SHV-1}$	*aac(3)-IIa, aac(6′)-Ib-cr, aph (6)-Id, aph(3″)-Ib, aac(3)-IIe*	*aac(6′)-Ib-cr, oqxB, oqxA, gyrA, parC*	*sul2, sul1, qacE dfrA1, dfrA14, ompK37, ompA, uhpT, catII*
K33 (ST101)	KL17	wzc18, wzi137	O1/ O2v1	+	*bla*$_{TEM-79}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-9}$, *bla*$_{SHV-106}$, *bla*$_{SHV-1}$	*aac(3)-IIa, aac(6′)-Ib-cr, aph (6)-Id, aph(3″)-Ib, aac(3)-IIe*	*aac(6′)-Ib-cr, oqxB, oqxA, gyrA, parC*	*sul1, sul2, dfrA1, dfrA14, qacE, ompA, fosA6, ompK37, catII*
E14 (ST101)	KL17	wzc18, wzi-137	O1/ O2v1	+	*bla*$_{CTX-M-15}$, *bla*$_{SHV-28}$, *bla*$_{SHV-106}$	-	*oqxB, oqxA, gyrA, parC*	*catA2, catII ompK37, ompA fosA*
K31 (ST985)	KL39	wzc39, wzi39	O1/ O2v2	+	*bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-187}$	*aac(3)-IIa, aac(6′)-Ib-cr, aph (6)-Id, aph(3″)-Ib, aac(3)-IIe*	*aac(6′)-Ib-cr, oqxA, oqxB, gyrA,*	*acrR, dfrA14, sul2, fosA, catB3, ompA*
K32 (ST15)	KL112	wzc923, wzi93	O1/ O2v1	+	*bla*$_{CTX-M-15}$, *bla*$_{OXA-181}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$, *bla*$_{SHV-106}$, *bla*$_{SHV-28}$	*aac(3)-IIa, aac(6′)-Ib-cr, aph (6)-Id, aph(3″)-Ib, aac(3)-IIe*	*aac(6′)-Ib-cr, oqxB, oqxA qnrB1, gyrA*	*sul2, dfrA14, mph(A), acrR, ramR, tet(A), catB3, fosA, ompA, ompK37*
K35 (ST17)	KL25	wzc26, wzi141	O5	+	*bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-11}$, *bla*$_{SHV-96}$, *bla*$_{SHV-94}$, *bla*$_{SHV-172}$	*aadA16, aac(3)-IIa, aac(6′)-Ib-cr, aph(6)-Id, aph(3″)-Ib, aac (3)-IIe*	*aac(6′)-Ib-cr, oqxB, oqxA, qnrB6*	*sul1, sul2, acrR, arr-3, fosA6, dfrA27, tet(D), qacE, ompA, ompK37*
K37 (ST152)	KL149	wzc928, wzi110	O4	+	*bla*$_{TEM-1B}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-1}$	*aadA16, aph(3′)-Ia, aac(3)-IIa, aac(6′)-Ib-cr, aph(6)-Id, aph (3″)-Ib, aac(3)-IIe*	*aac(6′)-Ib-cr, oqxB, oqxA, qnrB6, parC, gyrA*	*sul1, sul2, catA1, dfrA27, fosA6, rmtC, acrR, mph(A), qacE, arr-3, ompA, ompK37*
K. oxytoca								
K51 (ST450)	KL29	wzc30, wzi386	OL104	-	*bla*$_{OXY-2-5}$, *bla*$_{OXY-2-4}$	*baeR*	*oqxA, emrR, gyrB*	*adeF, ompA, fosA5,*
EC2 (ST450)	KL29	wzc30, wzi386	OL104	-	*bla*$_{OXY-2-5}$, *bla*$_{OXY-2-4}$	*baeR*	*oqxA, emrR, gyrB*	*ompA, fosA5*
EC7 (ST450)	KL29	wzc30, wzi386	OL104	–	*bla*$_{OXY-2-5}$, *bla*$_{OXY-2-4}$	*baeR*	*oqxA emrR, gyrB*	*acrR adeF, ompA fosA5*
A11 NF	KL29	wzc30, wzi386	OL104	-	*bla*$_{OXY-2-5}$	*baeR*	*oqxA, emrR, gyrB*	*acrR adeF, ompA, fosA5,*

K and O typing—Klebsiella surface polysaccharide capsule characterization typing scheme (K-loci and O-loci), wzc and wzi type—allelic typing; KL, ARGs, antibiotic resistance genes; ESBL, Extended spectrum β-lactamases; +/-, Presence/Absence, MLST, Multilocus sequence type; NF, Not found.

sequence (IS*1380*), or a recombinase. The Tn3 and a myriad of ISs were associated with the ESBLs (*bla*$_{CTX-M-15}$, *bla*$_{TEM}$) and aminoglycosides resistance genes (*aph(6)-Id*, *aph(3″)-Ib*). Insertion sequences IS3 and IS6 were most often associated with aminoglycoside (*aac(3)-IIe*), and trimethoprim (*dfrA14*) genes, respectively (Table 4). The resistance genes and MGEs in *K. pneumoniae* isolates showed (98–100 %) similarity with target sequences in the GenBank database. A total of 13 isolates had intact prophages, of which PHAGE_Salmon_SEN5 was the most prevalent (Table S6). No ARGs were carried by prophages.

3.8. Cassette arrays of class 1 integrons and genetic environment

Class 1 Integron (*intI1*) was found in eight isolates with a similar genetic environment (Table 4). The *IntI1* was flanked by insertion sequences, and contained ARGs conferring resistance to sulfonamides, aminoglycosides, quaternary ammonium compound disinfectant, trimethoprim, fluoroquinolones, rifampicin genes, and a recombinase (Table 4) indicating that the class 1 integron plays a vital role in conferring MDR among bacterial strains [23]. The trimethoprim gene *dfrA14*, IS*6* and relaxosome (*mobC*), and Tn3 transposon were associated with *IntI1* in five isolates (Table S4).

3.9. Phylogenomics

The phylogenetic analysis, combined with metadata, shows clear grouping patterns based on Multilocus Sequence Typing (MLST) and geographic origin. Isolates with similar MLST type clustered together, alongside country of isolation (Figs. 1 and 2). For instance, Fig. 1 revealed that the ST101 isolates from this study (K13-ST101, K33-ST101, E14-ST101) clusters with other ST101 isolates from different African countries, including Kenya, Egypt, and Nigeria, highlighting the broader geographic distribution and potential mobility of this MLST type across the continent. Similarly, ST17 isolate from this study clustered with ST17 isolates from various African countries such as South Africa, Ghana, Kenya, and Nigeria. Additionally, the ST152 isolate from this study (K37-ST152) is closely associated with other South African isolates, indicating minimal genetic divergence within this MLST type in the region, implying a strong regional lineage for ST152 in South Africa (Fig. 1). Phylogenomics findings may indicate a shared lineage or recent genetic exchange between these groups, pointing to complex evolutionary dynamics within these populations.

3.10. Discussion

We analysed *K. pneumoniae* and *K. oxytoca* isolates implicated in BSIs. Both *K. pneumoniae* and *K. oxytoca* isolates harboured several hypervirulence genes, but only the *K. pneumoniae* isolates had an assortment of ESBL, and other antibiotic-resistance genes. Our results revealed isolates with high genome "plasticity characterized by the presence of several virulence and resistance determinants in different

Table 4
Plasmid replicon types, class 1 integrons, and gene cassettes found in *K. pneumoniae* and *K. oxytoca* isolates.

Sample code (MLST)	Mobile genetic elements				pMLST	Integron Class	Integron	Cassette arrays	
	Plasmids replicon	Insertion sequences	Transposon	ICE				CG1[a]	CG2
*K. pneumoniae*rowhead									
K8 (ST25)	IncFIB(K), Col156, IncFII(K)	IS6, IS1380, IS481, IS630, IS3, IS5, IS110	Tn5403	–	[K8:A-:B-]	IntI1	In191	*dfr*A14	
E24 (ST25)	IncFIB(K)	IS1380, IS6, IS481, IS5, IS3, IS630, IS110	Tn3	–	_	IntI1	In191	*dfr*A14	
E28 (ST25)	IncFIB(K)	IS1380, IS6, IS481, IS5, IS3, IS630, IS110	Tn3	–	_	IntI1	In191	*dfr*A14	
K13 (ST101)	IncFII(K), ColRNAI, IncR, IncFIB(K)	IS110, IS6, IS481, IS3, IS5, IS1	Tn3	–	[K1:A-:B-]	IntI1	In191	*dfr*A14	
K33 (ST101)	IncFII(K), ColRNAI, IncR, Col440I Col (MG828), IncFIB (K)	IS110, IS3, IS5, IS6, IS1, IS481	Tn3	–	[K1:A-:B-]	IntI1	In191	dfrA14	
E14 (ST101)	IncFII(K), ColRNAI, IncFIB(K), Col440I, IncR	IS1380, IS481, IS1, IS3, IS110, IS630	Tn3	–	[K1:A-:B-]	_	–	-b	
K31(ST985)	IncFIB(Mar), IncFIB(K), IncFII (K), IncFIA(HI1)	IS6, ISNCY, IS5, IS1, IS110, IS3,	Tn5403	–	[K3:A-:B-]	_	In191	*dfr*A14	
K32 (ST15)	ColKP3, IncFII(K), IncFIB(K), ColRNAI, ColpVC, IncL/M (pMU407)	ISKra4, IS1380, IS3, IS5, IS1, IS481, IS110, IS630, IS6	Tn5403	ICEKp1	[K9:A-:B-]	IntI1		*sul1:qacE:aadA16: dfrA27:arr-3:aac(6′)-Ib-cr*	
K35 (ST17)	IncFII(K), IncR IncFIA(HI1), IncFIB(K)	IS5, IS6, IS3, IS1380, IS66, IS1	Tn3	–	[K13: A13:B-]	IntI1		*sul1:qacE:aadA16: dfrA27:arr-3:aac(6′)-Ib-cr5*	
K37 ST152	IncFII(Yp), ColRNAI, IncFIB (pB171), IncFIB(K)	IS1380, IS66, ISL3, IS3, IS481, IS110, IS6	Tn3	ICEKp1	[K12:A-: B36]	IntI1	In152	*aar-3*	*dfrA27*
K. oxytocarowhead									
K51 (ST450)	IncFIB(pKPHS1)	–	–		–	–		-b	
EC2 (ST450)		–	–		–	–		-b	
EC7 (ST450)	IncFIB(pKPHS1)	–	–		–	–		-b	
A11 (NF)	–	–	–		–	–		-b	

Note: [a] GC denotes gene cassettes. [b] denotes the missing cassette arrays due to the fragmented draft genomic sequences during the sequencing and assembling process into different contigs. MLST, Multilocus sequence type; ICE, integrative conjugative element, If applicable, the plasmid Inc-type, antimicrobial resistance genes, +/-, Presence/Absence.

permutations and combinations.

The emergence and spread of *Klebsiella* isolates harbouring both MDR and hypervirulence genes pose clinical challenges. A huge diversity of virulence genes often associated with hypervirulence in *Klebsiella* spp., were found in the genomes of isolates in this study (Table 2). Similar virulence factors have been identified by Russo et al. [24] from clinical *K. pneumoniae* isolates proposed as clear markers for hvKP. The presence of genes like *iroB* and *iucA*, which are part of the salmochelin and aerobactin gene clusters suggests a potential hypervirulent phenotype in our isolates [25–27]. The (*ybt, fyuA*) genes are responsible for iron acquisition in iron limiting conditions, adhesion, invasion, infection, and biofilm formation [22,24]. The presence of *mrk*, and *fim* genes have been shown to enhance resistance to the non--β-lactam antibiotics in a study that investigated the relationship between biofilm formation and surface adhesins genes in *K. oxytoca* isolates. Their results showed that the presence of adhesins enhance biofilm production, and restricts antibiotic penetration into the cells [28], which may result in limited treatment options. Most *ybt*, *fyuA*, irp1/2, *mrk* and *fim* genes were bracketed by ISs and integrase, suggesting that IS are important in the transfer of virulence genes (Table S3). Furthermore, the *fyuA,* and *irp2* were associated with ICEKp1 (Table 4) in two isolates, which compares with the results reported by Arena et al. (2022), in which two isolates carried ybt genes on ICEKp1. This finding is significant since ICEs are important MGEs responsible for mobilizing virulence genes including *ybt* gene cluster within the *K. pneumoniae* population [29].

Furthermore, the virulome analysis showed that the study isolates carried *aur*, *iro*, *irp1/2*, and *ybt* genes associated with hypervirulence, but lacked other genes linked to enhanced capsular polysaccharides including the plasmid-borne virulence factors *rmpA* and *rmpA2*, *magA* genes (Table 2). This is similar to what was observed in a study done in Saudi Arabia, where the analysed hospital *K. pneumoniae* strain isolated from patient with a recurrent urinary tract, did not have the *rmpA/rmpA2* genes. Their findings demonstrated that capsule production was mediated by *rcsA* and *rcsB* genes, suggesting that there are other virulent factors that may be involved in the capsular polysaccharides synthesis and hypervirulence [30]. The *rcsA* and *rcsB* genes found in our study isolates encode the regulator of capsule synthesis (Rcs) phosphorelay. The rcs is a complex signal transduction pathway involved in the regulation of CPS [31], and biofilm formation [32].

The detection of antibiotic resistance genes (ARGs) from WGS generally correlated with observed phenotypic resistance patterns. However, discrepancies were noted with azithromycin resistance. The *mphA* gene, which confers resistance to azithromycin, was detected in some isolates that were phenotypically susceptible, suggesting it may be unexpressed. Conversely, some isolates showed phenotypic resistance but lacked the *mphA* gene, indicating other resistance mechanisms may be involved. Such genotype-phenotype discordances are not uncommon [31], and can result from lack of gene expression, sequencing limitations, or issues with phenotypic detection methods [31,32]. These findings highlight the complexity of antimicrobial resistance and emphasize the importance of integrating both genotypic and phenotypic data to accurately assess resistance profiles.

Our study findings revealed different STs and capsular serotypes (Table 3). The ST101 (KL17) and ST25 (KL2) strains were dominant among *K. pneumoniae,* while *K. oxytoca* belonged to single sequence type (ST450) KL29. However, *K. oxytoca* (A11) isolate was not assigned to ST due to the missing locus (*pgi*), and thus considered to be a novel ST. ST101 and ST25 strains carrying multiple ARGs, including CTX-M-15 have been reported in South Africa [4,8]. It is important to note that the two globally reported ESBL producing STs (ST25 and ST15) carrying $bla_{CTX\text{-}M\text{-}15}$, co-harbored the carbapenem resistance $bla_{OXA\text{-}181}$ gene (Table 3). Similar ESBL and carbapenem carriage was reported by Lowe et al. (2019) in a study that investigated nosocomial outbreaks of ST307 with $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}181}$ within several South African provinces.

Also, we noticed that some ST25-K2 strains, and *K. oxytoca* ST450

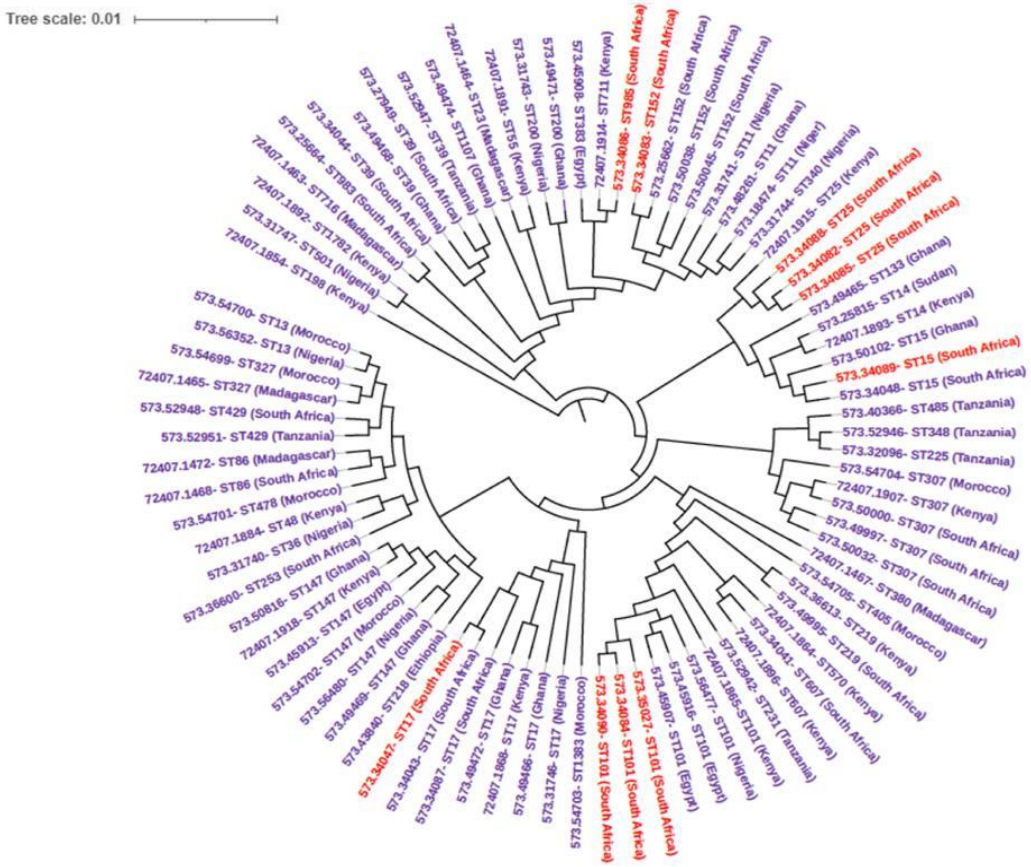

Fig. 1. Circular phylogenetic tree with colour annotations depicting the relationship between *K. pneumoniae* from this study (coloured in red), and African isolates from blood culture(coloured in purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

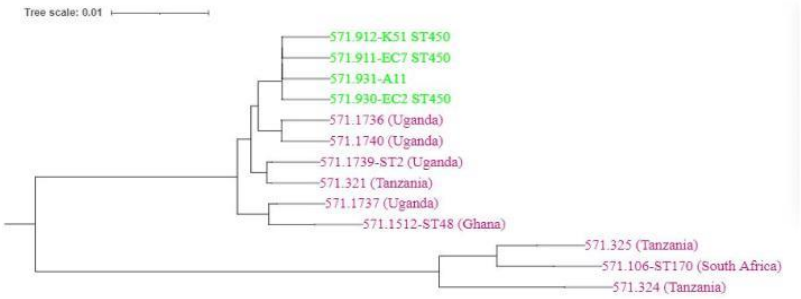

Fig. 2. Rectangular Phylogenomic tree with colour annotations depicting the relationship between *K. oxytoca* isolates from this study (coloured in green), and African isolates from diverse clinical sources (coloured in purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

strains were obtained from intensive care unit (ICU) (Table S1). ST25 strains have been isolated from the ICU in a study that was done in China that identified ST25 lineages from the neonatal ICU [34]. The isolation of ST25, and ST450 warrants further studies to investigate their prevalence in ICU environments. K1 and K2 strains are more virulent than other strains because of the ability to evade phagocytosis and intracellular killing by alveolar macrophages and neutrophils [10]. In South Africa, ST25 K2- hvKP strains similar to those reported here have been described in a study that analysed clinical isolates of *K. pneumoniae* obtained from six hospitals in Pretoria, South Africa [8]. In this study, the *K. oxytoca* isolate from the neonatal ICU contained only the $bla_{\text{OXY-2-5}}$ gene and did not harbor any ESBL genes. In contrast, reports of *K. oxytoca* strains associated with neonatal ICUs often describe ESBL producers that have been implicated in outbreaks. Therefore, our isolate is not similar to those strains [35]. The dissemination of MDR, in healthcare settings, especially in ICUs where there are immune-compromised patients poses a major problem.

The ST101, ST17, and ST152 are potentially multidrug resistant

clones, which have also been isolated from clinical isolates of *K. pneumoniae* in South Africa [8,37]. ST17 is one of the most widely distributed clones, predominant among clinical isolates [38], and faecal samples [39]. ST17 strains obtained from NICU outbreak strain were characterised by ESBL production, especially a $bla_{CTX\text{-}M\text{-}15}$ [38]. ST17 KL25/O5 identified herein co-carried assorted ESBL genes with other ARGs genes conferring resistance to trimethoprim, fluoroquinolones, aminoglycosides, and tetracyclines, posing an additional challenge to treatment (Table 3). ST152 isolates identified herein contained similar capsular serotypes to those reported in Kwa-Zulu Natal and Pretoria, South Africa [8,37,40], suggesting a possible epidemiological linkage between these isolates.

The study isolates were characterised by resistance to β-lactam, flouroquinolones, aminoglycosides, trimethoprim, and cephalosporins antibiotics, which was confirmed by the presence of various ARGs (Table 3). This is similar to a study done in South Africa, that studied ESBL producing *K. pneumoniae* isolated from clinical samples including sputum and urine obtained from hospitalised patients. WGS analysis of *K. pneumoniae* isolates revealed the occurrence of several TEM, SHV, and CTX-M types [41]. Although the variants of SHV were found in our isolates (Table 3), the SHV-1 was found in only three isolates. Previous studies have reported β-lactam resistance in *K. pneumoniae* isolates carrying other SHV variants, but lacking the $bla_{SHV\text{-}1}$ which is known to be constitutively expressed in *K. pneumoniae* [42]. *K. oxytoca* isolates carried variants of bla_{OXY} genes, which confers ampicillin resistance (Table 3). The $bla_{OXY\text{-}2\text{-}5}$, is among the most predominant bla_{OXY} types reported in clinical isolates, which has heightened ceftazidime hydrolysis action [43]. In this study, $bla_{CTX\text{-}M\text{-}15}$ was the most prevalent ESBL gene, consistent with the study by Silago and Mshana, (2022), which revealed 90.6 % of CTX-M-15 gene among 34 *Klebsiella pneumoniae* isolated from blood samples in Tanzania [44].

The genetic environment of ARGs' had transposases, ISs, and recombinase/integrase, which may aid their mobilisation via horizontal transmission (Table S4). Tn3 transposons often bracketed various ARGs, including $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, *aph(6)-Id*, *aph(3")-Ib*, *dfrA14*, and *sul*2 with many ISs in most isolates (Table S4). The $bla_{CTX\text{-}M\text{-}15}$ gene was found on genetic elements $bla_{CTX\text{-}M\text{-}15}$::Tn3 and $bla_{CTX\text{-}M\text{-}15}$:IS1380 (IS*Ec9*) on contigs that had closest nucleotide homology to plasmids from *Escherichia coli* YJ1 pYJ1-NDM5 DNA (AP023225.1), and *E. coli* p15_d1-58 DNA (LC846576.1) (Table S4). The $bla_{CTX\text{-}M\text{-}15}$ gene has become the most predominant CTX-M types responsible for cephalosporin resistance worldwide [45], and has also been reported in clinical isolates in South Africa [4,46]. The IS*Ec9* transposases have been shown to play a crucial role in the dissemination and global spread of $bla_{CTX\text{-}M\text{-}15}$ [47]. Similarly, a recent study done in South Africa reported that *K. pneumoniae* with $bla_{CTX\text{-}M\text{-}15}$ was consistently associated with IS1380 (IS*Ec9*) and Tn3 [46], implying that this insertion sequence is important in mobilizing $bla_{CTX\text{-}M\text{-}15}$ gene.

In this study, the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\ \ 1B}$ were closely associated with a recombinase and IS*91* or IS*3* surrounded by ARGs conferring resistance to aminoglycosides *aph(6)-Id*, *aph(3")-Ib),* and sulphonamide (*sul*2) (Table S4). These genes were found on genetic element recombinase:$bla_{TEM\text{-}1B}$:IS*91*:*aph(6)-Id*:*aph(3")-Ib*:*sul*2 and IS1380:recombinase:$bla_{TEM\text{-}1B}$:IS*91*:*aph(6)-Id*:*aph(3")-Ib*:*sul2* on contigs that had closest homology to plasmids from *K. pneumoniae* isolate INF277 genome assembly, plasmid: 2 (LR890205.1), and *K. pneumoniae* strain E17KP0053 plasmid pE17KP0053-2 (CP052219.1), suggesting that mobilization of these resistance genes' is plasmid-mediated (Table S4). It is important to note that the isolates associated with IS*91* were isolated from the regional hospital (from paediatric ward = 5, and ICU = 1), and some belonged to STs known to cause hospital outbreaks such as ST15, ST17, 101 associated with MDR (Table S1). ST15 is considered as an emerging international high-risk clone responsible for nosocomial outbreaks, associated with high levels of ESBLs production, mainly CTX-M-15 [48]. The carriage of multiple ARGs, and virulence genes among such strains with reduced susceptibility to multiple antibiotics in paediatric wards is alarming and can result in untreatable infections. The IS*91* is often associated with ARGs and can enable mobilization of adjacent sequences by a one-ended transposition process [49], which may potentially increase the risk of mobilizing multiple ARGs among patients admitted within the same ward and across different wards in the same hospital thereby, contributing to the dissemination of multidrug resistant strains.

In this study, the MDR ST25 and ST15 strains carried a $bla_{OXA\text{-}181}$ gene (Table 2). The detection of $bla_{OXA\text{-}181}$ was reported by Lowe et al. (2019), in which a high prevalence of $bla_{\text{OXA-181}}$ was observed among *K. pneumoniae* ST307 strains across six South African provinces. Interestingly, carbapenem resistance in our study isolates was not mediated by MBLs or KPC enzymes. This observation was unexpected, as previous reports from South Africa have detected various carbapenemase genes, including bla_{NDM}, bla_{VIM}, bla_{GES}, and bla_{KPC} [49], as well as bla_{NDM} and $bla_{OXA\text{-}48}$ [4]. The absence of these common carbapenemase genes in our isolates suggests regional variations in the mechanisms of carbapenem resistance and highlights the importance of ongoing local surveillance to detect and monitor these variations. This finding underscores the need for tailored antimicrobial stewardship and infection control strategies that consider the specific resistance mechanisms prevalent in different regions. The *K. pneumoniae* isolates identified (75 %) were CRE and most (52 %) carried $bla_{OXA\text{-}48/181}$ carbapenemase gene [42]. The $bla_{OXA\text{-}181}$ was found on genetic element recombinase:pRiA4b:recombinase: ISKpn19:ereA: $bla_{OXA\text{-}181}$ (Table 4). Similarly, a recent study by Ransamy et al. (2022), reported the $bla_{OXA\text{-}181}$ gene with the genetic context: $bla_{OXA\text{-}181}$:ereA::ISKpn19:recombinase:pRiA4b ORF-3. In both genetic contexts, the $bla_{OXA\text{-}181}$ was associated with insertion sequence, recombinases, erythromycin resistance gene, ISKpn19 and a recombinase. The presence of ISKpn19-based transposon on the region where $bla_{OXA\text{-}181}$ and *ere*(A) genes were located suggest the possibility of the spread of carbapenemase and erythromycin resistance caused by these genes. Also, the genetic context of $bla_{OXA\text{-}181}$ may suggest that its spread or mobility is associated with plasmids (Table 4). This phenomenon agrees with a study of Lowe et al. [33], which also found IncX3 plasmid and $bla_{OXA\text{-}181}$ in isolates belonging to ST307 in six provinces in South Africa. Furthermore, the observed variation in susceptibility patterns to carbapenems, such as imipenem and meropenem, in *K. pneumoniae* is not uncommon and has been documented in the literature [50]. For instance, Kayama et al. (2015) reported a *K. pneumoniae* strain producing OXA-181 that was susceptible to imipenem but resistant to meropenem [51]. These differences in carbapenem susceptibility can be attributed to variations in outer membrane porin structures and the expression of efflux pumps. Alterations in porin proteins can affect the permeability of the bacterial outer membrane to different carbapenems, while overexpression of efflux pumps can actively remove antibiotics from the cell, reducing their efficacy. This highlights the complexity of resistance mechanisms in *K. pneumoniae* and underscores the importance of comprehensive antimicrobial susceptibility testing to guide appropriate therapy.

In our study, we detected various plasmid replicons in the isolates, including conjugative IncFIB(K), IncFII(K), IncR, and Col plasmids, which are known reservoirs of acquired antibiotic resistance genes (ARGs) in *K. pneumoniae* (Table 4). IncFIB plasmids are conjugative and are commonly associated with the dissemination of ARGs, including ESBLs such as $bla_{SHV\text{-}12}$, $bla_{CTX\text{-}M\text{-}15}$, and $bla_{OXA\text{-}1}$, as well as carbapenemase genes like *bla*NDM-$_1$ and $bla_{OXA\text{-}181}$ [52]. We identified the *dfrA14* gene, which mediates trimethoprim resistance, on a contig with high homology to plasmid pIncFIBK_IncFIIK (accession number CP067373.1), suggesting that *dfrA14* may be located on a conjugative plasmid and thus could be transferable. Additionally, the $bla_{OXA\text{-}181}$ gene, associated with a high risk of rapid dissemination due to its transferability, was detected in our isolates. Our analysis revealed that $bla_{\text{OXA-181}}$ was located on a plasmid similar to pRiA4b, flanked by recombinase genes and the insertion sequence ISKpn19, on a contig with high similarity to the *E. coli* plasmid pEco15-4 (accession number CP047714.1). The presence of these mobile genetic elements suggests

potential for mobilization of $bla_{OXA-181}$ via transposition or recombination, contributing to the spread of carbapenem resistance through alternative mechanisms. This highlights the importance of monitoring plasmid-mediated resistance and underscores the need for comprehensive genomic surveillance to inform infection control strategies.

Phylogenomic analyses revealed that the *K. pneumoniae* samples in this study clustered mainly with clinical *K. pneumonia* isolates from other African countries (Fig. 1). The ST101, ST15, and ST17 isolates were closely related to other ST101 isolates from other African countries (Fig. 1), suggesting that their dissemination in hospital environments could be critical. ST101 and ST17 strains are commonly associated with multidrug-resistant *K. pneumoniae* in South Africa [8,30], as was the case in this study. ST17 isolate in this study was closely related to several ST17 isolated from South Africa, indicating a potential spread of this strain in hospitals. ST17 is the most widely distributed clone, predominant among clinical isolates [38], associated with MDR hospital infections. Concerning *K. oxytoca*, phylogenomic analyses revealed that our study isolates were not closely related to other isolates from other countries (Fig. 2), suggesting that the occurrence of ST450 strains could be local.

Despite the valuable insights provided by WGS data analysis, the absence of clinical and demographic data hindered the ability to analyze relationships between bacterial species, hospital wards, and genomic profiles. Moreover, resource constraints prevented the inclusion of colistin, and ceftazidime-avibactam in our susceptibility testing panel, limiting our capacity to differentiate between MBLs and SBLs. Additionally, we were unable to perform phenotypic tests such as the string test for hypervirulence and conjugation assays to assess the virulence potential and transmission of carbapenemase and ESBL genes. Also, due to resource constraints were unable to perform phenotypic tests to detect carbapenemase or ESBL. Future studies should consider incorporating comprehensive clinical data, including colistin, and ceftazidime-avibactam in susceptibility testing, and performing phenotypic assays with WGS data to enhance the understanding of resistance mechanisms and transmission dynamics.

4. Conclusion

This study revealed the presence of diverse ARGs, MGEs and virulence genes in MDR *K. pneumoniae* and *K. oxytoca* strains obtained from BSIs in various departments in two hospitals in uMgungundlovu District within Kwa-Zulu Natal province. Many ARGs, virulence genes, and or MGEs in different permutations and combinations present challenges to clinical management and infection prevention and control measures. These results advocate the need for ESBL, and hvKp screening programme, and continuous molecular surveillance to identify and contain hypervirulent and ESBL-producing *Klebsiella* spp.

Supplementary Materials: Table S1: Demographic characteristic of patients with BSIs caused by *K. pneumoniae* and *K. oxytoca*; Table S2: Genome and assembly characteristics of sequenced *K. pneumoniae* and *K. oxytoca* isolates from blood cultures; Table S3: MGE associated with virulence genes in the *K. pneumoniae* and *K. oxytoca* isolates; Table S4: MGE associated with resistance genes in the *K. pneumoniae* and *K. oxytoca* isolates; Table S5: Point mutations on the fluroquinolone and tigecycline antibiotic resistance genes located on the chromosome; Table S6: Distribution of intact prophage regions among the *K. pneumoniae* and *K. oxytoca* isolates.

Institutional review Board Statement

This study was approved by the UKZN Biomedical Research Ethics Committee (BREC) (Ref No. BCA444/16.). Permission was granted by the KwaZulu-Natal Department of Health, the uMgungundlovu District Manager, the hospital Chief Executive Officer.

CRediT authorship contribution statement

Bakoena A. Hetsa: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Jonathan Asante:** Writing – review & editing, Data curation. **Joshua Mbanga:** Writing – review & editing, Formal analysis. **Daniel G. Amoako:** Writing – review & editing, Validation, Supervision, Formal analysis, Project administration, Methodology, Conceptualization. **Akebe L.K. Abia:** Writing – review & editing, Supervision. **Arshad Ismail:** Writing – review & editing, Supervision, Resources. **Sabiha Y. Essack:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data Availability Statement

All analysed data have been included in the manuscript. The nucleotide sequences of the fourteen isolates analysed in this study were deposited in the NCBI GenBank database in the BioProject number (PRJNA669537): under the Accession numbers; JADIWP000000000, JADIWQ000000000, JADIWR000000000, JADIWS000000000, JADIWT000000000, JADIWU000000000, JADIWV000000000, JADIWW000000000, JADIWX000000000, JADIWY000000000, JADIWZ000000000, JADQTD000000000, JADQTF000000000, and JADQTG000000000.

Declaration of competing interest

S.Y.E. is a chairperson of the Global Respiratory Partnership and member of the Global Hygiene Council, both supported by unrestricted educational grants from Reckitt (Pty.) Ltd, UK All other authors have no competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2024.107272.

Data availability

Data will be made available on request.

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Table S1: Demographic characteristic of patients with BSIs caused by *K. pneumoniae* and *K. oxytoca*

Isolate ID	Species	Sample	Department	Location	Gender	Age
K8 (ST25)	*K. pneumoniae*	Blood	Intensive care unit	Tertiary hospital	F	<1 year
E24 (ST25)	*K. pneumoniae*	Blood	Intensive care unit	Regional hospital	F	<1 year
E28 (ST25)	*K. pneumoniae*	Blood	Paediatric ward (ICU)	Regional hospital	M	<1 year
K13 (ST101)	*K. pneumoniae*	Blood	Renal unit	Tertiary hospital	F	37 years
K33 (ST101)	*K. pneumoniae*	Blood	Intensive care unit	Regional hospital	F	<1 year
E14 (ST101)	*K. pneumoniae*	Blood	Paediatric outpatient department	Tertiary hospital	M	<1 year
K31 (ST985)	*K. pneumoniae*	Blood	Paediatric ward	Regional hospital	M	3 years
K35 (ST17)	*K. pneumoniae*	Blood	Paediatric ward	Regional hospital	M	<1 year
K32 (ST15)	*K. pneumoniae*	Blood	Intensive care unit	Regional hospital	F	59 years
K37 (ST152)	*K. pneumoniae*	Blood	Paediatric ward	Regional hospital	M	<1 year
K51 (ST450)	*K. oxytoca*	Blood	Burns ward	Regional hospital	M	30 years
EC2 (ST450)	*K. oxytoca*	Blood	Paediatric ward	Regional hospital	F	<1 year
EC7 (ST450)	*K. oxytoca*	Blood	Surgical intensive care unit	Regional hospital	M	59 years
A11 (ST450)	*K. oxytoca*	Blood	Intensive care unit	Tertiary hospital	F	<1 year

Table S2: Genome and assembly characteristics of sequenced *K. pneumoniae* and *K. oxytoca* isolates from blood cultures

Isolate ID	Accession no.	Sex*	Age (Years)	Hospital	Species	Size (Mb)	GC%	Contigs	No. of RNAs	No of coding Sequences	N50	L50
K. pneumoniae												
K8	JADIWY000000000	F	<1 year	Tertiary	*K. pneumoniae*	5.6	57.1	147	90	5601	105113	15
E24	JADIWR000000000	F	<1 year	Regional	*K. pneumoniae*	5.5	57.1	140	88	5552	117441	16
E28	JADIWQ000000000	M	<1 year	Regional	*K. pneumoniae*	5.5	57.1	146	91	5564	127529	17
K13	JADIWT000000000	F	37	Tertiary	*K. pneumoniae*	5.6	57.1	132	85	5643	133665	15
K33	JADIWX000000000	F	<1 year	Regional	*K. pneumoniae*	5.9	56.9	548	86	6305	137072	14
E14	JADQTG000000000	M	<1 year	Tertiary	*K. pneumoniae*	5.1	57.1	907	44	5741	9666	144
K31	JADIWW000000000	M	3	Regional	*K. pneumoniae*	5.7	56.5	105	89	5729	202686	9
K35	JADIWV000000000	M	<1 year	Regional	*K. pneumoniae*	5.6	57.2	170	87	5679	234183	9
K32	JADIWZ000000000	F	59	Regional	*K. pneumoniae*	5.6	57	118	90	5616	189299	9
K37	JADIWU000000000	M	<1 year	Regional	*K. pneumoniae*	5.5	57.3	131	88	5492	201585	9
K. oxytoca												
K51	JADIWS000000000	M	30	Regional	*K. oxytoca*	5.9	55.1	76	85	5744	300862	7
EC2	JADQTF000000000	F	<1 year	Regional	*K. oxytoca*	5.9	55.1	76	85	5744	300862	7
EC7	JADIWP000000000	M	59	Regional	*K. oxytoca*	5.9	55.1	74	84	5744	221632	10
A11	JADQTD000000000	F	<1 year	Tertiary	*K. oxytoca*	4.9	55.2	56	68	4799	300860	6

Key: N50 = smallest contig of the size-sorted contigs that make up at least 50% of the respective assembly; L50 = number of contigs that make up at least 50% of the respective total assembly length.

Table S3: MGE associated with virulence genes in the *K. pneumoniae* and *K. oxytoca* isolates

Contig	Synteny of resistance genes and MGEs	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
	K. pneumoniae	
08	IS3::IS481-like element ISKpn28:type I toxin-antitoxin system toxin:	*K. pneumoniae* isolate KSB2_9B chromosome (100%), accession 1(LR890710.1)
27	**::*iroE*::**	*K. pneumoniae* strain NK_H12_034 chromosome (100%), accession (CP152948.1)
28	*tssE*:*tssJ*:*tssF*::::*vgrG:ompA*::*tssK*	*K. pneumoniae* strain SMU18037509 chromosome (99.96%), accession (CP045661.1)
01	***mrkA*:*mrkB*:*mrkC*:*mrkD*:*mrkF*::*mrkI*:*mrkH***:ompk26	*K. pneumoniae* strain KP_NORM_URN_57042 (99.99%), accession (CP153326.1)
31	IS1:transposase:*silE*:*silS*:*silR*:*silC*::*silB*:*silA*::*silP*	*K. pneumoniae* strain ARLG-4568_pC685_1 (99.99%), accession (CP067557.1)
49	integrase:ybtS:ybtX:ybtQ:ybtP:ybtA:***irp2*:*irp1***:ybtU:ybtT:ybtE:***fyuA***	*K. pneumoniae* strain CriePir120 chromosome (99.99%), accession (CP063008.1)
05	ompK26:***mrkH***:***mrkI***:***mrkF***:***mrkD***:***mrkC***:***mrkB***:***mkrA***::***fimB***:***fimA***	*K. pneumoniae* strain KP_NORM_URN_57042 (99.99%), accession (CP153326.1)
33	IS1:silE:silS:silR:*silC*:cusF:silB:silA:silP:pcoA:pcoBpcoC:pcoD:pcoR:pcoE::IS L3 transposase:arsH:arsC:arsB:::*hha*:*hlb*:::IS1:clpK:hsp20:IS5	*K. pneumoniae* strain ARLG-4568 pC685_1 (99.99%), accession (CP067557.1) (plasmid)
47	***fyuA***:ybtE:ybtT:ybtU:i***rp1*:*irp2***:ybtA:ybtP:ybtQ:ybtX:ybtS:integrase	*E. coli* strain K71-77 chromosome (99.99%), accession (CP040886.1)
01	ompK26::***mrkH***:***mrkI***::***mrkF***:***mrkD***:***mrkC***:***mrkB***:***mrkA***::***fimB***:***fimE***:***fimA***:	*K. pneumoniae* strain KP_NORM_URN_57042 (99.99%), accession (CP153326.1)
23	IS630:::Cag12:virB11:::virB9:virB3:virB2:::***fyuA:ybtE*:*ybtT:irp1:irp2:ybtA:ybt P:ybtQ:ybtX:ybtS***:integrase	*K. pneumoniae* ST2017:950142398 chromosome (100%), accession (CP023553.1)
36	IS1:transposase:*silE*:*silS*:*silR*:*silC*::*silB*:*silA*:*silP*::ISL3::arsH:arsD:arsA:arsC:ars B::arsR::*hha*::ISNCY::clpK:hsp20:transposase	*K. pneumoniae* p1_IncFIB(K) (99.99%), accession (CP071253.1) (plasmid)
30	recombinase/integrase:***ybtS:ybtX:ybtQ:ybtP:ybtA:irp2:irp1:ybtU:ybtE:fyuA***: *virB2*::::*virB3*::*virB9*:*virB11*::Cag12:mobC:::IS630	*K. pneumoniae* ST101:960186733 chromosome (100%), accession (CP023487.1)
34	IS1:*silE*:*silS*:*silR*:*silC*::*silB*:*silA*:*silP*:::ISL3:*arsH:arsC:arsB*:::*hha:hlb*::ISNCY transposase:IS*3*:clpK:hsp20:IS*1*	*K. pneumoniae* E17KP0053 pE17KP0053-1 (100%), accession (CP052218.1) (plasmid)
100	***mrkD*:*mrkC*:*mrkB*:*mrkA*::*fimB*:*fimE*:*fimA***	*Klebsiella pneumoniae* strain F16KP0014 chromosome (100%), accession (CP052192.1)
09	***mrkH*:*mrkI*:*mrkF*:*mrkD*:*mrkC*:*mrkB*:*mrkA*:::*fimB*:*fimE*:*fimA***	*K. pneumoniae* NICU_1_P3 chromosome (100%), accession (CP059379.1)
27	cag12protein:virB11::virB9:vriB3:::**fyuA:ybtE:ybtT:ybtU:irp1:irp2:ybtA:ybt P:ybtQ:ybtX:ybtS**:integrase	*K. pneumoniae* strain Iso00073 chromosome (99.95%), accession (CP095150.1)
30	IS1:*silE:silS:silR:silC:cusF:silB:silA:silP*::::ISL3:*arsH:arsC:arsB*:::*hha:hlb*:::I SNCY:IS3:clpK:hsp20:IS1	*K. pneumoniae* strain Iso00267 plasmid pIso00267_01 (99.99%), accession (CP095133.1)
01	cag12protein:virB11::virB9:virB3::***fyuA:ybtE:ybtT:ybtU:irp1:irp2:ybtA:ybtP:y btQ:ybtX:ybtS***:integrase	*K. pneumoniae* strain Iso00073 chromosome (99.95%), accession (CP095150.1)

25	***fimA:fimE:fimB***:::***mrkA***:***mrkB***:***mrkC:mrkD:mrkF:mrkI***	*Klebsiella pneumoniae* strain AR376 chromosome (100%), accession (CP029137.1)
40	*silP::silA:silB:::silC*:*silR*:*silS*:*silE*:IS1 transposase	*K. pneumoniae* pF16KP0014-1 (100%), accession (CP052193.1)
31	transposase:*merE:merD:merA:merC:merP:merT*:IS*3*:::transposase:IS*66*	*K. pneumoniae* p1-MV931658 (99.96%), accession (CP165960.1) (plasmid)
41	Tn5403:recombinase::colicin release lysis protein:cloacin:*exc2*	*Klebsiella pneumoniae* strain INF281 plasmid pINF281.7 (99.92%), accession (CP110600.1)
	K. oxytoca	
18	***fimD***:***fimA***:integrase:::***mrkA***	*Klebsiella oxytoca* strain KONSF-1 chromosome (99.52%), accession (CP090245.1)
28	IS4 transposase::::*ompA*	*Klebsiella oxytoca* strain KONSF-1 chromosome (99.51%), accession (CP090245.1)
28	*::arcA*:::*arcAB::*	*Klebsiella oxytoca* strain 2022CK-00499 chromosome (99.46%), (CP114308.1)
22	*tssF*:*tssG*:*tssJ*:*tssE*:type 1 fimbrial ::*zntB::uspF*:*ompC::hslJ*	*Klebsiella oxytoca* strain KONSF-1 chromosome (99.65%), accession (CP090245.1)
25	*nirB:nirD:nirC*:*cobA*:::*pilN:::hslR:hs10*	*Klebsiella oxytoca* strain ST34 chromosome (99.75%), accession (CP103690.1)
26	capsule assembly *wzi* protein:*wzm* (O-antigen)	*Klebsiella oxytoca* strain NCTC13727 chromosome (99.69%), accession (LR134333.1)
30	::*zntR*:::*trkA*	*Klebsiella oxytoca strain* FDAARGOS_500 chromosome (100%), accession (CP033844.1)

Mobile genetic elements (MGEs); virulence genes.

Table S4: MGE associated with resistance genes in the *K. pneumoniae* and *K. oxytoca* isolates

Sample ID	Contig	Synteny of resistance genes and MGEs	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
		K. pneumoniae	
K8	16	:::*blaSHV-11*::::	*K. pneumoniae* isolate KSB2_9B chromosome (99.98%), accession (LR890710.1)
	50	Tn3::*bla*$_{CTX-M-15}$:IS*1380*(IS*Ec9*)	*K. pneumoniae* strain KP_NORM_URN_57042 chromosome (100%), accession (CP153326.1)
	81	recombinase:*bla*$_{TEM-1B}$:IS91:*aph(6)-Id*:*aph(3")-Ib*:*sul2*	*K. pneumoniae* pE17KP0053-2 (100%), accession (CP052219.1) (plasmid)
	87	*tet(A)*:*tetR(A)*:relaxase	*E. coli* pNDM-5-1001 (100%), accession (MH985167.1) (plasmid)
	91	*mph(A)*:*tetR*	*E. coli* strain AH62 pAH62-3 (99.98%), accession (CP055262.1) (plasmid)
	92	IntI1:*dfrA14*:mobC:IS*6* (IS*6100*)	*K. pneumoniae* pKPNU_58394.1 (100%), accession (CP153287.1) (plasmid)
	94	IS3::*aac(3)-IIe*	*K. pneumoniae* pVKpST395_NDM_3082 (100%), accession (PQ126478.1) (plasmid)
E24	**19**	:::*blaSHV-11*::::	*K. pneumoniae* isolate KSB2 9B chromosome (99.98%), accession (LR890710.1)
	70	IS1:IS6:mobC(relaxosome):*dfrA14*:IntI1::recombinase:Tn3	*K. pneumoniae* strain ARLG-4568 pC685_1 (99.99%), accession (CP067557.1) (plasmid)
	71	*sul2*:*aph(3")-Ib*:*aph(6)-Id*:IS*9*:*bla*$_{TEM-1}$:recombinase:: IS*1380* (IS*Ec9*):*bla*$_{CTX-M-15}$::Tn3	*K. pneumoniae* strain B16KP0141 pB16KP0141-1 (99.99%), accession (CP052538.1) (plasmid)
	87	*aac(3)-IIe*::IS*3*	*K. pneumoniae* subsp. pneumoniae strain M40 pM40-NDM5 (100%), accession (PQ247031.1) (plasmid)
E28	9	*bla*$_{SHV-11}$:::*marB*:*marA*:*marR*::IS1	*K. pneumoniae* strain Bckp021 chromosome (99.95%), accession (CP050834.1)
	68	Tn3-like (Tn*As3*):recombinase:IntI1:*dfrA14*: mobC:IS6	*K. pneumoniae* strain B17KP0069 pB17KP0069-1 (99.99%), accession (CP052491.1) (plasmid)
	69	Tn3::*bla*$_{CTX-M-15}$: IS*1380* (IS*Ec9*)::recombinase:*bla*$_{TEM-1}$:IS91:*aph(6)-Id*:*aph(3")-Ib*:*sul2*	*K. pneumoniae* pNK_H12_086.1 (100%), accession (CP152918.1) (plasmid)
	87	IS3:*aac(3)-IIe*	*K. pneumoniae* strain 3082_ pVKpST395_NDM_3082 (100%), accession (PQ126478.1) (plasmid)
K13	57	Tn3:IS*5075*::*sul2*:*aph(3")-Ib*:*aph(6)-Id*:IS*91*	*K. pneumoniae* ST101:960186733 chromosome (99.99%) accession (CP023487.1)
	62	IS*1*::IS*6* (IS*6100*):*mobC*:*dfrA14*	*K. pneumoniae* strain ATCC BAA-2146 plasmid pHg (100%), accession (CP006662.2)
	69	*blaCTX-M*-15:Tn3	*Escherichia coli* YJ1 pYJ1-NDM5 DNA (100%), accession (AP023225.1) (plasmid)
	40	TrbB::::IncFII(K): IS30	*K. pneumoniae* ST2017:950142398 p18-43_02 (100%), accession (CP023555.1) (plasmid)
K33	58	IS9:*aph(6')-Id*: *aph(3')-Ib*:*sul2*:IS*110*(IS*507*):Tn3	*K. pneumoniae* ST101:960186733 (99.99%), accession (CP023487.1) (chromosome)
	65	*aac(3)-IIe*::IS*3* (IS*Kpn11*)::IS*5* (IS*Kpn12*)	*K. pneumoniae* pKP16103-MCR-1 (100%), accession (MH733011.1) (plasmid)
	63	*dfrA14*:relaxase:IS*6*(IS*6100*)::IS1	*K. pneumoniae* strain ST101:960186733 p19-10_01 (100%), accession (CP023488.1) (plasmid)
	75	*blaCTX-M-15*::Tn3	*Escherichia coli* YJ1 pYJ1-NDM5 DNA (100%), accession (AP023225.1) (plasmid)
	76	*qacE*:*sul1*::IS*21* (IS*1326*)	*K. pneumoniae* p19051-FIIK (100%), accession (MN823997.1) (plasmid)
E14	489	Tn3:::*blaCTX-M-15*: IS*1380* (IS*Ec9*): recombinase	*K. pneumoniae* pNK_H21_037.1 (100%), accession (CP152731.1) (plasmid)
	299	*oqxA*:*oqxB20*:	*K. pneumoniae* strain F16KP0014 (99.04%) , accession (CP052192.1) (chromosome)
	1556	:::*blaSHV-106*::::	*Klebsiella pneumoniae* strain F16KP0014 (99.94%), accession (CP052192.1) (chromosome)
K31	44	IS*1*::IS*6* (IS*6100*):*mobC*:*dfrA14*:IntI1	*K. pneumoniae* strain BA30371 pIncFIBK_IncFIIK, (99.81%), accession (CP067373.1) (plasmid)

	45	IS*1380* (IS*Ec9*)::recombinase:*bla*$_{TEM-1B}$:IS91:*aph(6)-Id:aph(3'')-Ib:sul2*	*K. pneumoniae* pINF277.2 (100%), accession (CP110610.1) (plasmid)
	50	Tn3::*blaCTX-M-15*	*K. pneumoniae* pINF277.2 (100%), accession (CP110610.1) (plasmid)
	56	*aac(3)-IIe*::IS*3*	*E. coli* strain 13BC160093101 (100%), accession (CP138487.1) (chromosome)
K32	30	recombinase:::pRiA4b:recombinase:IS*Kpn19*:ereA:*bla*$_{OXA-181}$	*E. coli* pEco15-4 (99.01%), accession (CP047714.1) (plasmid)
	48	recombinase:*bla*$_{TEM-1B}$: IS91:*aph(6)-Id: aph(3'')-Ib:sul2*	*K. pneumoniae* pNK_H12_086.1 (100%), accession (CP152918.1) (plasmid)
	57	*bla*$_{CTX}$*-M-15*:IS*1380* (IS*Ec9*)	*E. coli* p15_d1-58 DNA, (100%) accession (LC846576.1) (plasmid)
	58	IS*3*::*aac(3)-IIa*	*Klebsiella pneumoniae* pMS14393B (100%), accession (CP054305.1) (plasmid)
	59	relaxosome:*dfrA14*:IntI1	*K. pneumoniae* strain 3082_kpn pVKpST395_NDM_3082 (100%), accession (PQ126478.1) (plasmid)
	40	IS*6* (IS6*100*)::::*sul1*:*qacE*::*qnrB6*::IS9:*sul1*:*qacE*:*aadA16*:*dfrA27*:*arr-3*:*aac(6')-Ib-cr*:IntI1:recombinase	*Raoultella planticola* strain S25 plasmid pS25-68, (99.97%), accession (CP044120.1) (plasmid)
	68	:::*qnrB1*:	*K. pneumoniae* pNK_H12_086.1 (100%), accession (CP152918.1) (plasmid)
K35	14	*marR:marA:marB*:::*blaSHV-11*	*K. pneumoniae* strain NK_H3_007 (99.99%), accession (CP152628.1) (chromosome)
	40	IS*6* (IS6*100*)::*sul1*:*qacE:qnrB6*::IS91:*sul1*:*qacE*:*aadA16*:*dfrA27*:*arr-3*:*aac(6')-Ib-cr5*:IntI1:recombinase	*Raoultella planticola* pS25-68, (99.97%), accession (CP044120.1) (plasmid)
	44	recombinase:*bla*$_{TEM-1B}$*:aac(3)-IIe*::IS*3* (IS*Kpn11*)::IS5IS*Kpn12*)	*K. pneumoniae* pKp2177_1 (100%), accession (CP075592.1) (plasmid)
	45	Tn3 (IS*Pa38*)::*aph(6)-Id:aph(3'')-Ib:sul2*	*K. quasipneumoniae* pAR8538_4 (99.98%), accession (CP081830.1) (plasmid)
	68	*bla*$_{CTX-M-15}$:IS*1380* (IS*Ec9*)	*E. coli* p15_d1-58 DNA, (100%) accession (LC846576.1) (plasmid)
K37	01	::*blaSHV-1*::	*K. pneumoniae* strain NK_H3_007 chromosome (99.99%), accession (CP152628.1)
	49	Tn3::*bla*$_{CTX-M-15}$:IS*1380* (I*Ec9*):recombinase:*bla*$_{TEM-1B}$:IS91:*aph(6)-Id: sul2*: IS110:Tn3	*Enterobacter* sp. pCRENT-193_1 (100%), accession (CP024813.1) (plasmid)
	52	IS3:IS30:: IS9:recombinase:::*qacE*	*Enterobacter cloacae* pECL3-NDM-1 (99.92%), accession (KC887917.2) (plasmid)
	70	*aac(3)-IIe*::IS3	*K. pneumoniae* pM40-NDM5 (100%), accession (PQ247031.1) (plasmid)
	91	*sul1:qacE*	*Escherichia coli* strain 13BC160093101 pFQ71_NDM-5 (100%), accession (CP138488.1)
			K. oxytoca
K51	07	:::*mdtI:mdtJ*::	*K. oxytoca* strain NCTC13727 chromosome (99.99%), accession (LR134333.1)
EC2	59	*blaOXY-2-4:::ramA*	*K. oxytoca* strain KO190245 chromosome (99.71%), accession (CP144730.1)
	95	*marB:marA:marR*	*K. oxytoca* strain 3238 chromosome (99.65%), accession (CP169719.1)
EC7	14	:::*fosA*::::	*Klebsiella oxytoca* strain 2022CK-00499 chromosome (99.46%), accession (CP114308.1)
	22	::*oqxB*::	*Klebsiella oxytoca* strain KONSF-1 chromosome (99.65%), accession (CP090245.1)
A11	32	*emrB:emrA*	*Klebsiella oxytoca* strain KONSF-1 chromosome (99.97%), accession (CP090245.1)

MLST, Multilocus sequence typing, (MGEs); antibiotic resistance genes (ARGs)

Table S5: Point mutations on the fluoroquinolone and tigecycline antibiotic resistance genes located on the chromosome

Isolate ID (MLST)	Species	Gene Mutation(s) in:					
		GyrA	***GyrB***	***ParC***	***ParE***	***acrR***	***ramR***
K8[μ] (ST25)	*K. pneumoniae*	S83Y, D87G,	-	S80F, F410Y	-	F197I, L195V, P161R, F172S,R173G, G164A, K201M	-
E24 (ST25)	*K. pneumoniae*	-	-	-	-	P161R,G164A, R173G, K201M, F197I, F172S, L195V	-
E28 (ST25)	*K. pneumoniae*	-	-	-	-	R173G, F197I, P161R, F172S, L195V, G164A, G164A, K201M	-
K13[μ] (ST101)	*K. pneumoniae*	S83Y, D87G,	-	S80I	-	A162S, N495D, D596E	-
K33[#] (ST101)	*K. pneumoniae*	D87G, S83Y	-	S80I	-	-	-
E14[#] (ST101)	*K. pneumoniae*	S83Y, D87G	-	S80I	-	-	-
K31[μ] (ST985)	*K. pneumoniae*	A331G, W334M, S328V, R327Q	-	P170T, S172I	-	F172S, F197I, P161R, L195V, G164A, R173G, K201M	-
K35[μ] (ST17)	*K. pneumoniae*	-	-	-	-	P161R,G164A, L195V, F172S,R173G, K201M, F197I	-
K32[μ] (ST15)	*K. pneumoniae*	S83F, D87A	-	S80I	-	-	A19V
K37[μ] (ST152)	*K. pneumoniae*	-	-	S80I	-	D87A, S83Y	-
K51 (ST450)	*K. oxytoca*	-	-	-	-	-	-
EC2 (ST450	*K. oxytoca*	-	-	-	-	-	-
EC7 (ST450)	*K. oxytoca*	-	-	-	-	-	-
A11	*K. oxytoca*	-	-	-	-	-	-

Mutations in (*GyrA, GyrB, ParC* and *ParE*), and tigecycline (*acrA, ramA) in the K. pneumoniae and K. oxytoca isolates.*

[μ]*Isolates resistant to both fluoroquinolones and tigecycline.*

[#]*Isolates resistant to fluoroquinolones only*

Table S6: Distribution of intact prophage regions among the *K. pneumoniae* and *K. oxytoca* isolates

Isolate ID	No. of prohage	Region	Length (Kb)	No. CDS	GC%	Phage (Hit genes count)
				K. pneumoniae		
K8	2	1	40.7	40 775	52.87	PHAGE_Cronob_ENT47670
		2	22.1	22 143	54.75	PHAGE_Salmon_RE_2010
E24	2	2	21.7	21 736	55.00	PHAGE_Salmon_RE_2010_NC_
		4	20.4	20 486	51.97	PHAGE_Entero_HK544_NC_
E28	2	2	21.7	21 736	55.00	PHAGE_Salmon_RE_2010
		4	20.9	20 996	52.03	PHAGE_Entero_HK544
K13	3	2	30.6	30 599	51.43	PHAGE_Escher_phiV10
		7	40.9	40 936	54.95	PHAGE_Entero_P88
		9	15.9	15 990	51.28	PHAGE_Salmon_118970_sal3
K33	4	2	30.6	30 599	51.43	PHAGE_Escher_phiV10
		7	40.9	40 936	54.95	PHAGE_Entero_P88
		8	25.8	25 824	53.61	PHAGE_Klebsi_phiKO2
		10	15.9	15 990	51.28	PHAGE_Salmon_118970_sal3
E14	0	-	-	-	-	-
K31	1	2	33.9	33 904	50.89	PHAGE_Salmon_Fels_2_NC
K35	5	1	32.8	32 840	54.44	PHAGE_Salmon_Fels_2
		2	45.9	45 935	50.99	PHAGE_Pectob_ZF40
		3	38.2	38 209	50.13	PHAGE_Salmon_SEN5
		4	24.5	24 512	51.94	PHAGE_Entero_mEp235
		6	26.6	26 689	53.84	PHAGE Salmon SEN5
K32	3	1	35.8	35 822	51.45	PHAGE_Salmon_RE_2010
		2	37.8	37 854	50.42	PHAGE_Salmon_SEN5
		3	33.8	33 822	52.03	PHAGE_Klebsi_phiKO2
K37	3	2	37.5	37 584	52.58	PHAGE_Klebsi_phiKO2
		3	39.8	39 890	52.15	PHAGE_Salmon_SEN5
		7	23.9	23 960	51.62	PHAGE_Salmon_118970_sal3
				K. oxytoca		
K51	3	1	49.9	49 942	51.23	PHAGE_Entero_c_1
		2	36	36 081	51.40	PHAGE_Salmon_SEN5
		3	102.5	102 554	49.47	PHAGE_Salmon_SSU5
EC2	2	1	44.4	44 429	50.54	PHAGE_Entero_c_1
		2	36	36 065	51.39	PHAGE_Salmon_SEN5
EC7	2	1	44.4	44 429	50.54	PHAGE_Entero_c_1_NC_
		2	36	36 065	51.39	PHAGE_Klebsi_ST16_OXA48phi5.4_NC_
A11	2	1	44.4	44 429	50.54	PHAGE_Entero_c_1
		2	36	36 065	51.39	PHAGE_Salmon_SEN5

CHAPTER 4- GENOMIC CHARACTERISATION OF METHICILLIN-RESISTANT AND METHICILLIN-SUSCEPTIBLE *STAPHYLOCOCCUS AUREUS* IMPLICATED IN BLOODSTREAM INFECTIONS, KWAZULU-NATAL, SOUTH AFRICA: A PILOT STUDY

Author contributions

- **Bakoena Ashton Hetsa** as principal investigator, co-conceptualised the study, did laboratory work, analysed the data, and drafted the manuscript.

- **Dr Jonathan Asante** undertook critical revision of the manuscript.

- **Dr J. Mbanga**– As co- supervisor co-conceptualised the study, supervised the laboratory work and critically revised the manuscript.

- **Dr A.L.K Abia** – As co- supervisor co-conceptualised the study, supervised the laboratory work.

- **Dr D.G Amoako** – As co-supervisor co-conceptualised the study, supervised the laboratory work, and critically revised the manuscript

- **Professor S.Y Essack** – As the main supervisor co-conceptualized the study, guided the literature review and ethical clearance application, enabled data collection and analysis and undertook the critical revision of the manuscript.

Objectives met – This paper answers objectives 4, 5 and 6.

antibiotics

MDPI

Article

Genomic Characterization of Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Implicated in Bloodstream Infections, KwaZulu-Natal, South Africa: A Pilot Study

Bakoena A. Hetsa [1,*], Jonathan Asante [1,2], Joshua Mbanga [1,3], Arshad Ismail [4,5], Akebe L. K. Abia [1], Daniel G. Amoako [1,6] and Sabiha Y. Essack [1,7]

[1] Antimicrobial Research Unit, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa; jonathan.asante@ucc.edu.gh (J.A.); mbangaj@ukzn.ac.za (J.M.); abiaakebel@ukzn.ac.za (A.L.K.A.); amoakod@ukzn.ac.za (D.G.A.); essacks@ukzn.ac.za (S.Y.E.)
[2] School of Pharmacy and Pharmaceutical Sciences, University of Cape Coast, PMB, Cape Coast, Ghana
[3] Department of Applied Biology & Biochemistry, National University of Science and Technology, Corner Cecil Avenue & Gwanda Road, Bulawayo 263, Zimbabwe
[4] Sequencing Core Facility, National Institute for Communicable Diseases, Division of the National Health Laboratory Service, Johannesburg 2193, South Africa; arshadi@nicd.ac.za
[5] Department of Biochemistry and Microbiology, Faculty of Science, Engineering and Agriculture, University of Venda, Thohoyandou 0950, South Africa
[6] Department of Pathobiology, University of Guelph, Guelph, ON N1G 2W1, Canada
[7] School of Pharmacy, University of Jordan, Amman 11942, Jordan
* Correspondence: 218085934@stu.ukzn.ac.za

Abstract: *Staphylococcus aureus* is an opportunistic pathogen and a leading cause of bloodstream infections, with its capacity to acquire antibiotic resistance genes posing significant treatment challenges. This pilot study characterizes the genomic profiles of *S. aureus* isolates from patients with bloodstream infections in KwaZulu-Natal, South Africa, to gain insights into their resistance mechanisms, virulence factors, and clonal and phylogenetic relationships. Six multidrug-resistant (MDR) *S. aureus* isolates, comprising three methicillin-resistant *S. aureus* (MRSA) and three methicillin-susceptible *S. aureus* (MSSA), underwent whole genome sequencing and bioinformatics analysis. These isolates carried a range of resistance genes, including *blaZ*, *aac(6′)-aph(2″)*, *ant(9)-Ia*, *ant(6)-Ia*, and *fosB*. The *mecA* gene, which confers methicillin resistance, was detected only in MRSA strains. The isolates exhibited six distinct *spa* types (t9475, t355, t045, t1265, t1257, and t7888) and varied in virulence gene profiles. Panton–Valentine leukocidin (Luk-PV) was found in one MSSA isolate. Two SCC*mec* types, IVd(2B) and I(1B), were identified, and the isolates were classified into four multilocus sequence types (MLSTs), with ST5 (n = 3) being the most common. These sequence types clustered into two clonal complexes, CC5 and CC8. Notably, two MRSA clones were identified: ST5-CC5-t045-SCC*mec*_I(1B) and the human-associated endemic clone ST612-CC8-t1257-SCC*mec*_IVd(2B). Phylogenomic analysis revealed clustering by MLST, indicating strong genetic relationships within clonal complexes. These findings highlight the value of genomic surveillance in guiding targeted interventions to reduce treatment failures and mortality.

Keywords: *Staphylococcus aureus*; bloodstream infections; whole-genome sequencing; antibiotic resistance; virulence; bioinformatics

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1. Introduction

Staphylococcus aureus is a Gram-positive bacterium inhabiting healthy individuals' nostrils and skin. However, it has become an important opportunistic pathogen in communities and hospitals [1]. It causes severe skin infections, pneumonia, endocarditis, and bloodstream infections (BSIs) [2]. BSIs caused by *S. aureus* infections have high morbidity

and mortality if not treated timeously [3]. The most significant risk factors for *S. aureus* BSIs are intravascular devices, surgical procedures, and a debilitated immune system [4].

Methicillin-resistant *S. aureus* (MRSA) has become a significant cause of BSIs. MRSA poses a major public health threat because of multidrug resistance to different antibiotic classes that limit treatment options [5]. Methicillin resistance in MRSA strains is mediated by the *mecA* gene, found on a mobile genetic element (MGE) known as the staphylococcal cassette chromosome *mec* (SCC*mec*) [6]. Methicillin-susceptible *S. aureus* (MSSA) is also emerging as a causative agent of BSIs [7] and has been reported to display high virulence and multidrug resistance [8].

The pathogenicity of *S. aureus* depends on its ability to produce a wide array of virulence factors involved in adhesion, invasion of host tissues, immune system evasion, and biofilm formation [9,10]. Also, *S. aureus* produces metallophores that enable bacteria to scavenge metal ions such as iron and zinc essential for bacterial metabolism and pathogenicity [11]. Virulence factors and multiple resistance genes can be transmitted by horizontal gene transfer (HGT) [12] on diverse MGEs, amongst which plasmids are reported as the primary sources for dissemination [4].

The epidemiology of *S. aureus* strains indicates that its molecular characteristics continually change over time, resulting in new clones, which vary by region. In a study in the United States, ST5 and ST8 were the most prevalent sequence types [13]. In South Africa, ST612 is dominant in the hospital environment [14]. The ST612-IV [2B], belonging to *spa* type t1257, was identified as a typical clone in clinical settings [15] and sporadically in poultry settings [16]. The ST5 and ST8 clones are commonly associated with BSIs and the pandemic lineages of *S. aureus*, such as the clonal complex CC8 and CC5 [17]. Notably, the sequence types ST612, ST5, ST8, and ST72 have displayed high resistance to most antibiotic drug classes and are challenging to treat [17].

Multidrug-resistant (MDR) *S. aureus* infections pose a serious clinical concern. A high incidence of pathogenic MDR MRSA has been reported, and the data suggest that its prevalence is increasing in Africa [18]. A recent South African study investigating the genetic relatedness of hospital-acquired-associated MRSA isolates in two hospitals revealed that all isolates were resistant to aminoglycosides and β-lactams. All the isolates carried the *aacA-aphD* and *mecA*-resistant genes and clusters of virulence genes [19]. This pilot study aimed to comprehensively characterize the genomic profiles, resistance mechanisms, virulence factors, pathogenicity, phylogenomic relationships, and clonal diversity of *Staphylococcus aureus* clinical strains implicated in BSIs at a regional hospital in KwaZulu-Natal, South Africa.

2. Results

2.1. Patient Demographics and Characteristics

The 6 isolates investigated in this study were obtained from patients who visited a regional hospital in the uMgungundlovu District in the KwaZulu-Natal Province. Three of the six isolates were recovered from the neonatal ICU (n = 3, 50%), two from surgical wards, and one isolate from the pediatric ward. Four patients were male, while two were female. The age distribution of patients ranged from 0 to 33 years old, and the mean age was 8.83 years (Table 1). The demographic details of the source participants of the isolates that were selected for WGS are shown in Supplementary Table S1.

Table 1. Antibiotic susceptibility profiles, age, and demographic characteristics of patients with BSIs attributed to *S. aureus*.

Isolate ID	Species	Sex	Ward	Age	Antibiotics																			
					PEN	AMP	FOX	CIP	MXF	LEV	GEN	AMK	ERY	CLI	TET	DOX	TGC	CHL	NIT	SXT	VAN	RIF	LZD	TEC
S11	MRSA	F	Surgical ward	17 years	R	R	R	R	R	R	R	R	R	R	R	R	R	I	S	R	S	R	R	R
S29	MRSA	M	Pediatric ward	<1 year	R	R	R	R	R	R	R	R	I	R	R	R	S	I	S	R	S	I	R	R
S31	MRSA	F	Surgical ward	3 years	R	R	R	R	R	R	R	R	R	R	R	R	S	R	S	R	S	R	R	R
S24	MSSA	M	ICU	33 years	R	S	S	R	R	R	S	S	R	R	R	I	S	I	R	R	S	R	I	I
S13	MSSA	M	ICU	<1 year	R	S	S	R	R	R	I	R	I	R	I	R	S	S	S	I	S	S	I	R
S34	MSSA	M	NICU	<1 year	R	S	S	R	R	R	I	R	I	R	R	R	S	I	S	S	S	I	S	S

Key: PEN, penicillin; AMP, ampicillin; FOX, cefoxitin; CIP, ciprofloxacin; MXF, moxifloxacin; LEV, levofloxacin; GEN, gentamicin; AMK, amikacin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; DOX, doxycycline; TGC, tigecycline; CHL, chloramphenicol; NIT, nitrofurantoin; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin; RIF, rifampicin; LZD, linezolid; TEC, teicoplanin. R, resistant; I, intermediate; S, susceptible; M, male; F, female; NICU, neonatal intensive care unit; ICU, intensive care unit.

2.2. Antibiotic Susceptibility Test Results

The isolates displayed varying phenotypic resistance profiles, with most being resistant to penicillin G (n = 6), tetracycline (n = 5), doxycycline (n = 5), clindamycin (n = 5), moxifloxacin (n = 5), rifampicin (n = 4), and erythromycin (n = 3). The lowest resistance was against nitrofurantoin, tigecycline, and chloramphenicol (n = 1) (Table 1).

2.3. Phenotypic and Genotypic Identification of MRSA Isolates

MRSA isolates were confirmed by phenotypic resistance to cefoxitin (Table 1) and the detection of the *mecA* gene using polymerase chain reaction (PCR).

2.4. Genomic Features

The genome size of our draft genomes ranged from 2.7 Mb to 2.9 Mb. The genomic characteristics of the sequences in relation to G + C content (%), number of RNAs, number of coding sequences, size, N50, L50, and coverage are shown in Table S2.

2.5. In Silico ARGs Analysis

Isolates harbored various permutations and combinations of ARGs, which included ARGs against β-lactams [*blaZ*], aminoglycosides [*aac(6′)-aph(2″)*, *aad(6′)*, *ant(9)-la*, *ant(6)-Ia*, *aph(2″)-Ia*, *aph(3′)-IIa*, *sat-4*], trimethoprim [*dfrG*, *dfr*C], macrolides [*erm(C)*, *erm(A)*], tetracycline [*tet(K)*, *tet(M)*, *mepR*, *mepA*], flouroquinolones [*parE*, *parC*, *grlA*, *gyrA*, *norA*, *norC* (multidrug efflux pumps)], rifampicin [*rpoB*] and fosfomycin [*fosB*, *murA*] (Table 2). Only the MRSA isolates harbored the *mecA* gene. There was good concordance between ARGs and phenotypic profiles for ARGs in all MRSA and MSSA isolates.

Table 2. Genotypic characteristics of *S. aureus* implicated in BSIs.

Isolate ID	MRSA/MSSA	MLST	*spa* Type	Resistome	Plasmid Replicon Type	Insertion Sequences	Confirmed CRISPRs (CAS)	Clonal Complex	* SCC*mec* Type	*agr* Type	Pathogenicity Score
S11	MRSA	ST8	t9475	*blaZ, mecA, aac(6')-aph(2''), parC, dfrG, erm(C), grlA, tetK, mepR, mepA, norA, norC, fosB*	rep10, rep7a, rep7c	-	6 (0)	CC8	NT	Type I	0.982 (882)
S29	MRSA	ST5	t045	*blaZ, mecA, aph(3')-III, aac(6')-aph(2''), ant(6)-Ia, ant(9)-Ia, aad(6'), erm(C), erm(A), qacA, mepR, fosB, norA, norC, sat-4*	rep10, rep21	IS6, IS256	12 (0)	CC5	SCC*mec* type I(1B)	Type II	0.98 (914)
S31	MRSA	ST612	t1257	*blaZ, mecA, aac(6')-aph(2''), aph(2'')-Ia, aad(6'), ant(6)-Ia, ant(9)-Ia, tet(M), mepR, mepA, dfrC, parC, erm(C), parE, gyrA, rpoB, fosB, norA, norC, murA*	rep7c, rep20	IS256, IS6	7 (0)	CC8	SCC*mec* type IVd(2B)	Type I	0.976 (978)
S24	MSSA	ST152	t355	*blaZ, dfrG, mepR, norC, murA*	rep16, rep5a	-	8 (0)	-	NA	Type IV	0.975(225)
S13	MSSA	ST5	t1265	*blaZ, norA, norC, fosB*	rep20	-	9 (0)	CC5	NA	Type II	0.985 (844)
S34	MSSA	ST5	t7888	*blaZ, norA, norC, mepR, fosB*	rep19, rep16, rep20, rep5a	1S6	7 (0)	CC5	NA	Type II	0.983 (871)

* SCC*mec* typing was predicted with the SCC*mec*Finder, MSSA—Methicillin-susceptible *Staphylococcus aureus*, MRSA—Methicillin-resistant *Staphylococcus aureus*—non-typeable (NT). The MSSA do not harbor SCC*mec* types, which is indicated as "None (NA)" to reflect their SCC*mec*-negative status.

2.5.1. MLST, spa Typing, and Clonal Complex

MLST revealed total four sequence types, i.e., ST5 (CC5, n = 3), ST152 (n = 1), ST612 (CC8, n = 1), and ST8 (CC8, n = 1). Two MRSA strains belonged to CC8 and one to CC5. Methicillin-susceptible (MSSA) isolates were identified as ST5 (n = 2) or ST152 (n = 1). The genetic diversity of the isolates was confirmed by *spa* typing, which revealed six different *spa* types: t9475, t1265, t355, t045, t1257, and t7888 (Table 2). CC and *spa* type combinations were CC8-t9475, CC8-t1257, and CC5-t045 among MRSA isolates, with CC5-t1265 belonging to one MSSA isolate. There was no association observed between STs, *spa* type, and CC. The grouping of the STs and *spa*-types yielded six genotypes, i.e., ST8-t9475, ST152-t355, ST5-t045, ST5-t1265, ST612-1257, and ST5-t7888, indicating that isolates were not clonally related.

The SCC*mec*Finder analysis identified two SCC*mec* types, i.e., IVd (2B) and I (1B), among the MRSA isolates (Table 2). One MRSA isolate was non-typeable (NT) for SCC*mec*. The combination of MLST, CC, *spa*, and SCC*mec* yielded the ST612-CC8-t1257-SCC*mec*_IVd (2B) and ST5-CC5-t045-SCCmec_I (1B), clones both of which have been reported in South Africa.

2.5.2. Mobilome (Plasmids, Insertion Sequences, Intact Prophages, and SCCmec Elements)

Analysis of the six isolate genomes identified various MGEs, including plasmid replicons, IS's, prophages, and SCC*mec* elements. A total of eight different plasmid replicons were detected, of which rep20 (n = 3) was the most prevalent (Table 2). There were no associations between plasmid replicons and STs. However, the rep7c was found in CC8 isolates in addition to other plasmid replicons, while rep16 and rep5a were found in isolates with the non-typeable CC. The rep20 plasmid replicon was associated with CC5 and CC8 isolates. The rep10 was carried in CC8 and CC5 isolates, while the re7a and rep21 were carried in CC8 and CC5 isolates, respectively. IS6 and IS256 were identified in three isolates, and their occurrence was not associated with any STs or CC (Table 2). The ddistribution of ISs and plasmid replicons detected among the isolates are shown in Supplementary Table S4. A total of six intact prophages were detected, of which the most identified were PHAGE_Staphy_phi2958PVL (n = 2) and PHAGE_Staphy_P282 (n = 2) (Table S5). PHAGE_Staphy_phiJB was associated with the *dfrG* gene.

2.5.3. Virulome and Pathogenicity of S. aureus Strains

A total of 82 virulence genes were detected across the isolates (Table S3). The virulence genes belonged to the five main virulence determinant classes of *S. aureus*: adherence factors, immune evasion, enzymes (exoenzymes), toxins, and the secretion system. It is noteworthy that the most prevalent toxins were hemolysins, i.e., gamma (*hlg*), delta (*hld*), alpha (*hly/hla*), staphylococcal enterotoxins (*se*, *set*, *sel*) genes, and leucocidin genes (*lukD/E*), while *lukS-PV* and *lukF-PV* genes were detected in two isolates (S24 and S29). The prediction of isolates pathogenicity towards humans yielded a high average probability score (Pscore ≈ 0.980).

2.6. Genetic Environment of the ARGs and Virulence Genes

The co-carriage of ARGs and virulence genes was evident across the isolates. Using NCBI annotation, we identified *blaZ* genes on five isolates in parallel with *cacD*, virulence genes, and the type 1 toxin–antitoxin system. Across the isolates, most *blaZ* genes were associated with regulator genes *blaR* and *blaI* and frequently found with either a recombinase, integrase, cadmium resistance (*cadD*) gene, or type I toxin–antitoxin system (Table 3). A similar genetic context was detected in the S13 isolate, where *blaZ*, *blaR*, and *blaI* were flanked by IS6, *cadD*, a type I toxin–antitoxin system, on a contig with the closest nucleotide homology to a plasmid from *S. aureus* pER10678.3A.1 (CP051928.1), suggesting that ARGs, heavy metal resistance genes (HMRGs), and virulence genes may be mobilized by plasmids (Table 3). It is noteworthy that IS1182 was associated with the *mecA*, *mecI*, and *mecR1* genes together with recombinases, while IS6 bracketed the *mecA* gene and its regulatory genes (*mecI* and *mecR*) in three MRSA isolates (Table 4). Most ARGs, including *erm(A)*, *ant(9)-Ia*, *dfrG*, and *tet(M)*, were associated with a recombinase and integrase. One isolate was found harboring the *dfrG* gene bracketed by ISL3 and recombinases (Table 4).

Regulatory Genes

The accessory gene regulator system (*agr*) involved in the regulation and expression of toxins, exoenzymes, and biofilm was detected in all isolates. Isolates carried *agr* type I and II. The distribution of the *agr* group in MRSA was: *agr* I (n = 1), *agr* II (n = 2), while in MSSA *agr* I (n = 2), and *agr* II (n = 1).

2.7. Phylogenomics

The phylogenetic analysis, integrated with metadata, reveals clear clustering patterns based on Multilocus Sequence Typing (MLST) and geographic origin. Isolates sharing the same MLST type generally clustered together, with additional grouping observed by country of isolation (Figure 1). For instance, the ST5 isolates from this study (S11-ST5, S34-ST5, and S29-ST5) are closely aligned with other South African isolates, indicating minimal genetic divergence within this MLST type in the region. This suggests a strong regional lineage for ST5 in South Africa. Additionally, this study isolates S24-ST152 clusters with other ST152 isolates from various African countries, including Kenya and Ghana, highlighting the broader geographic distribution and potential mobility of this MLST type across the continent (Figure 1). Notably, the tree analysis also reveals that ST8 and ST612 isolates are clustered together, suggesting close genetic relatedness despite being distinct MLST types (Figure 1). This finding could indicate a shared ancestry or recent genetic exchange between these groups, pointing to complex evolutionary dynamics within these populations.

The linkage between the *mecA* gene, SCC*mec* types, and clonal complexes is particularly notable, as it highlights the genetic mechanisms underlying methicillin resistance and the clustering of MRSA strains (Figure 2). The distinct clustering patterns observed for different isolates underscore the complex interplay between genetic background, resistance gene acquisition, and selective pressures in the evolution of these pathogens.

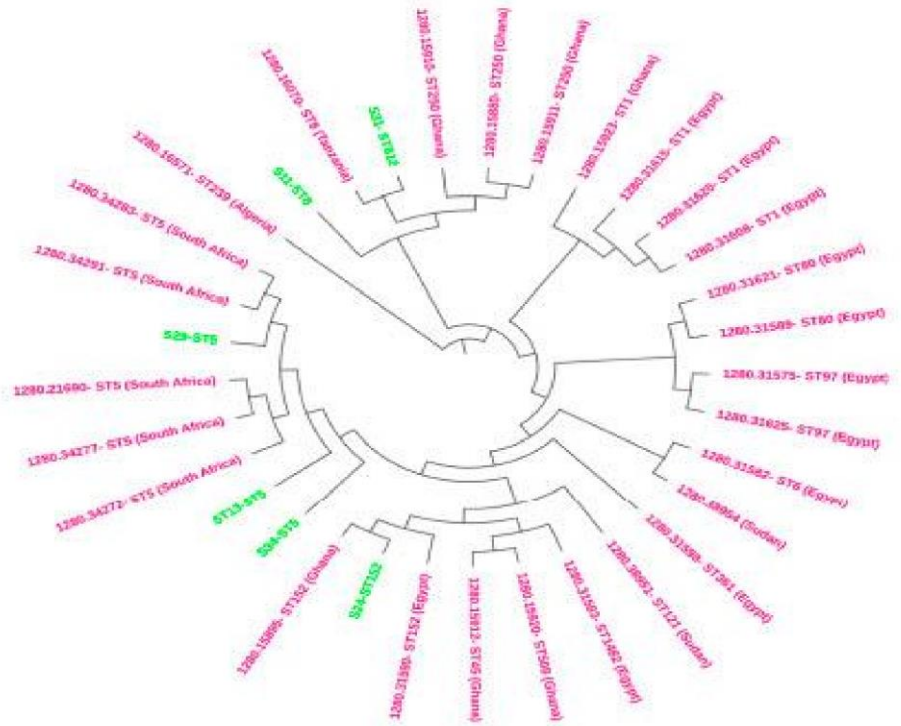

Figure 1. The circular phylogenetic tree provides a visual representation of the genetic relationships between *Staphylococcus aureus* isolates from this study (highlighted in green) and various African blood culture isolates (highlighted in purple). This tree illustrates how isolates cluster based on their Multilocus Sequence Typing (MLST) and geographic origin.

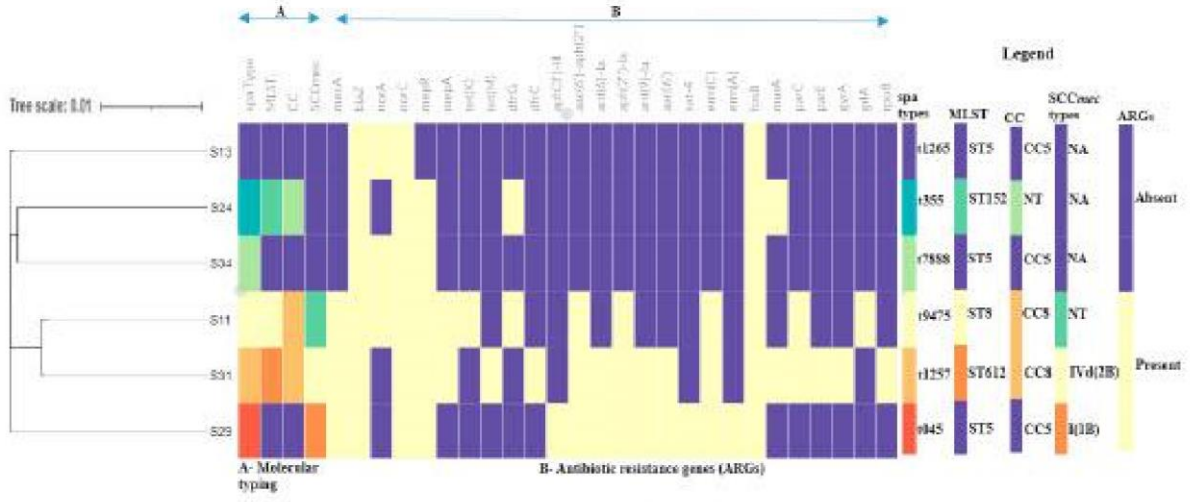

Figure 2. The phylogenetic branch and metadata, A—Molecular typing [*spa* type, sequence type (ST), clonal complex, SCC*mec* types], and B—Antibiotic resistance genes [ARGs], visualized using Phandango (https://github.com/jameshadfield/phandango/wiki accessed on 15 August 2024) in *S. aureus* isolates. The heat map in the middle indicates the presence (yellow) and absence (purple) of antibiotic resistance genes. NT indicates non-typeable. Isolates S13, S24, and S34 are MSSA and therefore do not harbor SCC*mec* types, which is indicated as "None (NA)" to reflect their SCC*mec*-negative status.

Table 3. Genetic context of virulence genes in *S. aureus* isolates.

Strain (MLST)	Strain	Contig	Synteny of Virulence Genes and MGEs	Plasmid/Chromosomal Sequence with Closest Nucleotide Homology (Accession Number)
S11 (ST8)	MRSA	4	pmtC:pmtB:pmtA:***eap***::***scn***::***sak***:::sph:: ***lukG***::***lukH***::intergrase::::agrB	*S. aureus* strain Laus385 chromosome (CP071350.1)
		6	***icaR***::***icaD***:***icaB***:***icaC***:vraD:vraE:vraH:: IS30:vraH::recombinase:IS6	*S. aureus* strain TF3198 chromosome, complete genome (CP023561.1)
		10	***lukE***:***lukD***::splA::epiE::***splA***:splB:splC: splD:splE:splF::pepA1:transposase	*S. aureus* strain 82 chromosome, complete genome (CP031661.1)
S29 (ST5)	MRSA	53	type I toxin–antitoxin system:IS6:cadD	*S. aureus* strain MIN-175 chromosome (CP086121.1)
		40	***clfA***:vwb:***emp***	*S. aureus* strain ER02693.3 chromosome, complete genome (CP030605.1)
S31 (ST612)	MRSA	11	pmtD:pmtC:pmtB:pmtA::***eap***:***scn***::***sak***	*S. aureus* strain 2395 USA500, complete genome (CP007499.1)
		15	***lukE***:***lukD***::::***splA***:***splB***:***splC***:***splF***::type I restriction-modification system	*S. aureus* strain NRL 02/947 chromosome, complete genome (CP103850.1)
		19	***lukG***:***lukH***:pathogenicity island:intergrase::phenol-soluble modulin:agrB	*S. aureus* strain 2395 USA500, complete genome (CP007499.1)
		22	***seq***:***sek***:integrase:::***emp***:***clfA***	*S. aureus* strain 2395 USA500, complete genome (CP007499.1)
		33	recombinase::universal stress protein::cadD::***seq***:***sek***:integrase:::***emp***:***clfA***	*S. aureus* plasmid SAP017A, complete sequence (GQ900382.1)
		64	***sea***:putative holin-like toxin	*S. aureus* strain R50 chromosome, complete genome (CP039167.1)
S13 (ST5)	MSSA	4	***sbi***:***hlgA***:***hlgC***:***hlgB***	*S. aureus* strain AR462 chromosome, complete genome (CP029086.1)
		5	scpA:::**eap**::***scn***:***sak***:::::intergrase:sph: ***lukH***:***sbi***:***hlgA***:***hlgC***:***hlgB***	*S. aureus* strain pt239 chromosome, complete genome (CP049467.1)
		15	IS6::cadD:::***sed***:***sej***:***ser***::recombinases: cpA::eap::***scn***:**sak**::integrase:sph:***lukH***	*S. aureus* strain ER10678.3 plasmid pER10678.3A.1 (CP051928.1)

Table 3. *Cont.*

Strain (MLST)	Strain	Contig	Synteny of Virulence Genes and MGEs	Plasmid/Chromosomal Sequence with Closest Nucleotide Homology (Accession Number)
S24 (ST152)	MSSA	8	arsB::crcB::***scn***:***sak***:::recombinase::type II toxin–antitoxin system toxin:intergrase	*S. aureus* strain UMCG579 chromosome, complete genome (CP091066.1)
		21	cadD:type toxin–antitoxin::integrase	*S. aureus* strain GHA13 chromosome (CP043911.1)
		11	BrxA/BrxB:::msrA:msrB:::norD::cspA:cvfB	*S. aureus* strain NGA84b chromosome, complete genome (CP051165.2)
S34 (ST5)	MSSA	7	***eap/map***::***scn***:***sak***:::***sea***:::type II toxin–antitoxin:integrase:sph:***lukG***:***lukH***	*S. aureus* strain HPV107 chromosome, complete genome (CP026074.1)
		8	clfA:vwb:emp::thermonuclease protein::**sek**:***seq***::pathogenicity island	*S. aureus* strain B4-59C chromosome, complete genome (CP042153.1)
		12	**sem**:**sei**:**seu**:**sen**:**seg**:::***lukE***:***lukD***::splA:splB:splC:splD:splF	*S. aureus* strain ER03588.3 chromosome, complete genome (CP030595.1)
		14	isdB:isdA:isdC:isdD:isdE:isdF::isdG::***ecb***::***efb***:***scb***	*S. aureus* strain B3-17D chromosome, complete genome (CP042157.1)
		20	SSL13:SSL12:**hyl**	*S. aureus* strain NAS_AN_239 chromosome, complete genome (CP062409.1)

Virulence gene(s) in bold.

Table 4. Genetic environment of antibiotic resistance genes in *S. aureus* isolates.

Isolate ID (MLST)	Strain	Contig	Synteny of Resistance Genes and MGEs	Plasmid/Chromosomal Sequence with Closest Nucleotide Homology (Accession Number)
S11 (ST8)	MRSA	4	*blaI*:*blaR1*:*blaZ*::recombinase/integrase	*S. aureus* strain ER02826.3 chromosome (CP030661.1)
		7	recombinase::dfrG:insertionelement:::ISL3::: recombinase:	*S. aureus* strain UP_403 chromosome (CP047849.1)
		59	IS6::*mecA*:*MecR1*:IS6::	*S. aureus* strain ER03868.3 chromosome (CP030403.1)
		123	Plasmid recombination:*tet(K)*	*S. epidermidis* isolate BPH0662 genome assembly, plasmid: 1 (LT614820.1)

Table 4. *Cont.*

Isolate ID (MLST)	Strain	Contig	Synteny of Resistance Genes and MGEs	Plasmid/Chromosomal Sequence with Closest Nucleotide Homology (Accession Number)
S29 (ST5)	MRSA	8	*ermA*:*ant(9)-Ia*:transposase:recombinase:integrase	*S. aureus* strain 628 chromosome (CP022905.1)
		11	*gyrB*:*gyrA*:::ligase	*S. aureus* strain MIN-175 chromosome (CP086121.1)
		38	recombinase:IS1182::*mecR1*:*mecA*:::IS6	*S. aureus* subsp. aureus strain FDAARGOS_5 chromosome (CP007539.3)
		51 *	recombinase:*blaI*:*blaR1*:*blaZ*:*cadC*:*cadA*	*S. aureus* plasmid pSK57, partial sequence (GQ900493.1)
		56	*ant(6)-Ia*:*sat4*:*aph(3′)-IIIa*	*S. pseudintermedius* strain MAD627 chromosome (CP039743.1)
		64	*qacA/B*:qacR	*S. aureus* strain MIN-175 chromosome (CP086121.1)
		67	*ermCL*:*erm(C)*	*S. epidermidis* strain TMDU-137 plasmid p5, complete sequence (CP093178.1)
S31 (ST612)	MRSA	23	*mecA*:*mecR1*::IS1182::recombinase	*S. aureus* strain 2395 USA500 (CP007499.1)
		17	integrase:::::tet(M):::IS256	*S. aureus* strain NRS120 chromosome, complete genome (CP026072.1)
S13 (ST5)	MSSA	15 *	IS6 IS6::*cadD*:::type I toxin–antitoxin::recombinase::*blaI*:*blaR1*:*blaZ*	*S. aureus* strain ER10678.3 plasmid pER10678.3A.1 (CP051928.1)
S24 (ST152)	MSSA	21	cadD:typetoxinantitoxin::recombinase:*blaI*:*blaR1*:*blaZ*:recombinase	*S. aureus* strain GHA13 chromosome (CP043911.1)
S34 (ST5)	MSSA	19	recombinase:blaZ:blaR1:blaI:recombinase:integrase	*S. aureus* strain UP_678 plasmid unnamed (CP047840.1)

* Co-occurrence of a heavy metal resistance gene (HMRG) and antibiotic resistance genes (ARGs).

3. Discussion

We studied the genomic characteristics of six MDR *S. aureus* isolates implicated in BSIs. This study analyzed the resistome, virulome, mobilome, phylogeny, and genetic environment of the resistance genes using WGS and bioinformatics. The genomes analyzed herein were predominantly recovered from ≤1-year-old patients.

There was a diversity of ARGs encoding resistance to different antibiotics and good concordance between the observed phenotypic and genotypic resistance. The incidence of ARG's encoding resistance to β-lactams, aminoglycosides, macrolides, fosfomycin, trimethoprim, tetracycline, and genes coding multidrug resistance (MDR) efflux pumps (*norA*, *mepR*, and *mgrA*) was not dependent on the clonal type. The *erm(C)* and *erm(A)* genes that are commonly found in macrolide–lincosamide–streptogramin B (MLS_B)-resistant *S. aureus* were found in erythromycin and clindamycin-resistant isolates (Table 2), which was expected since resistance to erythromycin co-selects resistance to other antibiotics, such as streptogramin B (MLS_B) and lincosamides [20]. The *ermC* gene is among the primary *erm* types that facilitate ribosome methylation of the 23S rRNA, triggering conformational changes resulting in drug binding inhibition [21], and has been reported in clinical *S. aureus* isolates from South Africa [22]. In this study, the *ermC* encoding macrolide resistance was carried on a plasmid, on a contig that had the closest nucleotide homology to plasmids from *S. epidermidis* strain TMDU-137 plasmid p5, complete sequence (CP093178.1), implying the likelihood of horizontal transfer of *ermC* genes in clinical *S. aureus* isolates. The *ermC* are often plasmid-mediated, resulting in high resistance to macrolides in *S. aureus* [23].

The *blaZ* gene, which inactivates penicillin through hydrolysis of the beta-lactam ring, was observed in all six isolates that were phenotypically resistant to penicillin. The *blaZ* genes have also been isolated in clinical isolates of Staphylococci in South Africa [24]. In this study, the *blaZ* genes were found on contigs with closest homology to either chromosomes or plasmids. This agrees with a study conducted in Spain that analyzed ARGs presence in chromosomes and plasmids from the genomes of *S. aureus*. WGS analysis of *S. aureus* revealed that *blaZ* (n = 2) was located on chromosomic contigs, while *blaZ* was found in plasmid contigs in three isolates [25]. It is important to note that most *blaZ* and associated MGEs from isolates belonging to ST5 (S13, S34) isolated from the intensive care unit (ICU) and pediatric ward (S29) were located on contigs that had the closest homology to plasmids, implying that plasmids play a crucial role in mobilizing the *blaZ* gene in clinical *S. aureus* isolates. The S29 isolate, belonging to the t045-CC5 lineage, carried an assortment of ARGs encoding resistance to different antibiotics (Table 4). Similar ARGs in MRSA lineage t045-CC5-MRSA were also reported in a study conducted in South Africa, where t045-CC5 MRSA lineages obtained from different clinical samples from South Africa and Nigeria reported that t045 lineages were MDR, suggesting that this lineage is hospital-associated and their multidrug resistance nature may compromise treatment [26].

Also, the *blaZ* genes, heavy metal genes, and associated MGEs were carried on either plasmid or chromosome. The *blaZ* and *cadAC* genes were found on the genetic element recombinase *blaI*:*blaR1*:*blaZ*:*cadC*:*cadA* for isolates S24 (MSSA) that was from the ICU and S29 (MRSA) from the pediatric ward, suggesting co-selection of heavy metal resistance dissemination and adaptation in different wards. The *cadA* gene confers a high resistance to cadmium and other heavy metals like zinc and lead in *S. aureus* isolates [27]. The *cadA* was associated with a plasmid, similar to the findings of a study that was conducted by Al-Trad et al. (2023) in Malaysia, who used WGS to analyze the plasmid content of clinical MRSA isolates and reported that heavy metal resistance plasmids harbored cadmium resistance genes, with the majority being *cadAC* [28]. The HMRGs have been reported to trigger a co-selection mechanism with antibiotics, which may complicate treatment [29]. This may pose a challenge, especially among patients in the ICU, where broad-spectrum antibiotics are often used.

Tetracycline resistance genes (*tetK* and *tetM*) were observed in two isolates. Isolate S11 carried *tet(K)* associated with the following genetic context: plasmid recombination *tet(K)*

that had a high similarity to *S. epidermidis* BPH0662, and plasmid 1 (LT614820.1), which could be significant in mobilizing TET-resistant genes. Also, the *tet(M)* was bracketed by integrase and IS256 in isolate S31. The IS*256* is a retrotransposon that can mobilize the resistance genes through a copy-and-paste mechanism and has been shown to confer a robust genomic plasticity in MRSA strains [30].

We found that ARGs and virulence genes were associated with MGEs, which may enable their transfer within and between plasmids and chromosomes [31]. In this study, the *mecA* gene was located on IS1182 in two MRSA isolates, surrounded by recombinase in the genetic context *mecA*:*mecR1*::IS1182::recombinase. The insertion sequence IS1182 was present in 2/3 MRSA strains that contained *mecA*. IS1182 has been shown to occur close to the SCC*mec* element and increase resistance through inactivating the *lytH* gene encoding a putative lytic enzyme in pathogenic MRSA isolates [32].

MLST, clonal complex typing, *spa* typing, and SCC*mec* typing were used to analyze the molecular characteristics of the *S. aureus* isolates. Four ST types and two clonal clusters (CCs) were found among the six clinical isolates in this study, with ST5 as the most predominant complex clonal CC5 and CC8. Generally, clonal lineages ST5, ST8, ST152, and ST612 are among the most commonly reported in hospital environments, along with other sequence types of *S. aureus* [33]. *S. aureus* ST5, belonging to CC5, was predominant in this study and was previously reported among patients with bloodstream infections at Ruijin Hospital in Shanghai [3]. The detection of clonal complexes CC5 and CC8 agrees with a study by Smith et al. [17], which also found CC8 and CC5 were predominant in a study that analyzed the genomic epidemiology of MRSA and MSSA from bloodstream infections in the USA. Their results revealed that the MDR phenotype observed in strains belonging to CC5 and CC8 was responsible for the occurrence of multidrug and methicillin resistance in the *S. aureus* population. MRSA strains belonging to CC8 and CC5 are frequently associated with global outbreaks and have been identified in Africa [34].

The *spa* typing revealed six different *spa* types, suggesting a non-clonal MRSA and MSSA distribution. The detection of *spa* types t1257, t045, and t355 agrees with a study conducted in South Africa, which analyzed the diversity of SCC*mec* elements and *spa* types in *S. aureus* isolates from blood culture in the Gauteng, KwaZulu-Natal, Free State, and Western Cape provinces [15], in which t037 and t1257 were the most common and predominated throughout the seven-year study period. In this study, some antibiotic resistance genes were associated with specific MRSA clones belonging to *spa* types t1257, t045, and t9475. Shittu et al. (2021) found the *spa* types t045 and t1257 to be the most prevalent and associated with genes conferring resistance to aminoglycosides, trimethoprim, macrolides, and tetracycline in clinical isolates of *S. aureus* from South Africa and Nigeria [26].

The analysis of SCC*mec* types revealed the presence of SCC*mec* type IVd (2B) and SCC*mec* type I (B) carrying the *mecA* gene, which occurred in tandem with *mecR1* in both MRSA isolates. However, one MRSA (S11) isolate had a non-typeable SCC*mec* element cassette due to the missing cassette chromosome recombinase (*ccr*) gene complex [35]. The *ccr* gene complex is an essential component required to facilitate the integration or excision of the SCC*mec* element in the staphylococcal chromosome, and their loss has also been reported [36]. The SCC*mec* IV detected in our study is associated with the *spa* type t1257, previously reported in South Africa in *S. aureus* obtained from poultry isolates [16], implying its possible transfer between humans and animals.

We found different MRSA genotypes, ST612-t1257-CC8, ST8-t9475-CC8, and ST5-t045-CC5, suggesting that MRSA isolates were not clonally and epidemiologically related. The ST612-t1257-CC8 identified in this study is an endemic MRSA clone that has been reported in animal and clinical settings [15,16]. The ST5-I-MRSA, known as the pandemic British EMRSA-3 clone, was detected in the pediatric ward. This is similar to a study conducted in South Africa, where the t045-MRSA strain occurred in pediatric patients [19]. The isolation of the t045-ST5-MRSA strain could confirm its successful persistence in the hospital and its capacity to cause infections in neonatal and pediatric wards [37].

Several virulence factors, including adherence, immune invasion, toxins, and exoenzymes associated with invasive infections, were detected in our isolates. The virulence genes encoding clumping factor proteins (*clfA* and *clfB*) are involved in the pathogenesis of *S. aureus*, including bacteremia [9]. Consistent with pathogenic *S. aureus* strains isolated in various environments globally, our isolates were characterized by the *icaADBC* operon and *sdrC*, *sdrD*, and *sdrE* involved in biofilm-forming genes [38]. Most strains harbored genes, including the alpha and gamma-hemolysin genes (*hlgA*, *hlgB*, *hlgC*, *hly/hla*, and *hlb*), and the *ica* operon associated with pathogenicity and adhesion. Additionally, our isolates were characterized by various toxins, including *lukE/D* genes and Panton–Valentine leukocidin (PVL) *lukS-PV*/*lukF-PV* genes in one MSSA and MRSA strain. The expression of these PVL toxin genes in *S. aureus* isolates lyses host cells and promotes virulence of the bacteria [39], which might worsen the outcomes of *S. aureus* infection. Consistent with clinical *S. aureus* strains, our isolates were characterized by a capsular polysaccharide (CP) serotype 8, which shields the bacterial pathogen from host immune defense mechanisms associated with increased virulence in BSIs [40].

Most virulence genes, including those encoding SEs, *sak*, *hlg*, *luk*, *scn*, *clfA*, *sbi*, and associated MGEs, were carried on chromosomes in the majority of isolates. The *ica* gene operon and *vra* genes were found to be associated with ISs (IS30, IS6) and recombinase for S11 (ST8) isolate from the surgical ward. The *ica* genes *vraDEH* genes have been shown to play an important role in biofilm formation [41] and daptomycin resistance in *S. aureus* [42], which could enhance antibiotic resistance traits and chronic infection. The occurrence of ST8-t9475 MRSA strains co-harboring *ica* genes and genes encoding daptomycin resistance in ST8 MRSA could be advantageous to the ST8-t9475 colonization, invasion, and survival in the surgical ward. The virulence genes encoding SEs, *eap*, *scn*, *sak*, *sph*, *lukH*, and *cadA*, were found on a contig that had high sequence similarity to *S. aureus* strain ER10678.3 plasmid pER10678.3A.1 (CP051928.1), implying that they are mobilized by plasmids. Virulence genes, including those encoding *hla*/*hld*, toxin production, and biofilm formation, are plasmid-mediated [43], thus could easily facilitate their transfer, resulting in highly pathogenic strains that may be difficult to treat.

Phylogenomic analyses revealed that the clinical isolates in this study clustered mainly with clinical isolates from hospital patients (Figure 2). ST5 study isolates were closely related to clinical isolates from South Africa, suggesting possible dissemination of ST5 strains and adaptation in hospital environments. Furthermore, the ST152 isolate was closely related to ST152 strains from Egypt and Ghana, implying a possible spread and epidemiological linkage between these isolates. ST152-PVL-producing *S. aureus* isolates are particularly frequent and widespread in West and Central Africa [44] and livestock [45]. The ST152-PVL-positive MSSA has also been reported from cutaneous abscesses among mine workers at a gold mine in Gauteng, South Africa [46]. Identifying ST152 in livestock and humans suggests animal–human transmission, which requires further investigation. ST8 and ST612 isolates were closely related to ST8 isolated from Tanzania, indicating that ST612 is a double-locus variant of ST8. ST8 and ST612 isolates are potentially multidrug-resistant and highly virulent strains associated with hospital outbreaks [47]. The integration of phylogenetic data with resistance profiles offers valuable insights into the epidemiology and evolutionary dynamics of these isolates, highlighting potential patterns of transmission and resistance development [48].

In light of the increasing resistance observed in *S. aureus* strains, innovative strategies are being explored to combat antimicrobial resistance. One promising approach focuses on targeting microbial metallophores, molecules that bacteria use to scavenge essential metals from their environment. By inhibiting metallophore function, it is possible to disrupt bacterial metabolism and enhance the effectiveness of existing antibiotics [49]. Additionally, alternative strategies, such as the use of bacteriophages, antimicrobial peptides, and immune system modulation, offer potential avenues for treating resistant infections [50]. Finally, combination therapy, which involves using multiple antibiotics or combining antibiotics with adjuvants that inhibit resistance mechanisms, is gaining attention as a way to

overcome multi-drug resistance and reduce the likelihood of treatment failure [51]. These emerging strategies represent critical avenues for future research and clinical application, aiming to curb the growing threat of antimicrobial resistance.

This study analyzed a limited number of *Staphylococcus aureus* isolates (n = 6), which may not fully represent the epidemiology of MRSA/MSSA in South Africa or bloodstream infections more broadly. The small sample size limits the generalizability of the findings to the wider population. Therefore, while this study offers valuable insights into the genomic characteristics and resistance profiles of these isolates, it should be viewed as a pilot study that lays the groundwork for larger, more comprehensive investigations. The small sample size also limits the ability to validate the findings, particularly concerning virulence factors and other genomic features. Future studies with larger sample sizes and experimental validation are necessary to confirm and extend these observations.

4. Materials and Methods

4.1. Ethical Consideration

The ethical approval for this study was issued by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal under the following reference number: BCA444/16.

4.2. Sample Collection and Bacterial Identification

A total of forty-five presumptive *Staphylococcus* isolates from blood cultures sourced from patients with BSIs at two hospitals in the uMgungundlovu district in the KwaZulu-Natal province from November 2017 to December 2018. All isolates were confirmed as *S. aureus* using the automated VITEK 2 system (BioMérieux, MarcyL'Etoile, France). We selected a subset of 10 MDR isolates for WGS based on their antibiotic-resistant profiles/patterns, but 4 isolates were excluded during the quality control process.

4.3. Antimicrobial Susceptibility and MRSA Detection

Isolates were tested for antibiotic susceptibility by disk-diffusion method on Mueller–Hinton agar as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [52] or Clinical and Laboratory Standards Institute (CLSI) [53]. The antibiotics tested and interpreted according to the EUCAST breakpoints (EUCAST, 2017) were penicillin G (10 µg), ampicillin (10 µg), cefoxitin (30 µg), tigecycline (15 µg), and nitrofurantoin (300 µg). The CLSI guidelines (CLSI, 2017) were used for the following antibiotics: ciprofloxacin (5 µg), levofloxacin (5 µg), moxifloxacin (5 µg), erythromycin (15 µg), gentamicin (10 µg), amikacin (30 µg), chloramphenicol (30 µg), tetracycline (30 µg), doxycycline (30 µg), sulphamethoxazole/trimethoprim (1.25 µg + 23.75 µg), teicoplanin (30 µg), linezolid (30 µg), clindamycin (2 µg), and rifampicin (5 µg). MRSA isolates were identified using a cefoxitin disk (30 µg). The antibiotic disks were obtained from Oxoid (Oxoid, Basingstoke, UK). *S. aureus* ATCC 29213, was used as the quality control strain. Multidrug resistance (MDR) was defined as resistance to three or more antibiotic classes [54].

4.4. Whole-Genome Sequencing (WGS) and Bioinformatic Analysis

Genomic DNA extraction was performed using the GenElute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. The quality of the DNA was assessed using NanoDrop 8000 (Thermo Fisher Scientific Waltham, MA, USA). Genome libraries were constructed using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced on the Illumina NextSeq Machine (Illumina, San Diego, CA, USA). The raw reads were trimmed using Sickle v1.33 (https://github.com/najoshi/sickle accessed on 15 August 2020) and assembled using the SPAdes v3.6.2 assembler (https://cab.spbu.ru/software/spades/ accessed on 15 August 2020). Assembled genome sequences were submitted to Genebank and assigned accession numbers under the BioProject number PRJNA400143.

MRSA isolates in this study. Table S4: Distribution of insertion sequences and plasmid replicon among the *Staphylococcus aureus* strains. Table S5: Distribution of intact prophage region among the *Staphylococcus aureus* strains.

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Institutional Review Board Statement: Ethical approval for this study was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal under the following reference number BCA444/16. The study isolates were part of a larger surveillance study using the Global Antimicrobial Resistance and Use Surveillance System (GLASS) guidelines.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in GenBank and assigned accession numbers under the BioProject PRJNA400143. [NCBI Genebank] [https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA400143 accessed on 15 May 2024] [PRJNA400143].

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Table S1: Patients demographics

Isolate ID	**Location/ Hospital**	**Department**	Gender	Age
S11	Regional hospital	Surgical ward	F	17 years
S24	Regional hospital	ICU	M	33 years
S29	Regional hospital	Paediatric ward	M	<1 year
S13	Regional hospital	ICU	M	<1 year
S31	Regional hospital	Surgical ward	F	3 years
S34	Regional hospital	NICU	M	<1 year

Table S2: Genomic characteristics of S. aureus strains

Isolate ID	Accession no	Size (Mb)	GC%	Contigs	No. of RNAs	No of coding Sequences	N50	L50	Coverage (X)
S11	JADQTH000000000	2.7	32.7	37	65	2629	421368	2	95.7
S24	JADIXC000000000	2.7	32.7	47	69	2571	330697	4	95.7
S29	JADIXA000000000	2.8	32.8	95	69	2798	77541	14	99.2
S13	JADIXB000000000	2.7	32.7	32	69	2550	695668	2	95.7
S31	JADIXE000000000	2.9	32.7	106	69	2942	155137	6	102.8
S34	JADIXD000000000	2.7	32.6	52	63	2635	273350	5	95.7

Table S3: Virulence genes identified in MSSA and MRSA isolates

Isolate ID	Adherence factors	Immune envasion	Enzymes	Iron sequestration	Capsule biosynthesis	Toxins	Type VII secretion
S11	*atl, ebh, clfA, ebp, efb, fnbA, fnbB, icaA, icaB, icaC, icaR, sdrC, sdrE, spa*	*adsA, chp, scn, sbi*	*sspB, sspC, hysA, geh, lip, sspA, splA, splB, splC, splD, splE, splF, coa, sak, nuc, aur*	*isdA, isdB, isdC, isdD, isdE, isdF, isdG*	*cap8A, cap8B, cap8C, cap8D, cap8E, cap8F, cap8G, cap8L, cap8M, cap8N, cap8O, cap8P*	*hlgA, hlgB, hly/hla, hlb, hld, selk, eta, sek set21, set30, set31, set34, set36, set37, set38, set39, set40, set8, hlgA, hlgB, hlgC, lukD*	*esaA, esaB, esaD, esaE, esaG, essA, essB, essC, esxA, esxB, esxC, esxD*
S24	*atl, cna, ebp, fnbA, fnbB, icaA, icaB, icaC, icaR, sdrE, sdrD, spa*	*adsA, chp, scn, sbi*	*sspB, sspC, hysA, geh, lip, sspA, coa, sak, nuc,* aur	*isdA, isdB, isdC, isdD, isdE, isdF, isdG*	*cap8A, cap8B, cap8C, cap8D, cap8E, cap8F, cap8G, cap8L, cap8M, cap8N, cap8O, cap8P*	*hlgA, hlgB, hlgC, hly/hla, hlb, hld, eta, set15, set16, set18, set34, set36, set4, set7, lukD, lukE, lukF-PV, lukS-PV*	*esaA, esaD, esaE, esaG, essA, essB, essC, esxA, esxB, esxC, esxD*
S29	*atl, ebh, clfA, clfB, ebp, efb, fnbA, icaA, icaB, icaC, icaR, sdrC, sdrE, spa*	*adsA, sbi*	*sspB, sspC, hysA, geh, lip, sspA, splA, splB, splC, splD, splF, coa, nuc,* aur	*isdA, isdB, isdC, isdD, isdE, isdF, isdG*	*cap8A, cap8B, cap8C, cap8D, cap8E, cap8F, cap8G, cap8L, cap8M, cap8N, cap8O, cap8P*	*hlgA, hlgB, hlgC, hld, seg, yent1, yent2, selk, selm, seln, selo, eta, set11, set13, set15, set34, set37, set39, set6, set7, set8, set9, lukD, cylR2*	*esaA, esaD, esaE, esaG, essA, essB, essC, esxA, esxB, esxC, esxD*
S13	*atl, ebh, clfB, ebp, , efb, fnbA, fnbB, icaA, icaB, icaC, icaR, sdrC, spa*	*adsA, chp, scn, sbi*	*sspB, sspC, hysA, geh, lip, sspA, splA, splB, splC, splD, splF, coa, sak, nuc,* aur	*isdA, isdB, isdC, isdD, isdE, isdF, isdG*	*cap8A, cap8B, cap8C, cap8D, cap8E, cap8F, cap8G, cap8L cap8M, cap8N, cap8O, cap8P*	*hlgA, hlgB, hlgC, hly/hla, hlb, hld, sea, sej, sed, ser, seo, yent1, yent2, selk, selm, seln, selo, eta, set11, set13, set15, set34, set37, set39, set6, set7, set8, set9, lukE, lukD*	*esaA, esaB, esaD, esaE, esaG, essA, essB, essC, esxA, esxB, esxC, esxD*
S31	*atl, ebh, clfA, clfB, ebp, efb, fnbA, icaA, icaB, icaC, icaR, sdrC, sdrD, sdrE, sdrE, spa*	*adsA, chp, scn, sbi*	*sspB, sspC, hysA, geh, lip, sspA, splA, splC, splD, coa, sak, nuc,* aur	*isdA, isdB, isdC, isdD, isdE, isdF, isdG*	*cap8A, cap8B, cap8C, cap8D, cap8E, cap8F, cap8G, cap8L cap8M, cap8N, cap8O, cap8P*	*hlgA, hlgB, hlgC, hly/hla, hlb, hld, sea, seb, selk, selq, eta, set18, set21, set30, set31, set34, set36, set37, set38, set39, set40, lukD*	*esaA, esaB, esaD, esaE, esaG, essA, essB, essC, esxA, esxB, esxC, esxD*
S34	*atl, clfA, clfB, ebp, efb, fnbA, fnbB, icaA, icaB, icaC, icaR, sdrE, spa*	*adsA, chp, scn, sbi*	*sspB, sspC, hysA, geh, lip, sspA, splA, splC, splD, splF, coa, sak, nuc, aur*	*isdA, isdB, isdC, isdD, isdE, isdF, isdG*	*cap8A, cap8B, cap8C, cap8D, cap8E, cap8F, cap8G, cap8L cap8M, cap8N, cap8O, cap8P*	*hlgA, hlgB, hlgC, hly/hla, hlb, hld, sea, seb, seg, yent1, yent2, selk, selm, seln, selo, selp, selq, eta, set11, set13, set15, set34, set37, set39, set6, set7, set8, set9, lukD*	*esaA, esaB, esaD, esaE, esaG, essA, essB, essC, esxA, esxB, esxC, esxD*

Table S4: Distribution of insertion sequences and plasmid replicon among the *S. aureus* strains

Isolate	ST	Insertion sequence (IS)	Plasmid replicon (s)
S11	ST8	-	Rep10
		-	Rep7a
		-	-
S13	ST5	-	Rep20
S24	ST152	-	Rep16
S29	ST5	-	Rep10
		IS6	-
		IS6	-
		IS256	-
S31	ST612	IS256	-
		IS256	-
		IS6	-
		-	Rep7c
		-	Rep20
S34	ST5	-	Rep19

Table S5: Distribution of intact prophage region among the *S. aureus* strains

Isolate ID	**No. of prophage**	**Region**	**Length (Kb)**	**No. CDS**	**GC%**	**Phage (Hit genes count)**	**Resistance**
S11	1	3	73.5	73 534	32.03	PHAGE_Staphy_P282	-
S13	1	2	45.2	45 286	33.01	PHAGE_Staphy_P282	-
S24	1	1	73.5	73 537	32.88	PHAGE_Staphy_phi2958PVL	-
S29	2	2	26.8	26 885	36.85	PHAGE_Staphy_SA13	-
		3	31.5	31 540	33.65	PHAGE_Staphy_phi2958PVL	-
S31	1	2	48.4	48 460	34.74	PHAGE_Staphy_phiJB	*dfrG*
S34	-	-	-	-	-	-	-

CHAPTER 5 – CONCLUSION

The study describes the molecular epidemiology of antibiotic resistant ESKAPE and *E. coli* pathogens isolated from bloodstream infections, in uMgungundlovu District, KwaZulu-Natal (KZN), South Africa, specifically their resistomes, virulomes, mobilomes, clonality and phylogenies.

5.1 Conclusions

The following were the main conclusions drawn from the study according to the study objectives.

- To ascertain the incidence of ESKAPEEc from hospitals in the uMgungundlovu district of the KwaZulu-Natal province from blood cultures routinely processed by the central microbiology laboratory using culture and biochemical techniques:

➢ The overall incidence rate of ESKAPEEc was 85.9% (159/185), distributed across various hospital departments including the ICU, NICU, paediatric ward, paediatric OPD, medical wards, emergency departments, surgical ICU, orthopedic out-patient department, burns ward, dermatology clinic, casualty, renal unit, and surgical ward. The high incidence of ESKAPEEc pathogens has the potential to increase hospital stay and expenses for both patients and the healthcare system.

- To identify the ESKAPEEc isolates using the automated VITEK 2 system, followed by real-time PCR using species-specific oligonucleotide primer sequences of the *E. faecium*

(*sodA*) *S. aureus* (*nuc*) *K. pneumoniae* (*khe*), *A. baumannii* (*sp4*), *P. aeruginosa* (*oprl*), *Enterobacter spp.* (*hsp60*), and *E. coli* (*uida*).

➢ The distribution of ESKAPE species determined by VITEK 2 and confirmed by PCR was *K. pneumoniae* (46, 28.9%), *S. aureus* (45, 28.3%), *E. coli* (25, 15.7%), *E. faecium* (15, 9.4%), *A. baumannii* (11, 6.9%), *P. aeruginosa* (8, 5.0%), *Enterobacter* species (5.7%).

• To determine the antibiotic resistance patterns of ESKAPEEc isolates by Kirby-Bauer disk diffusion according to the European Committee on Antimicrobial Susceptibility (EUCAST) and/or Clinical and Laboratory Standards Institute (CLSI) guidelines as MIC methods.

➢ Most *K. pneumoniae* (n=45) displayed high resistance to antibiotics in the access group, including cephalexin (78.3%), amoxicillin-clavulanic acid (69.6%), trimethoprim-sulfamethoxazole (67.4%), gentamycin (67.4%), tetracycline (65.2%) and chloramphenicol (65.2%), while higher resistance in the Watch group antibiotics was observed against cefotaxime (69.0%), ceftazidime (67.4%), ceftriaxone (67.4%), and ciprofloxacin (67.4%). The lowest resistance was against amikacin (39.1%), imipenem (30.4%), and meropenem (32.6%). *K. pneumoniae* isolates were highly susceptible to tigecycline (91.3%). *K. pneumoniae* was not tested for ampicillin due to intrinsic resistance to ampicillin because of the presence of the SHV-1 gene (Holt et al., 2015).

➢ *E. coli* (n=25) isolates were susceptible to tigecycline (92.0%) (reserve antibiotic), meropenem (80.0%), imipenem (80.0%) (Watch antibiotics), and chloramphenicol (68.0%). *E. coli* isolates showed resistance of 68.0% to antibiotics in the Watch group, including cefepime, ceftazidime, and ceftriaxone, while resistance against antibiotics in the Access group antibiotics was recorded against ampicillin (76.0%), amoxicillinclavulanic acid (68.0%).

➢ *E. cloacae* (n=9) were highly susceptible to tigecycline (88.9%). *E. cloacae* isolates exhibited high resistance to ampicillin (77.8%) (Access group), and cefoxitin (66.7%) (Watch category).

➢*A. baumannii* isolates showed high resistance to amoxicillinclavulanic acid (63.6%), cephalexin (63.6%), chloramphenicol (63.6%), and tetracycline (63.6%) (access category), while high resistance in the Watch category was recorded for cefoxitin (72.7%), ceftazidime (72.7%), ceftriaxone (72.7%), and ciprofloxacin (72.7%), *A. baumannii* isolates were highly susceptible to tigecycline (90.9%).

➢Most *P. aeruginosa* isolates (n=8) displayed resistance to antibiotics in the watch category including cefoxitin (62.5%), cefotaxime (62.5%), whereas the lowest resistance was recorded for piperacillin-tazobactam (12.5%). Isolates were highly susceptible to tigecycline (100.0%), amikacin (87.5%), and gentamycin (87.5%).

- *E. faecium* isolates showed the highest rate of resistance against the antibiotics in the access category, including ampicillin (80.0%), tetracycline (66.7%), and trimethoprim-sulfamethoxazole (66.7%), while resistance to antibiotics in the Watch group was recorded against erythromycin (66.7%), streptomycin (66.7%). Isolates showed the lowest resistance was against imipenem (33.3%). *E. faecium* isolates were highly susceptible to vancomycin (93.3%), and reserve antibiotics comprising linezolid (96.0%), tigecycline (93.3%), and quinupristin-dalfopristin (93.3%).

- Most *S. aureus* (n = 45) isolates displayed resistance to antibiotics belonging to the Access group, such as penicillin G (93.3%) and tetracycline (60.0%), while resistance in the Watch group antibiotics was observed against rifampicin (62.2%), moxifloxacin (60.0%), and ciprofloxacin (57.8%). The lowest resistance was recorded for chloramphenicol (17.8%). *S. aureus* isolates were highly susceptible to reserve antibiotics such as linezolid (95.6%), tigecycline (93.3%), and quinupristin-dalfopristin (93.3%). There was no resistance to vancomycin against *S. aureus*.

- High resistance rates observed among the ESKAPEEc pathogens may lead to high mortality rates due to the limited availability of effective antibiotics for the treatment of BSIs.

- Multidrug resistance was recorded in 151 (94.9%) isolates and overall MARI across all isolates ranged between 0.1 and 1.0 (mean = 0.56). Most isolates with MARI of ≥0.2 were obtained from ICU 35 **(**23.5%), of which *K. pneumoniae* and *A. baumannii* isolates had the highest MARI of 0.7 and 0.9, respectively. The highest MARI (0.951.0) was recorded in *A. baumannii* isolates resistant to all 20 antibiotics tested. The isolates displayed varying

resistance patterns that were grouped into 131 different antibiograms depicting high resistance of the isolates to commonly used antibiotics.

• To identify and characterize antibiotic-resistance and virulence genes in *K. pneumoniae, K. oxytoca*, and *S. aureus* and their associated MGEs and their genetic support/environment using whole genome sequencing and bioinformatics tools:

➢ In *K. pneumoniae* resistance genes conferring resistance to β-lactams [*bla*$_{\text{CTX-M-15,}}$ *bla*$_{\text{SHV-106}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{SHV-1}}$, *bla*$_{\text{SHV-81}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{SHV-187}}$, *bla*$_{\text{SHV-96,}}$ *bla*$_{\text{SHV-94}}$, *bla*$_{\text{SHV-172}}$, *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{TEM-1C}}$, *bla*$_{\text{TEM-79}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-9}}$, *bla*$_{\text{OXA-181}}$], aminoglycosides [*aac(3)-IIa*, *aph(3')-Ia*, *aph(6)-Id*, *aph(3'')-Ib*, *aac(6')-Ib-cr*, *aac*(3)-IIe, *aadA16*, *baeR*], trimethoprim [*dfrA1*, *dfrA14*, *dfrA27*], tetracycline [*tet(A)*, *tet(D)*] macrolides [*mph*(A)], sulfonamides [*sul1*, *sul*2], fosfomycin [*fosA*, *fosA5*, *fosA6*], rifampicin [*arr*3], phenicols [*cat*A2, *cat*II, *cat*B3], fluoroquinolones [*qnrB1*, *qnrB6*, *oqxB*, *oqxA*, *gyr*A, *parC*, *emrR*] and quaternary ammonium compound disinfectant [*qacE*] were detected.

➢ Mutations in chromosomal genes associated with quinolone/ ciprofloxacin (*gyrA* and/or *par*C) and tigecycline (*acrR*, *ramR*) resistance were found in some *K. pneumoniae* isolates.

➢ In *K. oxytoca* genes conferring resistance to β-lactams (*bla*$_{\text{OXY-2-4,}}$ *bla*$_{\text{OXY-2-5}}$), aminoglycosides [*baeR*], fluoroquinolones [*oqxA*, *emrR*, *gyrB*], fosfomycin [*fosA5*], and other ARGs [*acrR adeF*, *ompA*] were identified.

➢ Various virulence genes, including those involved in regulator of mucoid phenotype [*rcsA/B, phoP*], yersiniabactin [*ybtAEPQSTUX, irp1/2, fyuA*], aerobactin (*iutA*), enterobactin [*entABCDEFS, fepABCDG, fes*], salmochelin (*iroBDEN*), type I fimbriae (*fim*ABCDEFHIJ]and type 3 fimbriae [*mkr*ABCDFHIJ] were present in *Klebsiella* isolates.

➢ In *S. aureus* resistance genes conferring resistance to β-lactams [*blaZ, mecA*], aminoglycosides [*aac(6')-aph(2''), aad(6'), ant(9)-la, ant(6)-Ia, aph(2'')-Ia, aph(3')IIa, kdpD* sat-*4*], trimethoprim [*dfrG, dfrC*], macrolides [*erm(C), erm(A)*], tetracycline [*tet(K), tet(M), mepR, mepA*], fluoroquinolones [*parE, parC, mgrA, arlS, arlR, grlA, gyrA, norA, norC, sdrM*], rifampicin [*rpoB*] and fosfomycin [*fosB, murA*] were found.

➢ In *S. aureus s*ix mutations were found in *gyrA*, three in *gyrB,* four in *parC* and five in *parE*. We also found three mutations (H481N, I527M, F737Y) in the rpoB gene.

➢ In *S. aureus*, a total of 82 virulence genes were detected, including those encoding for biofilm formation/adherence (*icaA, icaB icaC, icaR, clfA, clfB*), hemolysins (*hlg, hld, hly/hla*), enterotoxins (*sea, sej, sed, seg ser, seo, seb, sel, set*), leucocidin genes (*lukD, lukE, lukS-PV, lukF-PV*), capsule biosynthesis (*cap8A*), immune evasion (*adsA, chp, scn, sbi*), iron sequestration (*isdA, isdB, isdC, isdD, isdE, isdF, isdG*), type VII secretion (*esa*), and enzymes (*ssp, hysA, geh, lip, sspA, spl, coa, sak, nuc, aur*) in isolates.

• To identify and characterise mobile genetic elements (MGEs) in clinical *K. pneumoniae, K. oxytoca* and *S. aureus* using WGS and bioinformatics tools.

➢ MGEs included insertion sequences (IS3, IS6, Tn5403and Tn3) and plasmid replicons (mostly IncFIB(K)), IncFIB(pKPHS1) were found in *K. oxytoca* only as were intact prophages, of which PHAGE_Salmon_SEN5 was the most prevalent. Other MGEs, such as transposons (Tn554) and IS257, were frequently found as part of the genetic support environment of resistance genes.

➢ In *K. pneumoniae*, class 1 integrons (*intI1*) were identified in 8 (80%) isolates, including In191 and In152. The ARGs were mostly associated with insertion sequences, transposons, and class 1 integrons. Some class 1 integrons carried an assortment of gene cassettes (including ARGs) and were bracketed by transposons. The *IntI1* was associated with insertion sequences and contained various ARGs, including the trimethoprim gene *dfrA14*, which were bracketed by either *IntI1* and IS6 or Tn3 and associated with relaxosome (*mobC*).

➢ The $bla_{\text{CTX-M-15}}$ gene was often associated with either recombinase, Tn3 transposase, insertion sequence (IS1380), or a recombinase. The Tn3 contained ISs (IS91 or IS3), which were associated with the ESBLs ($bla_{\text{CTX-M-15}}$, bla_{TEM}) and other ARGs conferring resistance to aminoglycosides (*aph(6)-Id*, *aph(3'')-Ib*) and sulphonamide (*sul*2).

➢ Most virulence genes were not associated with MGEs in *K. pneumoniae* except haemolysin (*hha*), human leukocyte antigen (*hla*), caseinolytic protein (*clpK*), heat shock protein (*hsp20*) that occurred together with heavy metal resistance genes such as silver (*silABCES*), copper (*pcoABCDER*), and arsenic (*arsACBDHR*), and had IS1 in their genetic environment.

➢ In *S. aureus* MGEs found included insertion sequences (IS6, IS256), plasmid replicons (mostly rep20) and phages HAGE_Staphy_phi2958PVL, with PHAGE_Staphy_P282 being most prevalent. PHAGE_Staphy_phiJB was associated with the *dfrG* gene conferring resistance to trimethoprim.

➢ Other ARGs were associated with ISs and recombinase/integrase. The *m*ecA gene was commonly associated with IS1182 and IS6 and surrounded by recombinase in the genetic environment.

➢ *blaZ* genes, heavy metal genes and associated MGEs were carried on either plasmids or the chromosome. The *bla*Z and cadmium (*cad*AC) gene was found on a contig with the closest homology to plasmid pER10678.3A.1 (CP051928.1). The convergence of ARGs and HMRGs is a cause for concern as it may potentially trigger co-selection of resistance to antibiotics, which may complicate treatment.

➢ Virulence genes in *S. aureus* were mainly associated with IS, including (IS6, IS30), suggesting that insertion sequences play a prominent role in transfering virulence genes in clinical isolates.

- To determine the clonal relatedness and phylogeny of isolates using whole-genome sequencing and bioinformatics tools to compare and contrast study strains with others.

➢ Phylogenomic analyses of *K. pneumoniae* revealed that isolates of the same MLST clustered together, mainly with South African and other African clinical isolates mostly from hospital patients.

➢ Phylogenomics in *K. oxytoca* revealed that our study isolates were not closely related to other isolates from other countries (Figure 2), suggesting that the occurrence of the ST450 strains could be local.

➢ Phylogenomic analysis of *S. aureus* integrated with metadata revealed clear clustering patterns based on Multilocus Sequence Typing (MLST) and geographic origin. Isolates sharing the same MLST type clustered together, with additional grouping observed by country of isolation (Figure 1). For instance, the ST5 isolates from this study (S11-ST5, S34-ST5, and S29-ST5) are closely aligned with other South African isolates, indicating minimal genetic divergence within this MLST type in the region. Also, S24-ST152 clusters with other ST152 isolates from various African countries, including Kenya and Ghana, highlighting the broader geographic distribution and potential mobility of this MLST type across the continent. Notably, the tree analysis also reveals that ST8 and ST612 isolates are clustered together, suggesting close genetic relatedness despite being distinct MLST types

➢ The rich diversity of MGEs in both bacterial species attests to the plasticity of their genomes and highlights the ease of transfer of ARGs and virulence factors via horizontal gene transfer (HGT) within and between different isolates. The fluidity and mobility of MGEs were evidenced by the different permutations and combinations of ARGs, virulence factors, and MGEs in isolates from different sources. The convergence of ARGs and virulence genes is a cause for concern as it may potentially complicate treatment.

5.2 Limitations

•The small numbers of some members of ESKAPEEc isolates obtained from specific hospitals or departments made it challenging to make statistically significant comparisons across the hospitals' departments.

• Considering the importance of identifying patients at risk of ESKAPEEc infection, the absence of detailed information such as the period of hospitalisation, previous antibiotic treatment, immune status of patients, clinical signs and symptoms and co-morbidities made it challenging to assess the true pathogenic potential of isolates recovered, even though WGS gave us important insights.

• The number of *S. aureus* and *Klebsiella* spp. (*K. pneumoniae* and *K. oxytoca*) isolates used in the genomic surveillance was limited and does not give a complete picture of the resistome, virulome, and mobilome in the investigated hospitals.

5.3. Recommendations

- Future studies should consider a large number of isolates to be able to explain trends observed with greater statistical significance.

- Future studies should be more patient-focused, with the provision of detailed patient-level data to assist in assessing other factors that contribute to pathogenicity/virulence and also to consider confounding factors.

- There should be increased education and awareness about antibiotic resistance and its spread to decrease its occurrence, particularly in the clinical setting.

- Future studies should use a larger sample size of antibiotic-resistant bacteria for genomic surveillance to provide a more comprehensive picture of the bacterial genomes from the hospitals in the Umgungundlovu district, KwaZulu-Natal, South Africa.

5.4 Significance of the research

Antibiotic resistance is a global public health threat driven by the indiscriminate use of antibiotics. The AMR threat continues to rise in both human and veterinary practice, resulting in the development of multidrug-resistant infections associated with increased morbidity and mortality. The increased level of multidrug resistance observed in this study, and the rich repertoire of resistance genes, virulence genes and MGEs necessitate real-time monitoring and evaluation in the South African health sector when dealing with ESKAPEEc. There is still limited data regarding the role of ESKAPEEc in bloodstream infections, mainly in relation to molecular epidemiology in Africa, with most studies reported from Europe and the USA. In

Africa, few studies have been done collectively on ESKAPEEc and ARGs from bloodstream infections, using genomic approaches. This study provides a comprehensive picture of antibiotic resistance using a combination of phenotypic and genomic approaches in ESKAPEEc from bloodstream infections. This study provided critical information on the epidemiology of infection to inform clinical and public health decisions.

APPENDICES:

APPENDIX I

Ethical approval

health
Department:
Health
PROVINCE OF KWAZULU-NATAL

330 Langalibalele street,
Private Bag X9051 PMB, 3200
Tel: 033 395 2805/3189/3123 Fax: 033 394 3782
Email: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

DIRECTORATE:
Health Research & Knowledge Management (HKRM)

Reference: HRKM108/17
KZ_2017RP3_437

29 March 2017

Dear Prof S Y Essack
(University of KwaZulu-Natal)

Subject: Approval of a Research Proposal

1. The research proposal titled **'The "One Health" Approach to Containing Antibiotic Resistance'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby **approved** for research to be undertaken at Edendale, Greys & Appelsbosch Hospitals and also Bruntville, Appelsbosch Gateway, East Boom & Imbalenhle Clinics.

2. You are requested to take note of the following:
   a. Make the necessary arrangement with the identified facility before commencing with your research project.
   b. Provide an interim progress report and final report (electronic and hard copies) when your research is complete.

3. Your final report must be posted to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za

For any additional information please contact Ms G Khumalo on 033-395 3189.

Yours Sincerely

[redacted]

Dr E Lutge
Chairperson, Health Research Committee
Date: 29/03/17.

Fighting Disease, Fighting Poverty, Giving Hope