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DEPARTMENT OF MEDICAL MICROBIOLOGY

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Naledi Shakoane Masalane

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A dissertation submitted in fulfilment for the degree Master of Medical Science (Medical Microbiology) in the School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal

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A dissertation submitted to the School of Laboratory Science and Medical Sciences, College of Health Science, University of KwaZulu-Natal, Westville Campus, for the degree of Master of Medical Science (Medical Microbiology).

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DECLARATION

I, Naledi Shakoane Masalane, declare that:

i. The research reported in this dissertation is my original work, except where otherwise indicated.
ii. This dissertation has not been submitted for any degree or examination to the University of KwaZulu-Natal or any other institution.
iii. The content of this dissertation does not contain other persons' data, pictures, graphs, and other information, unless specifically acknowledged as being sourced from other researchers.
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Signed:  Date: 17/01/2025

DEDICATION

This study is dedicated to my loving family (mom, dad, sisters, aunt and grandmother). Your belief in me gave me the strength to complete this study. On a personal note, I would like to dedicate this work to the cherished memories of my cousin, aunt, and grandfather, whose kindness, inspiration, and belief in my potential have always motivated me. Your memory is a continuous source of strength, inspiration, and perseverance in my life.

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LIST OF ABBREVIATIONS

AME	Aminoglycoside-Modifying Enzymes
AMR	Antimicrobial Resistance
ANT	Aminoglycoside Nucleotidyl Transferases
APH	Aminoglycoside Phosphotransferases
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Gene
ATCC	American Type Culture Collection
BREC	Biomedical Research Ethics Committee
BV-BRC	Bacterial and Viral Bioinformatics Resource Centre
CAI	Community Acquired Infection
CARD	Comprehensive AMR Database
CF	Cystic Fibrosis
CLSI	Clinical & Laboratory Standards Institute
CRAB	Carbapenem resistant *Acinetobacter baumannii*
CRKP	Carbapenem Resistant *Klebsiella pneumoniae*
DNA	Deoxyribonucleic Acid
ECC	*Enterobacter cloacae* complex
ESBL	Extended Spectrum β-lactamase
ESBL-EC	Extended spectrum β-lactamase producing *Escherichia. coli*
ESKAPEE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp,* and *Escherichia coli*
FAO	Food and Agriculture Organization of the United Nations
GAP	Global Action Plan
HA-MRSA	Hospital-Acquired Methicillin Resistant *S. aureus*
HIC	High Income Country
HWW	Hospital Wastewater
ICU	Intensive Care Unit

iTOL	Interactive Tree of Life
LMIC	Low-and-Middle-Income Country
MBL	Metallo- β-lactamase
MDR	Multi-Drug Resistance
MGE	Mobile Genetic Element
MRSA	Methicillin Resistant *Staphylococcus aureus*
NCBI	National Centre for Biotechnology Information
NHLS	National Health Laboratory Service
NICD	Nation Institute for Communicable Diseases
OECD	Organisation for Economic Co-operation and Development
OMP	Outer Membrane Porin
PCR	Polymerase Chain Reaction
PDR	Pan-Drug Resistant
PHREC	Provincial Health Research Ethics Committee
SCAI	Simmons Citrate Agar supplemented with Inositol
TSB	Tryptone Soy Broth
UTI	Urinary Tract Infections
VAP	Ventilator-Associated Pneumonia
VRE	Vancomycin Resistant *Enterococcus faecium*
VRSA	Vancomycin Resistant *Staphylococcus aureus*
WBE	Wastewater-Based Epidemiology
WGS	Whole Genome Sequencing
WHO	World Health Organization
WOAH	World Organization for Animal Health
WWTP	Wastewater Treatment Plant
XDR	Extensively Drug Resistant

ABSTRACT

Background: The ESKAPEE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp,* and *Escherichia coli*) is a group of Gram-negative and -positive pathogens that exhibit antimicrobial resistance (AMR) to commonly used antibiotics. **Aim:** This study compared clinical ESKAPEE isolates from patients and hospital effluent in terms of antibiotic resistance patterns, antibiotic resistance genes, mobile genetic elements (MGEs) and phylogenomic relationships. **Methodology:** Samples were collected and pooled from the final effluent point of a regional hospital in the uMgungundlovu district, Kwa-Zulu Natal, South Africa. Clinical isolates were also collected from the same hospital. Selective culture media was used for isolation and identification. Antimicrobial susceptibility testing (AST) was performed using the VITEK® 2 system. DNA was extracted using the GenElute extraction kits prior to whole genome sequencing. The resistome, mobilome and phylogenetic lineages of sequenced isolates were assessed using bioinformatics analysis. ResFinder, PlasmidFinder, INTEGRALL and PGAP & ISFinder were used to annotate and identify resistance genes, plasmids, integrons and insertion sequences and transposons, respectively. MLST was used to identify sequence types, BV-BRC was used to construct the phylogenetic trees, and iTOL was used to view, edit and annotate the generated phylogenetic trees. **Results:** A total of 112 presumptive ESKAPEE constituted the sample of which 42 were clinical isolates and 70 were isolates from hospital effluent. Of these, 36 isolates consisting of 16 *K. pneumoniae*, 9 *E. faecium*, 7 *Enterobacter hormaechei*, 3 *E. coli* and 1 *P. aeruginosa* were positively identified as ESKAPEE pathogens by WGS. The effluent *E. faecium* isolates were totally resistant to six of the antibiotics tested (tetracycline, doxycycline, erythromycin, azithromycin, fosfomycin and levofloxacin). They also harboured antibiotic resistance genes (ARGs) that confer resistance to aminoglycosides (*aac(6')-Ii, aac(6')-aph(2''), aph(2'')-Ia*), macrolides (*msr(C)*), tetracycline (*tet(M), tet(L)*), and trimethoprim (*dfrG),* none of which were carried on any MGEs. In the *K. pneumoniae* isolates, ARGs conferring resistance to ß-lactam antibiotics were the most common among the clinical isolates while effluent *K. pneumoniae* carried markedly fewer ARGs. Aminoglycoside resistance genes (*aph(6)-Id, aph(3'')-Ib, aac(6')-Ib-cr aadA16*), quinolone (*qnrB1, qnrB6, qnrS1, OqxA* and *oqxB*), ß-lactam (bla_{SHV} group, $bla_{CTX\text{-}M\text{-}15}$, and bla_{TEM} group) and trimethoprim-sulfamethoxazole (*sul1, sul2,* and *dfrA27, drfA14)* were common among the clinical *K. pneumoniae* isolates. ARGs were associated with diverse MGEs, particularly in the clinical isolates and a common plasmid replicon IncFIB(K) was identified in both clinical and effluent isolates. MLST showed no relation between the *K. pneumoniae* clinical and effluent isolates. The clinical *P. aeruginosa* isolate harboured ß-lactam [$bla_{OXA\text{-}50}$, bla_{PAO}], aminoglycoside *(aph(3')-Iib)*, and fluoroquinolone *(crpP)* ARGs that were not associated with MGEs. The isolate had a sequence type ST275, which showed no relation with other isolates it was compared with. The majority of clinical *E. hormaechei* isolates showed total resistance to aminoglycosides, ß-lactams and tetracycline antibiotics. The *E. hormaechei* isolates, except one, harboured [$bla_{OXA\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{ACT\text{-}5}$ *and* $bla_{TEM\text{-}1B}$], *dfrA14, tet(A),* [*aph(6)-Id, aph(3'')-Ib, aac(3)-Iia, aac(6')-Ib-cr*] and *sul2*

ARGs conferring resistance to ß-lactams, trimethoprim, tetracycline, aminoglycoside, and sulfamethoxazole, respectively. Clinical and effluent isolates of *E. coli* displayed similar resistance patterns. ARGs conferring resistance to ß-lactam (*bla*$_{CTXM-15}$ and *bla*$_{OXA-1}$), aminoglycosides (*aph(6)-Id, aph(3")-Ib, aac(6')-Ib-cr, aadA5 and aac(3)-IId*), trimethoprim-sulfamethoxazole (*dfrA14, dfrA17, sul1* and *sul2*), and tetracycline (*tet(A)* and *tet(B)*) were observed among the clinical isolates; while the effluent isolate harboured ß-lactam (*bla*$_{CTXM-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1B}$ and *bla*$_{OXA-10}$), aminoglycosides (*aac(3)-Iid, aac(3)-IIa, aac(6')-Ib-cr, aph(6)-Id, aph(3")-Ib*), trimethoprim-sulfamethoxazole (*dfrA14, dfrA23, sul1* and *sul2*), and tetracycline (*tet(A)*). The clinical *E. coli* isolates had sequence types ST69 and ST131 and the effluent isolate belonged to ST10. The clinical and effluent isolates from this study that did not cluster together were not closely related and belonged to different sequence types. **Conclusion:** These findings demonstrate the prevalence of ESKAPEE pathogens in hospital effluent. While the effluent did not mirror AMR in the clinical setting, presence of antibiotic-resistant bacteria (ARB) in the effluent cannot be overlooked. This study highlights the need for continuous monitoring of the effluent to track the spread of resistant bacteria from the hospital to the environment.

Keywords: ESKAPEE, antimicrobial resistance, effluent

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Antibiotics are the most significant class of pharmaceuticals that have been used to cure human or animal infectious diseases by either killing or inhibiting the growth of bacteria and are one of the most influential medical inventions of the 20th century (Gould et al., 2000; Ribeiro da Cunha et al., 2019; Uddin et al., 2021a). The introduction of antibiotics into clinical use has made a major contribution and revolutionised the management of infectious diseases, saving millions of lives (Hutchings et al., 2019; Katz and Baltz, 2016; Sabri et al., 2020).

Antibiotics have allowed the development of several fields of medical practice; in addition to treating infectious diseases. They have made many modern medical procedures possible, including organ transplantation, surgical procedures, chemotherapy and other immunosuppressive therapies that rely on antibiotic prophylaxis (Dodds, 2017; Hutchings et al., 2019). However, the spread of antibiotic resistance in bacterial communities presents a significant challenge to all healthcare systems worldwide (Uddin et al., 2021b) and has led to a steady decline in the efficacy of antibiotics or even reversed their progress (Hubeny et al., 2022; Marston et al., 2016).

Hospitals are typically settings where patients consume a lot of antibiotics (Cai et al., 2021). Until recently, it was thought that the main sources of the spread of antibiotic resistance were healthcare facilities (Hubeny et al., 2022), but research has shown that the environment, animal husbandry and agriculture (Holmes et al., 2016), and aquaculture also contribute to this spread (Hubeny et al., 2022). About 30–90% of the antibiotics used are eliminated through urine and faeces because they are not completely destroyed by either people or animals (Gao et al., 2012; Sabri et al., 2020). Due to the excretion of utilized antibiotics and the disposal of unneeded substances, significant volumes of antibiotics may be discharged into hospital effluent (Lien et al., 2016).

Antibiotics used in hospitals that are discharged into effluent expose bacteria in the environment to selection pressure for the development or escalation of resistance (Asfaw et al., 2017). Effluent from hospitals additionally contains high numbers of resistant bacteria, antibiotic resistance genes (ARGs) and associated mobile genetic elements (MGEs) (Hubeny et al., 2022). The effluent has thus been suggested as hotspots for the prevalence and dissemination of resistance through horizontal gene transfer, allowing antibiotic resistance genes to spread between various bacterial species (Karkman et al., 2018). Antibiotic-resistant bacteria are particularly frequent in hospitals, where they are linked to nosocomial infections (Hocquet et al., 2016; Nishiyama et al., 2021).

The application of wastewater-based epidemiology (WBE) has gained traction as a cost effective tool in AMR surveillance with studies showing a correlation between the presence of resistant bacteria from hospital sewage and patients (Cai et al., 2021; Chau et al., 2022; Clarke et al., 2024; Siri et al., 2024;

Tiwari et al., 2022). The high similarities observed between AMR profiles in wastewater and clinical settings in these studies highlight that monitoring wastewater could serve as a reliable tool to effectively predict clinical implications. Whilst such studies suggest that hospital effluent analysis can be a useful tool to detect clinical cases, there are limitations to it (Clarke et al., 2024). The relationship between AMR in hospital effluent and clinical resistance depends on the antimicrobial agent and bacterial species studied (Perry et al., 2021).

Nosocomial pathogens, are frequently members of the "ESKAPEE" group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp*, and *Escherichia coli*) (Nishiyama et al., 2021; Santajit and Indrawattana, 2016). They include both Gram-positive and Gram-negative species that express drug resistance mechanisms that allow them to "escape" antibiotics and they are significant sources of life-threatening hospital-acquired infections (Bhatia et al., 2021; Nishiyama et al., 2021).

1.2. LITERATURE REVIEW

1.2.1 Antimicrobial Resistance (AMR)

Antimicrobial resistance (AMR) is the ability of microorganisms to resist the effects and counteract the action of antimicrobial agents that previously killed them. Resistant microorganisms are able to multiply in the presence of therapeutic levels of the antimicrobial agents (Nadeem et al., 2020; Pulingam et al., 2022; Zaman et al., 2017). This is a serious public health threat worldwide (Kang and Song, 2013). In total 1.27 million deaths were linked to bacterial AMR in 2019 (Murray et al., 2022). AMR is associated with the selection pressure driven by the widespread use and misuse of antibiotics in humans, animals and agriculture as well as their disposal into the environment (Harbarth et al., 2015).

Resistance was first identified in *Staphylococcus*, *Streptococcus*, and *Gonococcus* species after the first commercialised antibiotic, penicillin was introduced into clinical practice in the early 1940s (Uddin et al., 2021a). Penicillin-resistant strains of *Staphylococcus aureus* evolved right away, as early as 1942 (Foster, 2017). AMR, however, was documented even before the beginning of the use of the antibiotics in fighting infections; long before antimicrobials were discovered, produced, or introduced to the market (Morrison and Zembower, 2020; Zaman et al., 2017). For example, the antibiotic tetracycline has been recovered from human skeletal remains of ancient Sudanese Nubian tracing back to 350–550 AD (Nelson et al., 2010). Antibiotic resistance genes have accumulated in human populations, revealing their historical roots (Carrano et al., 2018).

Although the overuse or misuse of antibiotics are primary drivers of the emergence of AMR, other multiple interconnected factors contribute to its prevalence and spread (WHO, 2022). AMR is a global problem that is powered by clinical, biological, political, economic, social, and environmental factors. It not only affects humans but animals, as well as ecosystems (Vikesland et al., 2019). Given that a number of antibiotics come from the same class of medications, resistance towards a single antibiotic

agent can spread throughout the whole related class (WHO, 2017). Resistance that occurs in a single microorganism can spread quickly through the interchange of genetic material between various microorganisms and can impact antimicrobial therapies for many different illnesses and infections (WHO, 2022).

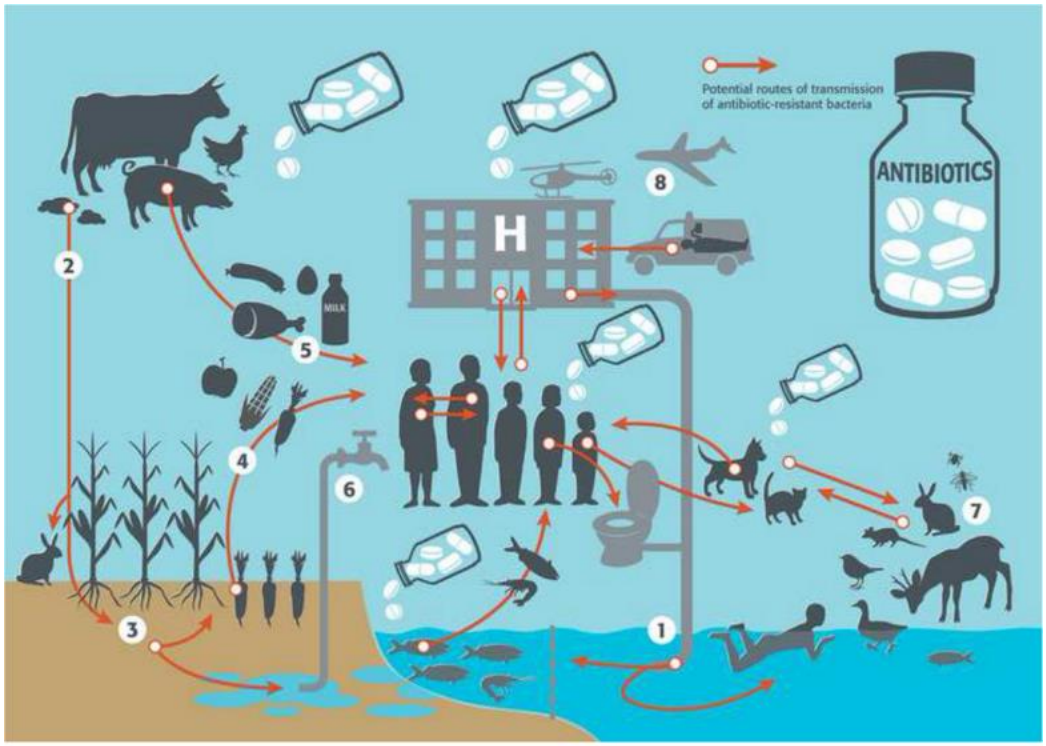

Figure 1: Routes of spread of AMR. Legend: "Humans in the community or in hospitals, pets, livestock, and fish farms rely on similar classes of antibiotics to fight infectious diseases. Both pathogenic and non-pathogenic bacteria evolve or exchange the ability to survive when exposed to these antibiotics. They spread into the environment through different routes, such as water sanitation systems (1), as wastewater treatment facilities do not entirely remove antibiotic resistant bacteria before releasing water into the environment. Another common route is through the application of manure to fields with cultivated crops (2), where antibiotic resistant bacteria can readily develop on the plants (3). The uptake of these resistant bacteria can then happen through the food chain, when humans later consume these plants (4) or the contaminated flesh of animals and fish harbouring resistant bacteria (5). As bacteria can easily reach water reserves, water distribution infrastructure is also a potential route for the spread of these germs (6). Even wildlife, insects and other bugs are potential carriers of antimicrobial resistance (7). Tourism, migrations and food imports (8) are nevertheless reported as the fastest way of spreading resistant strains of bacteria across borders. At the healthcare facilities level, resistant bacteria can spread by contact between patients or with healthcare staff, or through contaminated surfaces and medical devices."
Source: (Harbarth et al., 2015)

Current global estimates of the burden of AMR do not give adequate and detailed information about the economic impact of AMR (de Kraker et al., 2016). Reliable data is needed to improve AMR control measures especially with respect to thorough population-based monitoring systems from low-, middle, and high- income countries (de Kraker et al., 2016). Each year, antibiotic resistance claims 700 000 lives, and the death toll is projected to rise to 10 million deaths each year globally by 2050 (O'Neill,

2014; Shankar, 2016). O'Neill (2014) estimated that by 2050 the world population would at least be between 11 million and 444 million lower than it would otherwise be in the absence of AMR. Similarly, in 2019, Murray et al. (2022) estimated 4·95 million deaths that were associated with bacterial antibiotic resistance, and that included 1·27 million deaths directly attributable to bacterial AMR.

AMR is a burden on the global economy, resulting in financial losses from less production driven by illness in both humans and animals; and increased treatment expenses (WHO, 2022). In 2017, the World Bank, depicted a few scenarios to highlight the effects of AMR. The high AMR scenario would cause an economic recession significantly greater than the 2008 global financial crisis where more than 28 million people will be forced into poverty by 2050. In addition, there would be an increase in healthcare costs of between $340 billion and $1 trillion annually. The low AMR scenario would result in roughly 8 million people, predominantly in low-income countries, slipping into extreme poverty due to lower productivity and higher health-care costs (Jonas et al., 2017). At a global level, awareness of the growing urgency nature of AMR has brought about the establishment of many programs to mitigate AMR (Elton et al., 2020).

The tripartite organizations, the World Health Organization (WHO), the Food and Agriculture Organization (FAO) of the United Nations and the World Organization for Animal Health (WOAH) developed the Global Action Plan (GAP) on AMR. The GAP sets out five strategic objectives to improve awareness and understanding of AMR; improve knowledge through surveillance and research; reduce the possibility of infection; make the most effective use of antibacterial agents; to ensure sustainable investment in countering AMR (WHO Geneva, 2017). However, there is today considerable awareness of the need for, and political support for, action to combat AMR (WHO Geneva, 2017). In most countries, antibiotics can be purchased in markets, shops, pharmacies or over the Internet without prescription or involvement of a health professional or veterinarian. Reducing AMR will require the political will to adopt new policies, including controlling the use of antimicrobial medicines in human health and animal and food production. Laws to ensure that medicines are of assured quality, safe, effective, and accessible to those who need them have to be enacted and enforced (WHO, 2017).

AMR is a global concern that affects all countries; even those with lower antibiotic consumption (Cecchini et al., 2015). Antimicrobial-resistant infections are highly prevalent in high-income countries (HICs) (G7 and Organisation for Economic Co-operation and Development (OECD)) as they are in low-and-middle-income countries (LMICs) (Cecchini et al., 2015). In lower-income countries, easily accessible antibiotics are essential for effective treatment of infectious diseases (WHO, 2017). However, effective antimicrobial treatment for drug-resistant infections usually remains out of reach for many LMICs due to bad legislation, a lack of enforced regulation, a lack of understanding about responsible antibiotic use, and insufficient distribution of treatment recommendations (Bloom et al.,

2017). Major data gaps remain on the issue of AMR in Africa including the actual burden of AMR in the community, hospital settings, animals, and the environment (Elton et al., 2020).

Addressing the global spread of AMR is a challenging concept that requires multisectoral collaborative action to develop strategies to tackle the growing threat (Ahmed et al., 2024; Stewart Williams and Wall, 2019; Uchil et al., 2014). Research that focuses on actionable strategies is crucial for developing approaches that could effectively mitigate AMR (Aslam et al., 2018; Sulis et al., 2022). Primarily, there is a need for addressing excessive use of antimicrobials. Regulations governing the use of antimicrobials in agriculture and veterinary settings and implementation of antimicrobial stewardship programs in healthcare facilities can help reduce the overuse of antimicrobials (Agyare et al., 2024; Huang et al., 2022). Improved surveillance systems are required for monitoring and tracking the spread of resistant microorganisms (Delesalle et al., 2022). Uchil et al. (2014) discussed the effective strategies for combating AMR at different levels. These include, at international, national, community, hospital, individual and patient levels;- rational usage of antimicrobials, AMR awareness motive, infection prevention and control, AMR surveillance, research and development of new drugs and/or vaccines, and national policy, amongst other measures.

1.2.2 AMR in ESKAPEE pathogens

In February 2017, the World Health Organisation (WHO) released a list of global priority antibiotic resistant bacteria for prioritisation of research and development of new, effective antibiotics (WHO, 2017). Among those were the ESKAPEE pathogens. These bacteria are frequently isolated from clinical settings where they are linked to several potentially fatal nosocomial infections with limited or no antimicrobial treatment options, and they considerably contribute to the global burden of disease in both HICs and LMICs (Navidinia et al., 2017; Sakkas et al., 2019).

The ESKAPEE pathogens, also named ‘superbugs’, are increasingly associated with antibiotic resistance, which poses a serious threat to public health (Hubeny et al., 2022). They harbour genes that encode resistance and have developed resistance mechanisms to the most popular classes of antibiotics, including oxazolidinones, lipopeptides, macrolides, fluoroquinolones, tetracyclines, β-lactams, β-lactam– β-lactamase inhibitor combinations, amongst others; and antibiotics that are the last line of defence, including carbapenems, glycopeptides, and clinically unfavourable polymyxins (De Oliveira et al., 2020; Hubeny et al., 2022; Kalpana et al., 2023).

Carbapenem resistant *A. baumannii* and *P. aeruginosa*, extended spectrum β-lactamase (ESBL)-producing or carbapenem resistant *K. pneumoniae, Enterobacter spp* and *E. coli* are listed in the critical priority list of pathogens, whereas listed in the high priority group are vancomycin resistant *E. faecium* (VRE) and methicillin and vancomycin resistant *S. aureus* (MRSA and VRSA) (Mulani et al., 2019). The production of enzymes, that irreversibly inactivate β-lactam antibiotics, e.g., ESBL and carbapenemases like metallo- β-lactamase (MBL), has contributed to the success of Gram-negative

members of ESKAPEE pathogens in clinical settings (Pandey et al., 2021). Multidrug resistance is identified among multiple Gram-negative bacteria as a result of the widespread use of antibiotics in hospitals and communities (Founou et al., 2018). On the same ground, drug-resistant strains of *S. aureus*, mainly MRSA, vancomycin-resistant *Enterococcus* (VRE) and β-lactam resistant pathogens have shown to be among the greatest clinical potential risks (Pandey et al., 2021).

The inefficiency of antibiotics against these pathogens is due to the fact that these pathogens exhibit drug resistance through a variety of mechanisms, such as using enzymes to irreversibly cleave and inactivate the drug, decreasing drug permeability, modifying the drug-binding site, and/or increasing drug efflux to decrease drug accumulation (Motiwala et al., 2022; Pandey et al., 2021; Santajit and Indrawattana, 2016). With each year passing, the general supply of antibiotics effective against ESKAPEE is decreasing and it is pushing the world to a future with inadequate and ineffective antibiotics (Mulani et al., 2019).

Clinical ESKAPEE isolates are frequently multi-drug resistant (MDR), extensively drug-resistant (XDR), or pan-drug-resistant (PDR) which is one of the biggest challenges in clinical practice (Pandey et al., 2021; Trastoy et al., 2018) resulting in a dramatic increase in morbidity and mortality in infected patients, hence their prompt detection is vital (Trastoy et al., 2018). Understanding of virulence, resistance, transmission and pathogenicity of these microbes will inform innovative strategies for the development of new antimicrobial options (Navidinia et al., 2017). The ability to detect antibiotic-resistance genes and their low turnaround times has made molecular methods a reference for the diagnosis of multidrug resistance (Trastoy et al., 2018).

Antibiotic therapy to effectively treat infections involves the use of antibiotics either singly or in combination (Mulani et al., 2019). Alternative therapies that are currently in practice or under trial include the use of antibiotics in combination with adjuvants, bacteriophage therapy, use of antimicrobial peptides, photodynamic therapy, antibacterial antibodies, phytochemicals, and nanoparticles as antibacterial agents (Mandal et al., 2014; Mulani et al., 2019). To improve the efficiency and/or minimize the limitations of any therapy/therapeutic agents, combinatorial approaches are suggested. It involves use of two or more monotherapies together (co-administered, functionalized, or conjugated). Altogether, combinatorial approach could be a prospect for exploring novel alternate solutions against ESKAPEE pathogens (Mulani et al., 2019).

A combination of drugs broadens the spectrum of protection against serious infections caused by pathogenic bacteria; and has been proven to be effective (Baronia and Ahmed, 2014; Vazquez-Grande and Kumar, 2015). Fosfomycin and daptomycin, in combination, has shown to successfully clear infections caused by the Gram-positive members of the ESKAPEE, *E. faecium* and *S. aureus* (Mulani et al., 2019). The antibiotic colistin is a last resort antibiotic used to treat infections caused by Gram-negative bacteria. In recent years, research studies showed positive outcomes for treatment of infections

caused by *K. pneumoniae* and *A. baumannii* using combinations of different antibiotics and colistin or tigecycline (Mulani et al., 2019). In fact, a study by Ramsamy et al. (2018) showed a very low resistance rate (4%) to colistin alone in *A. baumannii*. Similarly, Sakkas et al. (2019) also found that the majority (77.8%) of the multidrug resistant *P. aeruginosa* strains were susceptible to colistin.

1.2.3 AMR in *Enterococcus faecium*

Enterococcus faecium is a Gram-positive facultative anaerobe that is part of the normal gut microbiota of animals and humans (Santajit and Indrawattana, 2016). *Enterococcus faecium* acquired characteristics that allowed it to thrive as a nosocomial pathogen (Zhou et al., 2020). It is an important source of healthcare-associated infections, and vancomycin resistance is rising in hospital-adapted lineages (De Oliveira et al., 2020).

Enterococcus spp. exhibit intrinsic and acquired resistance to various antibiotics, particularly to vancomycin (Sakkas et al., 2019). Resistance against vancomycin can be acquired through nine different gene clusters: *van* A, B, C, D, E, G, L, M, and N. The most predominant types worldwide are *van*A and *van*B (Navidinia et al., 2017; Sakkas et al., 2019). Gene *van*A, which confers high resistance to teicoplanin and vancomycin, is usually seen in vancomycin-resistant *Enterococcus faecium.* Gene *van*B is associated with high vancomycin resistance levels, but susceptibility to other glycopeptides such as teicoplanin (Kang et al., 2014; Navidinia et al., 2017; Werner et al., 2012).

The intrinsic resistance to different antibiotics and spread of resistant genes by horizontal gene transfer plays a major role in the emergence of MDR Enterococci (Haghi et al., 2019). Vancomycin-resistant enterococci (VRE) emerged as the second most common hospital-acquired pathogen in the 1990s due to the use of vancomycin to stop the spread of infections (Mancuso et al., 2021; Ramos et al., 2020; Zaheer et al., 2020). *E. faecium* is considered a MDR bacteria since it is intrinsically resistant to aminoglycosides (gentamicin), β-lactams (cephalosporins), and glycopeptides (vancomycin) (Hollenbeck and Rice, 2012) , and it is capable of producing aminoglycoside-modifying enzymes (AMEs) including aminoglycoside nucleotidyl transferases (ANTs), aminoglycoside acetyltransferases (AACs), and aminoglycoside phosphotransferases (APHs) (Mancuso et al., 2021).

Studies conducted in South Africa have characterised *Enterococcus* spp from clinical settings (Lochan et al., 2016), and effluent sources (Ekwanzala et al., 2020; Hamiwe et al., 2019; Iweriebor et al., 2015). Lochan et al. (2016), investigated vancomycin-resistant *Enterococcus* in a hospital and revealed the spread of the *vanA* and *vanB* resistance genes in the hospital. A study on molecular detection of antibiotic resistance and virulence factors of *Enterococcus* spp from aquatic sources in the Eastern Cape detected high levels of resistance in rifampicin (83.6%), erythromycin (64.2%) and tetracycline (52.2%) (Adeniji et al., 2021).

1.2.4 AMR in *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium forming irregular clusters of cocci and a normal commensal of healthy individuals, mainly found on the skin and mucous membrane (Otto, 2010; Rasheed and Hussein, 2021). It plays a significant role in causing both hospital and community infections and is known for its ability to become resistant to antibiotics (Chambers and DeLeo, 2009; Rasheed and Hussein, 2021).

It can be represented by two genotypes: methicillin-resistant *S. aureus* (MRSA) harboring the *mecA* gene, and vancomycin-resistant *S. aureus* (VRSA) harbouring the *vanA* gene (Hubeny et al., 2022). Methicillin-resistant *S. aureus* (MRSA) was first isolated in the 1960s in the hospital environment, therefore, it was referred to as hospital-acquired MRSA (HA-MRSA) (Ramessar and Olaniran, 2019). After the first clinical reports of methicillin resistant *S. aureus* (MRSA) in the 1960s, via *mecA* gene expression which encodes for the production of a low affinity penicillin-binding protein (PBP2a) which renders a number of antibiotics ineffective (Navidinia et al., 2017; Thompson et al., 2013), MRSA has extended resistance against β-lactam antibiotics including all β-lactams such as penicillins, cephalosporins, and carbapenems (Navidinia et al., 2017).

Few studies have highlighted the presence of MRSA associated with bacteraemia in hospital settings (Fortuin-de Smidt et al., 2015; Moodley and Perovic, 2017; Segal et al., 2023). A study in South Africa investigated the prevalence, AMR profiles and genotypic and phenotypic characterisation of MRSA in frequently touched surfaces at a hospital (Mkhize et al., 2021). The study showed that MRSA can contaminate various surfaces, allowing the dissemination of the bacteria within the hospital environment which will act as reservoirs in the transmission of MRSA. In a study investigating antibiotic resistance profiles of MRSA recovered from treated effluent and receiving surface water, MRSA isolates showed a high frequency of resistance to oxacillin, ampicillin, and penicillin (Ramessar and Olaniran, 2019).

First-line drugs for infections due to MRSA are mostly glycopeptide antibiotics such as vancomycin or teicoplanin. Unfortunately, intensive selective pressure has caused the emergence of vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *S. aureus* (VRSA) (Navidinia et al., 2017). It is known that *S. aureus* and MRSA may gain a temporary residence within hospitals therefore posing the possibility of dissemination into the environment through routes such as the hospital effluent (Thompson et al., 2013).

1.2.5 AMR in *Klebsiella pneumoniae*

Klebsiella pneumoniae is an opportunistic pathogen that belongs to the order Enterobacterales and is described as a Gram-negative, non-motile bacterium that has been implicated as one of the most common causes of hospital and community acquired infections (CAI) (Le et al., 2021; Ssekatawa et al., 2021). It causes infections at multiple sites such as the urinary tract, the lungs and surgical sites, and

has emerged as a major pathogen of worldwide concern as a result of the increasing infections caused by carbapenem-resistant strains (Chang et al., 2021).

Klebsiella pneumoniae has also acquired a variety of β-lactamase enzymes (including bla_{NDM} and bla_{KPC} genes) and this pathogen is now capable of hydrolysing penicillins, cephalosporins, as well as carbapenems, which are regarded as drugs of last resort (Hubeny et al., 2022). Typically, MDR *K. pneumoniae* have been resistant to several different classes of antibiotics such as aminoglycosides, quinolones, β-lactams including β-lactams/β-lactamase inhibitor combinations (Jamil et al., 2014; Navidinia et al., 2017).

The most important β-lactamases conferring resistance to carbapenems that have already been detected in Enterobacterales are *K. pneumoniae* carbapenemases (KPC, Ambler class A), metallo-β-lactamases (MBL, Ambler class B) and members of class D β-lactamases such as OXA-48 (Sakkas et al., 2019). The worldwide spread of carbapenemase-producing *K. pneumoniae* is a latent threat due to its capacity for causing and spreading healthcare-associated infections (HAIs) (Galarde-López et al., 2022). Carbapenems were considered the last resort antibiotics used to treat multidrug resistant infections, however, Carbapenem Resistant *Klebsiella pneumoniae* (CRKP) has emerged and CRKP has been reported to be resistant to a number of antibiotics used clinically (Sheu et al., 2019). *Carbapenem*-resistant *K. pneumoniae* strains are the most clinically prominent carbapenem-resistant Enterobacterales (Mancuso et al., 2021).

An evolutionary epidemiological study assessing molecular mechanisms of resistance and virulence factors in clinical *K. pneumoniae* revealed a largely diverse virulome, mobilome and resistome; and the isolates were resistant to several clinically important antibiotics except for carbapenems, colistin and tigecycline (Mbelle et al., 2020a). One study on molecular characterization of antibiotic resistance determinants in *K. pneumoniae* recovered from hospital effluents was conducted in the Eastern Cape province in South Africa (Okafor and Nwodo, 2023). It showed a high percentage (82.7%) of the *K. pneumoniae* isolates that are multi-drug resistant with the most resistance observed against Ampicillin. It also highlighted the concerning widespread presence of resistance genes in hospital effluents and the need for effective water treatment and surveillance systems. Other studies also reported the presence of MDR *Klebsiella pneumoniae* in hospital effluents (Ekwanzala et al., 2019; King et al., 2020a).

1.2.6 AMR in *Acinetobacter baumannii*

Acinetobacter baumannii is a Gram-negative aerobic bacillus (Mancuso et al., 2021). *A. baumannii* is widely distributed in all clinical settings and predominantly causes diverse nosocomial infections including ventilator-associated pneumonia (VAP), urinary tract infections (UTI), bacteraemia, and wound infections (Kyriakidis et al., 2021). It affects mostly immunocompromised and intensive care unit (ICU) patients. MDR *A. baumannii* infections have been associated with prolonged hospitalisations and higher rates of morbidity and mortality (Yadav et al., 2020). This pathogen can easily acquire and

transfer ARGs to other bacteria, causing infections expressing broad-spectrum drug resistance that have been reported worldwide (Hubeny et al., 2022; Zhang et al., 2014).

A. baumannii has acquired several resistance mechanisms to escape the effects of antibiotics (Agyepong et al., 2023). The most common mechanisms include expression of efflux pumps and the production of hydrolytic enzymes, such as extended spectrum β-lactamases (ESBLs) and carbapenemases (Wilson, 2014). Carbapenem resistant *A. baumannii* (CRAB) was ranked in 2018 by WHO as number one priority for antibiotic research and development (Kyriakidis et al., 2021). It harbours Ambler class B and D genes that confer resistance to carbapenems, and it poses a significant problem in the medical sector (Hubeny et al., 2022). The most important mechanism of carbapenem resistance in *A. baumannii* involves OXA-type carbapenemases, which are encoded by bla_{OXA} lineage genes (Hrenovic et al., 2019).

Studies on analysis and characterisation of *A. baumannii* isolates from bloodstream infections have been conducted in South Africa (Lowe et al., 2022; Perovic et al., 2022). Lowe et al. (2022) detected Multidrug resistance in more than 80% of the *A. baumannii* isolates. Other South African studies published on antibiotic resistant *A. baumannii* strains recovered from effluents (Eze et al., 2022, 2021; Mapipa et al., 2022). Eze et al. (2021), examined the relationship between antibiotic resistance and biofilm formation in *A. baumannii* recorded a prevalence of 81.7% of MDR and XDR isolates recovered from hospital effluents. Mapipa et al. (2022) also showed a high level of resistance, especially to piperacillin (93%).

Colistin and tigecycline are the major antibiotics active against *A. baumannii* and have become the last resort of treatment for multidrug-resistant multidrug infections (Vázquez-López et al., 2020). Another study investigating genetic determinants of multi-drug resistant *A. baumannii* in a hospital in South Africa also found colistin and tigecycline to be the most effective against their investigated isolates (Nogbou et al., 2021). However, although colistin resistance is low in South Africa, Lowe et al. (2022) highlighted the need for understanding the mechanisms of colistin resistance in *A. baumannii* after reporting a 9.4% resistance rate to colistin.

1.2.7 AMR in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic bacillus that naturally inhabits environments such as the soil, water and plants (Diggle and Whiteley, 2020). This human opportunistic pathogen is one of the most common causal agents of many severe acute and chronic nosocomial infections and is known as the leading cause of high morbidity and mortality in patients with cystic fibrosis (CF) (McClean et al., 2021; Moradali et al., 2017; Wood et al., 2023). It is a widespread hospital-acquired pathogen of the respiratory and urinary tracts, and is responsible for causing infections in immunocompromised patients, especially in intensive care units (Hocquet et al., 2016; Wilson and Pandey, 2024). The ability

of *P. aeruginosa* to develop resistance to almost all effective antibiotics as well as its inherent resistance to several kinds of antibiotics makes treating infections more difficult (Hocquet et al., 2016).

Strains of *P. aeruginosa* are known to show resistance to many antibiotics due to their high levels of intrinsic and acquired resistance mechanisms (Mancuso et al., 2021; Pang et al., 2019). The intrinsic mechanisms of resistance include expression of efflux pumps and the production of antibiotic inactivating enzymes (β-lactamases, such as OXA-50 and AmpC) (Langendonk et al., 2021), whereas the acquired mechanisms can include horizontal gene transfer (Pang et al., 2019). Endogenous β-lactamase such as AmpC β-lactamase can be induced by several β-lactams such as benzylpenicillin and imipenem (Mancuso et al., 2021).

The treatment of MDR *P. aeruginosa* involves colistin in combination with an anti-pseudomonal agents like imipenem, piperacillin, ceftazidime or ciprofloxacin (Mancuso et al., 2021). However, Hosu et al. (2021) revealed an alarming burden of resistance against these antibiotics used to treat MDR *P. aeruginosa* (ceftazidime, cefepime, imipenem and piperacillin). Studies reporting the emergence of MDR *P. aeruginosa* in South Africa have been published (Adjei et al., 2018; Dame et al., 2020; Singh-Moodley et al., 2018). Singh-Moodley et al. (2018), described the antibiotic susceptibility profiles of *P. aeruginosa* in patients with bacteraemia. They reported a 90% and above susceptibility rate in the antibiotics amikacin, gentamicin, imipenem, meropenem and tobramycin. A study determining the antibiogram and virulence determinants of *P. aeruginosa* isolated from hospital effluents revealed *P. aeruginosa* strains showing multiple antibiotic resistance, with a 100% susceptibility to gentamicin, amikacin and imipenem (Mapipa et al., 2021).

1.2.8 AMR in *Enterobacter* species

Enterobacter spp., named for their enteric recovery are Gram-negative (Sass and Fisher, 2009), rod-shaped, facultative anaerobic bacteria that form part of the normal flora of the gastrointestinal tract in mammals, and can also be found on human skin, water, foods, soil, and sewage (Cooney et al., 2014; Davin-Regli and Pagès, 2015). These species are responsible for causing many nosocomial infections like UTIs, respiratory tract infections and endocarditis, however, not all species are known to be human pathogens (Ramirez and Giron, 2024). The most common opportunistic species of *Enterobacter* causing infections in ICU patients, especially those on mechanical ventilation are *Enterobacter aerogenes* and *Enterobacter cloacae* (Mezzatesta et al., 2012).

The introduction of resistance genes in Gram-negative bacteria in the ESKAPEE group is via genetic mobile elements (Partridge et al., 2018) and they are able to acquire numerous genetic mobile elements that strongly contribute to antibiotic resistance (Davin-Regli and Pagès, 2015). The production of β-lactamase enzymes is the most important mechanism responsible for resistance to β-lactam antibiotics in most of the *Enterobacter* species (Davin-Regli and Pagès, 2015). These species have an intrinsic

resistance to penicillin and first- and second-generation cephalosporins, due to the expression of AmpC β-lactamases (Bouza and Cercenado, 2002; Davin-Regli et al., 2019a).

Infections due to *Enterobacter* species are mainly nosocomial, therefore most isolates present broad resistance to first- and second-generation cephalosporins, penicillins, and quinolones (Davin-Regli et al., 2019b; Davin-Regli and Pagès, 2015). Treatment with a third-generation cephalosporin may be effective in some strains of *Enterobacter*, however, it can also lead to multi-resistant infections and therefore the use of third-generation cephalosporins in the treatment of severe strains of *Enterobacter* is not recommended (Ramirez and Giron, 2024). The use of fourth generation cephalosporins like cefepime and cefpirome seems to be more effective, mostly due to their activity against AmpC-hyperproducing *Enterobacter* strains (Davin-Regli et al., 2019b). Carbapenems are also very efficient against a wide variety of AmpC-overproducing *Enterobacter*ia (Harris et al., 2016; Ramirez and Giron, 2024). The two clinically relevant species, *E. cloacae* and *E. aerogenes* have been shown to be susceptible to meropenem and Imipenem (Ramirez and Giron, 2024).

(Singh-Moodley et al., 2015) characterised *E. cloacae* expressing $bla_{\text{OXA-48-}}$, bla_{IMP}- and bla_{VIM} and showed a 96% resistance rate and total susceptibility to meropenem and imipenem. Very few studies in South Africa have isolated *Enterobacter* spp from effluents (Maguvu and Bezuidenhout, 2021; Mbanga et al., 2021b); and as part of the Enterobacterales Fadare and Okoh (2021), who found *Enterobacter* spp to show resistance to 12 out of the 18 investigated antibiotics. Mbanga et al. (2021b), investigating genomic analysis of three members of the *Enterobacter cloacae* complex (ECC), namely *Enterobacter kobei*, *Enterobacter ausburiae*, and *Enterobacter hormaechei* revealed that *E. ausburiae* harboured the fosfomycin resistant gene (*fosA*) and *E. kobei* harboured both (*fosA*) and the colistin resistance gene *mcr-10*.

1.2.9 AMR in *Escherichia coli*

Escherichia coli, is a Gram-negative, rod-shaped, facultative anaerobe that is found in the environment and forms part of the normal microbiota in the colon and naturally inhabits the gastrointestinal tract of humans and warm-blooded animals (Basavaraju and Gunashree, 2023; Korzeniewska et al., 2013). It is a leading cause of a variety of infections such as community and hospital acquired bloodstream and urinary tract infections (Ludden et al., 2021; Wu et al., 2021). Pathogenic *E. coli* strains can be divided according to site of infection into intestinal and extraintestinal (Wu et al., 2021), where both strains harbour resistance genes that may be transferred to other opportunistic and pathogenic bacteria (Sora et al., 2021).

E. coli acts as a donor and a recipient of antibiotic resistance genes in the order Enterobacterales, therefore can acquire resistance genes from other bacteria and can also pass on its resistance genes to other bacteria (Poirel et al., 2018; Puvača and Frutos, 2021). It possesses genes encoding multiple

resistance, and strains of *E. coli* have become highly resistant to a number antibiotic classes and are therefore described as multidrug-resistant (Korzeniewska et al., 2013).

The most common mechanism of resistance in *E. coli* is the production of extended-spectrum β-lactamases (ESBLs) (Wu et al., 2021), which confers resistance to β-lactams including cephalosporins and penicillin (Puvača and Frutos, 2021). Extended spectrum β-lactamase producing *E. coli* (ESBL-EC) are transferred between humans and animals but may also spread in aquatic environments and potentially contaminate and infect exposed individuals (Jørgensen et al., 2017). *E. coli* is an example of a multidrug-resistant and ESBL-producing bacterium that can be the source of extremely severe infections in which ESBLs are produced and usually have quite an unpredictable course (Puvača and Frutos, 2021).

Of particular concern are the rising rates of infection with antibiotic-resistant *E. coli*, especially those ESBLs that hydrolyse third generation cephalosporins, and *E. coli* with acquired resistance to carbapenems and colistin (Raven et al., 2019). Third-generation cephalosporin-resistant and carbapenem-resistant Enterobacterales appear in the highest category on WHO's list of priority pathogens for research and development of new antibiotics (Raven et al., 2019). Treatment for *E. coli* infection has been increasingly complicated by the emergence of resistance to most first-line antibiotics (Rasheed et al., 2014). Carbapenem-resistant and ESBL bacteria-related infections are mainly treated using tigecycline and polymyxins in combination with other antibiotics (Wang et al., 2023).

A study by Mbelle et al. (2020a) delineated the resistome, mobilome, virulome and phylogenetics of *E. coli* from clinical isolates. The study classified the bacterial strains as MDR as they exhibited resistance multiple antibiotics including penicillins, cephalosporins, and aminoglycosides. Multiple studies have been conducted on the occurrence of *E. coli* in South African effluent sources (Adefisoye and Okoh, 2016; Gumede et al., 2021; Igwaran et al., 2018; Makuwa et al., 2023; Mbanga et al., 2021a). Igwaran et al. (2018), investigated the molecular characteristics and resistance patterns of *E. coli* isolated from effluent treatment plants (WWTP). They recorded a high resistance particularly to clindamycin and erythromycin (100%) and amoxicillin (94.5%). It further highlights the burden of *E. coli* in WWTP as a potential risk to human health. A similar study investigated the prevalence of antibiotic resistant *E. coli* from treated effluent effluents and showed the highest resistance to tetracycline (60.1%), and a 100% susceptibility to both meropenem and imipenem (Adefisoye and Okoh, 2016).

1.3 AMR in hospital effluent

Hospital effluents constitute the perfect scenario for exchange of resistance genes between clinical and environmental pathogens; given the mixed population of various bacteria, antimicrobial agents, and nutrients in the sewage environment that provides a selective pressure for the emergence of antibiotic resistance strains or new resistant mutations in bacteria (Akya et al., 2020; Kalaiselvi et al., 2016). A number of studies (Cai et al., 2021; Hocquet et al., 2016; Tiwari et al., 2022; Wang et al., 2022)

monitored antibiotic resistance and associated genes in untreated hospital wastewater (HWW) and others compared between treated and untreated HWW (Asfaw et al., 2017; Katouli et al., 2012).

Hospitals generally release their effluents into sewer systems without prior treatment, therefore hospital effluents are a great source of antibiotic resistant bacteria (ARBs) and ARGs (Hocquet et al., 2016). Both primary and secondary treatment fail to effectively remove multidrug-resistant bacteria from sewage, according to studies on the effectiveness of effluent treatment plants (Sakkas et al., 2019). The role of aquatic ecosystems as the final point of entry of effluents from effluent treatment plants (WWTPs) or the direct disposal of sewage of human or animal origin makes them crucial for understanding how the AMR develops and spreads globally (Singh et al., 2022).

1.4. AIM AND OBJECTIVES

1.4.2 Aim

The aim of the study was to delineate the prevalence and antibiotic resistance profiles of ESKAPEE bacteria from hospital effluent in comparison with clinical isolates in terms of their resistome, virulome, mobilome and phylogeny.

1.4.3 Objectives

- To isolate and identify ESKAPEE pathogens from hospital effluent using the membrane filtration technique, selective culture media and confirmatory biochemical tests for comparison with clinical ESKAPEE isolates routinely processed at the National Health Laboratory Service (NHLS).
- To determine the antibiotic susceptibility profiles of clinical and effluent ESKAPEE isolates using the VITEK® 2 AST System (bioMérieux, North Carolina, USA).
- To compare and contrast the resistome, virulome, mobilome, clonality and phylogenies of clinical and effluent to establish associations (if any).

1.5. STUDY DESIGN AND METHODOLOGY

1.5.1 Study Design

This was a point-prevalence study to ascertain the prevalence and resistance profiles of the ESKAPEE pathogens from clinical and hospital effluent isolates at a regional hospital, in the uMgungundlovu District of KwaZulu-Natal province. The hospital has a bed capacity of 897 beds. The study was conducted during the month of November 2023. No patient data was collected.

1.5.2 Ethical Considerations

Ethical approval was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) (Reference No.: BREC/00003640/2021) (Appendix 1) and the Provincial Health Research Ethics Committee (Reference No.:KZ_202203_023) (Appendix 2). Permission to collect sewage samples was granted by the Chief Executive Officer of a regional hospital in the uMgungundlovu District, KwaZulu-Natal Province. Clinical isolates were obtained with permission from the National Health Laboratory Services (NHLS), a South African public institute for laboratory service, research and training (reference : PR2225862) (Appendix 3).

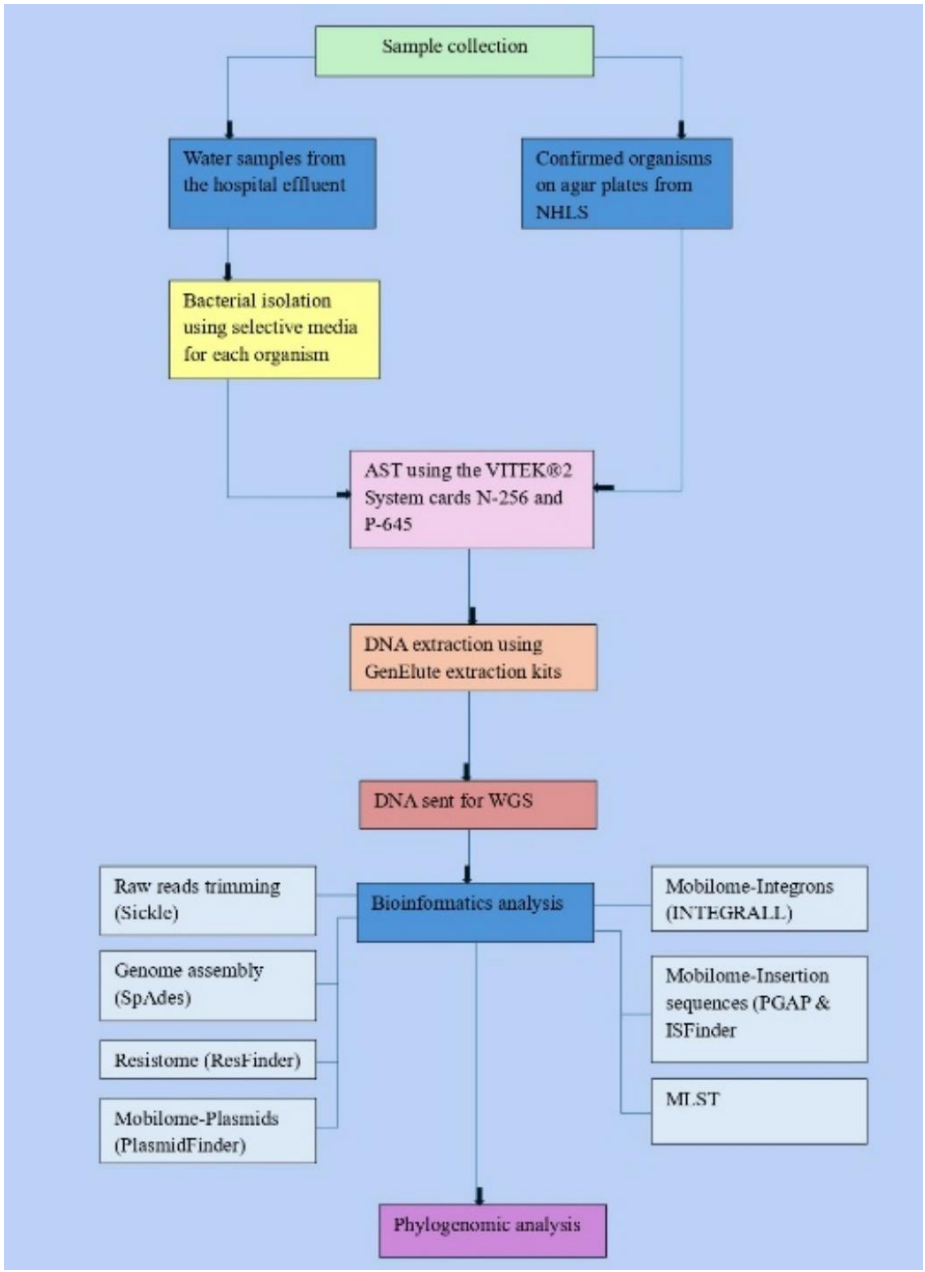

Figure 2: Summary of study methodology

1.5.2 Sample Collection

Sample size calculation was done using the randomised control trial (RCT) sample calculator as shown in Appendix 6. Hospital effluent samples were collected once from the final effluent discharge point that receives effluent from all the hospital wards and buildings. A composite sampling method was used, where water samples were manually grabbed over a period of 3 hours (30 seconds intervals); and pooled to create one sample. Clinical isolates on agar plates were received weekly during the month of November 2023 from the NHLS.

1.5.3 Bacterial isolation and identification

The membrane filtration technique was used to process the effluent samples where water samples were passed through a porous membrane (0.45 µm) (ISOLAB Laborgerāte GmbH, Eschau, Germany) using a filter funnel and vacuum system (ISOLAB Laborgerāte GmbH, Eschau, Germany). The membrane filter was applied onto agar plates containing selective media. *Enterococcus faecium* ChromoSelect Agar (Merck, Darmstadt, Germany) was used for the selective chromogenic detection of *E. faecium*, forming blue to blue-green colonies after incubation at 42ºC for 48 hours. Mannitol salt phenol-red agar (Merck, Darmstadt, Germany) was used as selective media for *S. aureus* where it appears as yellow colonies with yellow zones around them after 24 hours incubation at 37ºC. A coagulase test (Bactident™ Coagulase) (Merck, Darmstadt, Germany) was performed for further confirmation of *Staphylococcus aureus* species. For *K. pneumoniae* identification, Simmons Citrate Agar (Merck, Darmstadt, Germany) supplemented with Myo-Inositol (SCAI) and adjusted to a pH of 7.2 was used. After 24 hours at 37 ºC, yellow, moist colonies were produced. Eosin Methylene Blue (EMB) agar (Merck, Darmstadt, Germany) was used for identification of *A. baumannii*, and *Enterobacter* species. Colonies appeared as a faded-blue colour and dull pink with dark centres, respectively after 24 hours incubation at 37ºC. For *Pseudomonas aeruginosa*, Cetrimide agar (Merck, Darmstadt, Germany) was used on which colonies appeared in a blue-to-green pigment after incubation at 37ºC for 24 hours. CHROMagar™ ECC (CHROMagar™, Paris, France) was used for isolation and identification of *E. coli*. Dark blue colonies were observed after 24 hours incubation at 37ºC. The presumptive colonies were stored in Tryptone Soy Broth (TSB) supplemented with 20% glycerol at -80ºC for future use.

1.5.4 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed using the VITEK® 2 System AST (bioMérieux, North Carolina, USA) card N-256 for Gram-Negative and P-645 for Gram-Positive isolates. The antibiotic panel for the Gram-negative bacteria consisted of ampicillin, cefuroxime, ceftazidime, cefotaxime, cefepime, amoxicillin/clavulanic acid, piperacillin/tazobactam, imipenem, doripenem, meropenem, ertapenem, gentamicin, tobramycin, amikacin, ciprofloxacin, trimethoprim/sulfamethoxazole and tigecycline. For the Gram-positive bacteria, the antibiotic panel consisted of gentamicin, tetracycline, erythromycin, vancomycin, teicoplanin, ciprofloxacin and linezolid. Tigecycline, doxycycline, erythromycin, fosfomycin and levofloxacin was added for *E. faecium* only. Quality control organisms were processed according to the test card setup procedure. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used for card N-256. *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 were used for card P-645. The results were interpreted as resistant, intermediate or susceptible according to the Clinical & Laboratory Standard Institute (CLSI) guidelines (CLSI, 2022).

1.5.5 Whole Genome Sequencing and Bioinformatics

Genomic deoxyribonucleic acid (DNA) was extracted from selected isolates exhibiting phenotypic resistance to at least one antibiotic. DNA was extracted using the GenElute bacterial genomic DNA kit (Sigma Aldrich, St. Loius, MO, USA). The quality of the DNA was assessed using NanoDrop Lite Plus (Thermo Fisher Scientific, Wilmington, DE, USA) at 260/280 nm wavelength. WGS using the Illumina Miseq machine (Illumina, San Diego, CA, USA) was performed at the Nation Institute for Communicable Diseases (NICD) Sequencing Core Facility. Raw reads were trimmed using Sickle (https://github.com/najoshi/sickle) for quality trimming and assembled using the SPAdes assembler. Assembled sequences were annotated using ResFinder (http://genepi.food.dtu.dk/resfinder) at threshold ID (90%) and minimum length (60%) to identify resistance genes. PlasmidFinder 2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/) was used to identify the plasmid replicons. The INTEGRALL database was used to identify and classify integrons and gene cassettes. NCBI's PGAP and ISFinder (https://isfinder.biotoul.fr/) were used to annotate transposons and insertion sequences. MLST 2.0 (https://cge.food.dtu.dk/services/MLST/) was used to identify the sequence types of the isolates.

1.5.6 Phylogenomic analysis

Whole Genome Sequences of ESKAPEE isolates from this study were compared to clinical and environmental isolates from African countries curated at the BV-BRC (https://www.bv-brc.org/) website. The downloaded sequences for each organism were used alongside the sequences from this study to construct a phylogenetic tree on BV-BRC. The constructed phylogenetic trees were annotated, viewed and edited using iTOL (https://itol.embl.de/).

1.6 DISSERTATION STRUCTURE

This dissertation is presented in three chapters as follows:

- **Chapter 1. Introduction and Literature review:** this section covers the history of antibiotics and antibiotic resistance, AMR in the ESKAPEE pathogens, and AMR in hospitals and effluent. It also includes the aims objectives and methodology.
- **Chapter 2. Drug resistant *Klebsiella pneumoniae* from patients and effluent: s correlation:** this chapter reports on the antimicrobial susceptibility profiles of *K. pneumoniae* from patients and hospital effluent, the presence of ARGs and their association with MGEs, and their phylogenomic relationships in a manuscript submitted to the BioMed Central (BMC) Microbiology and is under peer review (Appendix 7). Results of the remaining ESKAPEE pathogens are presented in the appendices due to the small number of clinical and effluent isolates obtained for each organism.
- **Chapter 3. Conclusion:** includes the conclusion, limitations and recommendations from the study.

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CHAPTER TWO

This dissertation is in a manuscript format (as per the requirement of the College of Health Sciences at the University of KwaZulu-Natal) as follows:

Drug resistant *Klebsiella pneumoniae* from patients and hospital effluent: a correlation?

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Contributions:

Ms Naledi Masalane, as the principal investigator, co-conceptualized the study, undertook sample collection, laboratory work, results analysis, and manuscript drafting.

Dr Linda Bester, as the co-supervisor, guided on literature review antimicrobial susceptibility testing, review of the manuscript.

Prof Sabiha Essack, as the co-supervisor, co-conceptualized the study, guided literature review and ethical clearance, undertook critical review of the manuscript.

Dr Joshua Mbanga, as the main supervisor, co-conceptualized the study, guided literature review, assisted with sample collection, laboratory work and data analysis, and critically reviewed the manuscript.

Drug resistant *Klebsiella pneumoniae* from patients and hospital effluent: a correlation?

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Abstract

Background: *Klebsiella pneumoniae* is a major cause of hospital-acquired infections and implicated in urinary, respiratory and bloodstream infections, as well as infections in patients in intensive care units. Hospital effluent serves as a channel for the disposal of waste materials, including, faecal matter, clinical and biological wastes, and used clinical supplies that could potentially be contaminated with pathogenic bacteria. **Aim:** This study compared *Klebsiella pneumoniae* isolates from patients and hospital effluent in terms of antibiotic resistance patterns, antibiotic resistance genes (ARGs), mobile genetic elements (MGEs) and phylogenomic relationships. **Methodology:** This point-prevalence study was conducted by collecting pooled effluent samples from the final effluent point of a regional hospital in the uMgungundlovu district, Kwa-Zulu Natal, South Africa and presumptively identified on selective media. Clinical isolates were also collected concurrently from the same hospital. Antimicrobial susceptibility testing (AST) was performed using the VITEK® 2 system. DNA was extracted using the GenElute extraction kits prior to whole genome sequencing. The resistome, mobilome, and phylogenetic lineages of sequenced isolates were assessed using bioinformatics analysis. ResFinder, PlasmidFinder, INTEGRALL and PGAP & ISFinder were used to annotate and identify resistance genes, plasmids, integrons and insertion sequences & transposons, respectively. MLST was used to identify sequence types. BV-BRC was used to construct the phylogenetic tree, and iTOL was used to view, edit and annotate the generated phylogenetic tree. **Results:** A total of 10 randomly selected presumptive and 10 clinical *K. pneumoniae* constituted the sample and were subjected to AST. Whole Genome Sequencing (WGS) positively confirmed 10 effluent and six clinical isolates as *K. pneumoniae*. High resistance rates (100%) were observed in the clinical samples to cefuroxime, cefotaxime, piperacillin/tazobactam, gentamicin, tobramycin and trimethoprim/sulfamethoxazole . The effluent isolates exhibited total susceptibility to most antibiotics but showed resistance to amoxicillin/clavulanic acid (100%), piperacillin/tazobactam (100%) and tigecycline (10%). The effluent isolates did not exhibit a diverse resistome, while the clinical isolates harboured genes conferring resistance to aminoglycoside (*aph(6)-Id, aph(3")-Ib, aac(6')-Ib-cr*, *aadA16*), ß-lactam (*bla*$_{SVH}$ group, *bla*$_{OXA}$ group, *bla*$_{TEM}$ group), and fluoroquinolone (*oqxA, oqxB*) antibiotics. Most clinical isolates had ARGs associated with MGEs with the most common plasmid replicon IncFIB(K) identified in both clinical and effluent isolates. *Bla*$_{CTX-M-15}$ was associated with Tn3 and co-carried with insertion sequences ISEcp1, ISKpn11, ISKpn12, IS3. Only class 1 integrons were identified. ST152 was the most commonly reported sequence type. Phylogenetic analysis revealed that effluent isolates from this study were not closely related to the clinical isolates. **Conclusion:** While the effluent is a simple way to monitor the spread of antibiotic-resistant bacteria from hospitals to the environment, this study showed no correlation between the resistance profiles of the clinical isolates and the profiles of the effluent isolates. Findings from this study highlight the need to further monitor the hospital effluent to track the spread of resistance from clinical isolates.

Keywords: *Klebsiella pneumoniae*, antimicrobial resistance, effluent

2.1 Introduction

Klebsiella pneumoniae is a Gram-negative opportunistic bacteria and normal flora of the skin, mouth and gut belonging to the order Enterobacterales, and a major cause of hospital-acquired infections mainly responsible for urinary, respiratory and bloodstream infections (Abbas et al., 2024; Aires-de-sousa et al., 2019). It is a part of the *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp, and *Escherichia coli* (ESKAPEE), a group of pathogens that are increasingly associated with several multi-drug resistant nosocomial infections (Ruekit et al., 2022). They carry antimicrobial resistance (AMR) genes (ARGs) on mobile genetic elements (MGEs) that encode resistance to the most commonly-used classes of antibiotics (Hubeny et al., 2022; Ragupathi et al., 2019). These ARGs and MGEs are present in various environments, and can spread among different species of microorganisms, humans and animals, soil, and water (Endale et al., 2023). Carbapenem-resistant and 3rd-generation cephalosporin-resistant Enterobacterales are on the World Health Organization's (WHO) bacterial priority pathogens list for research and development of new antibiotics (WHO, 2024).

The development and rapid spread of AMR increases the risk of common infections becoming impossible to treat (WHO, 2023). AMR, primarily caused by use, misuse or overuse of antimicrobial agents, is a serious global health concern claiming above 700 000 lives each year (O'Neill, 2014). AMR is an increasing threat to healthcare systems resulting in the reduced ability of antimicrobial therapies to effectively combat infections and leading to increased morbidity and mortality rates (Meybodi et al., 2021). Drug-resistant bacteria are usually spread by direct contact of the infected patient with staff, other patients, and visitors, while clinical surfaces and hospital effluent are potential reservoirs of multi-drug resistant (MDR) bacteria (Gashaw et al., 2024).

Hospital effluent serves as a channel for the disposal of waste materials, including, faecal matter, clinical and biological wastes, and used clinical supplies that could potentially be contaminated with pathogenic bacteria (Gashaw et al., 2024; Nakamura-Silva et al., 2022b). Such effluent contains large quantities of diverse ARGs and their associated MGEs (Hubeny et al., 2022). This makes them reservoirs and hotspots for the prevalence and dissemination of drug-resistant strains via effluent (Karkman et al., 2018; Nakamura-Silva et al., 2022b). Hospital effluents are an ideal environment for exchange of resistance genes between clinical and environmental pathogens; given the mixed population of various bacteria, antimicrobial agents, and nutrients in the sewage environment (Akya et al., 2020; Kalaiselvi et al., 2016).

This study delineates the prevalence and antibiotic resistance profiles of *Klebsiella pneumoniae* from hospital effluent in comparison with clinical isolates by whole genome sequencing and bioinformatics to delineate their resistomes, mobilomes and phylogeny in a regional hospital, KwaZulu-Natal.

2.2 Methodology

Ethical Considerations

Ethical approval was granted by the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC) (Reference No.: BREC/00003640/2021) (Appendix 1) and the Provincial Health Research Ethics Committee (Reference No.:KZ_202203_023) (Appendix 2). Permission to collect sewage samples was granted by the Chief Executive Officer of a regional hospital in the uMgungundlovu District, KwaZulu-Natal Province. Clinical isolates were obtained with permission from the National Health Laboratory Services (NHLS), a South African public institute for laboratory service, research and training (reference : PR2225862) (Appendix 3). No patient data was collected.

Sample Collection

Water samples were collected from the final effluent discharge point that receives effluent from all the hospital wards and buildings at a regional hospital which has a bed capacity of 897 beds. A composite sampling method was used, where water samples were manually grabbed over a period of 3 hours (30 seconds intervals); and pooled to create one sample. Clinical isolates on agar plates were received weekly for the duration of the study from the National Health Laboratory Services (NHLS). Sampling was conducted during November 2023.

Bacterial isolation and identification

The membrane filtration technique was used to process the effluent samples where water samples were passed through a porous membrane (0.45 µm) (ISOLAB Laborgerāte GmbH, Eschau, Germany) using a filter funnel and vacuum system (ISOLAB Laborgerāte GmbH, Eschau, Germany). The membrane filter was applied onto agar plates containing selective media for *K. pneumoniae* identification, Simmons Citrate Agar (Merck, Darmstadt, Germany) supplemented with Myo-Inositol (SCAI) and adjusted to a pH of 7.2 was used. After 24 hours at 37 ºC, yellow, moist colonies were produced.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was performed using the VITEK® 2 System AST (bioMérieux, North Carolina, USA) card N-256 for Gram-Negative for the following panel of antibiotics: cefuroxime, ceftazidime, cefotaxime, cefepime, amoxicillin/clavulanic acid, piperacillin/tazobactam, imipenem, doripenem, meropenem, ertapenem, gentamicin, tobramycin, amikacin, ciprofloxacin, trimethoprim/sulfamethoxazole and tigecycline.

Whole Genome Sequencing and Bioinformatics

Genomic deoxyribonucleic Acid (DNA) was extracted from selected isolates exhibiting phenotypic resistance to at least one antibiotic. DNA was extracted using the GenElute bacterial genomic DNA kit (Sigma Aldrich, St. Loius, MO, USA). The quality of the DNA was assessed using NanoDrop Lite Plus (Thermo Fisher Scientific, Wilmington, Delaware, USA) at 260/280 nm wavelength. WGS using the

Illumina Miseq machine (Illumina, San Diego, California, USA) was performed at the Nation Institute for Communicable Diseases (NICD) Sequencing Core Facility. Raw reads were trimmed using Sickle (https://github.com/najoshi/sickle) for quality trimming and assembled using the SPAdes assembler. Assembled sequences were annotated using ResFinder (http://genepi.food.dtu.dk/resfinder) (Bortolaia et al., 2020; Camacho et al., 2009) at a threshold ID of 90% and minimum length of 60% to identify resistance genes. PlasmidFinder 2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/) was used to identify the plasmid replicons. The INTEGRALL database was used to identify and classify integrons and gene cassettes. NCBI's PGAP and ISFinder (https://isfinder.biotoul.fr/) were used to annotate transposons and insertion sequences. The Comprehensive Antimicrobial Resistance Database (CARD) was used to identify the efflux pump proteins (https://card.mcmaster.ca/) (Alcock et al., 2023). MLST 2.0 (https://cge.food.dtu.dk/services/MLST/) was used to identify the sequence types of the isolates. The sequences were submitted to GenBank under BioProject PRJNA1143548.

Phylogenomic analysis

Whole genome sequences of *K. pneumoniae* isolates from this study were compared to clinical and environmental isolates from South Africa and other African countries curated at the BV-BRC (https://www.bv-brc.org/) website. The downloaded sequences for each organism were used alongside the sequences from this study to construct a phylogenetic tree on BV-BRC. The constructed phylogenetic tree was annotated, viewed and edited using iTOL (https://itol.embl.de/).

Data analysis

Data was arranged and analysed using Microsoft® Excel® version 2501 (Microsoft Corporation, Redmond, Washington State, USA). A bar chart representing the resistance rates of the clinical and effluent isolates was produced. Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 29 (IBM, Armonk, NY, USA). An independent samples t test was run on the percentage resistance rates between the clinical and hospital effluent isolates. Two-tailed $p < 0.05$ was considered significant.

2.3 Results

Prevalence of *Klebsiella pneumoniae* in clinical samples and hospital effluent

A total of 20 *Klebsiella pneumoniae* isolates from clinical (10) and hospital effluent (10) were subjected to antimicrobial susceptibility testing and WGS. Sixteen of the isolates were positively identified as *K. pneumoniae* by WGS. These consisted of 10 effluent and 6 clinical isolates. The non- *K. pneumoniae* isolates identified after WGS included *Klebsiella quasi-pneumoniae*, *Klebsiella variicola* and *Klebsiella aerogenes* (2). The results presented relate only to the positively identified *K. pneumoniae* isolates.

Antibiotic Susceptibility profiles

The clinical *K. pneumoniae* isolates showed overall high resistance rates compared to the effluent isolates, which can be explained by use of these antibiotics in the clinical setting. All the clinical isolates were resistant to at least seven or more antibiotics belonging to three or more classes, while the effluent isolates were resistant to at least two antibiotics. High (100%) resistance rates were observed in the clinical *K. pneumoniae* against cefuroxime, cefotaxime, piperacillin/tazobactam, gentamicin, tobramycin and trimethoprim/sulfamethoxazole (**Figure *1***).

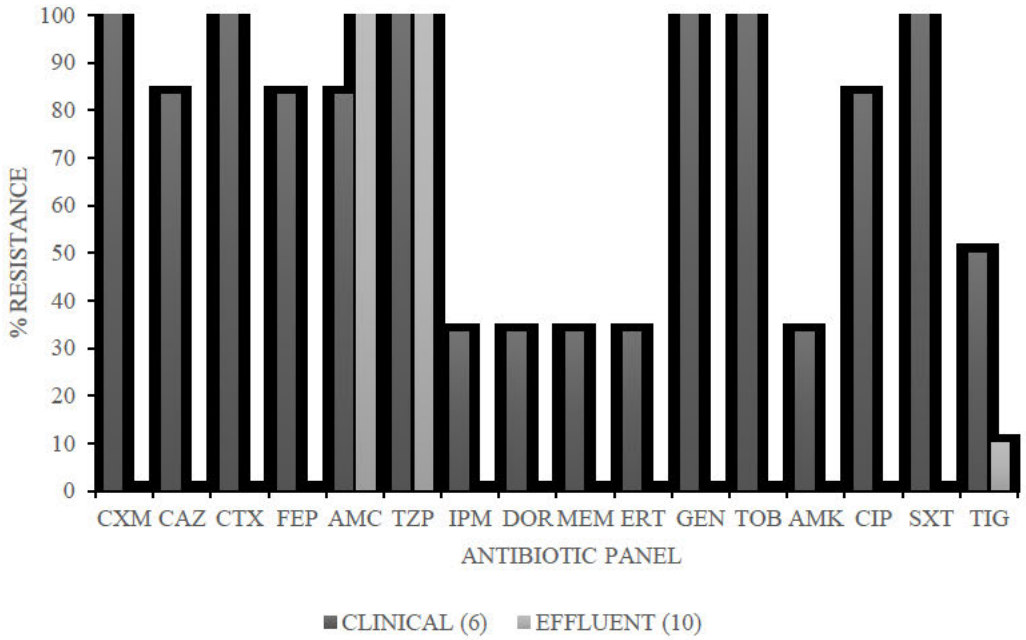

Figure 1: Antibiotic resistance rates of the *K. pneumoniae* from human and hospital effluent.

CXM=cefuroxime, CAZ=ceftazidime, CXT=cefotaxime, FEP=cefepime, AMC=amoxicillin/clavulanic acid, TZP=piperacillin/tazobactam, IPM=imipenem, DOR=doripenem, MEM=meropenem, ERT=ertapenem, GEN=gentamicin, TOB=tobramycin, AMK=amikacin, CIP=ciprofloxacin, SXT=trimethoprim/sulfamethoxazole, TIG=tigecycline.

The effluent isolates exhibited total susceptibility against all tested antibiotics except amoxicillin/clavulanic acid and piperacillin/tazobactam to which they were 100% resistant and

tigecycline (10%). Only 33.33% of the clinical isolates were resistant to the carbapenem antibiotics and the same isolates showed resistance to amikacin (**Figure 1**). Statistical analysis revealed that there was a significant difference between the resistance rates of the clinical isolates compared to the effluent isolates, $p < 0.001$.

Genomic Characteristics

The genomic features of the *Klebsiella pneumoniae* are presented in terms of genome size, GC content, sequence length, L50 and N50 values in the supplementary material (**Table S 1**).

Antibiotic resistance genes

The clinical *K. pneumoniae* isolates harboured more resistance genes compared to the effluent isolates. All the clinical isolates carried several ß-lactam resistance genes, with the most common genes being $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1B}$ found in all six isolates. In the effluent isolates, only 50% harboured the ß-lactam resistance gene $bla_{SHV\text{-}1}$, which is responsible for intrinsic resistance to ampicillin in *K. pneumoniae,* whereas in the clinical isolates, five different bla_{SHV} genes were identified ($bla_{SHV\text{-}81}$, $bla_{SHV\text{-}94}$, $bla_{SHV\text{-}96}$, $bla_{SHV\text{-}110}$, $bla_{SHV\text{-}172}$). $Bla_{SHV\text{-}187}$ was found only in one effluent isolate. The $bla_{NDM\text{-}1}$ gene was only present in the two (33.33%) clinical isolates. The extended-spectrum β-lactamase (ESBL) bla_{SHV} *(SHV-172, SHV-96, SHV-94, SHV-110* and *SHV-81)*, $bla_{CTX\text{-}M\text{-}15}$, and bla_{TEM} *(TEM-206, TEM-141, TEM-1B* and *TEM-214)* genes were co-carried in the clinical isolates (**Table 1**).

Aminoglycoside resistance genes (*aph(6)-Id, aph(3")-Ib, aac(6')-Ib-cr* and *aadA16*) were the most common among the clinical isolates. The effluent isolates did not harbour any aminoglycoside resistance genes, which was in line with their phenotypic resistance patterns (**Table 1**).

Table 1: Antibiotic resistance genes, resistance patterns, efflux resistance mechanisms and plasmids found in the *Klebsiella pneumoniae* pathogens from clinical and hospital effluent samples

Isolate ID	Source of isolation	Resistance pattern	*Resistance genes*	Plasmid replicons	Efflux mechanism of resistance
CKP1	Clinical	CXM-CTX-TZP-TOB-CIP-SXT-TIG	*blaTEM-206, blaTEM-141, blaTEM-1B, blaOXA-1, blaSHV-110, blaSHV-81, blaCTX-M-15, blaTEM-214, aph(6)-Id, aph(3")-Ib, aac(6')-Ib-cr, sul2, dfrA14, qnrB1*	IncFIB(K), IncFIB(pKPHS1), IncFII(K)	*Klebsiella pneumoniae* KpnF, LptD, H-NS, emrR, *Klebsiella pneumoniae* KpnG, *Klebsiella pneumoniae* KpnH, marA, *Klebsiella pneumoniae* KpnE
CKP2	Clinical	CXM-CAZ-CTX-FEP-AMC-TZP-IPM-DOR-MEM-ERT-GEN-TOB-AMK-CIP-SXT	*blaSHV-172, blaSHV-96, blaSHV-94, blaCTX-M-15, blaTEM-1B, blaOXA-181, blaNDM-1, aac(3)-IIa, aph(6)-Id, aac(6')-Ib-cr, aph(3")-Ib, aadA16, armA, aadA1, sul1, sul2, dfrA27, qnrS1, OqxB, OqxA, qacE*	ColKP3, IncFIA(HI1), IncFIB(K), IncFIB(pNDM-Mar), IncFII(K), IncHI1B(pNDM-MAR), IncR, IncX3	*Klebsiella pneumoniae* KpnE, *Klebsiella pneumoniae* KpnF, oqxA, cmlA5, qacEΔ1, AcrAB-TolC
CKP4	Clinical	CXM-CAZ-CTX-FEP-AMC-TZP-GEN-TOB-CIP-SXT-TIG	*blaCTX-M-15, blaOXA-1, blaTEM-1B, aph(6)-Id, aph(3")-Ib, aac(3)-Iia, aadA16, aac(6')-Ib-cr, sul2, sul1, dfrA27, OqxB, OqxA, qnrB6, tet(D), qacE*	ColRNAI, IncFIB(K), repB(R1701)	*Klebsiella pneumoniae* KpnF, LptD, oqxA, tet(D), qacEΔ1, adeF, H-NS, AcrAB-TolC, CRP
CKP5	Clinical	CXM-CAZ-CTX-FEP-AMC-TZP-GEN-TOB-SXT	*blaCTX-M-15, blaTEM-1B, blaSHV-172, blaSHV-96, blaSHV-94, blaSCO-1, aadA16, aac(6')-Ib-cr, aac(3)-Iia, aph(6)-Id,, aph(3")-Ib, sul1, sul2, dfrA27, OqxB, OqxA, qacE*	IncFIA(HI1), IncFIB(K), IncFII(K), IncR	*Klebsiella pneumoniae* KpnE, *Klebsiella pneumoniae* KpnF, oqxA, LptD, qacEΔ1, adeF, *Klebsiella pneumoniae* KpnG
CKP8	Clinical	CXM-CAZ-CTX-FEP-AMC-TZP-IPM-DOR-MEM-ERT-GEN-TOB-AMK-CIP-SXT	*blaCTX-M-15, blaTEM-1B, blaSHV-172, blaSHV-96, blaSHV-94, blaOXA-181, blaNDM-1, aac(3)-Iia, , aph(6)-Id, aph(3")-Ib, armA, aadA16, aac(6')-Ib-cr, sul1, sul2, dfrA27, aadA1, qnrS1, OqxB, OqxA, qacE*	ColKP3, IncFIA(HI1), IncFIB(K), IncFIB(pNDM-Mar), IncFII(K), IncHI1B(pNDM-MAR), IncR, IncX3	*Klebsiella pneumoniae* KpnE, *Klebsiella pneumoniae* KpnF, oqxA, cmlA5, qacEΔ1

CKP10	Clinical	CXM-CAZ-CTX-FEP-AMC-TZP-GEN-TOB-CIP-SXT-TIG	*blaCTX-M-15, blaTEM-1B, blaSHV-172, blaSHV-96, blaSHV-94,aac(3)-Iia, aph(6)-Id, aph(3")-Ib, aadA16, aac(6')-Ib-cr, sul2, sul1, dfrA27, OqxB, OqxA, qnrB6, qacE*	IncFIA(HI1), IncFIB(K), IncFII(K), IncR	OqxA, *Klebsiella pneumoniae* KpnE, *Klebsiella pneumoniae* KpnF, qacEΔ1,
SEB1	Effluent	AMC-TZP	*blaSHV-1, OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxA, marA, KpnEF, H-NS, adeF, KpnH
SEB2	Effluent	AMC-CTX-TZP	*blaSHV-1, OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxAB, marA, KpnEF, H-NS, adeF, KpnH
SEB3	Effluent	AMC-TZP-TIG	*OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxA, tet(D), H-NS, adeF, emrR, qacG, KpnEF, LptD, marA
SEB4	Effluent	AMC-TZP	*blaSHV-1, OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, marA, tet(D), oqxA, KpnG, KpnEF
SEB5	Effluent	AMC-TZP	*OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxA, tet(D), H-NS, adeF, emrR, qacG, KpnEF, LptD, marA
SEB6	Effluent	AMC-TZP	*blaSHV-1, blaSHV-187, OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxA, tet(D), H-NS, adeF, emrR, qacG, KpnEF,
SEB7	Effluent	AMC-TZP	*OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxA, tet(D), H-NS, adeF, emrR, qacG, KpnEF, LptD, marA
SEB8	Effluent	AMC-TZP	*OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxA, tet(D), H-NS, adeF, emrR, qacG, KpnEF, LptD, marA
SEB9	Effluent	AMC-TZP	*OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxA, tet(D), H-NS, adeF, emrR, qacG, KpnEF, LptD, marA

SEB10	Effluent	AMC-TZP	*blaSHV-1, OqxB, OqxA, tet(D)*	IncFIB(K)	MdtQ, oqxA, tet(D), H-NS, adeF, emrR, qacG, KpnEF, LptD

CXM=cefuroxime, CAZ=ceftazidime, CXT=cefotaxime, FEP=cefepime, AMC=amoxicillin/clavulanic acid, TZP=piperacillin/tazobactam, IPM=imipenem, DOR=doripenem, MEM=meropenem, ERT=ertapenem, GEN=gentamicin, TOB=tobramycin, AMK=amikacin, CIP=ciprofloxacin, SXT=trimethoprim/sulfamethoxazole, TIG=tigecycline

The clinical isolates also harboured the fluoroquinolone resistance genes *qnrB1*(1), *qnrB6*(2) and *qnrS1*(2). *OqxA* and *oqxB* that encode the multidrug efflux pump *oqxAB* was found in five (83.33%) of the six clinical isolates. The effluent isolates, although not phenotypically resistant to a number of the tested antibiotics, also harboured the genes encoding the multidrug efflux pump *oqxAB*. Trimethoprim-sulfamethoxazole resistance genes *sul1*, *sul2*, and *dfrA27* were carried by five of the clinical isolates, while the other one harboured *sul2* with *dfrA14*.

No tetracycline resistance genes were carried by the clinical isolates that showed resistance to tigecycline, while the effluent *K. pneumoniae* showed the tetracycline resistance gene *tetD* , although only one showed phenotypic resistance to tigecycline. Five of the clinical isolates carried the *qacE* gene that encodes resistance to quaternary ammonium compounds.

Mobile Genetic Elements and their association with ARGs

The effluent *K. pneumoniae* isolates had only one plasmid replicon type present (IncFIB(K)), which was also common in the clinical isolates. Other plasmid replicon types in the clinical isolates included IncFIA(HI1), IncFII(K), IncHI1B(pNDM-MAR), repB(R1701), colKP3, IncR, and IncFIB(pKPHS1). Resistance genes were carried on insertion sequences and/or transposable elements, and just a few carried on *intI1* integron. Most ARGs on the effluent *K. pneumoniae* isolates were not associated with mobile genetic elements. Resistance gene *blaCTX-M-15* was caried on the transposon Tn3 on all the clinical isolates that harboured the gene and co-carried in combination with other resistance genes (*aac(3)-IIa* and *blaTEM-1B*) on insertion sequences (ISEcp1, ISKpn11, ISKpn12, IS3) on three of the isolates (CKP2, CKP5 and CKP8). This combination of ARGs was also bracketed by insertion sequences IS1, ISKpn26 on CKP and CKP8 and co-carried by ISNCY and IS1X2 on CKP8.

Table 2: Mobile genetic elements associated with antibiotic resistance genes

SAMPLE ID	MLST	CONTIGS	SYNTENY OF RESISTANCE GENES AND MGEs	PLASMID/CHROMOSOMAL SEQUENCE WITH CLOSEST NUCLEOTIDE HOMOLOGY (ACCESSION #)
CKP1	ST348	4	*blaSHV-110*:*blaSHV-11*::IS3	*Klebsiella pneumoniae* strain Kp_2016-319 chromosome (CP085101.1) (99.58%)
		41	IS1::*DfrA14*:Intl1	*Klebsiella pneumoniae* strain 1050 plasmid pKp1050-4 (CP023420.1) (100%)
		44	ISEcp1: *blaCTX-M-15*:Tn3	*Klebsiella pneumoniae* strainUHD-33_plasmid pESBL-PH-33 (CP121138.1) (100%)
		47	*QnrB1*:IS3000:Tn3	*Klebsiella pneumoniae* isolates 307 plasmid: P1 (OW48947.1) (100%)
		48	*blaTEM-206*:*blaTEM-141*:*blaTEM-1B*:*blaTEM-214*:IS91:*APH(6)-Id*:*APH(3")-Ib*:*sul2*	*Klebsiella pneumoniae* isolates 307 plasmid P:1 (OW48947.1) (100%)
CKP2	ST17	30	Tn3:*blaCTX-M-15*:ISEcp1:*blaTEM-1B*:*AAC(3)-IIa*:ISE3:ISKpn11:ISKpn12:ISKpn26:IS1:ISNCY	*Klebsiella pneumoniae* strain KpST17-2117 plasmid pKp2117_1 (CP075592.1)
		41	Tn3:IS3000:*blaOXA-181*::ISKpn19:QnrS1	*Klebsiella pneumoniae* strain RIMV _C019688 plasmid pRIVM_C019688_3 (CP068958.1) (100%)
		42	IS110:ISPa38:*APH(6)-Id*;*APH(3")-Ib*:*Sul2*	*Klebsiella pneumoniae* strain 1613015451 plasmid p3-1613015451 (CP083069.1) (99%)
		43	ISEc33:*blaNDM-1*:IS91	*Klebsiella pneumoniae* C105 plasmid pC105-NDM1-IncHI3 (MN240794.1) (99%)
		44	*Mph(E)*:*msr(E):* ISEc29:IS5:ISCR1	*Klebsiella pneumoniae* strain BA10835 plasmid pvirulence_VBA10835 (CP053766.1) (99%)
		74	*Sul1*:QacEΔ1	*Klebsiella pneumoniae* E196 plasmid pE196_IMP6 DNA (AP019405.1) (100%)
CKP4	ST152	56	*blaTEM-1B*:IS91:*APH(6)-Id*:*APH(3")-Ib*:*sul2*	*Klebsiella pneumoniae* isolate 392 plasmid: P1 (OW968235.1) (100%)
		59	*QnrB6:*ISCR1	*Klebsiella pneumoniae* strain KP32558 plasmid pKP32558-2-mcr8 (CP076032.1) (100%)
		60	Tn3:*blaCTX-M-15*	*Klebsiella pneumoniae* isolate 307 plasmid: P1 (OW848947.1) (100%)
		61	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)

		62	MphR(A)::*Mph(A)*	*Klebsiella pneumoniae* isolate 11 plasmid: P1 (OW969612.1) (100%)
		66	IS3:*AAC(3)-IIa*	*Klebsiella pneumoniae* isolate 307 plasmid: P1 (OW848947.1) (100%)
		72	IS1:*catA1*	*Klebsiella pneumoniae* isolate 11 plasmid: P1 (OW969612.1) (100%)
		79	*Sul1*:QacEΔ1	*Klebsiella pneumoniae* E196 plasmid pE196_IMP6 DNA (AP019405.1) (100%)
CKP5	ST17	27	IS1:::*Sul1*:QacE:*AadA16*:*DfrA27*:*Arr-3*:*AAC(6')-Ib-cr*:IntI1	*Klebsiella pneumoniae* isolate 307 plasmid: P2 (OW849061.1) (99.99%)
		28	ISKpn12:ISKpn11:IS3:*AAC(3)-IIa*:*blaTEM-1*:ISEcp1:*blaCTX-M-15*:Tn3	*Klebsiella pneumoniae* strain KpST17-2177 plasmid pKp2177_1 (CP075592.1) (100%)
CKP8	ST17	18	IS1:IS3:IS1X2:ISKpn25:ISL3:ISNCY:ISKpn26:ISKpn12:ISKpn11:*AAC(3)-IIa*:*blaTEM-1*:ISEcp1:*blaCTX-M-15*:Tn3	*Klebsiella pneumoniae* strain KpST17-2177 plasmid pKp2177_1 (CP075592.1) (100%)
		39	Tn3:IS3000:*blaOXA-48*:EreA:ISKpn19	*Klebsiella pneumoniae* strain RIVM_C019688 plasmid pRIVM_C019688_3 (CP068958.1) (100%)
		40	ISAba125:ISEc33:*blaNDM-1*:IS91	*Klebsiella pneumoniae* strain CDC 0106 plasmid unitig_1 (CP022612.1) (100%)
		41	ISpa38:*APH(6)-Id*:*APH(3")-Ib*:*Sul2*	*Klebsiella pneumoniae* strain MAKM-3381 plasmid (CP129540.1) (99.98%)
		42	*Mph(E)*:*Msr(E)*:ISEc29:IS5:ISCR1	*Klebsiella pneumoniae* strain BA10835 plasmid pvirulence_VBA10835 (CP053766.1) (99%)
		43	*AadA16*:*dfrA27*:*Arr-3*:*AAC(6')-Ib-cr*:IntI1:Tn3:IS4321	*Klebsiella pneumoniae* KP17 plasmid pKP17_NDM1 DNA (LC521852.1) (98.85%)
		72	*Sul1*:QacEΔ1	*Klebsiella pneumoniae* E196 plasmid pE196_IMP6 DNA (AP019405.1) (100%)
CKP10	ST17	26	*BlaCTX-M-5*:ISEcp1:*blaTEM-1*:*AAC(3)-IIe*:IS3:ISKpn11:ISKpn12:ISKpn26:IS1:ISNCY:	
		33	ISpa38:*APH(6)-Id*:*APH(3")-Ib*:*Sul2*	*Klebsiella pneumoniae* strain MAKM-3381 plasmid (CP129540.1) (99.98%)
		35	IS1:::*Sul1*:QacEΔ1	*Klebsiella pneumoniae* isolate 307 plasmid: P2 (OW849061.1) (100%)
		36	*AadA16*:*DfrA27*:*Arr-3*:*AAC(6')-Ib-cr*:IntI1	*Klebsiella pneumoniae* strain HUM7199 plasmid pHUM7199_B (CP093316.1) (100%)

		39	*QnrB6*:ISCR1	*Klebsiella pneumoniae* strain KP32558 plasmid pKP32558-2-mcr8 (CP076032.1) (100%)
SEB1	ST29	40	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		42	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)
		40	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
SEB2	ST29	5	Recombinase:*OqxB*:*OqxA*	*Klebsiella pneumoniae* isolate INF171-sc-2280028 chromosome:1 (LR890446.1) (99.93%)
		37	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		41	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)
SEB3	ST29	37	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		39	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)
SEB4	ST29	39	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		42	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)
		39	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
SEB5	ST29	38	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		40	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)
SEB6	ST29	36	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		39	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)
SEB7	ST29	38	IS1B:*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		41	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)

SEB8	ST29	38	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		40	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)
SEB9	ST29	38	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 512 plasmid: P1 (OW967602.1) (100%)
		40	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)
SEB10	ST29	38	*CatA1*:TnAs3	*Klebsiella pneumoniae* isolate 307 plasmid: P1 (OW848947.1) (100%)
		39	TetR(D):*Tet(D)*	*Klebsiella pneumoniae* strain S166-1 plasmid pS166-1.1 (CP063946.1) (100%)

In all the effluent isolates, the *catA1* gene was carried on TnAs3, and co-carried on insertion sequence IS1B in one isolate; while only 1 of the clinical isolates harboured the gene and carried on insertion sequence IS1. Only one clinical isolate harboured *blaOXA-48* that was carried on Tn3 transposon and IS300 and ISKpn19 insertion sequences. All the effluent isolates did not have the class 1 integron, while 4 out of 6 clinical isolates had the class 1 integrase gene intI1(Table 3). In1021, which contained the gene cassette *aacA4cr-arr3-dfrA27-aadA16* was common in 3 of the clinical *K. pneumoniae*, the other isolate harboured the *dfrA14* gene cassette.

Table 3: Class 1 integrons and gene cassettes found in the clinical *K. pneumoniae* isolates

ISOLATE ID (MLST)	**INTEGRON**	**Cassette arrays**			
		GC1	**CG2**	**GC3**	**GC4**
CKP1 (ST348)	None	*dfrA14*	-	-	-
CKP5 (ST17)	In1021	*aacA4cr*	*arr-3*	*dfrA27*	*aadA16*
CKP8 (ST17)	In1021	*aacA4cr*	*arr-3*	*dfrA27*	*aadA16*
CKP10 (ST17)	In1021	*aacA4cr*	*arr-3*	*dfrA27*	*aadA16*

MLST and Phylogeny

MLST showed that all the effluent *K. pneumoniae* isolates had a common sequence type ST29. Four of the clinical isolates had a common sequence type ST17 while two had unique sequence types ST348 and ST152. Phylogenetic analysis revealed that clinical and effluent isolates from this study were not closely related and did not cluster together. In contrast, the effluent isolates clustered together according to their sequence type and formed a clade with South African environmental isolates. CKP2, CKP5, CKP8 and CKP10 (ST17) all clustered together with South African isolates from human and environmental settings according to sequence types. Clinical CKP1 formed a separate clade with 2 clinical isolates and an environmental isolate from South Africa. Clinical isolate CKP4 was also closely related and clustered with South African clinical and environmental isolates according to sequence type and formed a separate clade with an isolate from a water source in Tunisia (Figure 2).

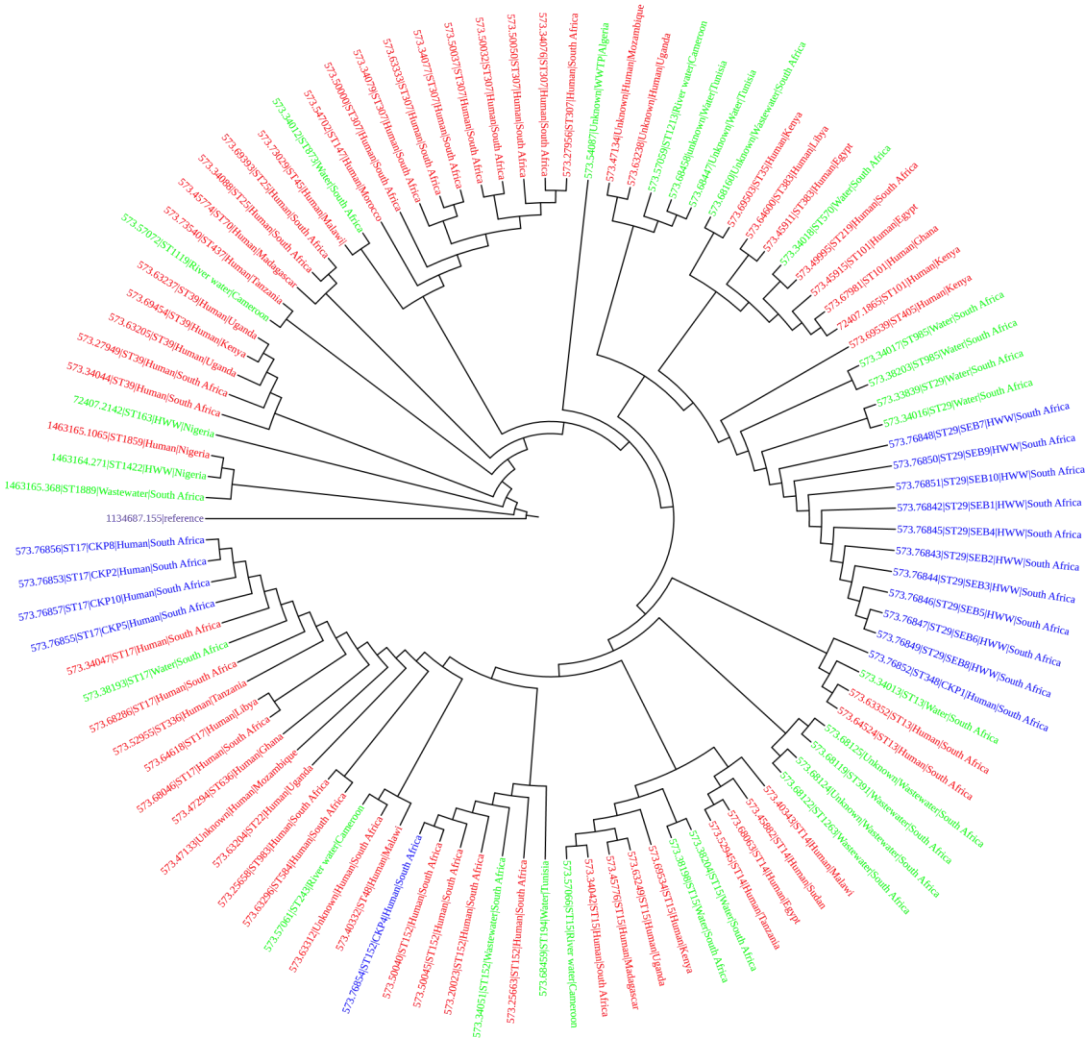

Figure 2: Phylogenomic tree showing the relationship between clinical and effluent *K. pneumoniae*. *Isolates from this study (blue) and isolates from other African isolates in red (human) and green (environmental).*

2.4 Discussion

This point-prevalence study investigated the phenotypic and genomic antibiotic resistance profiles of *Klebsiella pneumoniae* isolates from patients and hospital effluent. The frequency and characteristics of antibiotic resistant bacteria in hospital effluent is a simple technique to monitor the spread of resistance from the clinical setting into the environment (Tiwari et al., 2022) and there are a number of studies that report that hospital sewage is increasingly associated with multidrug resistant bacteria (Cai et al., 2021; Hocquet et al., 2016; Mustafa et al., 2022). While no such similarities were observed in our study, we report on the complexity and diversity of resistance observed in clinical isolates compared with isolates from hospital effluent that did not harbour major antibiotic resistance, nor resistance genes.

Klebsiella pneumoniae is a major cause of hospital-acquired infections and is implicated in urinary, respiratory and bloodstream infections as well as infections in intensive care unit (ICU) patients (Aires-de-sousa et al., 2019). Accurate identification of bacteria is essential for appropriate antibiotic treatment. Common diagnostic procedures include microbiological culture and biochemical tests (Cai et al., 2021). The ability of selective culture media to accurately identify bacterial organisms is questionable, especially when it comes to organisms belonging to the same genus or family of bacteria. SCAI achieved 80% accurate identification of *K. pneumoniae* in this study but also misidentified other *Klebsiella* species as *K. pneumoniae*. This highlights the need for new and improved phenotypic bacterial identification techniques.

This study did not reveal any relationship in the resistance in the *K. pneumoniae* from the hospital sewage and clinical samples. Higher resistance across all tested antibiotics were observed in the clinical isolates from patients at the hospital. The low levels of phenotypic antibiotic resistance in the *K. pneumoniae* recovered from hospital effluent in this study contradicts some studies that discovered above 40% resistance rates against the tested antibiotics on *K. pneumoniae* from hospital effluent (Okafor and Nwodo, 2023; Seguni et al., 2023).

The antibiogram of the clinical isolates showed multidrug resistance to commonly used antibiotics, with higher resistance rates to cefuroxime, cefotaxime, piperacillin/tazobactam, gentamicin and trimethoprim/sulfamethoxazole (100%) compared with others. Resistance rates above 70% were observed to ceftazidime, cefepime, amoxicillin/clavulanic acid and ciprofloxacin. Similar resistance rates have been described in other studies. Fatima et al. (2021) reporting on virulent and multidrug-resistant *K. pneumoniae* from clinical samples in Pakistan showed similar high resistance rates in cefotaxime and amoxicillin/clavulanic acid (100%), and gentamicin (76.3%); while Nakamura-Silva et al. (2022a), investigating multidrug-resistant *K. pneumoniae* in patients admitted to Brazilian hospitals also reported higher resistance rates of 85.7% and 90.4% against trimethoprim/sulfamethoxazole and cefuroxime, respectively. However, Fatima et al. (2021) also reported lower resistance against piperacillin/tazobactam and Chakraborty et al. (2016) evaluating antibiotic sensitivity patterns of *K.*

pneumoniae and *K. oxytoca* from clinical samples reported lower resistance in ciprofloxacin (37.5%) and gentamicin (31%) compared with our study. Moini et al. (2015) who investigated antibiotic resistance patterns and MDR *E.coli* and *K. pneumoniae* from patients in Iran also reported lower resistance rates in ciprofloxacin (23.3%) and gentamicin (35.3%) compared with our study. Another study analysing the genotypic and phenotypic antibiotic resistance patterns of *K. pneumoniae* from clinical samples in Iran, Khamesipour and Tajbakhsh, (2016) reported similar resistance rates against trimethoprim/sulfamethoxazole (36.7%) and gentamicin (26.7%) as observed by Chakraborty et al. (2016) (trimethoprim/sulfamethoxazole, 44%; gentamicin, 31%). These results were even lower than observed in this study. Only 33.3% of the clinical isolates were resistant to amikacin, similarly reported by Khamesipour and Tajbakhsh, (2016) at 32.3% and Moini et al. (2015) at 31%. Resistance to the carbapenem antibiotics, imipenem, ertapenem, doripenem and meropenem was found to be 33.3% among the clinical isolates.

Ninety percent of the effluent isolates were susceptible to almost all tested antibiotics, and resistant against one class of antibiotics, the ß-lactams. High resistance rates were only observed against amoxicillin/clavulanic acid and piperacillin/tazobactam in all the effluent isolates. This is indicative of inhibitor resistance phenotype, which is characterized by resistance to ß-lactam-ß-lactamase inhibitor combinations and susceptibility to cephalosporins (Chaïbi et al., 1999; Russ et al., 2020). Contrary to the high resistance against piperacillin/tazobactam in this study, Okafor and Nwodo, (2023), characterizing antibiotic resistance determinants in *K. pneumoniae* recovered from hospital effluent in the Eastern Cape province of South Africa; and King et al., (2020), investigating antibiotic resistant Klebsiella species from the hospital, hospital effluent and effluent treatment plants in Kwazulu-Natal found high susceptibility in the effluent isolates against piperacillin/tazobactam.

No resistance to the carbapenem antibiotics imipenem, meropenem, ertapenem and doripenem was observed in the effluent isolates in this study. This is similar to what King et al. (2020) reported in their study with 100% susceptibility against imipenem, ertapenem, meropenem, and less than 10% resistance to doripenem. Okafor and Nwodo, (2023) also reported high susceptibility to imipenem (95%), ertapenem (88%) and meropenem (77%). Ebomah and Okoh, (2020) who determined the occurrence of carbapenem-producing *Klebsiella* species in environmental niches in South Africa found similar lower resistance against meropenem (34%), ertapenem (35%), doripenem (35%) and imipenem (51%) in *K. pneumoniae* from hospital effluent. Another study by Sakkas et al. (2019) who investigated AMR and detection of carbapenemases in *K. pneumoniae* isolates from raw hospital sewage reported that 79.2% of the isolates in their study showed resistance to carbapenems. Only one (10%) of the effluent isolates showed resistance to tigecycline. Low resistance in tigecycline (5.3%) was also reported by Sakkas et al. (2019). While the effluent isolates in this study were susceptible to a number of antibiotics, including the last resort antibiotics, the carbapenems, the clinical isolates showed extensive resistance to the

commonly used antibiotics. This shows the limited efficacy of such antibiotics to effectively treat patients infected with *K. pneumoniae* (Khamesipour and Tajbakhsh, 2016).

Hospital effluent has been suggested as a hotspot for the prevalence and dissemination of resistance through horizontal gene transfer (Karkman et al., 2018), and an important source of antibiotic resistant bacteria (ARB) and ARGs (Wang et al., 2018). A systematic review by Tiwari et al. (2022) reviewed studies that compared effluent surveillance of AMR and ARGs in parallel to clinical evidence. Acosta et al. (2023) assessed hospital effluent to determine if the resistome of the effluent differed from the general population, and Wang et al. (2018) evaluated the diversity of AMR profiles in untreated hospital effluent to identify potential risks of dissemination of clinical resistant bacteria. These studies noted that investigation of AMR in untreated hospital effluent is thought to be a helpful and effective tool to aide hospital-based infection control and for understanding how clinical antibiotic resistance affects environmental ARB.

Clarke et al. (2024), reviewing wastewater-based epidemiology (WBE) for AMR surveillance has highlighted that while it may be a promising technique for AMR monitoring, challenges such as sampling inconsistencies may occur, affecting the ability to accurately depict AMR dynamics in clinical settings. While effluent analysis can be a useful tool to provide data on AMR at a population level (Munk et al., 2022), it may not be a source of direct correlation and an ideal indicator of clinical AMR at the individual level (Perry et al., 2021). Despite these limitations, researchers still argue that WBE can be a useful technique to provide broad understanding into clinical AMR trends (Clarke et al., 2024; Karkman et al., 2020; Munk et al., 2022; Tiwari et al., 2022).

The phenotypic resistance characteristics correlated with known ARGs which were located on mobile genetic elements. Genes encoding multidrug efflux pumps were identified in most isolates. Multidrug efflux pumps play an important role in mediating bacterial resistance to multiple antibiotic classes and heavy metals due to their ability to remove diverse molecules from inside the cell (Gaurav et al., 2023; Kumawat et al., 2023). There is increasing evidence that over-expression of multidrug efflux pumps is significantly associated with bacterial resistance to clinically relevant antibiotics (Gaurav et al., 2023; Soto, 2013; Sun et al., 2014).

Common efflux pumps found in both the clinical and effluent isolates included KpnEF and OqxA, while *tet(D)* was common among the effluent isolates, and AcrAB-TolC was identified in only two of the clinical isolates (CKP2 and CKP4). AcrAB-TolC, a common efflux pump reported in other studies, is a resistance-nodulation-division (RND) efflux pump found in Gram-negative bacteria that contributes to resistance to tetracyclines, fluoroquinolones, ß-lactams, macrolides and chloramphenicol (Bialek-Davenet et al., 2015; Ni et al., 2020; Jang, 2023). OqxAB is also a member of the RND efflux pump family that has been shown to confer resistance to quinolones, nitrofurantoin, tigecycline, and chloramphenicol, detergents, and disinfectants (Bharatham et al., 2021; Li et al., 2019; Ni et al., 2020). Resistance to both tigecycline and quinolones was observed in three clinical (CKP1, CKP4, and CKP10)

and one effluent isolate, (SEB3), the latter showing phenotypic resistance to tigecycline. A study in China reported on the AcrAB-TolC and OqxAB over-expression of efflux pumps that were associated with tigecycline resistance in *Klebsiella pneumoniae* (Zhong et al., 2014). However, the presence of *tetD* in all but one effluent isolate (SEB3) and the presence of *oqxA* and *oqxB* in all the effluent isolates did not show the commensurate phenotypic resistance and it was postulated that these isolates harboured these ARGs as silent or minimally expressed genes (Deekshit and Srikumar, 2022). *K. pneumoniae* is known to harbour silent genes (Stasiak et al., 2021). Xu et al., (2014) reported on the presence of $bla_{CTX,}$ $bla_{SHV,}$ and bla_{TEM} without phenotypic resistance to cephalosporin in clinical strains of *K. pneumoniae*.

Several different ß-lactam resistance genes delineated in the clinical isolates correlated with the resistance observed, especially against cephalosporin antibiotics. For example, resistance to cefuroxime, cefotaxime and ceftazidime was evident in isolates CKP2, CKP4, CKP5, CKP8 and CKP10 while CKP1 showed resistance against cefuroxime and cefotaxime only, with the ESBL ARGs, bla_{TEM}, bla_{CTXM-1}, and bla_{SHV} group. Carbapenem resistance could be attributed to the Bla_{NDM-1} and $bla_{OXA-181}$ ARGs in the two clinical isolates, CKP2 and CKP8. Al-Agamy et al. (2018), characterizing carbapenemases, ESBLs plasmid mediated quinolone resistance (PMQR) in carbapenem resistant *K. pneumoniae* reported the rate of bla_{NDM-1} to be 33.3% among the *K. pneumoniae* from patients. Other studies reported on low percentage of *K. pneumoniae* in hospital effluent harbouring carbapenem genes (Ebomah and Okoh, 2020; Okafor and Nwodo, 2023). The clinical isolates carried he bla_{TEM} group (CKP1, CKP2, CKP4, CKP5, CKP8 and CKP10), bla_{CTXM-1} (CKP1, CKP2, CKP4, CKP5, CKP8 and CKP10), and bla_{SHV} group (CKP1, CKP2 , CKP5, CKP8 and CKP10) ESBL genes. A study by Ferreira et al. (2019) investigated antibiotic resistance profiles, pathogenicity and clonal relationships of *K. pneumoniae* isolated from patients and ICU settings reported on the abundance of the *K. pneumoniae* carrying ESBL encoding genes.

The prevalence of the aminoglycoside resistance genes was high in isolates CKP2, CKP4, CKP5, CKP8 and CKP10, that were resistant to at least two of the tested aminoglycoside antibiotics. These genes included the common ones observed among these isolates, *aph(6)-Id, aph(3")-Ib, aac(6')-Ib-cr, aadA16,* and *aac(3)-Iia*. ARGs *aadA1* and *armA* were only observed in the isolates that were resistant to amikacin (CKP2 and CKP8). Among the aminoglycoside resistant *K. pneumoniae* isolates from patients, *aadA16* was associated with gentamicin resistance in five (83.3%) of the isolates. The *aac(6')-Ib-cr* gene, which confers resistance to aminoglycosides and fluoroquinolones was present in all the clinical isolates. Fluoroquinolone resistance genes *qnrS* (2/6), *qnrB* (3/6), *oqxA* (6/6) and *oqxB* (5/6) were present among the clinical isolates. Isolates SEB1-SEB10 did not show phenotypic resistance to both aminoglycoside and fluoroquinolone antibiotics although they harboured the *oqxA* and *oqxB* genes. Okafor and Nwodo, (2023) have also reported on the prevalence of aminoglycoside and fluoroquinolone resistance genes in the hospital effluent.

The sulphonamide and trimethoprim resistance genes *sul1* (5/6, 83.3%) in CKP2, CKP4, CKP5, CKP8 and CKP10; *sul2* (6/6, 100%) in CKP1, CKP2, CKP4, CKP5, CKP8 and CKP10; *dfrA14* (1/6, 16.7%) in CKP1; and *dfrA27* (5/6, 83.3%) in CKP2, CKP4, CKP5, CKP8 and CKP10 were detected among the clinical isolates. The susceptibility of the effluent isolates to trimethoprim-sulfamethoxazole is supported by the absence of these ARGs. Studies show the presence of genes encoding trimethoprim-sulfamethoxazole resistance in clinical and in effluent isolates (Al-Marzooq et al., 2015; Mbelle et al., 2020a; Okafor and Nwodo, 2023). Mbelle et al. (2020) who investigated the pathogenomics and evolutionary epidemiology of MDR clinical *K. pneumoniae* in South Africa identified *sul1* (78%), *sul2* (86%), and *drfA27* in 19% of the isolates. Al-Marzooq et al., (2015), investigating antibiotic resistance determinants and plasmids in MDR *K. pneumoniae* from patients at a Malaysian medical centre detected *sul1* (53.8%) as the most common gene encoding trimethoprim-sulfamethoxazole resistance, while Okafor and Nwodo, (2023) reported on the presence of the sulphonamide genes in effluent isolates to be high with the most common being *sul1* (68.4%).

Antibiotic resistance genes are carried on mobile genetic elements such as plasmids, integrons transposons, and can be transferred from organism to organism or between different species through conjugation, transformation and transduction (Tang et al., 2014). ARGs were associated with a repertoire of MGEs in the clinical isolates but not in the effluent isolates. The $bla_{OXA-181}$ gene was carried on plasmid replicon ColKp3 on isolates CKP2 and CKP8. This finding was supported by another study, (Xanthopoulou et al., 2020) analysing antibiotic resistance and MGEs in XDR *K. pneumoniae* from patients, reporting similar results. The most common plasmid type, IncFIB(K), was present in all the clinical and effluent isolates in this study. IncFII(K) plasmid types are associated with dissemination of ESBL and carbapenemases in *Klebsiella pneumoniae* clinical isolates (Dolejska et al., 2012; Mansour et al., 2015). Dolejska et al., (2012) investigated ESBL-producing Enterobacterales at a paediatric oncology hospital and reported that the transfer of ESBL plasmids was most frequent in *K. pneumoniae*. Mansour et al., (2015) reported on the molecular features of ESBL-producing *K. pneumoniae* from hospital and community acquired infections and found that the $bla_{CTX-M-15}$ gene was mostly carried on plasmid IncFII(K). We did not observe this in our study.

Only the class 1 integron integrase gene was harboured in 66% of the clinical isolates. The *IntI1* was flanked by the insertion sequence IS1 in two isolates (CKP1 and CKP5). Class 1 integrons are often associated with *sul1* and *qacEΔ1* genes (Nowrotek et al., 2017), which was observed in isolate CKP5 that carried the sul1:qacEΔ1 gene cassette, on the same contig as *IntI1*; while the other isolates (CKP1, CKP8 and CKP10) carried this gene cassette on different contigs surrounding the *IntI1*. Transposable element Tn3 was the most common among the clinical isolates, and was associated with $bla_{CTX-M-15}$ in CKP1, CKP2, CKP4 and CKP5. A study by Ramsamy et al. (2022), delineated molecular epidemiology of carbapenem resistant Enterobacterales (CRE) reported on Tn3 transposon commonly carrying the

bla$_{CTX-M-15}$ or CTX-M-3, and *bla*$_{TEM1B}$. Our findings were similar where *bla*$_{CTX-M-15}$ was mostly carried on Tn3 and flanked by ISEc9.

MLST revealed no common sequence types among the clinical and the effluent isolates. Moreover, the effluent isolates all clustered together and were related to other isolates from South Africa. Sequence type ST17 was more common among the clinical isolates (CKP2, CKP5, CKP8 and CKP10) while CKP4 (ST152) and CKP1 (ST348) were unique. ST152 and ST17 have been reported in other South African studies (Founou et al., 2019; Mbelle et al., 2020a; Ramsamy et al., 2022). Founou et al. (2019) characterised ESBL-producing *K. pneumoniae* from two hospitals and found ST152 in carriage and clinical isolates while ST17 was only observed in carriage isolates. ST152 *bla*$_{NDM-1}$ was an outbreak-related clone that has been reported by Magobo et al. (2023) who conducted an outbreak investigation of CRKP from bloodstream infections in a neonatal unit. This strain was found account for more than 60% of deaths. In this study, the NDM-1 and OXA-181 producing isolates, CKP2 and CKP8, were of the sequence type ST17.

Runcharoen et al. (2017), used WGS to reveal epidemiological relationships between clinical and environmental *K. pneumoniae* and reported that phylogeny analysis revealed some clinical and hospital sewage isolates in their study resided in the same clade, suggesting that they rise from a common ancestor. Another study by (Popa et al., 2021) investigating the transmission of MDR *K. pneumoniae* from the hospital to hospital effluent and its existence after chlorine treatment, showed 11 *K. pneumoniae* strains isolated from hospital effluent and clinical sector were clonally related. Davidova-gerzova et al. (2024) investigated the prevalence and phylogenetic relationships of MDR *K. pneumoniae* from patients, hospital effluent and surface waters. The phylogenetic analysis revealed a low similarity among the isolates in their study, even with those of the same sequence type. Siri et al. (2024), investigated the prevalence of antibiotic-resistant bacteria in hospital effluent and receiving waters as a reflection of antibiotic prescription and infection cases. They generally highlighted a correlation between hospital infection cases and MDR in effluent. The genomic characteristics, supported by evolutionary representation of the isolates suggest that the clinical and the effluent isolates in this study are not closely related.

2.5 Conclusion

K. pneumoniae clinical isolates showed higher rates of antibiotic resistance, including to the last resort antibiotics, compared with isolates from the hospital effluent. There was no evidence to show that the effluent reflects the clinical setting in the case of *K. pneumoniae* as there was no correlation between the resistome, mobilome and phylogenies of the clinical and effluent isolates.

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Laboratory work and data analysis: NSM

Manuscript draft: NSM

Manuscript review and revision: SYE, JM & NSM

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Supplementary material

Table S 1: Genomic characteristics of the *Klebsiella pneumoniae* from human and effluent isolates

ISOLATE ID	SOURCE	SEQUENCE LENGTH	GC CONTENT	NO OF CONTIGS	N50 VALUE	L50 VALUE
CKP1	Clinical	5513088	57.0	67	247332	9
CKP2	Clinical	5775620	56.7	99	304236	5
CKP4	Clinical	5329242	57.5	101	180171	11
CKP5	Clinical	5538071	57.3	62	369837	6
CKP8	Clinical	5774194	56.7	91	308375	6
CKP10	Clinical	5505863	57.3	58	304333	6
SEB1	Effluent	5542381	57.2	60	389055	6
SEB2	Effluent	5546575	57.2	56	389055	6
SEB3	Effluent	5545800	57.2	58	389055	6
SEB4	Effluent	5542901	57.2	59	387949	6
SEB5	Effluent	5545266	57.2	58	389055	6
SEB6	Effluent	5546809	57.2	58	389055	6
SEB7	Effluent	5542970	57.2	55	385652	6
SEB8	Effluent	5541174	57.2	56	389055	6
SEB9	Effluent	5544764	57.2	56	389055	6
SEB10	Effluent	5546423	57.2	54	389055	6

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CHAPTER THREE

This study delineated the AMR profiles, resistomes, mobilomes and phylogenies of the ESKAPEE pathogens from patients and hospital effluent to establish associations/correlations (if any) in terms of:

3.1 Conclusion

The conclusion encompasses the full study on all the ESKAPEE pathogens and is not limited to the findings presented in the manuscript in chapter two. The conclusions are presented in relation to the objectives:

- A total of 112 presumptive ESKAPEE isolates were subjected to antimicrobial susceptibility testing and 63 ESKAPEE were selected for WGS based on phenotypic resistance to at least one antibiotic. Of the 63, 36 isolates were positively identified as ESKAPEE by WGS. *K. pneumoniae* and *E. coli* were the only species with both clinical and effluent isolates.
- Of the 42 clinical isolates, 21 were selected for WGS, and 16 of the isolates were identified as ESKAPEE, suggesting possible misidentification and misdiagnosis at the clinical level.
- Of the 70 presumptive ESKAPEE from hospital effluent, 42 were selected for WGS and 20 were positively identified as ESKAPEE *Acinetobacter baumannii* identified, suggesting the inaccuracy of selective media in identifying ESKAPEE.
- All the effluent *E. faecium* isolates harboured similar resistance genes including aminoglycoside, macrolide, trimethoprim and tetracycline ARGs. While no ARGs were carried on transposable elements nor insertion sequences, plasmid repUS12 was usually associated with ARGs. These isolates did not harbour complex MGEs, showing that resistance in the hospital effluent among this species is mainly via conjugative plasmids. The isolates had the same sequence type ST80, with phylogenetic analysis confirming the same evolutionary history.
- *K. pneumoniae* from the effluent and clinical isolates displayed different resistance patterns, showing that clinical AMR is essentially a continuing health threat, while the prevalence of ß-lactamase inhibitor resistance among the effluent isolates can lead to future resistance to more antibiotics. Clinical *K. pneumoniae* isolates had more resistance genes than effluent isolates, with aminoglycoside resistance genes (*aph(6)-Id, aph(3")-Ib, aac(6')-Ib-cr* and *aadA16*) being the most common. The effluent isolates did not harbour any aminoglycoside resistance genes, which aligns with their phenotypic resistance. The effluent isolates had one plasmid replicon type, IncFIB(K). Other plasmid replicon types in the clinical isolates included IncFII(K) and colKP3. Resistance genes were carried on insertion sequences and/or transposable elements, with few carried on intI1 integron. Most ARGs on the effluent *K. pneumoniae* isolates were not

associated with mobile genetic elements. In1021, which contained the gene cassette *aacA4cr-arr3-dfrA27-aadA16* was common in the clinical *K. pneumoniae.* The effluent isolates had a common sequence type ST29, while the clinical isolates had a common sequence type ST17. Phylogenetic analysis revealed that clinical and effluent isolates were not closely related and did not cluster together. There was no correlation between the clinical and effluent isolates in terms of resistome, mobilome and phylogeny.

- *P. aeruginosa* harboured ß-lactam, aminoglycoside, and fluoroquinolone ARGs that were not associated with any MGEs. The MLST analysis gave a sequence type ST275, while the phylogenetic analysis revealed that the isolate in this study did not cluster with any other isolates because it had a unique sequence type in relation to the other isolates.
- Six *E. hormaechei* isolates harboured the same ß-lactam resistance genes (bla_{ACT-5}, $bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{OXA-1}) and displayed similar ARGs associated with mobile genetic elements except one isolate, CPA8. These MGEs include transposable element Tn3 that was associated with $bla_{CTX-M-15}$, insertion sequence IS1 and integron class 1. One sequence type ST 78 was observed among these isolates, showing close relation with each other and other South African isolates. However, CPA1 with a similar sequence type, was not closely related to the other *E. hormaechei* isolates.
- While the *E.coli* isolates from clinical were not too different from the effluent isolate in terms of resistance, the effluent isolate was resistant to more antibiotics than the clinical isolates, suggesting the presence of MDR *E. coli* in the hospital effluent. The effluent *E. coli* isolate had a more diverse resistome compared to the two clinical isolates. ARGs *aph(6)-Id*, *aph(3'')-Ib, aac(6')-Ib-cr, bla_{OXA-1}, $bla_{CTX-M-15}$, and tet (A)* were common in the clinical and effluent isolates. The ARGs were carried on MGEs. The *tet(A)* ARG on SEC1 was carried on transposon Tn3 on one contig, as well as the $bla_{CTX-M-15}$ and *AAC(3)-IId* on a different contig. The clinical isolates, however, did not have any resistance genes carried on transposons. SEC1 also carried the *sul1-qacEΔ1* gene cassette on one contig without the class 1 integron-integrase present, while clinical isolate CEC5 carried In54, which harboured the gene cassette *dfrA17-aadA5*. MLST analysis revealed the clinical *E. coli* isolates had unique sequence types, ST69, ST131while the effluent isolate belonged to ST10. The clinical and effluent isolates from this study did not cluster together and were not closely related, as they had different sequence types. This, however, is still indicative of the presence of MDR *E. coli* in clinical settings and hospital effluent, where spread of resistance among this species can occur.

3.2. Limitations

- The main limitations to this study were the short study period, and the small sample size.
- The isolates from this study were from one region of the province, hence the results cannot be generalized to all South African hospitals.

- One of the challenges faced in this study was the lack of clinical ESKAPEE from the NHLS.
- The isolates were only identified using culturing on selective media. More tests could have been done e.g biochemical tests.

3.3 Recommendations

- Continuous monitoring of the hospital effluent for longer study period including a larger sample size and covering more hospitals and associated effluents in the country to further track the spread of resistant bacteria from clinical settings to the environment via water sources.
- More biochemical tests (e.g., catalase and oxidase test) and Polymerase Chain Reaction (PCR) should be done to verify the identity of the bacterial species before WGS to avoid misidentification. Confirmation of isolates using biochemical and molecular tests strengthens the chances of sequencing positively identified microbes. Restricting identification of microbes to only selective culture media presents the risk of sequencing wrongly identified microbes.
- *E. faecium* from hospital effluent showed extensive resistance patterns and ARGs. Unfortunately, no clinical isolates were obtained in this study. Isolation of antibiotic resistant *E. faecium* from clinical samples and comparison to hospital effluent isolates is thus recommended.

APPENDICES

Appendix 1: Biomedical Research Ethics Committee (BREC) approval letter

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

27 March 2022

Prof Sabiha Yusuf Essack (3951)
School of Health Sciences
Westville

Dear Prof Essack,

Protocol reference number: BREC/00003640/2021
Project title: One Health Alternate Antimicrobial Resistance Monitoring System in a Coronavirus World Non-Degree Purposes

EXPEDITED APPLICATION: APPROVAL LETTER

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application.

The conditions have been met and the study is given full ethics approval and may begin as from 27 March 2022. Please ensure that any outstanding site permissions are obtained and forwarded to BREC for approval before commencing research at a site.

This approval is subject to national and UKZN lockdown regulations, see (http://research.ukzn.ac.za/Libraries/BREC/BREC_Amended_Lockdown_Level_1_Guidelines.sflb.ashx). Based on feedback from some sites, we urge PIs to show sensitivity and exercise appropriate consideration at sites where personnel and service users appear stressed or overloaded.

This approval is valid for one year from 27 March 2022. To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2020) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be noted by a full Committee at its next meeting taking place on 12 April 2022.

Yours sincerely,

[redacted]

Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

1 / 1

Appendix 2: Provincial Health Research Ethics Committee (PHREC) approval letter

KWAZULU-NATAL PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

DIRECTORATE:
Health Research & Knowledge Management Unit

Postal Address: Private Bag X9050
Physical Address: 330 Langalibalele Str; PM Burg; 3201
Tel: 0333953189/3123/2805 Fax: 033-3943782
Email address: hrkm@kznhealth.gov.za
www.kznhealth.gov.za

NHRD Ref: KZ_202203_023

Dear Professor S Essack
(UKZN)

Approval of research

1. The research proposal titled **'One Health Alternative Antimicrobial Resistance Monitoring System in Coronavirus World'** was reviewed by the KwaZulu-Natal Department of Health (KZN-DoH).

   The proposal is hereby approved for research to be undertaken at East Boom & Imbalenhle clinics and Edendale, Northdale and Greys hospitals.

2. You are requested to take note of the following:

   a. *All research conducted in KwaZulu-Natal must comply with government regulations relating to Covid-19. These include but are not limited to: regulations concerning social distancing, the wearing of personal protective equipment, and limitations on meetings and social gatherings.*
   b. *Kindly liaise with the facility manager BEFORE your research begins in order to ensure that conditions in the facility are conducive to the conduct of your research. These include, but are not limited to, an assurance that the numbers of patients attending the facility are sufficient to support your sample size requirements, and that the space and physical infrastructure of the facility can accommodate the research team and any additional equipment required for the research.*
   c. *Please ensure that you provide your letter of ethics re-certification to this unit, when the current approval expires.*
   d. *Provide an interim progress report and final report (electronic and hard copies) when your research is complete to **HEALTH RESEARCH AND KNOWLEDGE MANAGEMENT, 10-102, PRIVATE BAG X9051, PIETERMARITZBURG, 3200** and e-mail an electronic copy to hrkm@kznhealth.gov.za*
   e. *Please note that the Department of Health shall not be held liable for any injury that occurs as a result of this study.*

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

Yours Sincerely

[redacted]

Dr E Lutge
Chairperson, Health Research Committee
Date: 22/03/2022.

Appendix 3: National Health Laboratory Service approval letter

NATIONAL HEALTH LABORATORY SERVICE

Academic Affairs and Research
1 Modderfontein Road, Sandringham, 2031
Tel: +27 (0)11 555 0367/0406
Email: [redacted]
academic.research@nhls.ac.za
Web: www.nhls.ac.za

28 November 2022

Applicant: Sabiha Essack
Institution: University of KwaZulu-Natal
E-mail Address: essacks@ukzn.ac.za
Tel: 031 260 7785 **Cell:** [redacted]

CC: Sumayya Haffejee

Project Title: One Health AIARMS in the COVID-19 World
Reference Number: PR2225862

Research Application Type(s):
1. Request for Data

RE: APPROVAL LETTER: REQUEST TO ACCESS NHLS RESOURCES FOR RESEARCH PURPOSES

This letter serves to advise that the application requesting permission to conduct the above-mentioned research using the listed NHLS resources has been reviewed and **"Approved"**. Please note that the approval is granted on the condition that you comply with the NHLS Research Material and Data Access Policy and requirements stated below.

1. All material and data requested shall be used as per the research protocol submitted to the NHLS and as approved by the relevant Health Research Ethics Committee (HREC) in South Africa.
2. Access to the NHLS material and/or data shall be limited to the minimum required for successful completion of the approved study and shall be made available ***without patient names and other patient identifiers (including, but not limited to, national identity numbers, hospital/clinic file numbers, addresses and telephone numbers)***.
3. Confidentiality shall be maintained at the participant and institutional level and there shall be no disclosure of personal information or confidential information.
4. Data and/or material shall not be shared with other parties unless approved by the NHLS
5. The material and/or data obtained from the NHLS shall be anonymised and not, for any reason, be used to track or recruit patients as no pre-approval/consent is obtained from patients.
6. Processes shall be discussed with the relevant NHLS departments (i.e. Corporate Data Warehouse (CDW), NHLS Laboratory Management, Operations Office, etc.) and agreed upon.
7. Any amendments to the study requirements, including the use of the material and/or data for purposes not initially disclosed to the NHLS) shall be cleared by an approved HREC and submitted to the NHLS for approval via the AARMS system – https://aarms.nhls.ac.za.
8. The NHLS shall be acknowledged as a source of material and/or data in any output, such as abstracts and journal articles, emanating from the project.
9. A final report of the research study and any published output resulting from this study shall be submitted to the NHLS via AARMS

Please note that this letter constitutes approval by the NHLS Academic Affairs and Research Office. The NHLS entities tasked with providing the material and/data may have additional requirements for access. Data related queries may be directed to NHLS CDW, email: zarina.sabat@nhls.ac.za; contact number: 011 386 6074 and sample related queries (if applicable) shall be directed to the relevant business manager.

[redacted]
Dr Babaly[redacted]Kgokong
National Manager: Academic Affairs and Research

Appendix 4: TRREE Certificate

TRREE

Zertifikat

Certificat

Certificado

Certificate

Promouvoir les plus hauts standards éthiques dans la protection des participants à la recherche biomédicale
Promoting the highest ethical standards in the protection of biomedical research participants

Certificat de formation - Training Certificate

Ce document atteste que - this document certifies that

Clinical Trials Centre
The University of Hong Kong

Naledi Shakoane Masalane

a complété avec succès - has successfully completed

Introduction to Research Ethics

du programme de formation TRREE en évaluation éthique de la recherche
of the TRREE training programme in research ethics evaluation

[redacted]

Release Date: 2023/07/30

CID : 6qnbYTkzG

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

APPROVED BY SIWF FMH ISFM

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[REV : 20220217]

Appendix 5: Other ESKAPEE results

Table 5.1: Antibiotic resistance genes, resistance patterns and plasmid replicons of the ESKAPEE pathogens from clinical and hospital effluent samples

Isolate ID	Source of isolation	Resistance pattern	Resistance genes	Plasmid replicons
E. faecium				
SEF2	Effluent	TET-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	rep18a, repUS43, repUS15, repUS12
SEF3	Effluent	TET-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	repUS15, repUS43, repUS12, rep18a, repUS15, repUS43, repUS12, rep18a
SEF4	Effluent	TEC-TET-VAN-LZD-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	rep18a, repUS15, repUS43, repUS12
SEF5	Effluent	TEC-TET-VAN-LZD-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	rep18a, repUS15, repUS43, repUS12
SEF6	Effluent	TEC-TET-VAN-LZD-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	rep18a, repUS15, repUS12, repUS43
SEF7	Effluent	TET-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	repUS12, repUS43, repUS15, rep18a
SEF8	Effluent	TET-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	rep18a, repUS12, repUS15, repUS43
SEF9	Effluent	TEC-TET-VAN-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	repUS15, repUS43, rep18a, repUS12
SEF10	Effluent	TET-AZM-ERY-LVX-DOX-FOS	*aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia, msr(C), tet(M), tet(L), dfrG*	repUS15, repUS43, rep18a, repUS12
P. aeruginosa				
CPA9	Clinical	FEP-CAZ-TZP	*aph(3')-IIb, blaOXA-50, blaPAO, crpP*	
E. hormaechei				
CEC3	Clinical	AMP-FEP-CTX-CAZ-CIP-GEN-TZP-TIG	*aph(6)-Id, aph(3")-Ib, aph(3")-Ib, aph(3")-Ib, aac(3)-IIa, aac(6')-Ib-cr, blaOXA-1, catB3, catB3, blaACT-5, fosA, fosA, qnrB1, sul2, tet(A), dfrA14*	Col(pHAD28), Col440I, IncN3, IncR
CEC4	Clinical	AMP-FEP-CTX-CAZ-CIP-GEN-TZP-TIG	*aac(3)-IIa, aac(6')-Ib-cr, blaOXA-1, catB3, catB3, aph(6)-Id, aph(3")-Ib, aph(3")-Ib, aph(3")-Ib blaACT-5, fosA, blaCTX-M-15, blaTEM-1B,qnrB1, sul2, tet(A), dfrA14*	Col(pHAD28), Col440I, IncN3, IncR
CEC6	Clinical	AMP-FEP-CTX-CAZ-CIP-GEN-TZP-TIG	*aph(6)-Id, aph(3")-Ib, aac(3)-IIa, aac(6')-Ib-cr, blaOXA-1, catB3, catB3,*	Col(pHAD28), Col440I, IncN3, IncR

			blaACT-5, blaCTX-M-15, blaTEM-1B, fosA, fosA, qnrB1, sul2, tet(A), dfrA14	
CEB1	Clinical	AMP-FEP-CTX-CAZ-CIP-GEN-TZP-TIG	*aph(6)-Id, aph(3")-Ib, blaTEM-1B, aac(3)-IIa, aac(6')-Ib-cr, blaOXA-1, catB3, catB3, blaACT-5, blaCTX-M-15, fosA, qnrB1, sul2, tet(A), dfrA14*	Col(pHAD28), Col440I, IncN3, IncR
CPA1	Clinical	FEP-CAZ-CIP-TZP-TOB	*aph(6)-Id, aph(3")-Ib, aac(3)-IIa, aac(6')-Ib-cr, blaOXA-1, catB3, catB3, blaTEM-1B, blaCTX-M-15, blaACT-5, fosA, qnrB1, sul2, tet(A), dfrA14*	Col(pHAD28), Col440I, IncN3, IncR
CPA3	Clinical	AMK-FEP-CAZ-CIP-TZP-TOB	*aph(6)-Id, aph(3")-Ib, aac(3)-IIa, aac(6')-Ib-cr, blaOXA-1, catB3, catB3, blaACT-5, fosA, fosA, blaCTX-M-15, blaTEM-1B, qnrB1, sul2, tet(A), dfrA14*	Col(pHAD28), Col440I, IncN3, IncR
CPA8	Clinical	FEP-CAZ-TZP	*blaCMH-3, fosA*	IncFIB(pECLA), Col440I, IncFII(pECLA)
E. coli				
CEC2	Clinical	AMP-FEP-CTX-CAZ-TZP	*aph(6)-Id, aph(3")-Ib, sul2, blaCTX-M-15, erm(B), mph(A), tet(B), dfrA14*	IncFIA, IncFIB(AP001918), IncFII, Col(MG828), Col156
CEC5	Clinical	AMK-AMP-FEP-CTX-CAZ-CIP-GEN-TZP	*aadA5, mph(A), sul1, dfrA17, aph(6)-Id, aph(3")-Ib, sul2, tet(A), aac(6')-Ib-cr, blaOXA-1, aac(3)-IId, blaCTX-M-15, blaTEM-1B*	Col156, IncFIB(AP001918), IncFII(29), IncX4, Col(MG828), Col440I
SEC1	Effluent	AMK-AMP-CFX-FEP-CTX-CAZ-CIP-GEN-TZP-TOB	*aadA1, blaOXA-10, cmlA1, ARR-2, sul1, dfrA23, aac(3)-Iid, blaCTX-M-15, aac(3)-IIa, aac(6')-Ib-cr, blaOXA-1, catB3, aph(6)-Id, aph(6)-Id, aph(3")-Ib, blaTEM-1B, qnrB1, sul2, tet(A), dfrA14*	Col(BS512), IncC, IncFIB(AP001918, IncFII(pRSB107), IncI1-I(Alpha), Col156

A total of 112 presumptive ESKAPEE isolates Constituted the final sample, of which 70 were isolated from effluent and 42 from clinical isolates. The isolates were subjected to antimicrobial susceptibility testing and a total of 63 ESKAPEE were selected for WGS based on phenotypic resistance to at least one antibiotic. A total of 36 isolates consisting of *K. pneumoniae* (16), *E. faecium* (9), *Enterobacter hormaechei* (7), *E. coli* (3) and *P. aeruginosa* (1) were positively identified as ESKAPEE pathogens by WGS. No clinical *E. faecium*, effluent *Enterobacter* and *P. aeruginosa* effluent isolates were provided, and no *S. aureus* and *A. baumannii* were confirmed through WGS. The non-ESKAPEE isolates identified after WGS included *Klebsiella quasi-pneumoniae*, *Aeromonas hydrophila*, and *Pseudomonas monteilli*. The results presented relate only to the positively identified ESKAPEE isolates.

E. faecium

Nine *E. faecium* isolates were recovered from the hospital effluent with no clinical isolates provided. The effluent isolates showed total (100%) non-susceptibility to 6 antibiotics, tetracycline, doxycycline, erythromycin, azithromycin, fosfomycin and levofloxacin. Resistome analysis revealed that the effluent *E. faecium* isolates harboured similar resistance genes. These included the aminoglycoside *aac(6')-Ii, aac(6')-aph(2"), aph(2")-Ia*, macrolide *msr(C), erm(T)*, tetracycline *tet*(M), *tet*(L), and trimethoprim

resistance *(dfrG)* ARGs. Plasmidfinder revealed that all the *E. faecium* isolates had 4 different plasmid replicon types (rep18a, repUS43, repUS15, repUS12). Replicon type repUS12 is usually associated with ARGs *tet (L*), *tet* (M) (**Table 5.*1***). No ARGs were carried on transposable elements nor insertion sequences, and no class 1 integrons were detected in the *E. faecium* isolates. MLST analysis revealed that all the effluent *E. faecium* isolates had a common sequence type ST80. Phylogenetic analysis revealed that all the isolates from this study were closely related to each other, clustered together according to sequence type and formed a clade with clinical isolates from Cameroon and Reunion, according to sequence type.

P. aeruginosa

Only one *P. aeruginosa* was recovered from the clinical isolates, and showed total resistance to cefepime, ceftazidime and piperacillin-tazobactam. However, it showed susceptibility to all the other antibiotics tested. The clinical *P. aeruginosa* isolate harboured ß-lactam [bla_{OXA-50}, bla_{PAO}], aminoglycoside *(aph(3')-Iib)*, fluoroquinolone *(crpP)* ARGS (**Table 5.*1***). The *P. aeruginosa* isolate did not carry any plasmid replicon types, and all the other *P. aeruginosa* resistance genes were not associated with any MGEs. The MLST analysis on the *P. aeruginosa* isolate gave a sequence type ST275, while the phylogenetic analysis revealed that the isolate in this study did not cluster with any other isolates that it was compared to.

E. hormaechei

Seven *Enterobacter hormaechei* was recovered from both clinical and effluent. The clinical *Enterobacter hormaechie* isolates showed high resistance rates with 100% resistance to ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, gentamicin, and tigecycline. Susceptibility was observed to doripenem, imipenem and meropenem, while the lowest resistance rate was observed in amikacin (14.28%). All the *Enterobacter* isolates (7/7) had at least 1 ß-lactam resistance gene, whereas 6 of them harboured the trimethoprim *(dfrA14)*, tetracycline *(tet(A))*, aminoglycoside [*aph(6)-Id, aph(3'')-Ib, aac(3)-Iia, aac(6')-Ib-cr*], and sulphonamide *(sul2)* ARGs. Six of the isolates also harboured the same ß-lactam resistance genes [bla_{ACT-5}, $bla_{CTX-M-15}$, bla_{TEM-1B}, bla_{OXA-1}], except one that harboured bla_{CMH-3} gene (***Table 5.1***). The synteny revealed that the *E. hormaechei* isolates displayed similar ARGs associated with mobile genetic elements except one isolate, CPA8. ARG $bla_{CTX-M-15}$ was carried on transposon Tn3 in CEC3, CEC4, CEC6, CEB1, CPA1 and CPA3. More than three contigs on each of the *E. hormaechei* carried resistance genes that were bracketed by insertion sequences and/or transposable elements. The *qnrB1* gene that confers resistance to fluoroquinolones was carried on IS3000. Isolates CEC3, CEC4, CEC6, CEB1, CPA1 and CPA3 had the class 1 integron integrase gene IntI1. The *Enterobacter hormaechei* isolates all belonged to a common sequence type ST78. Clinical isolates CPA3, CPA8, CEB1, CEC3, CEC4 and CEC6 were all clustered together according to sequence type, and also formed a separate clade with two *E. hormaechei* isolates from South Africa and Nigeria according to sequence type. However, CPA1 with a similar sequence type,

was not closely related to the other isolates in this study; but clustered with *E. cloacae* isolate from South Africa and formed a separate clade with *E. cloacae* from Nigeria.

E. coli

Only three isolates were recovered, two clinical and one from the effluent (1). The clinical *E. coli* isolates were resistant to a number of antibiotics, similar to the effluent isolate. Effluent *E. coli* was completely resistant to ampicillin, cefepime, cefotaxime, ceftazidime, ciprofloxacin and piperacillin-tazobactam. This similar trend was observed in the clinical isolates exhibiting complete resistance to these antibiotics. Clinical *E. coli* also showed resistance to amikacin and gentamicin (50%), while the effluent isolate was resistant to both antibiotics. Both the clinical and effluent isolates showed similar trends in susceptibility to the carbapenem antibiotics, as well as tigecycline. The effluent *E. coli* isolate carried more resistance genes compared to the two clinical isolates. Both the effluent and clinical isolates harboured at least one ß-lactam resistance gene. Clinical isolate CEC5 carried the ß-lactam resistance genes (*bla_{OXA-1}, $bla_{CTX-M-15}$, and bla_{TEM-1B}*), similar to the effluent isolate SEC1. The effluent isolate also carried *bla_{OXA10}* in addition. Aminoglycoside resistance genes included *aadA1, aac(3)-Iid, aac(3)-IIa, aac(6')-Ib-cr, aph(6)-Id, aph(3'')-Ib* in the effluent isolate, and *aph(6)-Id, aph(3'')-Ib, aadA5, aac(6')-Ib-cr, aac(3)-IId* among the clinical isolates. Only the effluent isolate carried the quinolone resistance gene *qnrB1* (**Table 5.*1***). The resistance genes of both the clinical and effluent *E. coli* isolates were carried on mobile genetic elements. Plasmid replicon type Col(MG828) was only unique to the clinical isolates, while Col(BS512), IncC, IncFII(pRSB107) and IncI1-I(Alpha) were unique to the effluent isolate. Other replicon types carried by the clinical isolates included IncFII(29), IncFIA, IncX4 and Col440I. All the *E. coli* isolates had the replicon type col156 in common. The tetracycline resistance gene *tet(A)* on SEC1 was carried on transposon Tn3 on one contig, as well as the *$bla_{CTX-M-15}$-AAC(3)-IId* on a different contig. The clinical isolates, however, did not have any resistance genes carried on transposons. The effluent isolate (SEC1) carried the *sul1-qacEΔ1* gene combination on one contig without the class 1 integron-integrase present, while 1 clinical isolate had the class 1 integron without the typical *sul1-qacEΔ1* combination. Clinical isolate CEC5 carried In54, which harboured the gene cassette *dfrA17-aadA5*. MLST analysis revealed that the clinical *E. coli* isolates had unique sequence types, ST69, ST131 while the effluent isolate belonged to ST10. The clinical and effluent isolates from this study did not cluster together and were not closely related, as they had different sequence types. Clinical isolate CEC2 was more closely related to *E. coli* isolates from effluent in South Africa; and clustered according to sequence types. It formed two separate clades with both effluent and clinical isolates from South Africa. CEC5 clustered closely and formed separate clades according to sequence type only with human isolates from South Africa. Effluent SEC1 clustered more closely with South African clinical isolates, but formed separate clades in both effluent and clinical isolates from South Africa, Ghana and Uganda according to their sequence type.

Table 5.2: Mobile genetic elements associated with resistance genes

SAMPLE ID	Species	MLST	CONTIGS	SYNTENY OF RESISTANCE GENES AND MGEs
CPA9	*P. aeruginosa*	ST275	1	*CrpP*:*FosA*:*aph(3')-IIb*:transposase
			11	*CatB3*:recombinase
CEC3	*E. hormaechei*	ST78	7	*FosA*:*blaACT-5*:*blaACT-24:SugE*
			37	IS1:IS6100:*DfrA14*:IntI1
			41	IS3000:*QnrB1*
			44	ISEcp1:*blaCTX-M-15*:Tn3
			45	DDE-type integrase:TetR(A):*tet(A)*
			49	IS91:*blaTEM-1*:recombinase
			50	IS3: *AAC(3)-IIa*
CEC4	*E. hormaechei*	ST78	38	IS1:IS6100:*DfrA14*:IntI1
			41	IS3000:*QnrB1*
			44	DDE-type integrase:TetR(A):*tet(A)*:IS26
			46	*blaCTX-M-15*:Tn3
			49	IS91:*blaTEM-1*:recombinase
			50	IS3: *AAC(3)-IIa*
CEC6	*E. hormaechei*	ST78	14	*SugE:blaACT-24:blaACT-5*
			36	IS1:IS6100:*DfrA14*:IntI1
			39	*APH(6)-Id*:*APH(3")-Ib*:IS1133:Tn3
			40	IS3000:*QnrB1*
			42	DDE-type integrase:TetR(A):*tet(A)*
			43	Tn3:*blaCTX-M-15*
			46	IS91:*blaTEM-1*
			47	IS3:*AAC(3)-IIa*
CEB1	*E. hormaechei*	ST78	40	IS1:IS6100:*DfrA14:*IntI1
			42	IS3000:*QnrB1*
			45	*blaTEM-1*:IS91:*APH(6)-Id*
			47	DDE-type integrase:TetR(A):*tet(A)*
			48	Tn3:*blaCTX-M-15*
			52	IS3:*AAC(3)-IIa*
CPA1	*E. hormaechei*	ST78	37	IS1:IS6100:*DfrA14:*IntI1
			40	IS3000:*QnrB1*
			43	DDE-type integrase:TetR(A):*tet(A)*:IS26
			45	Tn3:*blaCTX-M-15*
			50	IS3:*AAC(3)-IIa*
CPA3	*E. hormaechei*	ST78	42	IS1:IS6100:*DfrA14*:IntI1
			47	IS3000:*QnrB1*
			50	DDE-type integrase:TetR(A):*tet(A)*:IS26
			51	Tn3:*blaCTX-M-15*
			55	IS91:*blaTEM-1*
			57	IS3:*AAC(3)-IIa*
CEC2	*E. coli*	ST69	39	ISL3:ISEcp1:*blaCTX-M*-15:IS6:IS4
			74	*Sul2*:IS91:*APH(6)-Id*:*APH(3")-Ib*
			80	TetR(B):*tet(B)*::ISVsa5
			90	Erm(B):transposase
			95	MphR(A):*Mph(A)*
			99	*DfrA14*:IntI1
CEC5	*E. coli*	ST131	8	IS110:*CatB3*:*blaCTX-M-15*:ISEcp1
			39	*blaTEM-1*:IS1:IncFII
			42	IntI1:*DfrA17*:*AadA5:*QaceEΔ1:*Sul1*:IS6100:::*Mph(A)*
			44	TetR(A):*Tet(A)*:*APH(6)-Id*:*APH(3")-Ib*:*Sul2*
SEC1	*E. coli*	ST10	71	*Arr-2:cmlA5:blaOXA-10*:*AadA1:*QacEΔ1:*Sul1*:ISCR1:*DfrA23*
			83	*blaCTX-M-15*:Tn3
			89	IS1:IS6100:*DfrA14*
			100	TetR(A):*Tet(A)*:Tn3
			107	*blaTEM-1*:ISEcp1:IS903B
			122	IS3:*AAC(3)-IIa*
			154	*APH(6)-Id*:IS91

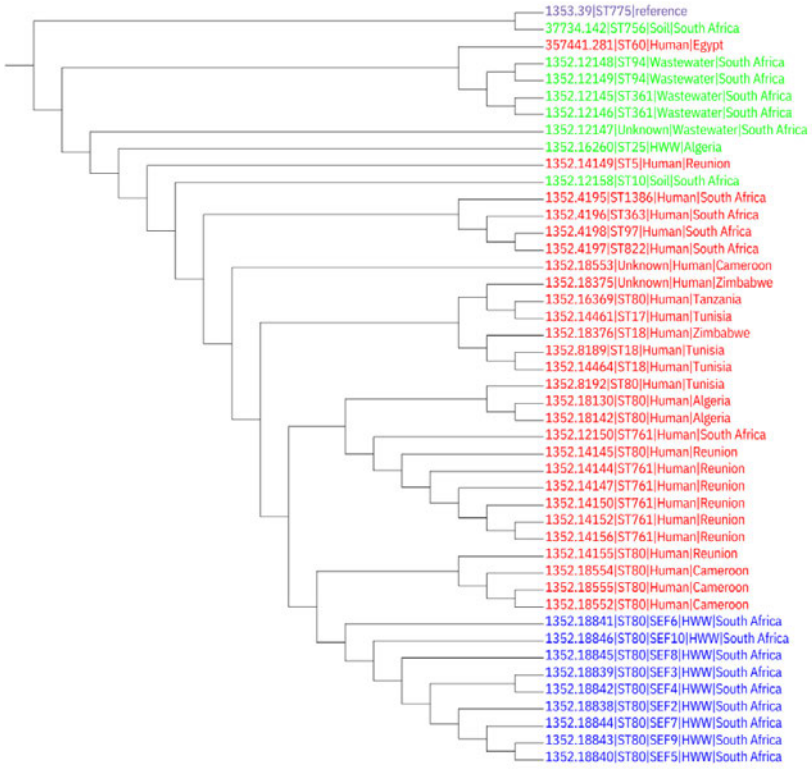

Figure 5.a: Phylogenomic tree showing the relationship of the effluent *E. faecium* from this study (blue) with human (red) and environmental (green) isolates from African countries.

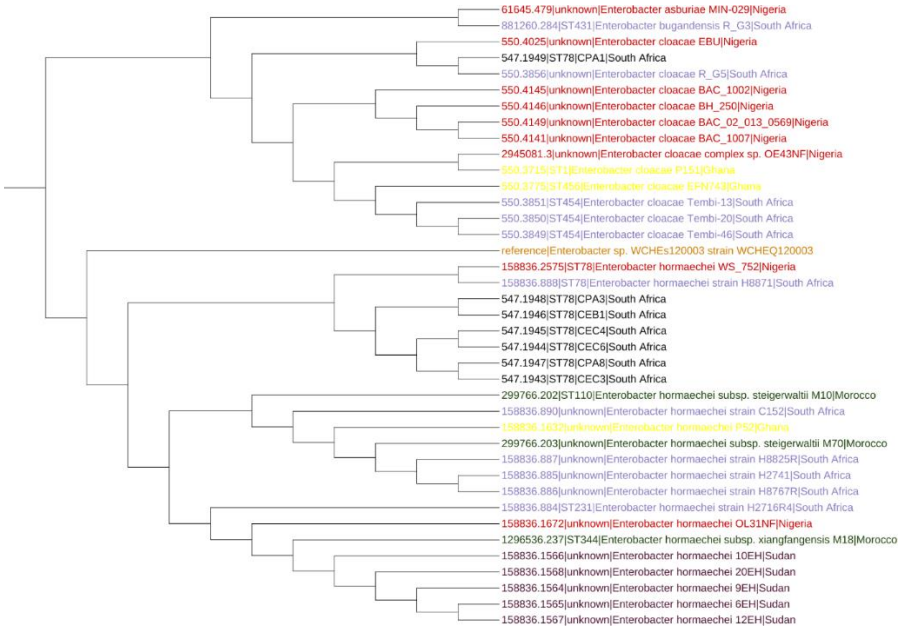

Figure 5.b: Phylogenetic tree showing the relationship between clinical *Enterobacter hormaechei*(black) in this study and other Enterobacter species from human samples in Africa

Appendix 6: Sample size calculation

$$n = \frac{(Z_{\alpha/2}+Z_{\beta})^2 \times [P_1 (1-P_1) + P_2 (1-P_2)]}{(P_1 - P_2)^2}$$

$$n = \frac{(1.96+0.84)^2 \times [0.65 (1-0.65) + 0.5 (1-0.5)]}{(0.65 - 0.5)^2}$$

$$n = 166$$

n= sample size
$Z_{\alpha/2}$= critical value of normal distribution at $\alpha/2$, (for a confidence level of 95%, α=0.05 and $Z_{\alpha/2}$= 1.96)
Z_{β}= critical value of normal distribution at ß, (for a power of 80%, ß=0.2 and Z_{β}=0.84)
P_1= expected resistance percentage in clinical samples (P_1=0.65)
P_2= expected resistance percentage in effluent samples (P_2=0.5)

Appendix 7: Proof of submission to BMC Microbiology