MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC-RESISTANT ESKAPEE PATHOGENS IN SURFACE WATER IN PROXIMITY TO INFORMAL SETTLEMENTS: A TALE OF TWO CITIES

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

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This is a dissertation by manuscript with an introduction and the final summary of the research.

This is to certify that the contents of this dissertation are the original research work by Miss Fulufhelo N. Mukwevho, supervised by:

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DECLARATION

I, Ms Fulufhelo Naomi Mukwevho, declare as follows:

i. That the work described in this dissertation has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

ii. That my contribution to the project was as follows:

   a. The research and findings reported in this dissertation is my original work.

   b. This dissertation does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.

iii. This dissertation does not contain other persons' writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. Their words have been rewritten, but the general information attributed to them has been referenced.

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Signed [redacted] Date 29/03/2025

DEDICATION

I dedicate this research to my dearest mother, Livhuwani Mukwevho, and family and friends for their unwavering love and support throughout this project.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

A. baumannii	*Acinetobacter baumannii*
ABC	ATP Binding Proteins
ABR	Antibiotic Resistance
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Genes
ARU	Antimicrobial Research Unit
BREC	Biomedical Research Ethics Committee
BSI	Bloodstream Infections
BV-BRC	Bacterial and Viral Bioinformatics Centre
CARD	Comprehensive Antibiotic Resistance Database
CF	Cystic fibrosis
CGE	Centre for Genomic Epidemiology
CLSI	Clinical and Laboratory Standards Institute
COVID	Corona Virus
CRAB	Carbapenem Resistant *Acinetobacter baumannii*
CRKP	Carbapenem Resistant *Klebsiella pneumoniae*
E. cloacae	*Enterobacter cloacae*
E. coli	*Escherichia coli*
E. faecium	*Enterococcus faecium*
E. kobei	*Enterobacter kobei*
ECC	Enterobacter cloacae Complex
ESBL	Extended spectrum beta-lactamase
ESKAPEE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp, Escherichia coli*
EUCAST	European Committee of Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
HGT	Horizontal Gene Transfer
IS	Insertion Sequence
KZN	KwaZulu-Natal
MATE	Multidrug and Toxic Compound Extrusion

MDR	Multidrug Resistance
MFS	Major Facilitator Superfamily
MGE	Mobile Genetic Elements
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSSA	Methicillin Susceptible *Staphylococcus aureus*
NICD	National Institute of Communicable Diseases
OMP	Outer Membrane Proteins
P. aeruginosa	*Pseudomonas aeruginosa*
PBP	Penicillin Binding Protein
PCP	Pneumocystis Pneumonia
PCR	Polymerase Chain Reaction
SMR	Small Multidrug Resistance
UK	United Kingdom
USA	United States of America
UTI	Urinary Tract Infections
VRE	Vancomycin Resistant Enterococci
WASH	Water, Sanitation and Hygiene
WGS	Whole Genome Sequencing
WHO	World Health Organisation
WWTP	Wastewater Treatment Plant
XDR	Extensively Drug Resistant

APPENDICES

ABSTRACT

Drug-resistant *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp and *Escherichia coli* (ESKAPEE) are increasingly identified in wastewater and surface water of rivers and streams, presenting a transmission risk to humans, animals, and plants. Using whole genome sequencing and bioinformatics analysis, we investigated the resistome, mobilome, and phylogenetic relationships of antibiotic-resistant ESKAPEE bacteria in surface water from two cities. Water samples (500 mL) from streams near informal settlements in Durban and Pietermaritzburg were filtered through a 0.45 µm filter membrane. The ESKAPEE were identified on selective media, purified and tested for antibiotic susceptibility using the VITEK® 2 platform. DNA was extracted from isolates for whole genome sequencing, followed by bioinformatics analysis using the open-source CARD, CGE, RAST, BV-BRC and PubMLST tools. Eleven *E. faecium*, 12 *E. coli*, four *K. pneumoniae* and one *Enterobacter* isolate were molecularly identified. Cephalosporin-resistant *E. coli* was found in Durban with the AcrAB-TolC efflux pump that conferred resistance to multiple antibiotic classes. The ARGs identified *in E. coli* were $bla_{\text{TEM-1}}$, *qnr*B19 and *qnr*S1, *sul*1, *sul*3, *dfr*A12, *tet*(A), *cml*A1, *aad*A1 and *aad*A2. ARGs a*ac(6)*-Ii, *ant(6)*-Ia and *aph(3")*-III, *tet*(M) and *tet*(L), *msr*(C) and *erm*(B) and *dfr*G were detected in *E. faecium*. The Durban *K. pneumoniae* isolates were MDR harbouring $bla_{\text{SHV-75}}$, $bla_{\text{SHV-110}}$, $bla_{\text{SHV-81}}$, $bla_{\text{CTXM-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, and $bla_{\text{OXA-1}}$. *E. kobei* only harboured bla_{ACT} and *tet*(A) genes that showed phenotypic resistance against piperacillin- tazobactam. ARGs and MGEs in *E. faecium* were mostly carried on chromosomes. Plasmid-carried ARGs were associated with IS1, IS1B, IS6, IS256 and ISKpn19, and the Tn3 transposons in *E. coli*. Of all identified ESBL genes in *K. pneumonaie*, only bla_{TEM}, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ were co-carried on plasmids and associated with ISKpn25, ISNCY, IS3, IS1, IS5075, IScep1, and Tn3. Phylogenetic analysis revealed close relationships with other South African human, animal and environmental isolates. The identified ARGs and their associations with MGEs present potential transmission routes of these resistance genes within and across bacterial species in aquatic environments, making these surface waters a potential reservoir for antibiotic resistance transmission.

CHAPTER ONE

Introduction and literature review

1.1. Introduction

The persistence of antibiotic resistance (AMR) continues to be a global health challenge. To emphasise the direness of the challenge, the World Health Organization (WHO) released a list of pathogens urgently needing new antibiotics in 2017 (WHO, 2017). These were categorised into three priorities: critical, high and medium. In 2024, this list was updated following new and updated ABR reports. The critical pathogens included carbapenem-resistant *A. baumannii*, carbapenem-resistant and third-generation cephalosporin-resistant Enterobacterales. Moreover, vancomycin-resistant *E. faecium*, carbapenem-resistant *P. aeruginosa*, and methicillin-resistant *S. aureus* were listed as high-priority pathogens (WHO, 2024). The acronym "ESKAPE" was formulated by the Infectious Diseases Society of America to emphasise these pathogens as the leading cause of hospital infections with the ability to "escape" antimicrobial activity (Pogue et al., 2015). As *E. coli* forms part of this study, the acronym becomes ESKAPEE.

ESKAPEE pathogens have been isolated from environmental reservoirs, including surface water, wastewater, food, and soil (Denissen et al., 2022). They are known to cause nosocomial infections, i.e., acquired infections when patients are under healthcare. Nosocomial infections are more prevalent in developing and developed countries (Khan, 2017; Raoofi et al., 2023). These infections affect many patients, increasing the mortality rate and financial loss. Moreover, different antibiotics are used to treat these infections (Khan, 2017). However, with uncontrolled usage and overconsumption, pathogens can develop resistance to a wide range of antibiotic classes (Yadav & Kapley, 2019)

Antibiotics are indiscriminately used in healthcare, agriculture and livestock production (Ghimpețeanu et al., 2019; Yadav & Kapley, 2019). Moreover, humans largely excrete antibiotics through faeces and urine (Larson, 2014). This increases their abundance in the environment, creating selection pressure for the development/escalation of ABR (Yadav & Kapley, 2019). The public and private health sectors both contribute to inappropriate antibiotic usage. In 2020, extended-spectrum penicillins were the most used antibiotics in the public sector in South Africa, accounting for 28% of total antibiotics. Trimethoprim-sulfamethoxazole and metronidazole accounted for 13% and 12%, respectively. Extended-spectrum penicillins, carbapenems (imipenem, meropenem, ertapenem and doripenem) and third-generation cephalosporins accounted for 41%, 20% and 13% usage in the private sector, respectively. Between 2018 and 2020, there was an increase in macrolide usage, which may reflect the impact of the Coronavirus Disease (COVID) pandemic in South Africa (Department of Health, 2021).

Different mechanisms of resistance have been studied in ESKAPEE pathogens. One of the mechanisms is drug inactivation or alteration by bacterial enzymes such as β-lactamases, chloramphenicol or aminoglycoside-modifying enzymes, which cause an irreversible modification and inactivation of the antibiotic (Santajit &

Indrawattana, 2016). β-lactamases are one of the well-characterised enzymes. Other mechanisms include modification of the drug binding sites to avoid recognition by the antibiotic and reduced intracellular drug accumulation by porin loss and efflux pumps (Santajit & Indrawattana, 2016). These mechanisms are mainly driven by the persistent selective pressure that the bacteria encounter in the environment, with genome plasticity playing a crucial role in the distribution of antibiotic-resistance genes (ARGs) (Das et al., 2022).

In the One Health approach, antibiotic-resistant bacteria are assessed considering their prevalence in humans, animals and the environment (Azuma et al., 2022). Highlighting the further importance of AMR, the South African Departments of Health and Agriculture, Forestry, and Fisheries initiated the South African Antimicrobial Resistance National Framework: A One Health Approach 2017-2024. This was initiated to provide AMR management structures in the human, animal and environment and limit infection increases by antibiotic-resistant bacteria while improving the population's health (Departments of Health, Agriculture, Forestry and Fisheries, 2017).

Informal settlements form a great part of human settlements in South Africa. Informal settlements develop, grow, and spread fast, limiting the city management's ability to keep up with service provision. As a result, the associated challenges, including air and water pollution, overcrowding, poor housing, and pollution by waste increase (Parikh et al., 2020, Zerbo, Delgado & Gonzalez, 2020). Sub-optimal access to safe potable water and inadequate sanitation increase communicable disease transmissions, specifically bacterial and viral infections. All these factors contribute to a highly negative impact on the health of the affected communities, impacting the health quality and life expectancy (Marais & Cloete, 2014).

Gqomfa, Maphanga and Shale (2022) investigated the impact of the Dunoon informal settlement on the nearby Diep River in Cape Town and the associated health impact. *E. coli* was used as a pollution indicator using the South African limits of 0 colony forming units (CFU)/100 ml in irrigation water and 0-130 CFU/100 ml in recreational water. Both these limits were surpassed as *E. coli* levels were recorded at 11737.5 CFU/100 ml in winter and 1435.3 CFU/100 ml in summer, indicating high pollution levels in the river. Although most of the interviewed residents mentioned they did not use the water, 41/249 reported using it for irrigation (Gqomfa, Maphanga & Shale, 2022). This is concerning as gastrointestinal diseases, urinary tract infections (UTI), and respiratory tract infections may arise from intentional or accidental ingestion of pathogenic bacteria from the water by people living in these areas (Mbanga et al. 2020; Higuita & Huycke, 2014; David & Daum, 2017).

There are multiple routes through which antibiotic-resistant bacteria (ARB) enter and remain in the environment and ecosystem. Their ability to persist and acquire ARGs regardless of the environmental conditions allows them to accumulate in the environment (Yadav & Kapley, 2019). Surface waters such as rivers, lakes and streams have been reported as antibiotic resistance hotspots and key reservoirs of ARGs of clinical importance (Ekwanzala et al., 2018). The total amount of resistance genes accumulated in an ecosystem is then regarded as the resistome (Yadav & Kapley, 2019). Antibiotic-resistant genes are carried on the mobilome termed the mobile genetic elements (MGEs), such as plasmids, integrons, gene cassettes, insertions sequences, and transposons

(Founou et al., 2018). Antibiotic resistance genes on these elements can be transferred from cell to cell by vertical and horizontal gene transfer (HGT) (Carr et al., 2021).

Mobile genetic elements such as insertion sequences, integrons and transposons are acquired by the plasmids and used in disseminating resistance genes. Integrons are characterised by the *int* integrase site-specific gene. They capture gene cassettes whose transcription is enabled by the *att1* recombination site and a *p* promoter gene. Gene cassettes in the integrons consist of single or multiple genes conferring resistance to several classes of antibiotics. While lacking mobility, integrons can be found on the bacterial chromosome and, therefore, regarded as chromosomal integrons or be mobile provided they are transposed on the plasmids (Mbelle et al., 2019)

The location of ARGs on MGEs makes it easy for ARGs to be transferred within a bacterial community, even when the taxonomic lineage is distant (Che et al., 2019). Certain ARGs have been detected by culture-dependent and culture-independent approaches and as high-risk ARGs (Raza et al., 2021; Zhang et al., 2021). The current study will test and analyse the antibiotic resistance profiles of ESKAPEE pathogens. The resistome, mobilome, and phylogenetic relationships will be delineated through whole genome sequencing to give a better understanding of antibiotic resistance in the ESKAPEE pathogens and their spread in surface water in proximity to informal settlements in the two cities, Durban and Pietermaritzburg.

1.2. Literature Review

1.2.1. Antibiotic-resistance

Antibiotics are critical in treating and preventing bacterial infections in human medicine. However, bacteria have developed the ability to resist antibiotic activity, referred to as antibiotic resistance (Stanton et al. 2020). Two types of antibiotic resistance are distinguished: intrinsic and acquired resistance.

1.2.2. Intrinsic resistance

Intrinsic resistance occurs naturally and exists in all bacterial species without previous antibiotic exposure. This resistance is attributed to neither selective pressure nor horizontal gene transfer of resistance genes. Low drug affinity to the bacterial target, the drug's inability to access bacterial cells, drug extrusion by efflux pumps and the production of antibiotic-degrading enzymes plays a critical role in intrinsic resistance (Kostyanev & Can, 2017).

a. Outer membrane

Gram-negative pathogens are characterised by the presence of an impermeable outer membrane. The morphology of the outer membrane is attributed to the presence of an asymmetric lipid bilayer with lipopolysaccharides and phospholipids positioned externally and internally (Ude et al., 2021). Hydrophobic and polar molecules are then prevented entry by the sturdy lateral interactions connecting the lipopolysaccharide molecules (Lehman & Grabowicz, 2019). Owing to the impermeability of the outer membrane, bacteria need to possess a mechanism for nutrient uptake. One mechanism is the presence of outer membrane proteins (OMP) called porins, which allow for the passage of nutrients necessary for bacterial growth but still restrict the influx

of antibiotics and decrease susceptibility in Gram-negative pathogens (Cox & Wright, 2013). *Enterobacterales, P. aeruginosa* and *A. baumannii* have the characteristic outer membrane; some antibiotics are prevented from entry and cannot locate nor reach the target for antimicrobial action (Ude et al., 2021).

The presence of porin proteins plays a role in the antibiotic influx. A decrease in the porin proteins will decrease antibiotic influx into the cell, allowing the bacteria to maintain antibiotic resistance. In *P. aeruginosa,* reducing the porin protein OprD enables resistance to imipenem. Similarly, in *A. baumannii,* loss of the 29-2KDa OMP reduces sensitivity to imipenem and meropenem. Lastly, in *K. pneumoniae*, the loss of the OMP ompK35 and ompK36 coupled with the production of resistance enzymes reduces the sensitivity to the β-lactams cephalosporins and carbapenems (Sanjatit & Indrawattana, 2016). However, the outer membrane sometimes slows down the influx of small molecules and does not entirely restrict entry, contributing only a certain level of intrinsic antibiotic resistance. An additional mechanism is the presence of efflux pumps, which ensures greater intrinsic antibiotic resistance (Cox & Wright, 2013).

b. Efflux pumps

Efflux pumps are the membrane proteins exporting antibiotics from the cell (Kapoor, Saigal & Elongavan, 2017). The intracellular concentrations of the antibiotics are reduced as efflux pumps function synergistically with other mechanisms of resistance to provide elevated levels of antibiotic resistance (Nishino et al., 2020). As the antibiotics enter the pathogen's cell, the efflux mechanism pumps out the antibiotic at the same speed. Although all bacteria have efflux pumps, they can also be antibiotic-specific. In contrast, others pump various unrelated antibiotics, including macrolides, tetracyclines and fluoroquinolones, contributing significantly to multidrug-resistant (MDR) organisms (Kapoor, Saigal & Elongavan, 2017). The efflux genes are located on the chromosomes and can be carried by plasmids (Nishino et al., 2020).

Bacteria possess five different classes of efflux pumps based on the structure and energy source. The first class is the ATP-binding cassette (ABC) family that uses energy from ATP hydrolysis to transport antibiotics (Pucelik & Dąbrowsk, 2022). The second one is the multidrug and toxic compound extrusion (MATE) family, which uses a sodium ion gradient as the energy source and extrudes most fluoroquinolones and some aminoglycosides. The small multidrug resistance (SMR) family efflux mainly consists of lipophilic cations. The major facilitator superfamily (MFS) transports macrolides and tetracyclines. MFS are substrate-specific but still have the greatest diversity. *A. baumannii* has separate pumps for erythromycin and chloramphenicol. The NorA pump in *S. aureus* transports fluoroquinolones and chloramphenicol, while another pump, LmrS transports linezolid, erythromycin, chloramphenicol, and trimethoprim (Reygaert, 2018). The last family is the resistance modulation cell division (RND), found almost exclusively in Gram-negative bacteria. Some are drug-specific; the Tet pump pumps tetracycline, while the Mef pump pumps out macrolides. The non-drug-specific MexAB-Oprm pump in

P. aeruginosa confers resistance to β-lactams, chloramphenicol, tetracycline, trimethoprim, and some fluoroquinolones (Reygaert, 2018). *E. coli* efflux systems include the RND pump, which confers resistance to tetracyclines, vancomycin, chloramphenicol, certain β-lactams and fluoroquinolones (Ullah & Ali, 2021).

1.2.3. Acquired resistance

Acquired resistance occurs when the bacterial chromosomal DNA undergoes mutations (Chattopadhyay, Das & Banerjee, 2023). Genetic mutations may arise from UV radiation, chemicals, and stress and are referred to as substitution and deletion of nucleotide bases changing the bacterial genome (Chattopadhyay, Das & Banerjee, 2023). The emergence of mutational resistance eliminates the antibiotic susceptible population while the resistant bacteria become increasingly prevalent (Munita & Arias, 2016). Acquired genetic changes facilitate resistance mechanisms, namely target modifications and antibiotic inactivation (Kostyanev & Can, 2017).

a. Target site modifications

In this mechanism, acquired changes in the target binding site prevent drug binding. Changes in the target site are often caused by spontaneous mutation of the bacterial gene on the chromosome. With a strictly site-specific antibiotic interaction, minor changes in the target site molecule can still significantly impact antibiotic binding (Kapoor, Saigal & Elongavan, 2017). Binding site modification follows three mechanisms: target site enzyme modification, ribosomal target site alteration and cell wall precursor alterations (De Oliveira et al., 2020).

Antibiotics such as macrolides, tetracyclines, and chloramphenicol affect the protein synthesis of the pathogen by altering the ribosomal 30S or 50S subunit. Mutations in the penicillin binding protein (PBP) reduce affinity to β-lactam antibiotics. *E. faecium* resistance to ampicillin is conferred by this mechanism (Kapoor, Saigal & Elongavan, 2017). Methicillin resistance in *S. aureus* is mediated by the acquisition and expression of the *mecA* gene, which encodes for PBP2a. This is the modified version of PBP with a low affinity for β-lactams, rendering most β-lactam antibiotics ineffective (De Oliveira et al., 2020). Mutations occur in the *mgrB* gene or the genes encoding the PhoPQ two-component system; these changes have been associated with *K. pneumonia's* resistance to colistin. In this case, phosphoethanolamine is added to the lipid A, preventing colistin from binding to the lipopolysaccharide (Kostyanev & Can, 2017). The *mcr-1* gene, discovered in Enterobacterales, is one of the phosphoethanolamine transferase enzymes responsible for phosphoethanolamine addition to lipid A (Kostyanev & Can, 2017).

b. Antibiotic inactivation

Antibiotic inactivation occurs through two mechanisms: the degradation of the antibiotic or the transfer of a chemical group to the antibiotic. In antibiotic degradation, β-lactamases play a significant role as a group of drug hydrolysing enzymes (Reygaert, 2018). Besides β-lactamases, such as aminoglycoside-modifying enzymes or chloramphenicol enzymes are produced to modify and inactivate these antibiotic classes. The highly prevalent β-lactamases hydrolyse the β-lactam ring, which is present in all penicillins, cephalosporins, monobactams, and carbapenems. Enzymes such as penicillinase, cephalosporinase, extended-spectrum beta- lactamases (ESBLs), and carbapenemases fall under the β-lactamase category, inactivating penicillins, third- generation

cephalosporins (ceftazidime, cefotaxime and ceftriaxone), aztreonam, and carbapenems. However, some of these enzymes can be inhibited by the β-lactamase inhibitors, clavulanic acid, sulbactam or tazobactam (Santajit & Indrawattana, 2016).

Enzymes may also be modified in a post-translational modification. Acetyl, phosphoryl and adenyl groups from bacterial enzymes are added in acetylation, phosphorylation and adenylation, respectively. These groups are added to specific sites on the antibiotics to chemically modify them while inactivating the antibiotic and losing its ability to bind to the target site (Abushaheen et al., 2020). Aminoglycoside, chloramphenicol, streptogramins and fluoroquinolones resistance is enabled through acetylation, while phosphorylation only confers resistance to aminoglycosides (Reygaert, 2018).

1.2.4. Antibiotic-resistant ESKAPEE pathogens

The term "ESKAPEE" is derived from the seven pathogens *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp and *E. coli.* Their importance is highlighted in their ability to develop and acquire drug resistance even to the last line of defence antibiotics, presenting a global health threat (Mulani et al., 2019). O'Neill (2014) estimates that by 2050, 10 million people will die from antimicrobial resistant pathogen infections if there are no actions taken to combat the active spread and effect of antimicrobial resistance.

1.2.5. Vancomycin-resistant *enterococci* (VRE)

According to Gao, Howden & Stinear (2018), Enterococci are mostly non-pathogenic human gastrointestinal microflora. However, reports from as early as the 1980s highlighted two distinct species, *Enterococcus faecalis* and *Enterococcus faecium,* as the pathogenic bacteria commonly isolated from healthcare settings. These pathogens are commonly associated with UTI, bacteraemia, and endocarditis (Higuita & Huycke, 2014). After the discovery of penicillin-resistant enterococci, a combination of penicillin and aminoglycosides yielded better results due to a synergistic activity produced (Miller, Munita & Arias, 2014; Winstanley & Hastings, 1989). This improved the cure rates from 40% to 88% (Miller, Munita & Arias, 2014). However, the persistence of penicillin-resistant enterococci highlighted the need for new and effective antibiotics, ultimately leading to vancomycin's discovery in the late 1950s.

Orally administered vancomycin was used to treat bacteria-associated diarrhoea in hospitals (Rice, 2001). In the late 1980s, the emergence of vancomycin-resistant enterococci (VRE) was reported in Europe and the United States of America (USA). The resistance was later reported in Australia in 1994 (O'Toole et al., 2023). Increases in VRE prevalence in hospitalised patients have since been reported in several European countries (Lucet et al., 2007; Egan et al., 2020; Gast et al., 2021).

Because of its bactericidal activity when binding to the bacterial cell envelope, vancomycin was immune to resistance, unlike most antibiotics that target a protein. However, the emergence of two complex resistance mechanisms comprising multi-enzyme pathways threatened vancomycin's efficacy in treatment. Here, an

intricate network of hydrogen bonds with the D-Ala-D-Ala region of the Lipid II is formed, interfering with the maturation process of the peptidoglycan layer. This natural precursor is then degraded and replaced with D-Ala-D-lac or D-Ala-D-Ser alternatives (Stogios & Savchenko, 2020). When D-Ala-D-D- Ala is replaced with –D-lac, the binding constant between vancomycin and peptidoglycan decreases 1000-fold as a single hydrogen bond is lost, conferring high vancomycin resistance levels. This resistance is conferred by the core resistance cassette composed of five genes, *van*A, *van*B, *van*D, *van*F and *van*M. Lower levels of resistance are conferred when additional hydroxyl groups are added for –D-Ser replacement, a steric hindrance for vancomycin interaction is introduced, lowering the drug affinity six-fold, conferred by *van*C, *van*E, *van*G, *van*L and *van*N genes (Stogios & Savchenko, 2020).

The first VRE isolates were reported in South Africa in 1997 (Budavari et al., 1997; Foka, Kumar & Ateba, 2018). In 2012, the National Institute of Communicable Diseases (NICD) reported an outbreak of VRE (Ekwanzala et al., 2018). And have since been transmitted and isolated in environmental sources. Adegoke et al. (2022) evaluated the occurrence of VRE in wastewater treatment plants (WWTP) in Durban and the associated receiving surface water. *E. faecium* and *E. faecalis* were the most isolated species. Vancomycin resistance was detected in 93.1% of the species. This study also reported a high prevalence of the *van*A gene, which confers resistance to vancomycin and teicoplanin. The gene was located on transposons, which means it is easily transferred and can be used to explain the isolation of VRE isolates in high numbers, posing a public health risk (Adegoke et al., 2022).

Multidrug-resistant (MDR) isolates were identified in that study, and most isolates were resistant to cefixime (100%), ciprofloxacin (90.1%), tetracycline (90.1%), and streptomycin (91.1%). Several isolates were reported as intermediate or resistant to quinupristin-dalfopristin (Adegoke et al., 2022). Resistance to quinupristin/dalfopristin, a currently approved therapeutic option for VRE infections (Parmanik et al., 2022), is of great concern. Quinupristin/dalfopristin, linezolid and daptomycin are currently approved as treatment options for VRE infections. Daptomycin's activity is concentration-dependent, and higher doses have been associated with better outcomes (Chuang et al., 2022). However, the United States Food and Drug Administration (FDA) has only approved doses (4-6 mg/kg) in clinical treatment, which has seen unsatisfactory clinical outcomes (Chuang et al., 2022). Chuang et al. (2022) conducted an observational study to determine the relation between daptomycin doses and efficacy and safety using 28-day mortality as the primary outcome. The study reported that patients who received high doses of daptomycin (>11 mg/kg) had a lowered 28-day mortality compared to those who received a lower dose (8-11 mg/kg). However, the high dose was associated with an increased risk of high creatine kinase levels, an enzyme associated with muscle damage (Nanavati et al., 2022). Chuang et al. (2022) also assessed the efficacy of combination therapy of β-lactams and daptomycin.
This is because a synergistic effect has been detected when daptomycin and β-lactams are combined, and this was supported in the study conducted by Chuang et al. (2022).

1.2.6. Methicillin-resistant *Staphylococcus aureus* (MRSA)

Staphylococci, known to colonise the human epithelia, cause numerous nosocomial infections as opportunistic pathogens. Virulent species of this group can cause severe or even fatal diseases (Stefani et al., 2012). *Staphylococcus aureus* common infections include skin, bone, bloodstream, and respiratory tract infections (David & Daum, 2017). Following the history of treating *S. aureus*-caused infections, it is highlighted that penicillin, accidentally discovered in 1928, was first used to treat these infections. This then led to the development of different classes of antibiotics (Khan, 2017). The usage of penicillin was supported by the 85% susceptibility of *S. aureus*. However, for patients who showed allergic reactions to this antibiotic, macrolides, lincosamides, and streptogramins were opted for (Schito, 2006).

A report of penicillin-resistant *S. aureus* was first published in 1940, leading to the development of methicillin (Khan, 2017). Aminoglycosides were introduced in 1944, but resistance was already reported by the 1950s. Quinolone, first introduced to treat Gram-negative infections, was soon used to treat *S. aureus* infections because of its Gram-positive spectrum of activity (Schito, 2006). According to Enright et al. (2002), methicillin was introduced in 1959 as a treatment option for penicillin-resistant *S. aureus* infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) was first reported in 1961 in the United Kingdom (UK). Soon after, the resistance was highlighted in European countries, followed by Japan, Australia and the United States and has since become a problem in hospitals and communities (Enright et al., 2002)

The characteristics of MRSA have been linked to an altered PBP that confers resistance to all penicillins, cephalosporins and carbapenems. This has, in turn, made β-lactams clinically ineffective (Gajdács, 2019). The resistance has also been to various other antibiotic classes, including macrolides, fluoroquinolones, lincosamides, tetracyclines and aminoglycosides (Algammal et al., 2020). MRSA has acquired MGEs carrying multiple resistance genes such as *blaZ, dfr*A and *dfr*K, *erm*C, *tet*K and *tet*L conferring resistance to penicillin, trimethoprim, erythromycin, and tetracycline, respectively. These genes have been identified in the insertion sequences, transposons, and plasmids in the MRSA and the methicillin-susceptible *Staphylococcus aureus* (MSSA) (Turner et al., 2019).

To track and highlight new antibiotic resistance profiles, environmentally isolated *S. aureus* pathogens must be continuously studied. Ramessar & Olaniran (2019) conducted an environmentally based study to characterise MRSA recovered from hospital and domestic treated wastewater effluent and the receiving surface water in Durban, South Africa. Eighty isolates were confirmed as MRSA by biochemical tests and the presence of the *mecA* gene. All 80 isolates were resistant to lincomycin, followed by oxacillin (79/80), cefoxitin and penicillin ((78/80), and ampicillin (77/80). Susceptibility was observed in amikacin (78/80), gentamicin (75/80), ciprofloxacin (73/80), and imipenem (72/80). Detection of these potentially pathogenic multidrug-resistant (MDR) pathogens in surface water could have detrimental health effects on individuals who encounter them, justifying the need for more environmental-based studies (Ramessar & Olaniran, 2019).

Akanbi et al (2017) conducted an aquatic environment-based study to determine the antimicrobial resistance patterns of *S. aureus* and MRSA from seawater and sand from selected beaches in Eastern Cape, South Africa. Thirty confirmed *S. aureus* isolates were screened for *bla*$_Z$ *rpo*B, *mec*A, *erm*A, *erm*C, *tet*K, *tet*M, *van*A, and *van*B genes. The *bla*$_Z$ gene, which encodes resistance to β-lactam antibiotics, was isolated in 16 of the isolates, *mec*A gene that codes for methicillin resistance was detected in 5 isolates, *rpo*B gene that codes for rifampicin resistance was detected in 11 isolates. The *erm*(B) gene that codes for erythromycin resistance was detected in 15 isolates. The *tet*M gene, which codes for tetracycline resistance, was detected in eight isolates. ARGs such as *erm*A, *erm*C, *tet*K, *van*A, and *van*B were undetected. The *erm*B, *mec*A, and *tet*M gene detection correlated with the resistance profiles detected when the isolates were tested against the respective antibiotics (Akanbi et al., 2017).

Currently, the treatment options for MRSA infections are linezolid, tigecycline, and daptomycin (Nandhini et al., 2022). These are novel antimicrobial drugs with significant action against MRSA infections. Linezolid works by resisting the initiation of protein synthesis in MRSA as a complete synthetic oxazolidinone. Because of the constant antibiotic resistance, research has also focused on combination therapy as an efficient treatment approach. In a study to determine the efficacy of combination therapy, combinations of carbapenem with linezolid, daptomycin, and fosfomycin and daptomycin and oxacillin showed a high synergistic effect against MRSA strains (Nandhini et al., 2022).

1.2.7. Carbapenem and third-generation cephalosporin-resistant Enterobacterales

β-lactams have been widely used to treat infections caused by Gram-negative bacteria worldwide (Sarojamma & Ramakrishna, 2011). Their usage dates back to the 1940s through the discovery of penicillin. Four classes of β-lactams are distinguished: penicillins, carbapenems and monobactams (Tooke et al., 2019). Cephalosporins have been used to treat mild to severe infections. Their spectrum of activity extends to both Gram-positive and Gram-negative MDR pathogens. Their mechanism of action inhibits the growth of bacterial cells by disrupting the cross-link between the peptidoglycan chains of the cell walls. Five generations of cephalosporins have been developed to date, each in response to resistance developed to the predecessor (Lin & Kück, 2022). The emergence and persistence of *E. coli* and *K. pneumoniae* resistance to third-generation cephalosporins is a grave concern. This has been linked to the production of ESBLs in Enterobacterales (Feng et al., 2022).

ESBLs hydrolyse third-generation oxyimino cephalosporins cefotaxime, ceftazidime and ceftriaxone while the pathogens remain susceptible to carbapenems and cephamycins (Gumede et al., 2021). The plasmid-mediated ESBL enzymes, TEM, SHV and CTX-M widely disseminate and are frequently isolated in Enterobacterales (*K. pneumoniae and E. coli,*) with a unique ability to extend their spectrum of activity as new antibiotics are introduced (Tooke et al., 2019 & Gumede et al., 2021). As antibiotics are continuously used, pathogens continue to adapt by mutating. Such mutations may be as simple as changing one amino acid and greatly altering the β-lactamases functionality, giving rise to an increased prevalence of ESBLs that render multiple and newly developed β-lactams ineffective (Sarojamma & Ramakrishna, 2011; Pandey & Cascella, 2022 & Smith &

Kendall, 2023). In addition to degrading cephalosporins, ESBLs undergo repeated mutations that have developed carbapenemases that hydrolyse carbapenem antibiotics (Sawa, Kooguchi & Moriyama, 2020). Carbapenemase enzymes NDM-1, VIM, KPC, OXA-48 and IMP production have been associated with carbapenem resistance and isolated in *K. pneumoniae, E. coli*, and *Enterobacter* spp. (Ebomah & Okoh, 2020; Smith & Kendall, 2023). The most prevalent carbapenemase-producing Enterobacterales is *K. pneumoniae* (Smith & Kendall, 2023).

1.2.8. Carbapenem & third generation cephalosporin-resistant *Klebsiella pneumoniae*

Klebsiella pneumoniae was first discovered in the late 19th century. Since then, it has become a global pathogen posing a significant threat to human health. A high concentration of *K. pneumoniae* is found in the gastrointestinal tract of humans and some in the nasopharynx through which the bacteria enter the blood circulation causing infection (Wang et al., 2020). There is a wide range of infections caused by *K. pneumoniae*; these are pneumonias, UTIs, bacteraemia as well as abscesses. Severe infections are caused in immunocompromised patients and are often accompanied by extended hospital stays, high treatment costs and mortality (Effah et al., 2020).

The discovery of penicillin more than 80 years ago significantly contributed to treating Gram-negative bacterial infections. *K. pneumoniae* was one of the pathogens that exhibited sensitivity to the antibiotic. However, this was soon challenged as the rapid emergence of β-lactamase-producing *K. pneumoniae* occurred. Penicillinase-producing *K. pneumoniae* continued to spread, leading to the need to develop cephalosporins. However, ESBLs–producing *K. pneumoniae* soon emerged, impacting the activity of both cephalosporins and penicillin, significantly limiting the treatment options (Rapp & Urban, 2012). To successfully treat infections caused by ESBL-producing *K. pneumoniae,* carbapenems were discovered, developed, recognised, and used by clinicians as the last line of defence. Challenges continued to arise as carbapenemase-producing *K. pneumoniae* emerged, leading to the spread of carbapenem-resistant *K. pneumoniae* (CRKP) (Falagas et al., 2007).

Production of carbapenemases OXA-48-like, NDM, KPC and VIM has been associated with the spread of CRKP (Pitout, Nordman & Poirel, 2015). OXA-48 was first discovered in a *K. pneumoniae* isolate in Turkey in the early 2000s (Lim et al, 2020). In the late 2000s, the first discovery of *K. pneumoniae* containing NDM-1 was reported in Mumbai (Moellering, 2010). *K. pneumoniae* strains producing KPC and VIM soon emerged, exacerbating the burden of antibiotic resistance (Won et al., 2011). Various variants of the genes encoding these resistance enzymes have been reported and described, influencing the persistence and spread of CRKP to various environments (Pitout, Nordman & Poirel, 2015). An alarming spread of carbapenemase-producing *K. pneumoniae* has been reported in African countries, including Angola, Mali, Nigeria and South Africa. NDM-1 and OXA-48 have been highlighted as the commonly detected carbapenemases (Ramsamy et al., 2020). 2016 Perovic et al (2016) reported a case of carbapenemase encoding genes in Enterobacterales from South Africa.

K. pneumoniae was the most common pathogen in a total of 1503 isolates. Carbapenemase-encoding genes were detected in 68% of the isolates, with bla_{NDM} being the most common, followed by $bla_{OXA\text{-}48}$. This report highlighted the presence of carbapenemase-encoding genes in South Africa (Perovic et al., 2016).

The increasing prevalence of carbapenem-resistant *K. pneumoniae* is a major concern; 25% of *K. pneumoniae* demonstrated ertapenem resistance in 2020, up from 8% in 2016. As of 2020, one in four *K. pneumoniae* isolates were reported as resistant to carbapenems (Department of Health, 2021). Current treatment options have mainly been effective when used in combination therapy; meropenem/varbobactam has been approved by the FDA for *K. pneumoniae* infections. Its treatment and patient tolerance enhancement has been proven in ongoing clinical trials compared to other therapies. Ceftazidime/avibactam, imipenem/relebactam and aztreonam/avibactam have been reported to have potency when used to treat carbapenem-resistant *K. pneumoniae* infections (Kumar et al., 2022).

1.2.9. Third-generation cephalosporin-resistant *Escherichia coli*

Not all *E. coli* strains are pathogenic; however, the common UTIs in humans are frequently caused by *E. coli*. Moreover, a high number of enteric and systemic infections, such as bacteraemia and nosocomial pneumonia, are caused by *E. coli* (Kaper, Nataro & Mobley, 2004; Lee, Lee & Choe., 2018 & Kao et al., 2023). β-lactams, fluoroquinolones, and aminoglycosides have been used to treat various *E. coli* infections because they inhibit bacterial cell wall synthesis, interfere with the DNA replication, and bind to the 50s subunit of the bacterial 70S ribosome, respectively, inhibiting the growth of *E. coli* effectively (Lee et al., 2018)

E. coli is said to have the highest rate of resistance against antibiotics that have been in use for a long time. This resistance dates to the first usage of sulfonamides in humans in 1930. Sulfonamide-resistant *E. coli* was reported in the 1950s (Galindo-Méndez, 2020). The introduction of penicillin was a short-lived victory against several pathogens, *E. coli* being one of them. In the early 1940s, penicillin-resistant *E. coli* isolates were reported, and a need for second-generation penicillin was highlighted. The 1960s introduced third-generation and broad-spectrum penicillins called aminopenicillins because of their high stability to penicillinases. Amoxicillin and ampicillin were effective against *E. coli* and a few other Gram-negative bacteria, but this was still followed by the development of resistance (Lobanovska & Pilla, 2017).

The introduction of first- and second-generation cephalosporins gave rise to the broad-spectrum enzymes TEM-1 and SHV-1. This then increased the usage of oxyimino-cephalosporins, particularly ceftazidime and cefotaxime. Inevitably, there began an evolution of new β-lactamases, the ESBLs, with hydrolysis activity against these drugs (Castanheira, Simner & Bradford, 2021). ESBLs were first described in the 1980s. Until the 2000s, TEM and SHV were the most predominant ESBLs; CTX-M became the most identified in ESBL-producing *E. coli* isolates, conferring resistance to third-generation cephalosporins (Poirel et al., 2018). CTX-M, originally from members of the *Klyvera* genus, spread to Enterobacterales via conjugative plasmids and have since become the most successful ESBL in Enterobacterales.

The first described CTX-M type was from a clinical isolate in the late 1980s (Rossolini, D'andrea & Mugnaioli, 2008). When they first emerged, CTX-M variants had efficient hydrolysing activity against cefotaxime and ceftriaxone. Unlike TEM- and SHV- type, CTX-M enzymes were not as efficient against ceftazidime. However, CTX-M variants with enhanced activity against ceftazidime were later described (Castanheira, Simner & Bradford, 2021). Environmental settings such as the soil, wastewater and surface water have been studied and found to harbour antibiotic-resistant *E. coli* and the associated ARGs (Gumede et al., 2021. The use of contaminated water may make way for the dissemination of ESBL-producing *E. coli* in the community. Moreover, the genes encoding the resistance enzymes are carried on the plasmids, complicating the problem further as these genes are continuously transferred across bacterial communities and species.

Nzima et al. (2020) conducted a study to evaluate the patterns of antibiotic resistance and ESBL genes in *E. coli* isolated from wastewater and the recipient surface water in South Africa. $bla_{\text{CTX-M}}$, $bla_{\text{SHV,}}$ and bla_{TEM} occurrences were determined using polymerase chain reaction (PCR). Confirmed *E. coli* isolates were a total of 140. Penicillin, tetracycline, cefotaxime, trimethoprim, ciprofloxacin, ceftazidime, imipenem, meropenem and ampicillin were used for susceptibility testing. *E. coli* isolates were frequently resistant to penicillin, tetracycline, and ampicillin. Resistance to cefotaxime, ceftazidime and trimethoprim was moderate (less than 50% of the isolates), while no resistance was observed to imipenem and meropenem. bla_{TEM} and $bla_{\text{CTX-M}}$ genes were detected in four isolates, and no *bla*SHV was detected. Resistance to cefotaxime and ceftazidime was highlighted in this study, indicating an escalating emergence of *E. coli* resistance to third-generation cephalosporins. Moreover, isolates that were not susceptible to cefotaxime expressed at least one type of the screened ESBL genes (Nzima et al., 2020).

Gumede et al. (2021) conducted a similar study to Nzima et al. (2020) by characterising ESBL-producing *E. coli* from wastewater. From this study, 80 isolates were identified as *E. coli*. Resistance was observed to penicillin, first, second, third and fourth-generation cephalosporins, and carbapenems. Resistance was considerably low in third- and fourth-generation cephalosporins and carbapenems. However, over 50% of the isolates were resistant to cefixime and cefotaxime, but less than 30% were resistant to ceftazidime and cefepime. There were 39 ESBL-producing *E. coli*, and ESBL ARGs were detected in 30 isolates. The most prevalent ARG was $bla_{\text{CTX-M}}$ (20) followed by bla_{TEM} (17) and bla_{SHV} (8). Cefixime and cefotaxime were the third-generation cephalosporins with the highest resistance. Moreover, some isolates carried more than one resistance gene, and ten were resistant to all the cephalosporins. As a priority antibiotic, such resistance to cephalosporins is concerning (Gumede et al., 2021).

Current treatment options for MDR *E. coli* infections rely on carbapenems and fluoroquinolone. Still, the spreading carbapenem resistance brings the need for alternative treatment options (Abavisani et al., 2023) such as polymixins (colistin), tigecycline, aminoglycosides and fosfomycin alone or in combination (Walker et al., 2022). Due to its potency against MDR Gram-negative bacteria, colistin has been increasingly used and is now regarded as the last line of defence antibiotic (Abavisani et al., 2023).

1.2.10. Carbapenem-resistant *Enterobacter cloacae complex*

Enterobacter cloacae complex (ECC) consist of nosocomial infection-causing pathogens, which are *Enterobacter cloacae, Enterobacter asbuarae, Enterobacter hormaechei, Enterobacter kobei, Enterobacter ludwigii* and *Enterobacter nimpressuralis*. (Mezzatesta, Gona & Stefani, 2012). These pathogens cause pneumonia, UTIs and septicaemia (Annavajhala, Gomez-Simmonds & Uhleman, 2019). *Enterobacter cloacae* complex was identified in the 1960s as a group of opportunistic pathogens inhabiting humans, animals, and environmental settings (Davin-Regli, Lavigne & Pagès, 2019). In the 1980s, these pathogens were recognised as clinically important as outbreaks caused by the MDR strains were reported in hospitals (Annavajhala, Gomez-Simmonds & Uhleman, 2019). *Enterobacter cloacae* complex has intrinsic resistance to penicillin, first- and second-generation cephalosporins. This is due to the constitutively expressed chromosomal *AmpC* gene (Annavajhala, Gomez-Simmonds & Uhleman, 2019).

Enterobacter cloacae complex species have become increasingly resistant to aminoglycosides, fluoroquinolones and third-generation cephalosporins (Liu et al., 2022). The first identification of plasmid- mediated ESBLs in ECC was in 1989. In these species, the presence of ESBLs has been associated with resistance to third-generation cephalosporins and monobactams (aztreonam) (Annavajhala, Gomez-Simmonds & Uhleman, 2019). The most concerning resistance is to carbapenems, as these are used to treat MDR ECC infections; therefore, special attention has been placed on these pathogens, particularly *E. cloacae* and *E. hormaechei* as the most isolated in human samples (Liu et al., 2022). Overexpression of *Amp*C combined with a disruption of the membrane permeability and acquisition of carbapenemase genes has been associated with resistance to carbapenems. Carbapenemase-encoding genes bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP} and $bla_{\text{OXA-48}}$ present on the plasmids are reported as the main cause of carbapenem resistance in ECC (Annavajhala, Gomez- Simmonds & Uhleman, 2019). Very few studies have focused on antibiotic resistance in ECC in environmental settings and this opens a gap for continued research on the current trends on these pathogens.

Ebomah and Okoh (2020) evaluated the occurrence of carbapenem-resistant *Enterobacter* species from various environmental samples (wastewater, soil, and surface water) in South Africa and determined antibiotic resistance profiles. Resistance was tested against doripenem, meropenem, imipenem, and ertapenem, and the study also detected carbapenem resistance genes by PCR. In totality, 142 isolates were confirmed *Enterobacter* species, and 115 were *E. cloacae*. The highest resistance was observed to doripenem, at 80%, followed by meropenem (75%), and imipenem (72%) and the lowest resistance was observed to ertapenem (66%). In this report, intermediate susceptible isolates were regarded as resistant. Carbapenem resistance genes were detected in 41 *E. cloacae* isolates, $bla_{\text{NDM-1}}$ was the most frequently isolated gene in 31 isolates, bla_{KPC} was carried by 9 isolates, while $bla_{\text{OXA-48-like}}$ was detected in 3 isolates. This study highlighted the escalating burden of carbapenem resistance. Moreover, using only PCR could have limited the results of this study. Using advanced technologies like whole genome sequencing (WGS) could detect more harboured ARGs and provide more information on the phylogenetic relationships to give light on the transfer of ARGs in different environments.

Resistance to several antibiotics has left carbapenems and the fourth-generation cephalosporins as antibiotics with some effectiveness in treating MDR *Enterobacter* spp infections (Denissen et al., 2022). The most potent antibiotics are carbapenems with reported effectiveness of meropenem and imipenem against *E. cloacae* (Ebomah & Okoh, 2020). With increasing reports of resistance, combination therapy with β-lactams is currently an effective option in treating antibiotic-resistant *Enterobacter* spp (Tompkins & van Duin, 2021).

1.2.11. Carbapenem-resistant *Acinetobacter baumannii*

Acinetobacter baumannii is an opportunistic pathogen associated with hospital-acquired infections. It manifests in immunocompromised patients admitted to the ICU and results in bloodstream infections (BSI) (Rangel, Chagas & De-Simone, 2021). *A. baumannii* has evolved significantly as a hospital pathogen as it resists desiccation, disinfectant action, and major antimicrobials (Isler et al., 2018), thereby facilitating the transmission of these infections in the hospital environment (Rangel, Chagas & De-Simone, 2021).

Until the 1980s, *A. baumannii* was recognised as a nosocomial infection-causing pathogen with little significance. In the 1990s, an emergence of MDR *A. baumannii* occurred, causing infections in intensive care units in European countries and then spreading worldwide (Doi, 2019). Carbapenem resistance in *A. baumannii* has been linked to the loss of outer membrane proteins, modification of the efflux pump and, most importantly, the acquisition of genes encoding oxacillinases (OXA) that hydrolyse carbapenems. Shortly after the introduction of carbapenems, a $bla_{\text{OXA-23}}$ gene was identified in *A. baumannii*. This is an acquired oxacillinase gene from *Acinetobacter radioresistens* and mobilised to *A. baumannii* by an insertion sequence. In 2003, $bla_{\text{OXA-58}}$ was discovered on a plasmid of an MDR *A. baumannii* isolate. Apart from acquired oxacillinases, *A. baumannii* also carries intrinsic bla_{OXA} genes known as $bla_{\text{OXA-58-like}}$ and is used as a marker for speciation. More variants of these genes have since been spread and identified worldwide (Hamidian & Nigro, 2019).

Numerous studies have been conducted where *A. baumannii* isolates were analysed for antibiotic resistance. Adewoyin, Ebomah & Okoh (2021) investigated the antibiotic resistance in *A. baumannii* from three rivers, Great Fish, Keiskamma and Tyhume rivers in the Eastern Cape Province. A total of 410 *A. baumannii* isolates were confirmed by the *recA* and *gyrB* genes from the 844 isolated *Acinetobacter* species. A panel of 11 antibiotics were tested, and resistance was observed against piperacillin-tazobactam, ceftazidime, cefotaxime, cefepime, imipenem, meropenem, amikacin, gentamicin, tetracycline, ciprofloxacin, and trimethoprim (Adewoyin, Ebomah & Okoh, 2021). In another study, Anane et al. (2020) investigated the prevalence of MDR *A. baumannii* and the frequency of carbapenemase-encoding genes. Samples were collected from an abattoir and aquatic environment (dam), in which 85 *A. baumannii* were isolated from the abattoir samples and 98 from the water samples. Resistance was observed in piperacillin/tazobactam, ceftazidime, ciprofloxacin, amikacin, imipenem, and meropenem. All isolates showed susceptibility to colistin and tigecycline. Carbapenem hydrolysing lactamase genes $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$, and $bla_{\text{OXA-23.}}$ were detected in carbapenem-resistant isolates (Anane et al., 2020).

Infections caused by carbapenem-resistant *Acinetobacter baumannii* (CRAB) are accompanied by high mortality rates resulting from treatment failure, leaving only a few treatment options, such as colistin (Piperaki et al., 2019). Therefore, it was of utmost importance that new alternatives were developed, such as minocycline/tigecycline, but resistance to these agents soon followed. Combination of ampicillin, sulbactam and carbapenem is now considered the best therapy for the treatment of MDR *A. baumannii* infections. Minocycline and colistin are used to treat minocycline-*resistant A. baumannii* (Mancuso et al., 2021). Polymyxins, mainly colistin, have been used to treat these infections as they have shown greater *in-vitro* activity against *A. baumannii* (Isler et al., 2018). Moreover, carbapenem-resistant *A. baumannii* strains are rapidly killed by a combination of trimethoprim-sulfamethoxazole and colistin (Mancuso et al., 2021).

1.2.12. Carbapenem-resistant *Pseudomonas aeruginosa*

Although *P. aeruginosa* forms part of the normal intestinal flora, it does not adhere well to the normal intact epithelium; therefore, it cannot cause infections in healthy individuals but causes infections in immunocompromised individuals as an opportunistic pathogen. It can survive on dry inanimate surfaces in the hospital surfaces and equipment for up to 6 months adapting to any environmental change (Pachori, Gothalwal & Gandhi, 2019). Nosocomial infections caused by *P. aeruginosa* include pneumonias, catheter-associated UTIs, and BSIs. Patients with cystic fibrosis (CF) and infected by *P. aeruginosa* suffer from an increased morbidity and mortality risk (Reynolds & Kollef, 2021).

P. aeruginosa's intrinsic resistance to chloramphenicol, macrolides and tetracyclines has been reported since the early 1990s and was found to be attributed to the efflux pump MexAB-Oprm. In the late 1990, isolates from patients with CF were found to be excessively resistant to aminoglycosides in both the UK and USA. Imipenem was the first carbapenem introduced in the mid-1980s, after which imipenem-resistant *P. aeruginosa* isolates were reported (Doi, 2019). Imipenem resistance has been attributed to the reduced outer membrane permeability because of the loss of OprD outer membrane porin which the antibiotic needs to enter the pathogen periplasmic space (Meletis et al., 2012). Unlike imipenem, meropenem is less prone to this kind of resistance mechanism, instead, the main mechanism employed has been reported as upregulation of the efflux pumps (Doi, 2019).

Production of carbapenemases in *P. aeruginosa* has been recently reported frequently. Most importantly, production of carbapenemases has been found to alter the effectiveness of commonly used antibiotics such as ceftazidime, cefepime, piperacillin-tazobactam, ceftolozane-tazobactam, imipenem-relebactam and ceftazidime-avibactam (Tenover, Nicolau & Gill, 2022). As *P. aeruginosa* naturally occurs in the soil and aquatic environments, dissemination of these MDR bacteria continues to be of great concern requiring further research to understand the current trends in the antibiotic resistance profiles, the mechanisms thereof and the public health threat.

Hosu et al., (2021) conducted a study to identify antibiotic resistance genes of *P. aeruginosa* recovered from non-clinical sites, an abattoir, a river and a dam and evaluated the implications to public health in Eastern Cape, South Africa. Of the fifty-five isolated microbes, 36 were confirmed to be *P. aeruginosa*. *P. aeruginosa* isolates

were highly resistant to aztreonam, ceftazidime, piperacillin and cefepime (31/36, 23/36, 21/36 and 20/36 respectively) and 20/36 isolates were regarded as MDR. The results of this study highlighted the importance of continued monitoring of MDR pathogens in non-clinical settings as these could harbour pathogenic *P. aeruginosa* and the implicated resistance genes while serving as a path for dissemination of the potentially harmful MDR bacteria (Hosu et al., 2021).

Analysing the molecular epidemiology of *P. aeruginosa* isolates has shown a high clonal diversity with unique genotypes. In recent years, there has been an increase in MDR and Extensively drug-resistant (XDR) *P. aeruginosa* strains (Horcajada et al., 2019). Because of its intrinsic resistance to various antibiotic classes, treating *P. aeruginosa* infections has been challenging. Cefiderocol has demonstrated *in vitro* activity against MDR and XDR *P. aeruginosa*. This antibiotic can evade hydrolysis by the AmpC enzyme. Moreover, efflux pumps and loss of porin proteins have been found to have little to no effect against this antibiotic (Karlowsky et al., 2022). Ceftolozane/tazobactam, ceftazidime/avibactam, imipenem/cilastatin/relebactam, and meropenem/varbobactam combination therapies are currently the available treatment options for MDR *P. aeruginosa* (Santos et al., 2022).

1.2.13. Horizontal gene transfer and the spread of antibiotic resistance

Horizontal gene transfer plays a significant role in spread and persistence of MDR and XDR pathogens. This process allows bacteria to exchange the acquired genetic changes and ARGs through conjugation, transformation and transduction (Sun et al., 2019). In conjugation, the genetic material is transferred from the donor to the recipient bacteria, mediated by physical contact between the two cells. In this manner, MGEs carrying ARGs are also transported to the recipient cell. The transfer of carbapenemase resistance genes is mediated by conjugation as bla_{KPC}, $bla_{\text{NDM-1}}$, and $bla_{\text{OXA-48}}$ are plasmid-mediated. In transformation, the recipient bacteria take up extracellular DNA from the lysed donor bacteria, which becomes integrated into the host genome, enabling the recipient bacteria to acquire new traits. In transduction, bacteriophages carry chromosomal and extrachromosomal DNA to the recipient bacteria. Antibiotic resistance genes coexist with bacteriophages and are transferred in this process (Tao et al., 2022). Most ARGs are found on plasmids that contribute to gene transfer as vehicles through which ARGs are transferred from one bacterial species to another (Sun et al., 2019).

1.2.14. Mobile genetic elements spreading antibiotic resistance and virulence genes

Mobile genetic elements are categorised into two. The first are mobile elements that can move from one cell to another, including the resistance plasmids, and conjugative transposons. The second category includes integrons, which can move between different or the same DNA molecules through recombination (Bennet, 2008). As part of the plasmids and transposons, integrons function in allowing the capture and expression of gene cassettes by bacteria.

Plasmids are circular, double-stranded DNA molecules enabling the cell to survive while exploiting any environmental changes, such as antibiotics, in this context. The plasmids, therefore, carry virulence and antibiotic-resistance genes that facilitate invasion and survival in a host (Bennet, 2008). Plasmids mediate the

development and spread of ABR in most Gram-negative bacteria. The spread of resistance to most known antibiotics, such as β-lactams, aminoglycosides, tetracyclines, chloramphenicol, macrolides, polymyxins and quinolones, is mediated by plasmids (Vrancianu et al., 2020). Resistance plasmids carry one or more antibiotic-resistance genes, while virulence plasmids possess one or more virulence genes (Bennet, 2008). As a type of plasmid, conjugative plasmids self-transfer and transfer other plasmids between bacterial cells. Their replication is independent of the main bacterial chromosome. Moreover, they are the main resistance drivers in Enterobacterales and *Enterococcus* (Bennet, 2008)

A wide range of different incompatibility *(Inc*) groups of plasmids are widely distributed in MDR Gram-negative bacteria. The groups associated with MDR include the types F, I, H (H11 and H12), L, C and N. These plasmids also play a role in the emergence and dissemination of ESBL *(bla*$_{\text{CTX-M}}$ type) and AmpC-type cephalosporinases *(bla*$_{\text{CMY-2}}$ and *bla*$_{\text{DHA-1}}$*)* (De Oliveira et al., 2020). Plasmids in *K. pneumoniae* have accumulated resistance genes that continuously increase resistance to cephalosporins, carbapenems, penicillin, aminoglycosides and fluoroquinolones (Bennet, 2008). Plasmids associated with ESBL-producing *E. coli* are *Inc*F11*, Inc*N and *Inc*C11. *Inc*C11 has been reported to contribute to the dissemination of the CTX-M type in ESBL-producing *E. coli* (Lee, Lee & Choe, 2019). Through the acquisition of the *mcr* gene, plasmid-mediated resistance to colistin has been reported in *E. coli* (Ullah & Ali, 2021).

Two classes of transposons are distinguished based on the transposons' role in the recombination and identification of sequences. The first class is the composite transposons, and the second is the complex transposons that constitute three interrelated families, Tn3, Tn21 and Tn2501 (Sultan et al., 2018). Complete insertion sequence (IS) elements acting as terminal repeats that flank the transposon cargo genes make up the composite transposons. Transposon mobilisation is similar to IS elements in that it uses transposase enzymes in their transposition (Noel, Petrey & Palmer, 2022).

The composite transposons, Tn5, Tn9, Tn10, Tn903, Tn1525 and Tn2350 are found in Gram-negative bacteria, while Tn4001 and Tn4003 are found in Gram-positive bacteria. The complex resistance transposons found in Gram-negative bacteria are Tn1, Tn3, Tn21, Tn501, Tn1721 and Tn3926, while in Gram-positive bacteria, they are Tn551, Tn917 and Tn445 (Sultan et al., 2018). Both these components can progress intramolecularly within a DNA molecule and intermolecularly from one DNA molecule to another (Sultan et al., 2018). IS10R and IS10L flank Tn10 which carries resistance genes *tet*A encoding tetracycline efflux pump production, *tet*R for tetracycline repressive protein, *tet*C for transcription regulation. Tn10 is found in *Pseudomonas* and *Klebsiella.* Resistance genes for chloramphenicol are carried on Tn9 flanked by IS1, while ampicillin resistance gene *bla*$_{\text{TEM}}$ is carried on Tn1. Tn4001 flanked by IS2560 was first isolated from *S. aureus*, carrying *aacA-aphD* gene conferring resistance to gentamicin and kanamycin (Babakhani & Oloomi, 2018).

Insertion sequences (IS) are important in transferring ARGs and mediating their expression through integration into their structure or providing an active promoter. A transposase enzyme is encoded when terminal inverted repeats in insertion sequences flank one or two open reading frames. The transposase excises and integrates IS

into the genome by recognising and binding the terminal inverted repeats. IS naming often includes the first three species' name letters; hence, *A. baumannii* IS elements are named *ISAba* (Noel, Petrey & Palmer, 2022). Several IS are associated with antibiotic resistance in *A. baumannii; ISAba1, ISAba2*, *ISAba3, ISAba4 and ISAba18*. These are commonly associated with the expression of the carbapenemase genes (Vrancianu et al., 2020). However, not all IS are significantly associated with ARGs; therefore, their underlying mechanisms are poorly understood (Galiot, Monger & Vincent, 2023).

As natural cloning and expression systems, integrons play a role in embedding the open reading frames, transforming them into functional genes when a promoter is present, thereby playing a vital role in acquiring and disseminating ARGs (Vrancianu et al., 2020). They are found on plasmids and transposons, allowing bacteria to capture and express gene cassettes. An *int*I gene encoding integrase, a site-specific recombinase and an attachment site and promoters are the key features forming a class 1 integron. The structure of gene cassettes comprises an open reading frame with no promoter sequence and a recombination site (*att*C), which the *intl* recombinase recognises. The *attC* is a critical part where the integration into an integron takes place. Integrons can exist as chromosomal integrons without involvement in antibiotic resistance and as antibiotic resistance mobile integrons (Noel, Petrey & Palmer, 2022). One after the other, gene cassettes can be inserted into the integron insertion site, producing long arrangements of ARGs that are, in turn, simultaneously transferred among the bacterial populations (Vrancianu et al., 2020).

Three of the five integron classes are associated with MDR (Vrancianu et al., 2020). These classes are classified based on the integrase *Int*1 gene sequence. Class 1 is highly prevalent in Gram-negative bacteria, *Acinetobacter, Enterobacter, Escherichia, Klebsiella and Pseudomonas,* carrying over 40 resistance genes conferring resistance to aminoglycosides, β-lactams, chloramphenicol, macrolides, and sulfonamides. Class II integrons are highly prevalent in *Acinetobacter* spp. Class III integrons are highly prevalent in *Acinetobacter spp, E. coli, K. pneumoniae and P. aeruginosa* (Sabbagh et al., 2021). Integrons play a vital role in the phylogeny of bacteria as they are ancient structures mediating the evolution of bacteria through the acquisition, storage, disposal and re-sorting of the reading frames in gene cassettes, increasing interspecies and intraspecies resistance transmission (Vrancianu et al., 2020).

Botelho, Cazares and Schulenburg (2023) conducted a study to inform on the MGE contribution to the spread of ARGs and virulence genes among the ESKAPE pathogens. This study included the analysis of plasmids, and integrative and conjugative elements in the ESKAPE pathogens. Complete genomes of 1782 ESKAPE pathogens were downloaded from the NCBI's RefSeq database, and only genomes with an average nucleotide identity of more than 95% were selected for further study. A total of 21 478 MGEs were analysed, comprising 16 153 prophages, 2685 conjugative and mobilizable elements and 2640 plasmids. *S. aureus* and *A. baumannii* genomes were found densely populated by prophages. Conjugative and mobilizable elements were found to be dominant in *P. aeruginosa*. Meanwhile, plasmids and prophages were prevalent in *K. pneumoniae* and

Enterobacter. All the analysed MGEs were highly prevalent in *E. faecium* (Botelho, Cazares & Schulenburg, 2023)

Antimicrobial inactivation and target site modification genes were studied on these MGEs. Although there were more prophages than plasmids, it was observed that the ARGs were spread broadly on the plasmids across all the ESKAPE pathogens. *K. pneumoniae* carried most ARGs on the plasmids and prophages, while most ARGs in *P. aeruginosa* were found within the conjugative and mobilizable elements. Moreover, virulence genes were broadly distributed across the ESKAPE pathogens' prophages (Botelho, Cazares & Schulenburg, 2023). This study highlighted the asymmetric distribution of MGEs across the ESKAPE pathogens and shed light on the role of the studied MGEs in ARG spread.

1.2.15. Informal Settlements in South Africa

More than 5 million people live in informal settlements in South Africa, which is reportedly 13.6% of the population by Statistics South Africa. The informal settlements are characterised by overcrowding, minimal/lack of basic services such as running water, clean toilets, and refuse removal. According to recent statistics, 89.9% of the 60.6 million population have access to piped water, 77.9% have access to improved sanitation, and 66% have access to refuse removal. In KwaZulu-Natal, only 73.9% of the population has access to improved and proper sanitation (Statistics South Africa, 2023). In the 2019/2020 Annual Performance Plan generated by the KwaZulu-Natal Department of Human Settlements, it was reported that the province had 743 informal settlements, of which 547 were found in the eThekwini Metro, and 97 were found in uMgungundlovu municipalities, Pietermaritzburg. The total number of households in eThekwini metro informal settlements reported was 258 559, and 48 286 in uMgungundlovu. These two municipal areas accounted for more than 80% of the total informal settlements in the province (KZN Department of Human Settlements, 2019).

The Quarry Road West informal settlement in Durban is administered by the eThekwini Municipality. The informal settlement is on a precarious site on the floodplain of the Palmiet River, which is about 26 Km long, originating in the Kloof escarpment (Williams et al., 2019). Chetty and Pillay (2019) assessed the impact of human activities on the Palmiet River. From this study, it was found that industrial and agricultural activities impact this river. Before the informal settlement, the northern part of the river was found to move past an industrial hub predominated by chemicals, plastic, food processing and vehicle manufacturing with elevated levels of metal pollution (Chetty & Pillay, 2019). Moodley et al (2016) assessed the quality of the Palmiet River relative to the human impacts and found elevated levels of *E. coli* in all points of study, including the points before, in and after the Quarry Road West informal settlement. Moreover, it was noted that high *E. coli* (+160 colonies per ml) and total coliform (+800 colonies per 100 ml) counts were recorded during the winter season, when there was little rainfall (Moodley et al., 2016). This brings to note the deterioration in the microbiological quality of the water and potential pollution by pathogenic microbes.

Informal communities often have communal toilets and washing areas that are not regularly cleaned. Moreover, these toilets may be constantly blocked, with broken taps and slow repair by the municipalities (Socio-Economic

Rights Institute of South Africa (SERI), 2018). Lacking waste and sewage disposal infrastructure makes sanitation a continuous concern in these informal settlements. Statistics SA released figures showing that these communal toilets were shared by about 68% of the households in informal settlements, 6.8% of the households relied on the bucket system and others practice open defecation (Socio-Economic Rights Institute of South Africa (SERI), 2018).

Poor sanitation is a major problem in informal settlements. Informal communities mostly use buckets or pit latrines, which frequently attract flies or overflow, causing faeces to spill near shacks and water taps. Consequently, many diseases in informal settlements are linked to a lack of sanitation. Most of the waste produced by informal communities is disposed of in the surrounding settlements because these areas lack the infrastructure to collect rubbish. Moreover, some of the waste is dumped near rivers, which leads to pollution issues and diseases like diarrhoea due to pathogenic bacteria (Department of Human Settlements, 2021). This makes communicable diseases an issue. Diseases in these areas may arise from intentional or accidental ingestion of contaminated water by people staying there (Gqomfa, Maphanga & Shale, 2022). This is termed intentional ingestion when the water is directly consumed from the rivers. In contrast, accidental ingestion may arise from using polluted water for household chores and recreational activities (Mbanga et al., 2020).

The development, acquisition and spread of antibiotic resistance amongst bacterial communities remains a global and costly One Health challenge. Indiscriminate usage of antibiotics contributes greatly to ABR development in humans, animals, plants and the environment (Yadav & Kapley, 2019). Special attention has been paid to the ESKAPEE pathogens because of their resistance to the last line of defence antibiotics. The ESKAPEE pathogens have various resistance mechanisms that enable them to resist several antibiotic classes, profiling them as MDR and XDR. Moreover, with the current limited treatment options and lack of recent discovery of new antibiotics, high mortality rates are projected in the coming years (O'Neill, 2014). ESKAPEE pathogens exist in almost every natural environment and the human and animal gut, where antibiotic resistance is actively developed and shared. Antibiotic-resistant bacteria in humans and animals are released through faecal and urinary matter and end up in the environment. Antibiotic resistance genes are mobilised by a variety of mobile genetic elements, creating reservoirs of resistance. Current reports indicate that urgent action is needed for enhanced AMR surveillance and the development of novel treatments needed to combat ABR.

1.3. Study Rationale

Antimicrobial resistance is a continuous problem with more complications than solutions. ESKAPEE pathogens have been associated with high mortality rates, and it has been estimated that at least 10 million people will die from AMR infections by 2050 if there is no action (O'Neill, 2014). Initially, research on these pathogens focused on human-associated infections. The One Health approach has recently been developed to determine the resistance profiles in humans, animals, and the environment. However, the National Institute of Communicable Diseases (NICD) does not focus on the environmental samples but the human and foodborne infections. This leaves a big gap in the national emphasis on the environment's role in disseminating antibiotic resistance.

Surface water receives pollution from several sources: hospital effluent, wastewater treatment plants, agricultural facilities, and human informal settlements (Silva et al., 2021). This makes the aquatic environment the primary source through which antibiotic-resistant bacteria enter and are transported in the environment. Therefore, surface water has a major potential for ARGs dissemination (Silva et al., 2021). Because of the genomic plasticity of the pathogens, this creates a reservoir of genes and the MGEs that can reshuffle depending on the selective pressures and are not hindered by the geographic, phylogenetic relationships and habitat boundaries (Sanderson et al., 2022).

Many ARGs detected in the environment have been linked to ARGs isolated from human samples in research using the One Health approach. One health approach considers AMR's emergence, dissemination and spread across humans, animals and the environment (Sanderson et al., 2022). Horizontal gene transfer is particularly an important way in which AMR is spread; MGEs carry the resistance genes that are transferred from species to species and within bacterial communities (Sanderson et al., 2022), with a high occurrence of this in water because of the high population of bacteria in the aquatic environment (Silva et al., 2021).

The development and spread of informal settlements in South Africa are concerning. These areas are characterised by pollution, overcrowding, poor housing and poor access to water, sanitation and hygienic services (WASH) (Parikh et al., 2020 & Zerbo, Delgado & Gonzalez., 2020). Such living conditions pose a huge threat to the communities' health because of possible outbreaks of AMR-associated infections. Moreover, public health threats in informal settlements have been studied, but the focus has been placed on HIV/AIDS, TB, diarrhoea, mental stress and cholera (Cornelius, 2019; Weimann & Oni, 2019; Chitsamatanga & Ntlama-Makhanya, 2021).

Because of the possible faecal contamination and upstream pollution, any consuming contaminated water, intentional or unintentionally, can be detrimental to human health, especially with infections by MDR and XDR pathogens. This brings to question why environmental settings, especially surface water, are not a focus in strategies to control and combat AMR in South Africa. Hence, this study will aim to answer the following research questions:

- How prevalent are ESKAPEE pathogens in the surface waters in the Durban and Pietermaritzburg rivers, near the informal settlements?
- What are the antibiotic resistance profiles and trends in these pathogens?
- What ARGs and associated MGEs are prevalent?
- How do the ABR profiles in the two areas compare?

1.4. Aims & objectives

This study aimed to compare and contrast the molecular epidemiology of antibiotic-resistant ESKAPEE pathogens in surface water in proximity to two informal settlements in two cities in KwaZulu-Natal.

The objectives are:

1. To determine the contamination with and prevalence of ESKAPEE pathogens in the surface water using selective media.
2. Determine the antibiotic resistance profiles of the ESKAPEE pathogens using the minimum inhibitory concentration (MIC) method according to Clinical and Laboratory Standards Institute (CLSI) guidelines.
3. Determine the ARGs, and MGEs associated with the antibiotic-resistant ESKAPEE pathogens using whole genome sequencing and bioinformatics.
4. Ascertain the clonality and phylogenies of the isolates using whole genome sequencing and bioinformatics.

1.5. Dissertation Outline

This study aimed to compare and contrast the molecular epidemiology of antibiotic-resistant ESKAPEE pathogens in surface water in proximity to two informal settlements in two cities in KwaZulu-Natal. The presentation of this research is in three chapters as follows:

Chapter 1: Background of the study, literature review, aims and objectives and study rationale

Chapter 2: Manuscript for publication (**STOTEN-D-25-00882**) reporting on the research findings on *E. coli* and *E. faecium*, as the largest number of molecularly confirmed bacterial species. The information *K. pneumoniae* and *Enterobacter* is presented in Appendices 1 and 2.

Chapter 3: Conclusions drawn from findings, recommendations and limitations

1.6. Summarised methodologies

Ethical considerations: Ethical approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC/00003640/2021) (Appendix 4)

Summarised methods: ESKAPEE were isolated from water samples collected from surface water near informal settlements in Durban and Pietermaritzburg. Isolates that were putatively identified on selective media were tested for antibiotic susceptibility using the VITEK®2 platform according to the Clinical and Laboratory Standards Institute (CLSI) (2023). The presumptive isolates were molecularly identified via whole genome sequencing. Antibiotic resistance genes, mobile genetic elements associated with the antibiotic-resistance genes, clonality and phylogenies of the ESKAPEE pathogens were determined using whole genome sequencing and bioinformatics.

References

Abavisani, M., Bostanghadiri, N., Ghahramanpour, H., Kodori, M., Akrami, F., Fathizadeh, H., Hashemi, A. & Rastegari-Pouyani, M. (2023). Colistin resistance mechanisms in Gram-negative bacteria: a Focus on *Escherichia coli. Letters in Applied Microbiology*, *76*(2), ovad023; doi.org/10.1093/lambio/ovad023.

Abushaheen, M.A., Fatani, A.J., Alosaimi, M., Mansy, W., George, M., Acharya, S., Rathod, S., Divakar, D.D., Jhugroo, C., Vellappally, S. & Khan, A.A. (2020). Antimicrobial resistance, mechanisms and its clinical significance. *Disease-a-Month*, 66(100971), doi.org/10.1016/j.disamonth.2020.100971.

Adegoke, A. A., Madu, C. E., Reddy, P., Stenström, T. A., & Okoh, A. I. (2022). Prevalence of vancomycin resistant *Enterococcus* in wastewater treatment plants and their recipients for reuse using PCR and MALDI-ToF MS. *Frontiers in Environmental Science*, *9(797992)*, doi.org/10.3389/fenvs.2021.797992.

Adewoyin, M. A., Ebomah, K. E., & Okoh, A. I. (2021). Antibiogram profile of *Acinetobacter baumannii* recovered from selected freshwater resources in the Eastern Cape Province, South Africa. *Pathogens*, 10(9), 1110; doi.org/10.3390/pathogens10091110.

Akanbi, O. E., Njom, H. A., Fri, J., Otigbu, A. C., & Clarke, A. M. (2017). Antimicrobial susceptibility of *Staphylococcus aureus* isolated from recreational waters and beach sand in Eastern Cape Province of South Africa. *International Journal of Environmental Research and Public Health*, *14*(9), *14, 1001*; doi:10.3390/ijerph14091001.

Algammal, A.M., Hetta, H.F., Elkelish, A., Alkhalifah, D.H.H., Hozzein, W.N., Batiha, G.E.S., El Nahhas, N. & Mabrok, M.A. (2020). Methicillin-Resistant *Staphylococcus aureus* (MRSA): one health perspective approach to the bacterium epidemiology, virulence factors, antibiotic-resistance, and zoonotic impact. *Infection and Drug Resistance,* 13, 3255-3265.

Anane A, Y., Apalata, T., Vasaikar, S., Okuthe, G.E. & Songca, S. (2020). Prevalence and molecular analysis of multidrug-resistant *Acinetobacter baumannii* in the extra-hospital environment in Mthatha, South Africa. *Brazilian Journal of Infectious Diseases,* 23, 371-380.

Annavajhala, M. K., Gomez-Simmonds, A., & Uhlemann, A. C. (2019). Multidrug-resistant *Enterobacter cloacae complex* emerging as a global, diversifying threat. *Frontiers in Microbiology*, *10*(44), doi.org/10.3389/fmicb.2019.00044.

Azuma, T., Murakami, M., Sonoda, Y., Ozaki, A., & Hayashi, T. (2022). Occurrence and quantitative microbial risk assessment of methicillin-resistant *Staphylococcus aureus* (MRSA) in a sub-catchment of the Yodo River Basin, Japan. *Antibiotics*, 11, 1355; doi.org/10.3390/antibiotics11101355.

Babakhani, S., & Oloomi, M. (2018). Transposons: the agents of antibiotic resistance in bacteria. *Journal of Basic Microbiology*, 58 (11), 905–917.

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British Journal of Pharmacology*, 153(1),347-357.

Botelho, J., Cazares, A., & Schulenburg, H. (2023). The ESKAPE mobilome contributes to the spread of antimicrobial resistance and CRISPR-mediated conflict between mobile genetic elements. *Nucleic Acids Research*, 51(1), 236-252.

Budavari, S.M., Saunders, G.L., Liebowitz, L.D., Khoosal, M. and Crewe-Brown, H.H., (1997). Emergence of vancomycin-resistant enterococci in South Africa. *South African Medical Journal*, 87(11), 1557.

Bush, K., & Bradford, P. A. (2020). Epidemiology of β-lactamase-producing pathogens. *Clinical Microbiology Reviews*, 33(2), doi.org/10.1128/cmr.00047-19.

Carr, V. R., Shkoporov, A., Hill, C., Mullany, P., & Moyes, D. L. (2021). Probing the Mobilome: Discoveries in the dynamic microbiome. *Trends in Microbiology*, 29(2), 158-170.

Castanheira, M., Simner, P. J., & Bradford, P. A. (2021). Extended-spectrum β-lactamases: an update on their characteristics, epidemiology and detection. *JAC-Antimicrobial Resistance*, *3*(3), dlab092; doi.org/10.1093/jacamr/dlab092.

Chattopadhyay, A., Das, N., & Banerjee, D. (2023). Mechanism of action of the antibiotics on the multidrug-resistant organisms. *In:* Joshi, S., Kar, R. K., Lahiri, D., & Nag, M (Eds). Advances in Biotechnology and Bioengineering, Lantibiotics as Alternative Therapeutics, Massachusetts, Academic Press.

Che, Y., Xia, Y., Liu, L., Li, A. D., Yang, Y., & Zhang, T. (2019). Mobile antibiotic resistome in wastewater treatment plants revealed by Nanopore metagenomic sequencing. *Microbiome*, 7(1), 1-13.

Chetty, S. and Pillay, L., (2019). Assessing the influence of human activities on river health: a case for two South African rivers with differing pollutant sources. *Environmental Monitoring and Assessment,* 191 (3), 168; doi.org/10.1007/s10661-019-7308-4.

Chitsamatanga, B. B., & Ntlama-Makhanya, N. (2021). Building on the evidence of the impact of coronavirus (COVID-19) in South African informal settlements. A mini-Review. *International Journal of Engineering, Management & Humanities,* 2(3),47-60.

Chuang, Y.C., Lin, H.Y., Yang, J.L., Lin, C.Y., Huang, S.H., Wang, J.T., Chen, Y.C. & Chang, S.C. (2022). Influence of daptomycin doses on the outcomes of VRE bloodstream infection treated with high dose daptomycin. *Journal of Antimicrobial Chemotherapy*, *77*(8), .2278-2287.

Clinical and Laboratory Standards Institute. (2023). 2023 Breakpoint Implementation Toolkit (2023 BIT). https://clsi.org/meetings/ast/breakpoints-in-use-toolkit/ Date of access: 16 August 2023.

Cornelius, G. S. (2016). Revisiting the approach to Informal Settlement Development in South Africa: with a special focus on the impact of HIV/AIDS (Doctoral dissertation, North-West University (South Africa), Potchefstroom Campus).

Cox, G., & Wright, G. D. (2013). Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. *International Journal of Medical Microbiology,* 303(6-7), 287-292.

Das, S., Bombaywala, S., Srivastava, S., Kapley, A., Dhodapkar, R., & Dafale, N.A. (2022). Genome plasticity as a paradigm of antibiotic resistance spread in ESKAPE pathogens. *Environmental Science and Pollution Research,* 29(27), 40507-40519.

David, M. Z. & Daum, R. S. (2017). Treatment of *Staphylococcus aureu*s infections. *In* Bagnoli F., Rappuoli R., & Grandi G (Eds). *Staphylococcus aureus*: microbiology, pathology, immunology, therapy and prophylaxis, Springer International Publishing., Berlin, Germany.

Davin-Regli, A., Lavigne, J. P., & Pagès, J. M. (2019). *Enterobacter spp*.: update on taxonomy, clinical aspects, and emerging antimicrobial resistance. *Clinical Microbiology Reviews*, 32(4), 02-19.

De Oliveira, D.M., Forde, B.M., Kidd, T.J., Harris, P.N., Schembri, M.A., Beatson, S.A., Paterson, D.L. & Walker, M.J. (2020). Antimicrobial resistance in ESKAPE pathogens. *Clinical Microbiology Reviews,* 33(3), https://doi.org/10.1128/cmr.00181-19.

Denissen, J., Reyneke, B., Waso-Reyneke, M., Havenga, B., Barnard, T., Khan, S. & Khan, W. (2022). Prevalence of ESKAPE pathogens in the environment: Antibiotic resistance status, community-acquired infection and risk to human health. *International Journal of Hygiene and Environmental Health*, 244 (114006), doi.org/10.1016/j.ijheh.2022.114006.

Department of Health of the Republic of South Africa. (2021). Surveillance for antimicrobial resistance and consumption of antimicrobials in South Africa. *Pretoria: National Department of Health*.

Department of Human Settlements of Republic of South Africa. (2021). Baseline Evaluation of Informal Settlements Targeted for Upgrading in the 2019-2024 MTSF, *Pretoria: Department of Human Settlements.*

Departments of Health, Agriculture, Forestry and Fisheries of the Republic of South Africa. (2017). South Africa Antimicrobial Resistance National Strategic Framework 2017-2024, *Pretoria: National Departments of Health, Agriculture, Forestry and Fisheries.*

Doi Y. (2019). Treatment Options for Carbapenem-resistant Gram-negative Bacterial Infections. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*, *69*(7), 565–575.

Ebomah, K. E., & Okoh, A. I. (2021). *Enterobacter cloacae* harbouring bla_{NDM-1}, bla_{KPC}, and $bla_{OXA-48-like}$ carbapenem-resistant genes isolated from different environmental sources in South Africa. *International Journal of Environmental Studies*, 78(1), 151-164.

Effah, C. Y., Sun, T., Liu, S., & Wu, Y. (2020). *Klebsiella pneumoniae*: an increasing threat to public health. *Annals of Clinical Microbiology and Antimicrobials*, 19(1), doi.org/10.1186/s12941-019-0343-8.

Egan, S.A., Corcoran, S., McDermott, H., Fitzpatrick, M., Hoyne, A., McCormack, O., Cullen, A., Brennan, G.I., O'Connell, B. and Coleman, D.C., (2020). Hospital outbreak of linezolid-resistant and vancomycin-resistant ST80 *Enterococcus faecium* harbouring an optrA-encoding conjugative plasmid investigated by whole-genome sequencing. *Journal of Hospital Infection,* 105(4), 726-735.

Ekwanzala, M. D., Dewar, J. B., Kamika, I., & Momba, M. N. B. (2020). Comparative genomics of vancomycin-resistant *Enterococcus spp*. revealed common resistome determinants from hospital wastewater to aquatic environments. *Science of the Total Environment*, 719, 137275; doi.org/10.1016/j.scitotenv.2020.137275.

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H., & Spratt, B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academy of Sciences,* 99(11), 7687-7692.

Falagas, M.E., Rafailidis, P.I., Kofteridis, D., Virtzili, S., Chelvatzoglou, F.C., Papaioannou, V., Maraki, S., Samonis, G. & Michalopoulos, A. (2007). Risk factors of carbapenem-resistant *Klebsiella pneumoniae* infections: a matched case–control study. *Journal of Antimicrobial Chemotherapy*, *60*(5), 1124-1130.

Feng, Y.C., Liou, C.H., Ng, W.V., Chen, F.J., Hung, C.H., Liu, P.Y., Liao, Y.C., Wu, H.C. & Cheng, M.F. (2022). Distribution and genomic characterization of third-generation cephalosporin-resistant *Escherichia coli* Isolated from a single family and home environment: A 2-year longitudinal study. *Antibiotics*, 11, 1152; doi.org/10.3390/antibiotics11091152.

Foka, F. E. T., Kumar, A., & Ateba, C. N. (2018). Emergence of vancomycin-resistant enterococci in South Africa: Implications for public health. *South African Journal of Science,* 114(9-10), 1-7.

Founou, L. L., Founou, R.C., Allam, N., Ismail, A., Djoko, C.F., & Essack, S.Y. (2018). Genome sequencing of extended-spectrum β–lactamase (ESBL)- producing *Klebsiella pneumoniae* isolated from pigs and abattoir workers in Cameroon. *Frontiers in Microbiology*, 9(2018), 33706; https://doi.org/10.3389/fmicb.2018.00188.

Gajdács, M. (2019). The continuing threat of methicillin-resistant *Staphylococcus aureus*. *Antibiotics*, 8(2), 52; doi.org/10.3390/antibiotics8020052.

Galindo-Méndez, M. 2020. Antimicrobial Resistance in *Escherichia coli*. In Rodrigo, L (Ed). *E. Coli* Infections. IntechOpen, Rijeka.

Galiot, L., Monger, X. C., & Vincent, A. T. (2023). Studying the Association between Antibiotic Resistance Genes and Insertion Sequences in Metagenomes: Challenges and Pitfalls. *Antibiotics*, 12(1), 175; doi.org/10.3390/antibiotics12010175.

Gao, W., Howden, B. P., & Stinear, T. P. (2018). Evolution of virulence in *Enterococcus faecium*, a hospital-adapted opportunistic pathogen. *Current Opinion in Microbiology*, 41, 76-82.

Gast, K. B., van Oudheusden, A. J. G., Murk, J. L., Stohr, J. J. J. M., Buiting, A. G., & Verweij, J. J. (2021). Successful containment of two vancomycin-resistant *Enterococcus faecium* (VRE) outbreaks in a Dutch teaching hospital using environmental sampling and whole-genome sequencing. *Journal of Hospital Infection,* 111, 132-139.

Ghimpețeanu, O.M., Pogurschi, E.N., Popa, D.C., Dragomir, N., Drăgotoiu, T., Mihai, O.D. & Petcu, C.D., (2022). Antibiotic use in livestock and residues in food—A public health threat: A review. *Foods*, *11*(10), 1430; doi.org/10.3390/foods11101430

Gqomfa, B., Maphanga, T., & Shale, K. (2022). The impact of informal settlement on water quality of Diep River in Dunoon. *Sustainable Water Resources Management*, 8, 27; doi.org/10.1007/s40899-022-00629-w.

Gumede, S. N., Abia, A. L., Amoako, D. G., & Essack, S. Y. (2021). Analysis of wastewater reveals the spread of diverse extended-spectrum β-lactamase-producing *E. coli* strains in uMgungundlovu District, South Africa. *Antibiotics*, 10(7), 860; doi.org/10.3390/antibiotics10070860.

Hamidian, M., & Nigro, S. J. (2019). Emergence, molecular mechanisms and global spread of carbapenem-resistant Acinetobacter baumannii. *Microbial Genomics*, *5*(10), https://doi.org/10.1099/mgen.0.000306.

Higuita, N. I. A., & Huycke, M.M. (2014). Enterococcal disease, epidemiology and implications for treatment. *In* Gilmore, M.S., Clewell, D.B., Ike, Y, & Shankar, N. Enterococci: From Commensal to Leading Causes Of Drug Resistant Infection. Boston, Massachusetts Eye and Ear Infirmary.

Horcajada, J.P., Montero, M., Oliver, A., Sorlí, L., Luque, S., Gómez-Zorrilla, S., Benito, N. & Grau, S., (2019). Epidemiology and treatment of multidrug-resistant and extensively drug-resistant *Pseudomonas aeruginosa* infections. *Clinical Microbiology Reviews,* 32(4), https://doi.org/10.1128/cmr.00031-19.

Hosu, M. C., Vasaikar, S., Okuthe, G. E., & Apalata, T. (2021). Molecular Detection of Antibiotic-Resistant Genes in *Pseudomonas aeruginosa* from Nonclinical Environment: Public Health Implications in Mthatha, Eastern Cape Province, South Africa. *International Journal of Microbiology*, *2021*, 8861074; doi.org/10.1155/2021/8861074.

Isler, B., Doi, Y., Bonomo, R. A., & Paterson, D. L. (2019). New treatment options against carbapenem-resistant *Acinetobacter baumannii* infections. *Antimicrobial Agents and Chemotherapy*, 63(1), doi.org/10.1128/aac.01110-18.

Kaper, J. B., Nataro, J. P., & Mobley, H. L. T. (2004). Pathogenic *Escherichia coli*. *Nature Reviews Microbiology,* 2(2), 123-14

Kapoor, G., Saigal, S., & Elongavan, A. (2017). Action and resistance mechanisms of antibiotics: A guide for clinicians. *Journal of Anaesthesiology, Clinical Pharmacology,* 33(3), 300-305.

Karlowsky, J.A., Walkty, A.J., Baxter, M.R., Adam, H.J., Lagacé-Wiens, P.R., Schweizer, F., Bay, D., Lynch III, J.P., Mulvey, M.R. & Zhanel, G.G. (2022). *In Vitro* activity of cefiderocol against extensively drug-resistant *Pseudomonas aeruginosa*: CANWARD, 2007 to 2019. *Microbiology Spectrum*, *10*(4), doi.org/10.1128/spectrum.01724-22.

Khan, M. F. (2017). Brief history of *Staphylococcus aureus*: a focus to antibiotic resistance. *EC Microbiology*, *5*(2), 36–39.

Kostyanev, T., & Can, F. (2017). The global crisis of antibiotic resistance. *In* Pulcini, C., Ergonel, O., Can, F., & Beovic, B (Eds). Antimicrobial Stewardship, 2nd edition, Massachusetts, Academic Press,.

Kumar, S., Anwer, R., Yadav, M., Vats, G., Thakur, S., Sehrawat, N., Kumar, V. & Singh, M. (2022). An update on advancements in treatment options for managing *Klebsiella pneumoniae* infections. *Current Pharmacology Reports*, *8*(6), 439-449.

KwaZulu-Natal Department of Human Settlements. (2019). 2019/2020 Annual performance plan. *Durban: KwaZulu-Natal Department of Human Settlements*.

Larsson, D.J., 2014. Antibiotics in the environment. *Upsala Journal of Medical Sciences*, *119*(2), 108-112.

Lee, D. S., Lee, S. J., & Choe, H. S. (2018). Community-acquired urinary tract infection by *Escherichia coli* in the era of antibiotic resistance. *BioMed Research International*, 2018, 7656752; doi.org/10.1155/2018/7656752.

Lehman, K. M., & Grabowicz, M. (2019). Countering gram-negative antibiotic resistance: Recent progress in disrupting the outer membrane with novel therapeutics. *Antibiotics*, 8(4), 163, https://doi.org/10.3390/antibiotics8040163.

Lim, F. H., Modha, D. E., Collins, E., Westmoreland, D., Ashton, C., & Jenkins, D. R. (2020). An outbreak of two strains of OXA-48 producing *Klebsiella pneumoniae* in a teaching hospital. *Infection Prevention in Practice*, *2*(3), 100033; doi.org/10.1016/j.infpip.2019.100033.

Lin, X. and Kück, U. (2022). Cephalosporins as key lead generation beta-lactam antibiotics. *Applied microbiology and biotechnology*, *106*(24), 8007-8020.

Liu, S., Huang, N., Zhou, C., Lin, Y., Zhang, Y., Wang, L., Zheng, X., Zhou, T. & Wang, Z. (2021). Molecular mechanisms and epidemiology of carbapenem-resistant *Enterobacter cloacae complex* isolated from Chinese patients during 2004–2018. *Infection and drug resistance*, 14, 3647-3658.

Lobanovska, M., & Pilla, G. (2017). Penicillin's Discovery and Antibiotic Resistance: Lessons for the Future?. *The Yale Journal of Biology and Medicine*, *90*(1), 135–145.

Lucet, J.C., Armand-Lefevre, L., Laurichesse, J.J., Macrez, A., Papy, E., Ruimy, R., Deblangy, C., Lozach, A., Lolom, I., Jarlier, V. & Andremont, A. (2007). Rapid control of an outbreak of vancomycin-resistant enterococci in a French university hospital. *Journal of Hospital Infection,* 67(1), 42-48.

Mancuso, G., Midiri, A., Gerace, E., & Biondo, C. (2021). Bacterial antibiotic resistance: The most critical pathogens. *Pathogens*, *10*(10), 1310; doi.org/10.3390/pathogens10101310.

Marais, L., & Cloete, J. (2014). "Dying to get a house?" The health outcomes of the South African low-income housing programme. *Habitat International*, 43, 48-60.

Mbanga, J., Abia, A.L.K., Amoako, D.G., & Essack, S.Y. (2020). Quantitative microbial risk assessment for waterborne pathogens in a wastewater treatment plant and its receiving surface water body. *BMC Microbiology,* 20(2020), 346; doi.org/10.1186/s12866-020-02036-7.

Mbelle, N. M., Feldman, C., Sekyere, J, O., Maningi, N. E., Modipane, L., & Essack, S.Y. (2019). The Resistome, Mobilome, Virulome and Phylogenomics of Multidrug-Resistant *Escherichia coli* Clinical Isolates from Pretoria, South Africa. *Scientific Reports*, 9, 16457; doi.org/10.1038/s41598-019-52859-2.

Meletis, G., Exindari, M., Vavatsi, N., Sofianou, D., & Diza, E. (2012). Mechanisms responsible for the emergence of carbapenem resistance in *Pseudomonas aeruginosa*. *Hippokratia*, *16*(4), 303-307.

Mezzatesta, M. L., Gona, F., & Stefani, S. (2012). *Enterobacter cloacae complex*: clinical impact and emerging antibiotic resistance. *Future Microbiology*, *7*(7), 887-902.

Miller, W. R., Munita, J. M., & Arias, C. A. (2014). Mechanisms of antibiotic resistance in enterococci. *Expert Review of Anti-Infective Therapy*, *12*(10), 1221–1236.

Moellering Jr, R. C. (2010). NDM-1—a cause for worldwide concern. *New England Journal of Medicine*, *363*(25), 2377-2379.

Moodley, K., Pillay, S., Patha, K., & Ballabh, H. (2016). Land use impacts on the water quality of the Palmiet River, Durban, KwaZulu-Natal, South Africa. *International Journal of Scientific Engineering and Applied Science,* 2(1), 339-348.

Mulani, M.S., Kamble, E.E., Kumkar, S.N., Tawre, M.S. and Pardesi, K.R. (2019). Emerging strategies to combat ESKAPE pathogens in the era of antimicrobial resistance: a review. *Frontiers in Microbiology*, *10*, 539; doi.org/10.3389/fmicb.2019.00539.

Munita, J. M., & Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Microbiology Spectrum,* 4(2), 481-511.

Nanavati, K., Rutherfurd-Markwick, K., Lee, S. J., Bishop, N. C., & Ali, A. (2022). Effect of curcumin supplementation on exercise-induced muscle damage: a narrative review. *European Journal of Nutrition*, *61*(8), 3835-3855.

Nandhini, P., Kumar, P., Mickymaray, S., Alothaim, A. S., Somasundaram, J., & Rajan, M. (2022). Recent developments in methicillin-resistant *Staphylococcus aureus* (MRSA) treatment: A review. *Antibiotics*, 11(5), 606; doi.org/10.3390/antibiotics11050606.

Nishino, K., Yamasaki, S., Nakashima, R., Zwama, M., & Hayashi-Nishino, M. (2021). Function and inhibitory mechanisms of multidrug efflux pumps. *Frontiers in Microbiology*, 12, 737288; doi.org/10.3389/fmicb.2021.737288.

Noel, H. R., Petrey, J. R., & Palmer, L. D. (2022). Mobile genetic elements in *Acinetobacter* antibiotic-resistance acquisition and dissemination. *Annals of the New York Academy of Sciences*, 1518(1),166-182.

Nzima, B., Adegoke, A. A., Ofon, U. A., Al-Dahmoshi, H. O. M., Saki, M., Ndubuisi-Nnaji, U. U., & Inyang, C. U. (2020). Resistotyping and extended-spectrum beta-lactamase genes among *Escherichia coli* from wastewater treatment plants and recipient surface water for reuse in South Africa. *New Microbes and New Infections,* 38, 100803; doi.org/10.1016/j.nmni.2020.100803.

O'Toole, R. F., Leong, K. W., Cumming, V., & Van Hal, S. J. (2023). Vancomycin-resistant *Enterococcus faecium* and the emergence of new sequence types associated with hospital infection. *Research in Microbiology*, 174(4), 104046; doi.org/10.1016/j.resmic.2023.104046.

Pachori, P., Gothalwal, R., & Gandhi, P. (2019). Emergence of antibiotic resistance *Pseudomonas aeruginosa* in intensive care unit; a critical review. *Genes & Diseases*, 6(2), 109-119.

Pandey N, Cascella M. Beta-Lactam Antibiotics. [Updated 2023 Jun 4]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK545311/ Date of access: 01 August 2023.

Parikh, P., Bisaga, I., Loggia, C., Georgiadou, M. C., & Ojo-Aromokudu, J. (2020). Barriers and opportunities for participatory environmental upgrading: Case study of Havelock informal settlement, Durban. *City and Environment Interactions*, 5, 100041; doi.org/10.1016/j.cacint.2020.100041.

Parmanik, A., Das, S., Kar, B., Bose, A., Dwivedi, G. R., & Pandey, M. M. (2022). Current treatment strategies against multidrug-resistant bacteria: a review. *Current Microbiology*, *79*, 388; doi.org/10.1007/s00284-022-03061-7.

Perovic, O, Britz, E, Chetty, V, & Singh-Moodley, A. (2016). Molecular detection of carbapenemase-producing genes in referral Enterobacteriaceae in South Africa: A short report. *South African Medical Journal*, 106(10), 975-977.

Piperaki, E. T., Tzouvelekis, L. S., Miriagou, V., & Daikos, G. L. (2019). Carbapenem-resistant *Acinetobacter baumannii:* in pursuit of an effective treatment. *Clinical Microbiology and Infection*, 25(8), 951-957.

Pitout, J. D., Nordmann, P., & Poirel, L. (2015). Carbapenemase-producing *Klebsiella pneumoniae*, a key pathogen set for global nosocomial dominance. *Antimicrobial agents and chemotherapy*, *59*(10),5873-5884.

Pogue, J.M., Kaye, K.S., Cohen, D. A., & Marchaim, D. (2015). Appropriate antimicrobial therapy in the era of multidrug-resistant human pathogens. *Clinical Microbiology and Infection,* 21(4), 302-312.

Poirel, L., Madec, J. Y., Lupo, A., Schink, A. K., Kieffer, N., Nordmann, P., & Schwarz, S. (2018). Antimicrobial resistance in *Escherichia coli*. *Microbiology Spectrum*, *6*(4), doi.org/10.1128/microbiolspec.arba-0026-2017.

Pucelik, B., & Dąbrowski, J. M. (2022). Photodynamic inactivation (PDI) as a promising alternative to current pharmaceuticals for the treatment of resistant microorganisms. *Advances in Inorganic Chemistry,* 79, 65-108.

Ramessar, K., & Olaniran, A. O. (2019). Antibiogram and molecular characterization of methicillin-resistant *Staphylococcus aureus* recovered from treated wastewater effluent and receiving surface water in Durban, South Africa. *World Journal of Microbiology and Biotechnology*, 35, 1-11.

Ramsamy, Y., Mlisana, K. P., Allam, M., Amoako, D. G., Abia, A. L. K., Ismail, A., Singh, R., Kisten, T., Han, K. S., Muckart, D. J. J., Hardcastle, T., Suleman, M., & Essack, S. Y. (2020). Genomic Analysis of Carbapenemase-Producing Extensively Drug-Resistant *Klebsiella pneumoniae* Isolates Reveals the Horizontal Spread of p18-43_01 Plasmid Encoding bla_{NDM-1} in South Africa. *Microorganisms,* 8(1), 137 ; doi.org/10.3390/microorganisms8010137.

Rangel, K., Chagas, T.P.G., & De-Simone, S.G. (2021). *Acinetobacter baumannii* infections in times of Covid-19 pandemic. *Pathogens*, 10(8), 1006; doi.org/10.3390/pathogens10081006.

Raoofi, S., Pashazadeh Kan, F., Rafiei, S., Hosseinipalangi, Z., Noorani Mejareh, Z., Khani, S., Abdollahi, B., Seyghalani Talab, F., Sanaei, M., Zarabi, F. & Dolati, Y. (2023). Global prevalence of nosocomial infection: A systematic review and meta-analysis. *PloS One,* 18(1), e0274248; doi.org/10.1371/journal.pone.0274248.

Rapp, R. P., & Urban, C. (2012). *Klebsiella pneumoniae* carbapenemases in Enterobacteriaceae: history, evolution, and microbiology concerns. *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, *32*(5), 399-407.

Raza, S., Jo, H., Kim, J., Shin, H., Hur, H. G., & Unno, T. (2021). Metagenomic exploration of antibiotic resistome in treated wastewater effluents and their receiving water. *Science of The Total Environment*, 765, 142755; doi.org/10.1016/j.scitotenv.2020.142755.

Reygaert, W. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology*, *4*(3), 482–501.

Reynolds, D. & Kollef, M. (2021). The Epidemiology and Pathogenesis and Treatment of *Pseudomonas aeruginosa* Infections: An Update. *Drugs*, 81, 2117–2131.

Rice L. B. (2001). Emergence of vancomycin-resistant enterococci. *Emerging Infectious Diseases*, *7*(2),183–187.

Rossolini, G. M., D'andrea, M. M., & Mugnaioli, C. (2008). The spread of CTX-M-type extended-spectrum β-lactamases. *Clinical Microbiology and Infection*, *14*, 33-41.

Sabbagh, P., Rajabnia, M., Maali, A., & Ferdosi-Shahandashti, E. (2021). Integron and its role in antimicrobial resistance: A literature review on some bacterial pathogens. *Iranian Journal of Basic Medical Sciences*, 24(2), 136-142

Sanderson, H., Gray, K. L., Manuele, A., Maguire, F., Khan, A., Liu, C., Rudrappa, C. N., Nash, J. H. E., Robertson, J., & Bessonov, K. (2022). Exploring the mobilome and resistome of *Enterococcus faecium* in a One Health context across two continents. *Microbial Genomics*, *8*(9), doi.org/10.1099/mgen.0.000880.

Santajit, S., & Indrawattana, N. (2016). Mechanisms of antimicrobial resistance in ESKAPE pathogens. *BioMed Research International*, 2016, 2475067; doi.org/10.1155/2016/2475067.

Santos, E. D., Jiménez, C. M., del Río-Carbajo, L., & Vidal-Cortés, P. (2022). Treatment of severe multi-drug resistant *Pseudomonas aeruginosa* infections. *Intensive Care Medicine*, 46(9), 508-520.

Sarojamma, V., & Ramakrishna, V. (2011). Prevalence of ESBL-producing *Klebsiella pneumoniae* isolates in tertiary care hospital. *International Scholarly Research Notices*, 2011, 318348; doi.org/10.5402/2011/318348.

Sawa, T., Kooguchi, K., & Moriyama, K. (2020). Molecular diversity of extended-spectrum β-lactamases and carbapenemases, and antimicrobial resistance. *Journal of Intensive Care,* 8, 1-13.

Schito, G. C. (2006). The importance of the development of antibiotic resistance in *Staphylococcus aureus*. *Clinical Microbiology and Infection*, 12, 3-8.

Silva, V., Ferreira, E., Manageiro, V., Reis, L., Tejedor-Junco, M.T., Sampaio, A., Capelo, J.L., Caniça, M., Igrejas, G. & Poeta, P. (2021). Distribution and Clonal Diversity of *Staphylococcus aureus* and Other Staphylococci in Surface Waters: Detection of ST425-t742 and ST130-t843 mec C-Positive MRSA Strains. *Antibiotics,* 10(11), 1416; doi.org/10.3390/antibiotics10111416.

Smith HZ, & Kendall B. Carbapenem Resistant Enterobacteriaceae. [Updated 2023 Jan 16]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK551704/ Date of access: 29 August 2023.

Socio-Economic Rights Institute of South Africa (SERI). (2018). Informal Settlements and human rights in South Africa. *Johannesburg: Socio-Economic Rights Institute of South Africa (SERI).*

Stanton, I.C., Bethel, A., Leonard, A.F., Gaze, W.H. and Garside, R., 2020. What is the research evidence for antibiotic resistance exposure and transmission to humans from the environment? A systematic map protocol. *Environmental Evidence*, *9*, 1-8.

Statistics South Africa. (2023) https://www.statssa.gov.za/?page_id=739&id=2&paged=2. Date of access: 1 August 2023

Stefani, S., Chung, D. R., Lindsay, J. A., Friedrich, A. W., Kearns, A. M., Westh, H., & MacKenzie, F. M. (2012). Meticillin-resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. *International Journal of Antimicrobial Agents,* 39(4), 273-282.

Stogios, P. J., & Savchenko, A. (2020). Molecular mechanisms of vancomycin resistance. *Protein Science*, 29(3), 654-669.

Sultan, I., Rahman, S., Jan, A. T., Siddiqui, M. T., Mondal, A. H., & Haq, Q. M. R. (2018). Antibiotics, resistome and resistance mechanisms: A bacterial perspective. *Frontiers in Microbiology,* 9, 2066; doi.org/10.3389/fmicb.2018.02066.

Sun, D., Jeannot, K., Xiao, Y., & Knapp, C. W. (2019). Editorial: horizontal gene transfer mediated bacterial antibiotic resistance. *Frontiers in Microbiology,* 10, 1933; https://doi.org/10.3389/fmicb.2019.01933.

Tao, S., Chen, H., Li, N., Wang, T., & Liang, W. (2022). The spread of antibiotic resistance genes in vivo model. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 2022, 3348695; doi.org/10.1155/2022/3348695.

Tenover, F. C., Nicolau, D. P., & Gill, C. M. (2022). Carbapenemase-producing *Pseudomonas aeruginosa* -an emerging challenge. *Emerging Microbes Infections*, 11(1):811-814.

The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 13.1, 2023. http://www.eucast.org Date of access: 16 August 2023

Tompkins, K. & van Duin, D. (2021). Treatment for carbapenem-resistant *Enterobacterales* infections: recent advances and future directions. *European Journal of Clinical Microbiology & Infectious Dis*eases, 40, 2053–2068.

Tooke, C. L., Hinchliffe, P., Bragginton, E. C., Colenso, C. K., Hirvonen, V. H., Takebayashi, Y., & Spencer, J. (2019). β-Lactamases and β-Lactamase Inhibitors in the 21st Century. *Journal of Molecular Biology*, 431(18), 3472-3500.

Turner, N.A., Sharma-Kuinkel, B.K., Maskarinec, S.A., Eichenberger, E.M., Shah, P.P., Carugati, M., Holland, T.L. & Fowler Jr, V.G. (2019). Methicillin-resistant *Staphylococcus aureus*: an overview of basic and clinical research. *Nature Reviews Microbiology*, 17(4), 203-218.

Ullah, W., & Ali, S. (2021). Antimicrobial Resistance in *Escherichia coli. In* Erjavec, M.S (Ed). *Escherichia coli.* London, IntechOpen.

Vrancianu, C. O., Popa, L. I., Bleotu, C., & Chifiriuc, M. C. (2020). Targeting plasmids to limit acquisition and transmission of antimicrobial resistance. *Frontiers in Microbiology*, 11, 761; doi.org/10.3389/fmicb.2020.00761.

Walker, M. M., Roberts, J. A., Rogers, B. A., Harris, P. N. A., & Sime, F. B. (2022). Current and Emerging Treatment Options for Multidrug Resistant *Escherichia coli* Urosepsis: A Review. *Antibiotics*, *11*(12), 1821; doi.org/10.3390/antibiotics11121821.

Wang, G., Zhao, G., Chao, X., Xie, L., & Wang, H. (2020). The characteristic of virulence, biofilm and antibiotic resistance of *Klebsiella pneumoniae*. I*nternational Journal of Environmental Research and Public Health,* 17(17), 6278; doi.org/10.3390/ijerph17176278.

Weimann, A., & Oni, T. (2019). A systematised review of the health impact of urban informal settlements and implications for upgrading interventions in South Africa, a rapidly urbanising middle-income country.*International Journal of Environmental Research and Public Health*, 16(19), 3608; doi.org/10.3390/ijerph16193608.

Williams, D. S., Máñez Costa, M., Sutherland, C., Celliers, L., & Scheffran, J. (2019). Vulnerability of informal settlements in the context of rapid urbanization and climate change. *Environment and Urbanization*, *31*(1), 157– 176.

Winstanley, T.G. and Hastings, J.G.M., (1989). Penicillin-aminoglycoside synergy and post-antibiotic effect for enterococci. *Journal of Antimicrobial Chemotherapy*, *23*(2), 189-199.

Won, S. Y., Munoz-Price, L. S., Lolans, K., Hota, B., Weinstein, R. A., Hayden, M. K., & Centers for Disease Control and Prevention Epicenter Program. (2011). Emergence and rapid regional spread of *Klebsiella pneumoniae* carbapenemase–producing Enterobacteriaceae. *Clinical Infectious Diseases*, *53*(6), 532-540.

World Health Organization. (2017). *WHO publishes list of bacteria for which new antibiotics are urgently needed*. https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed Date of access 03. May 2023.

WHO Bacterial Priority Pathogens List, 2024: bacterial pathogens of public health importance to guide research, development and strategies to prevent and control antimicrobial resistance. Geneva: World Health Organization; 2024. Licence: CC BY-NC-SA 3.0 IGO.

Yadav, S., & Kapley, A. (2019). Exploration of activated sludge resistome using metagenomics. *Science of The Total Environment,* 692, 1155-1164.

Zerbo, A., Delgado, R. C., & González, P. A. (2021). Water sanitation and hygiene in Sub-Saharan Africa: Coverage, risks of diarrheal diseases, and urbanization. *Journal of Biosafety and Biosecurity*, 3(1), 41-45.

Zhang, A.N., Gaston, J.M., Dai, C.L., Zhao, S., Poyet, M., Groussin, M., Yin, X., Li, L.G., van Loosdrecht, M.C., Topp, E. and Gillings, M.R., 2021. An omics-based framework for assessing the health risk of antimicrobial resistance genes. *Nature communications*, *12*(1), 4765; doi.org/10.1038/s41467-021-25096-

CHAPTER TWO

Potential environmental transmission of antibiotic-resistant *Escherichia coli* and *Enterococcus faecium* harbouring multiple antibiotic resistance genes and mobile genetic elements in surface waters close to informal settlements: a tale of two cities

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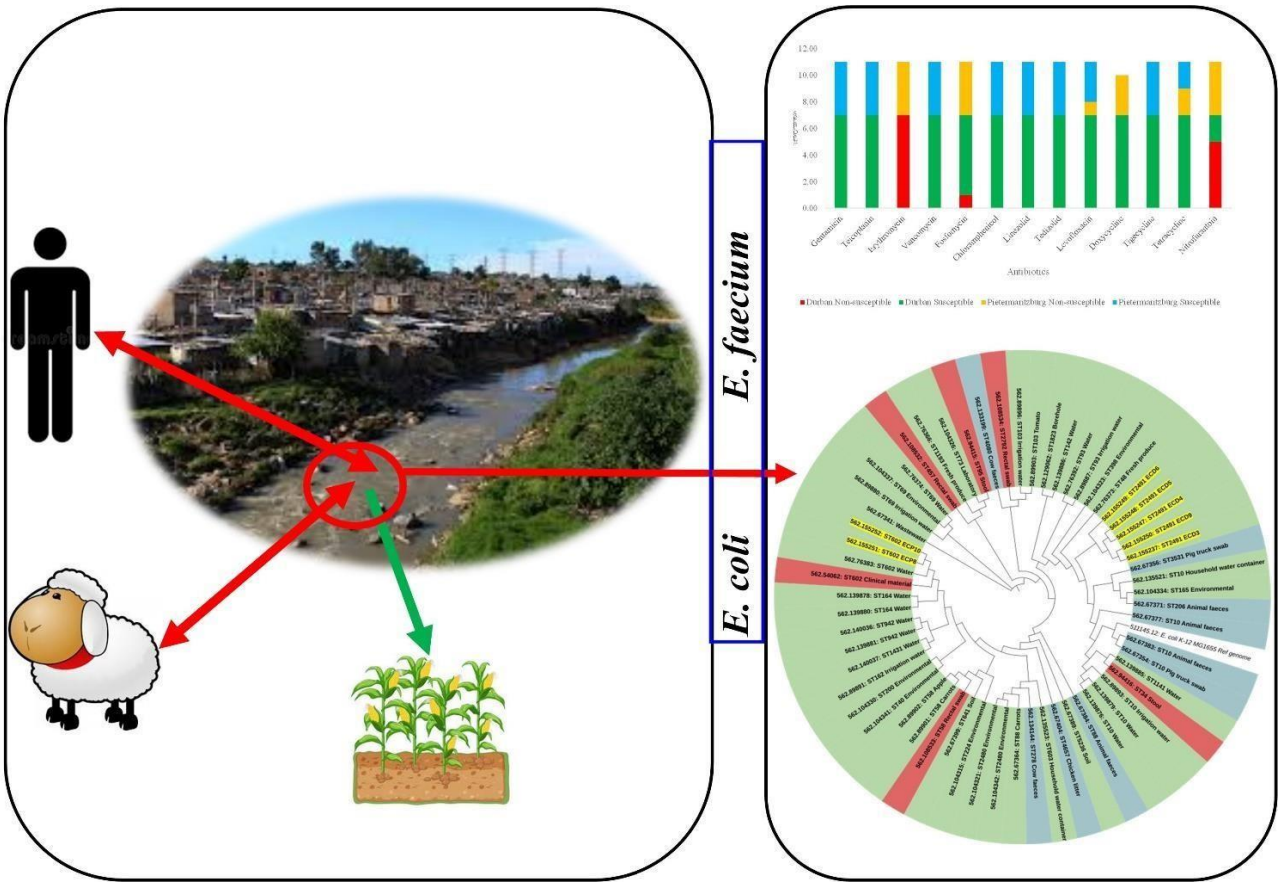
Graphical Abstract



Highlights

- We studied drug-resitant *E. coli* and *E. faecium* in surface water near informal settlemnts.
- Antibiotic-resistant *E. coli* belonged to 2 sequence types and *E. faecium* to 5.
- Third and fourth-generation cephalosporin-resistant *E. coli* were found in Durban.
- Both species harboured many antibiotic resistance genes and mobile genetic elements.
- Isolates were closely related to other human, animal and environmental ones.

ABSTRACT

Aquatic environments, including wastewater and surface water (rivers and streams), increasingly harbour third-generation cephalosporin-resistant *Escherichia coli* and drug-resistant *Enterococcus faecium*, presenting a transmission risk to humans, animals, and plants. We investigated the resistome, mobilome, and phylogenetic relationships of antibiotic-resistant *E. coli* and *E. faecium* in surface water from two cities using whole genome sequencing (WGS). Water samples (500 mL) from streams near informal settlements in Durban and Pietermaritzburg were filtered through 0.45 µm membrane filters. *E. faecium* and *E. coli* were identified on selective media and tested for antibiotic susceptibility using the VITEK® 2 platform. DNA was extracted from isolates for WGS to delineate the resistome, mobilome, multi-locus strain types (STs) and phylogenetic relationships using the open-source CARD, CGE, RAST, BV-BRC and PubMLST tools. Eleven *E. faecium* and 12 *E. coli* isolates were molecularly identified. Antibiotic resistance was observed in seven *E. coli* belonging to two STs and seven *E. faecium* belonging to five STs. Third and fourth-generation cephalosporin-resistant *E. coli* (3/7) were found in Durban. These isolates did not harbour extended-spectrum β-lactamase genes conferring cephalosporin resistance but had the AcrAB-TolC efflux pump for multiple antibiotic resistance. *E. coli* harboured *bla*TEM-1, *sul*1, *sul*3, and *dfr*A12, conferring resistance to amoxicillin-clavulanic acid, piperacillin-tazobactam and cotrimoxazole, respectively. Also, *E. faecium* harboured *qnr*B19, *qnr*S1, *tet*(A), *cml*A1, *aad*A1 and *aad*A2. *tet*(M), *tet*(L), *msr*(C) and *erm*(B) conferring resistance to tetracycline and erythromycin, respectively. ARGs and MGEs in *E. faecium* were mostly chromosome-borne. Plasmid-carried ARGs were associated with IS1, IS1B, IS6, IS256 and ISKpn19, and the Tn3 transposons in *E. coli*. Phylogenetic analysis revealed close relationships with other South African human, animal and environmental isolates. These ARGs, associated with MGEs, present possible transmission routes of these resistance genes within and across bacterial species in aquatic environments, making these surface waters potential reservoirs for antibiotic resistance transmission.

Keywords: Surface water resistome; antibiotic resistance transmission; public health; antibiotic resistance; resource-limited settings

1. Introduction

The spread of antibiotic resistance (ABR) remains a global public health crisis, imposing a significant financial strain on the health sector as the discovery and development of new antibiotics continue to decline (Iwu, Korsten & Okoh, 2020). Various factors contribute to the continuous development and spread of ABR, but the main drivers are the misuse and unregulated use of antibiotics (Iwu, Korsten & Okoh, 2020). Consequently, antibiotic-resistant bacteria (ARB) and antibiotics are discharged into the environment through human and animal waste, creating environmental reservoirs of antibiotic resistance. Such reservoirs enable the dissemination of antibiotic resistance within and between bacterial species by antibiotic resistance gene ARG)-carrying mobile genetic elements (MGEs) through horizontal gene transfer (Aslam et al., 2021).

Although various pathogenic bacteria populate many environments, some pose a greater threat to human and animal health because of their ability to resist antibiotic activity. There has been a consistent rise in β-lactams and broad-spectrum cephalosporin-resistant *E. coli*, mostly conferred by the production of extended-spectrum β-lactamase (ESBL) (Bezabih et al., 2021). ESBLs and overexpressed efflux pumps are some mechanisms associated with cephalosporin resistance (Tian et al., 2020). The carriage of ESBL genes on plasmids enables the dissemination of these ARGs. These genes can be co-carried with ARGs associated with resistance to other antibiotic classes, notably aminoglycosides and fluoroquinolones, conferring multidrug resistance in *E. coli* (Mcdonald et al., 2021; Soni et al., 2024; Athanasakopoulou et al., 2021).

E. faecium, like *E. coli,* forms part of the human and animal microbiota. However, these species were included in the World Health Organization's (WHO) updated list of priority pathogens for research and development in 2024 and categorised as high-priority and critical pathogens, respectively. Carbapenem-resistant and third-generation cephalosporin-resistant *E. coli* was categorised as critical with other Enterobacterales. Vancomycin resistance in *E. faecium* was reported since the 1980s and continues to pose a threat to immunocompromised patients on a global scale (O'Toole et al., 2023), hence its listing as a high-priority pathogen. Critical pathogens are considered the highest threat to public health because of the limited treatment options for the infections they cause. They have a high disease burden with increasing resistance trends. Moreover, there are limited, or no antibiotics being developed for them. High-priority pathogens are significantly difficult to treat, but potential treatments are available or being developed (World Health Organisation, 2024).

In treating and preventing the transmission of bacterial infections, proper healthcare and water, sanitation and hygiene (WASH) services play a vital role. However, not all communities can access WASH services and may depend on nearby natural water sources. Amongst these communities are informal settlements, where people's health is often compromised due to a lack of proper housing, inadequate access to safe and clean drinking water, and a lack of sanitation and drainage infrastructure (Nabirye et al., 2023). Many people use communal toilets and pit latrines, while some practice open defecation. This results in faecal pollution of the aquatic environment and transmission of bacteria, including drug-resistant ones, to humans and animals through intentional or unintentional ingestion of polluted water (Mbanga et al., 2020). Moreover, most informal settlements have a high population density, which may increase the incidence and transmission of bacterial infections, including drug-resistant ones (Nadimpalli et al., 2020; Nabirye et al., 2023).

In KwaZulu-Natal, South Africa, more than 260 000 households are in informal settlements (KZN-Human Settlements, 2020), usually proximate to surface water. The current study investigated the prevalence of faecal coliform bacteria, *E. coli* and *E. faecium,* isolated from surface water proximate to informal settlements in two cities, Durban and Pietermaritzburg, in KwaZulu-Natal. The study further delineated their resistomes, mobilomes and phylogenies to understand the health risk to inhabitants of informal settlements and to inform strategies to minimise the impact of antibiotic resistance on human health.

2. Materials and Methods

2.1. Ethical consideration

Ethical approval (BREC/00003640/2021) was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal.

2.2. Study site and study design

This was a point prevalence study where surface water samples were collected once-off from streams near the informal settlements in Durban and Pietermaritzburg in November 2023. In Durban, the water was sampled from the Palmiet River stream (-29°80'47''S 30°96'61''E) (Figure 1). The Palmiet River flows from the Kloof Escarpment and moves downstream, passing through the Palmiet Nature Reserve and residential and industrial

areas. It then feeds into the Umgeni River. Sampling was conducted where the stream passes through the Quarry Road Informal Settlement (Figure 1). As of 2022, the number of households at the Quarry Road Informal Settlement totalled 1169, with 1119 non-permanent structures. This informal settlement is located on a floodplain and pollutes the river upstream before the sampling point (Williams et al., 2018). On the other hand, the Kwapata stream (29°39'09''S 30°19'58'') in Pietermaritzburg originates in Kwapata and flows through residential and industrial areas before joining the Msunduzi River. The Kwapata township lacks proper waste management. Thus, the community uses this stream as a dumping site (Figure 1), and the waste is discharged into the Msunduzi River. There are no records of the number of people living in this area. Household infrastructures in this area are mostly mud houses.

Fig. 1 Study sites in Durban and Pietermaritzburg

2.3. Sample collection

Surface water samples were collected in triplicate using 500 mL plastic bottles. The grab sampling method (da Costa Filho, Duarte & Santos, 2022) was used. The collected samples were placed in a cooler box with ice and transported to the Antimicrobial Research Unit (ARU) at the University of KwaZulu-Natal for same-day processing. The weather conditions on the days of sampling were warm, sunny and moderate humidity with no precipitation.

The water samples were filtered using a vacuum filter set (ISOLAB Laborgeräte GmbH, Eschau, Germany) through a 0.45 µm membrane filter (ISOLAB Laborgeräte GmbH, Eschau, Germany) and cultured on selective media. *Enterococcus faecium* Chromoselect Agar base (Merck KGaA, Darmstadt, Germany) supplemented with *Enterococcus faecium* Selective supplement (Merck KGaA, Darmstadt, Germany) was used to selectively culture *E. faecium* and CHROMagar™ ECC (CHROMagar™ , Paris, France) was used for *E. coli*. Simmons Citrate Agar (Oxoid, Hampshire, England) supplemented with myo-inositol (Merck KGaA, Darmstadt, Germany) was used for selective culturing of *K. pneumoniae* and the *Enterobacter* spp were cultured on Eosin Methylene Blue Agar (Merck KGaA, Darmstadt, Germany).

E. coli and *Enterobacter* spp culture plates were incubated at 37°C for 24 hours, and the *E. faecium and K. pneumoniae* culture plates were incubated at 42°C for 48 hours. After incubation, the presumptive isolates were phenotypically identified by the colonies' colour appearance on the media, as indicated by the manufacturers' instructions. Single colonies were sub- cultured twice, firstly on selective media for further purification and then on Nutrient Agar (Merck KGaA, Darmstadt, Germany). Single colonies were stored at -80°C in cryogenic vials containing microbeads in 1 mL Tryptone Soy Broth (Oxoid, Hampshire, Germany) prepared with 20% glycerol (Minema Chemicals, Johannesburg, South Africa). Ten isolates of each species from the two sample sites constituted the final sample of 40 isolates. *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 were used as controls.

2.4. Antibiotic susceptibility testing

The minimum inhibitory concentrations (MICs) were determined on the VITEK® 2 system (bioMérieux, Durham, North Carolina, USA) using the VITEK® 2 AST-N256 card for the Gram-negative bacteria and the VITEK® 2 AST-P645 card for Gram-positive bacteria. Results were interpreted according to the Clinical and Laboratory Standards guidelines (CLSI, 2023). The antibiotic panel for the Gram-positive isolates included

chloramphenicol, doxycycline erythromycin, fosfomycin, gentamicin, levofloxacin, linezolid, nitrofurantoin, tedizolid, teicoplanin, tetracycline, tigecycline and vancomycin. Gram-negative isolates were tested against amikacin, amoxicillin-clavulanic acid, cefepime, ceftazidime, cefuroxime, ciprofloxacin, cotrimoxazole, doripenem, ertapenem, gentamicin, imipenem, meropenem, piperacillin-tazobactam, tigecycline, and tobramycin. Isolates were then classified as susceptible or non-susceptible (intermediate + resistant), and isolates resistant to one antibiotic in at least three different antibiotic classes were classified as multidrug-resistant (MDR) (Catalano et al., 2022).

2.5. DNA Extraction and Whole Genome Sequencing

DNA was extracted from the isolates using the GenElute™ -E DNA Bacterial Genomic DNA kit (Sigma-Aldrich, St Louis, Missouri, USA) following the manufacturer's instructions. The quantity and quality of the extracted DNA were determined using the Nanodrop Microvolume Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Multiplexed paired-end libraries (2 × 300 bp) were prepared using the Nextera XT DNA sample preparation kit (Illumina, San Diego, California, USA). The sequences were then determined on the Illumina MiSeq™ System (Illumina, San Diego, California, USA) with 100x coverage. Whole genome sequencing was done at the National Institute of Communicable Diseases in Johannesburg, South Africa.

2.6. Genomic analyses and annotation

Raw reads were trimmed for quality using Sickel v1.33 (https://github.com/najoshi/sickle). The trimmed and quality-checked reads were then spontaneously assembled using the SPAdes v3.6.2 Genome Assembler (https://github.com/ablab/spades). Genome characteristics were annotated with RAST SEEDVIEWER (https://rast.nmpdr.org/seedviewer.cgi). ARGs were identified on the Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/analyze/rgi) and ResFinder v4.5.0 (http://genepi.food.dtu.dk/resfinder), with a default 90% threshold ID and 60% minimum length. The isolates' sequence types were identified with MLST 2.0 (https://cge.food.dtu.dk/services/MLST/). PlasmidFinder v2.0.1 (https://cge.food.dtu.dk/services/PlasmidFinder/) was used to determine the plasmids. Integrons were delineated using RAST SEEDVIEWER (https://rast.nmpdr.org/seedviewer.cgi), and the integrons and gene

cassettes were further identified using INTEGRALL (http://integrall.bio.ua.pt/). ISFinder (https://isfinder.biotoul.fr/) was used to delineate insertion sequences. The isolates' pathogenicity probability was predicted with PathogenFinder 1.1 (https://cge.food.dtu.dk/services/PathogenFinder/). The genomes were then submitted to the National Center for Biotechnology Information (NCBI) GenBank under Bioproject **PRJNA1143543** (accession numbers are shown in Supplementary Table S1). The genetic environment of identified ARGs and the associated MGEs was determined using the General Feature Format (GFF3) files from Genbank.

2.7. Multilocus sequence typing (MLST) and phylogenetic relationships

The study isolates were compared with other South African *E. coli* and *E. faecium* genomes from humans, animals and the environment. Because of the limited number of molecularly confirmed *K. pneumoniae* and *Enterobacter* isolates, phylogenetic relationship analysis would not yield accurate comparisons and, therefore, was not be performed for these isolates. The genomes were downloaded, annotated, and analysed from the Bacterial and Viral Bioinformatics Resource Centre (BV-BRCB) (https://www.bv-brc.org/). The phylogenetic tree was constructed using the maximum likelihood method on BV-BRCB. The *E. coli* genomes included were reported in South Africa from 2016-2023 (n = 53), and the *E. faecium* genomes were reported from 2001-2019 (n = 26). Because of the limited availability of *E. faecium* genomes on BV-BRC, we included all available *E. faecium* genomes from South Africa. *Escherichia coli* K12-MG1655 and *Enterococcus faecium* SRRA4 were used as reference genomes for *E. coli* and *E. faecium* phylogenetic distance configuration, respectively. The generated phylogenetic trees were visualised, edited, and annotated using the interactive Tree of Life (iTOL) (https://itol.embl.de/).

3. Results

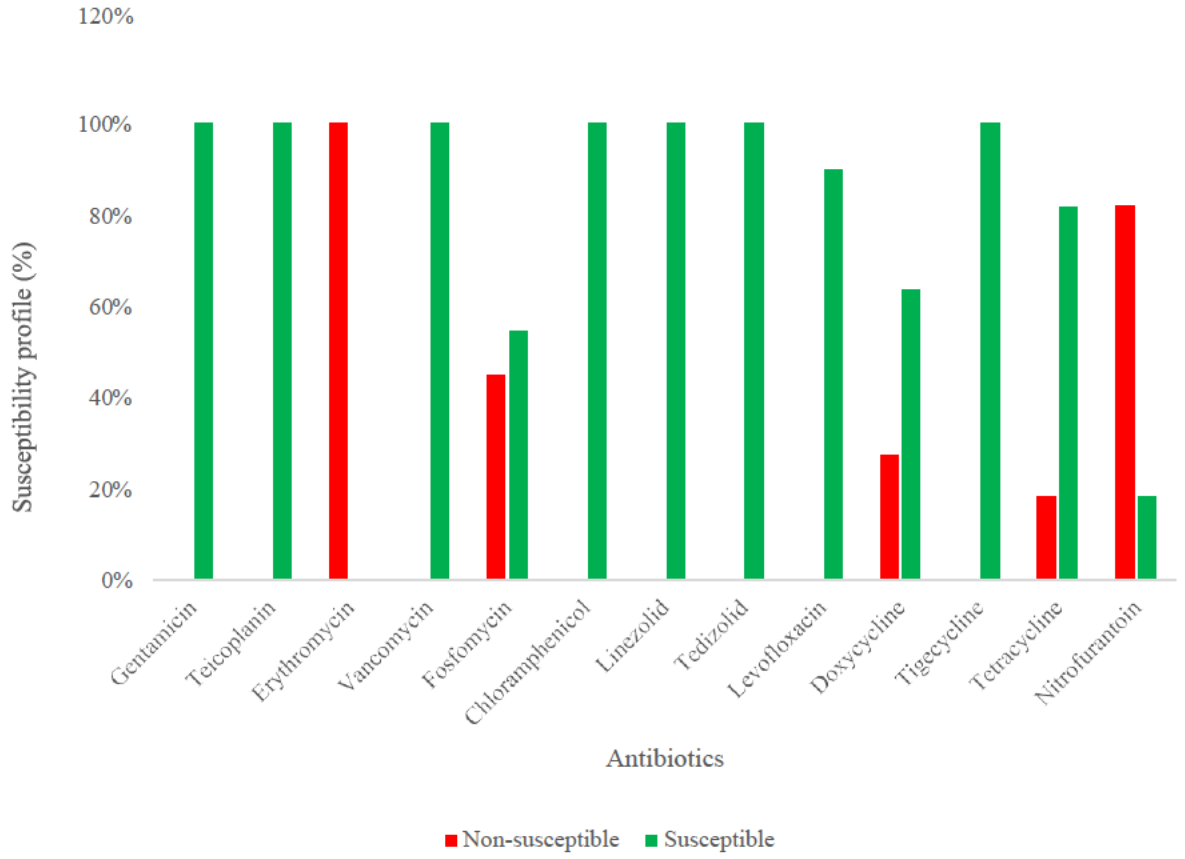
3.1. Faecal coliform bacteria prevalence in surface water

Of the 40 *E. coli* (n = 20) and *E. faecium* (n = 20) putatively identified on selective media, 12 and 11 isolates were molecularly confirmed via WGS, respectively. Other isolates were cross-contaminated with other species

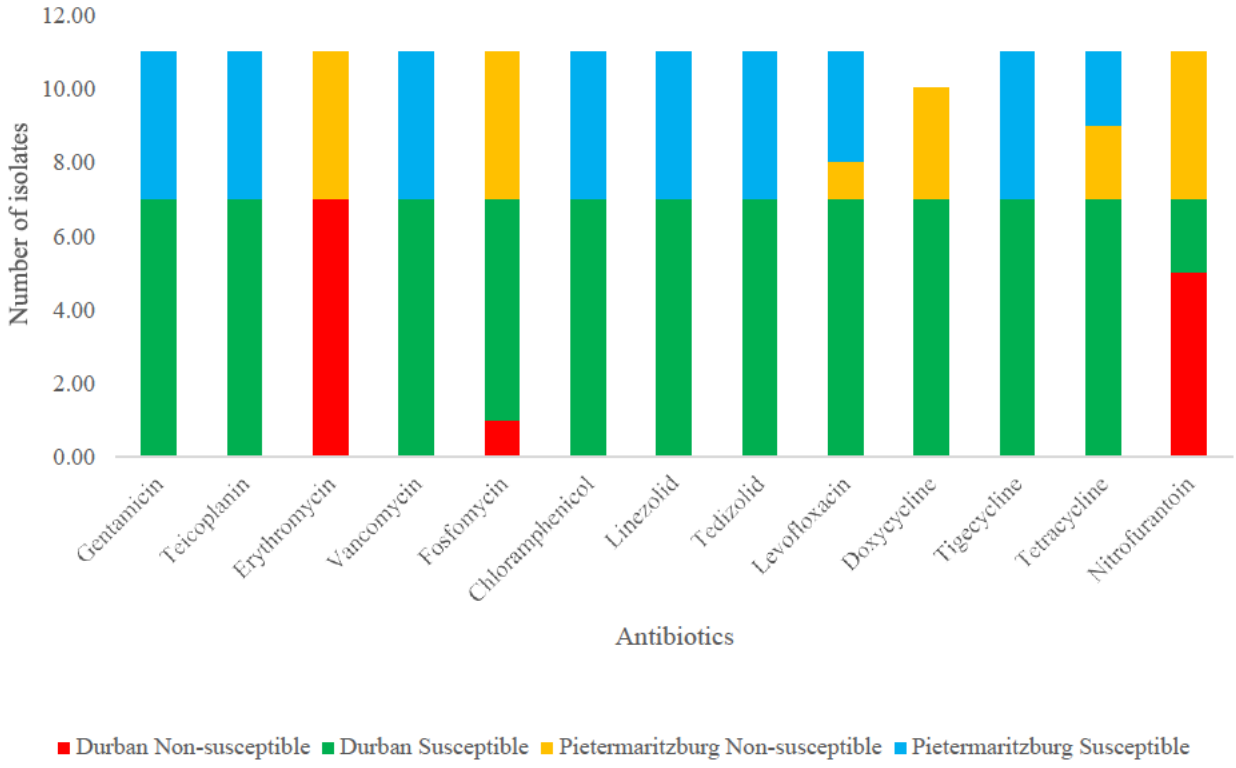
and were not part of the downstream analysis. Eight *E. coli* isolates were recovered from Durban and four from Pietermaritzburg. Seven *E. faecium* isolates were retrieved from Durban, while four were from Pietermaritzburg.

3.2. Antibiotic susceptibility profiles

The *E. faecium* isolates were all non-susceptible to erythromycin (100%, n = 11/11), and the least resistance was against tetracycline (18%, n = 2/11) (Figure 2A). Three Pietermaritzburg isolates were non-susceptible to doxycycline, and two were non-susceptible to tetracycline. The Durban *E. faecium* isolates were all susceptible to doxycycline and tetracycline (Figure 2B). All *E. faecium* isolates were susceptible to gentamicin when tested for high-level aminoglycoside resistance at 500 µg/mL and were fully susceptible to teicoplanin, vancomycin, linezolid, tedizolid, chloramphenicol and tigecycline (Figure 2A). Multidrug resistance was found in four (36.4%) *E. faecium* isolates (Table 1). The MDR patterns were against erythromycin, tetracyclines (tetracycline and doxycycline) and nitrofurantoin. (Table 1).


120%
100%
80%
60%
40%
20%
0%
Susceptibility profile (%)
Gentamicin
Teicoplanin
Erythromycin
Vancomycin
Fosfomycin
Chloramphenicol
Linezolid
Tedizolid
Levofloxacin
Doxycycline
Tigecycline
Tetracycline
Nitrofurantoin
Antibiotics
Non-susceptible
Susceptible

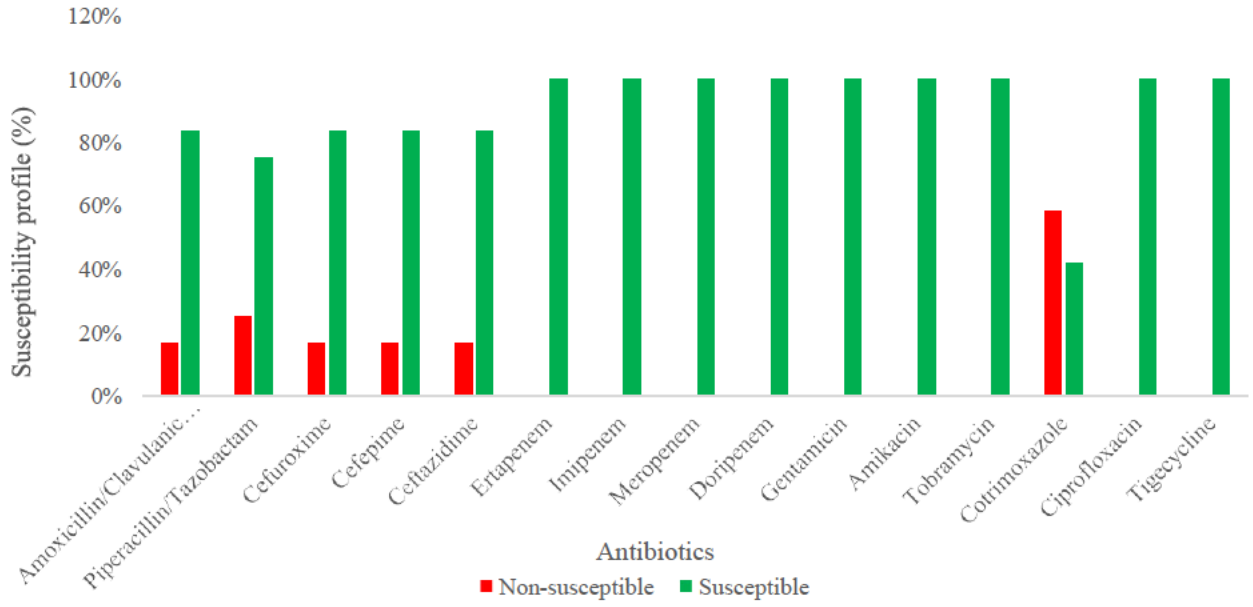
(A)


12.00
10.00
8.00
6.00
4.00
2.00
0.00
Number of isolates
Gentamicin
Teicoplanin
Erythromycin
Vancomycin
Fosfomycin
Chloramphenicol
Linezolid
Tedizolid
Levofloxacin
Doxycycline
Tigecycline
Tetracycline
Nitrofurantoin
Antibiotics
Durban Non-susceptible
Durban Susceptible
Pietermaritzburg Non-susceptible
Pietermaritzburg Susceptible

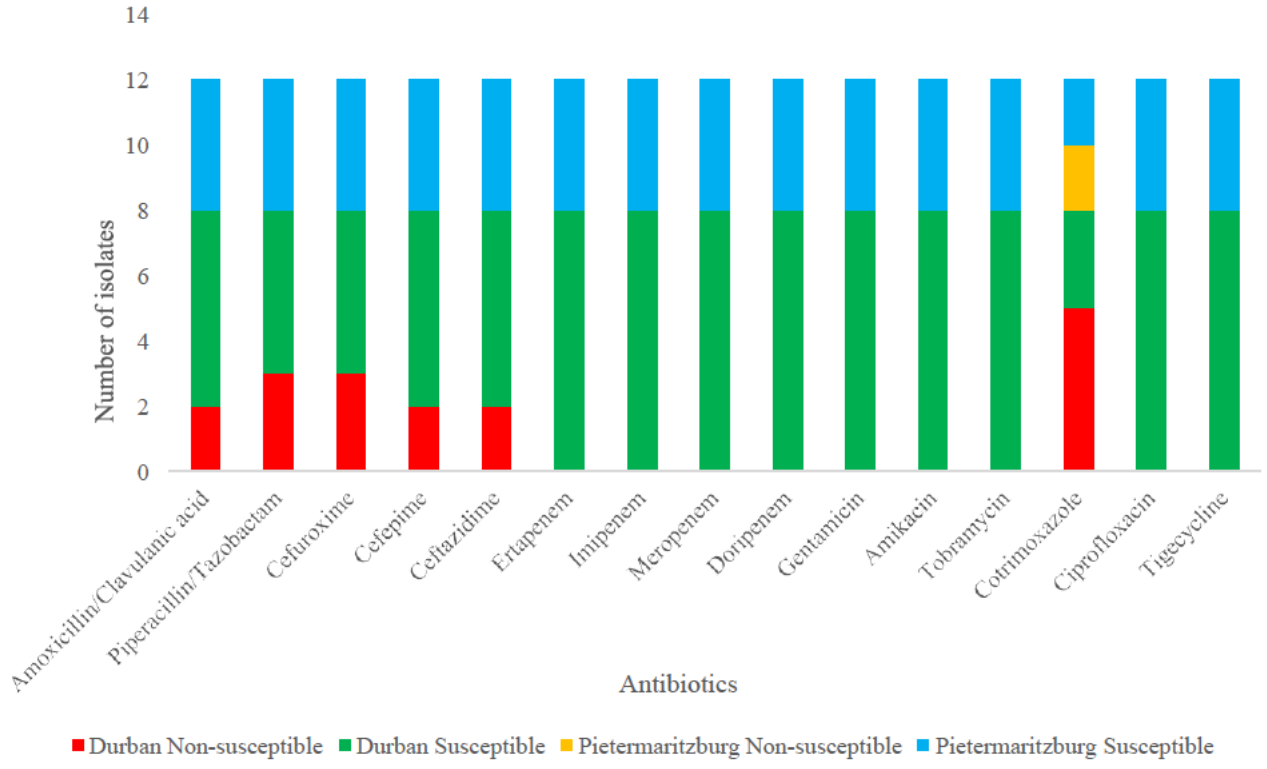
(B)

Fig 2. Percentage resistance of *E. faecium* (2A) and antibiotic resistance profiles from Durban and Pietermaritzburg (2B)

The highest non-susceptibility in *E. coli* was against cotrimoxazole (58%, n = 7/11), and only 17% (n = 2/12) of the isolates were not susceptible to amoxicillin-clavulanic acid, a third and fourth-generation cephalosporins ceftazidime and cefepime (Figure 3A). Non-susceptibility to amoxicillin-clavulanic acid, piperacillin- tazobactam, cefuroxime, cefepime and ceftazidime was observed only in the Durban isolates, and none were MDR (Figure 3B). The *E. coli* isolates were completely susceptible to the carbapenems, aminoglycosides, ciprofloxacin and tigecycline (Figure 3A**)**



(A)



(B)

Fig 3. *E. coli* antibiotic resistance profiles for the total resistance percentage for both cities (3A) and for both Durban and Pietermaritzburg (3B).

Table 1: Multidrug resistance profiles of E. faecium

Antibiogram	***E. faecium***
ERY-DOX-TET-NIT	1
ERY-DOX-NIT	2
ERY-TET-NIT	1

DOX-Doxycycline, ERY-Erythromycin, NIT-Nitrofurantoin, TET-Tetracycline,

3.3. Genome characteristics

The information on the accession numbers, number of contigs, longest contig size, N50 and L50 values is presented in Table S1 (Supplementary Material). The *E. faecium* total genome size ranged from 2.6-2.8 MB, and the GC content was between 37.9 and 38.3. *E. coli* genome size was between 4.5-5 MB, and the GC content was between 50.6 and 50.9.

3.4. Detected antibiotic resistance genes

The resistance observed in the *E. coli* isolates was attributed to the efflux pump belonging to the major facilitator superfamily (MFS), resistance nodulation cell division (RND) and ATP-binding cassette efflux pumps. Efflux pump genes *acr*A, *acr*B, and TolC are some identified genes encoding the AcrAB-TolC efflux pump to which cephalosporin resistance was attributed (Table 2). $bla_{\text{TEM-1B}}$ was the only identified β-lactam resistance gene in the *E. coli* isolates. Trimethoprim, sulfonamide and quinolone resistance genes were consistently identified in all *E. coli* isolates. The sulfonamide and trimethoprim resistance genes, *sul*1 and *dfr*A5 in Durban, *sul*3 and *dfr*A12 in Pietermaritzburg, corresponded with the observed cotrimoxazole resistance in all *E. coli* isolates. Only isolates from Pietermaritzburg harboured silent aminoglycoside [*aad*A1 and *aad*A2], and tetracycline resistance genes [*tet*A] in the absence of phenotypic resistance (Table 3).

Only EFP1 and EFP5 harboured the erythromycin [*erm*(B)] and acquired aminoglycoside [*ant*(6)-Ia and *aph*(3')-III] resistance genes. EFP1, EFP5, and EFP6 were all resistant to the tetracyclines and had corresponding resistance genotypes. However, EFP4 was resistant to doxycycline and tetracycline in the absence

of the tetracycline resistance genotype (Table 4). The observed resistance in EFP4 may be attributed to an overexpression of the MFS efflux pump, *efm*A identified in the isolate.

Table 2: Efflux pump genes identified in *E. coli*

Isolate ID	Species	Source	Resistance pattern	Efflux pump genes		
				MFS family	**RND Family**	**ABC Family**
ECD3	*E. coli*	Durban	AMC-TZP-CXM-FEP-CAZ-SXT	*evg*a, *H-NS, Tol*c	*acr*B, *acr*D, *mar*A, *mdt*F, *acr*E, *acr*F	*acr*S, *To*lC,
ECD4	*E. coli*	Durban	TZP-CXM-SXT	*emr*K, *evg*A, *mdt*F, *mdt*H, *emr*R, *emr*A, *emrB and H-NS*	*acr*B, *acr*D, *mar*A, *mdt*F, *acr*E, *acr*F	*acr*S, *To*lC,
ECD5	*E. coli*	Durban	SXT	*emr*K, *evg*A, *mdt*G, *mdt*H, *emr*R, *emr*A, *emr*B, *H-NS, Tol*C	*acr*B, *acr*D, *mar*A, *mdt*F, *acr*E, *acr*F	*acr*S, *To*lC, *msbA*
ECD6	*E. coli*	Durban	SXT	*Tol*C, *emr*K, *evg*A *and H-NS*	*acr*B, *acr*D, *mar*A, *mdt*F, *acr*E, *acr*F	*acrS, TolC,*

ECD9	*E. coli*	Durban	AMC-TZP-CXM-FEP-CAZ-SXT	*Tol*C, *H-NS and evg*A	*acrB, acrD,arA, mdtF, acrE, acrF*	*acrS, TolC,*
ECP8	*E. coli*	Pietermaritzburg	SXT	*evg*A, *H-NS, Tol*C, *mdt*G *TolC*	*acrB, acrD, marA, mdtF, acrE, acrF*	*acrS, TolC,*
ECP10	*E. coli*	Pietermaritzburg	SXT	*emr*K, *evg*A, *H-NS, TolC*	*acrB, acrD,marA, mdtF, TolC, acrE, acrF TolC*	*acrS, TolC*

AMC-amoxicillin-clavulanic acid, TZP- piperacillin-tazobactam, CXM-cefuroxime, FEP- cefepime, CAZ- ceftazidime, SXT- cotrimoxazole

Table 3: Antibiotic resistance genes, sequence types, plasmids, and insertion sequences in antibiotic-resistant *E. coli* isolates

Isolate ID	Source	Resistance pattern	Pathogenicity scores	Sequence Type	Antibiotic Resistance genes	Plasmid replicons	Insertion sequences
ECD3	Durban	AMC-TZP-CXM-FEP-CAZ-SXT	0.933	ST2491	$bla_{\text{TEM-1B}}$, *sul*1, *dfr*A5, *qnr*B19	Col(pHAD28), IncFIA(HI1), IncX1, IncY, Col8282	ISEc17, IS3, IS3F, IS3H, ISEc16, ISEaI1
ECD4	Durban	TZP-CXM-SXT	0.933	ST2491	$bla_{\text{TEM-1B}}$, *sul*1, *dfr*A5, *qnr*B19	Col(pHAD28), IncFIA(HI1), IncX1, IncY, Col8282	TnShfr1, ISSod9, ISHahy15, ISAs6, ISPpa7
ECD5	Durban	SXT	0.933	ST2491	$bla_{\text{TEM-1B}}$, *sul*1, *dfr*A5, *qnr*B19	Col(pHAD28), IncFIA(HI1), IncX1, IncY, Col8282	TnShfr1, ISSod9, ISHahy15, ISAs6, ISPpa7
ECD6	Durban	SXT	0.933	ST2491	$bla_{\text{TEM-1B}}$, *sul*1, *dfr*A5, *qnr*B19	Col(pHAD28), IncFIA(HI1),	ISEsa1, ISSen13, ISSba14, MITEEc1, ISKpn21

						IncX1, IncY, Col8282	
ECD9	Durban	AMC-TZP-CXM-FEP-CAZ-SXT	0.933	ST2491	*bla*$_{TEM-1B}$, *sul*1, *dfr*A5, *qnr*B19	Col(pHAD28), IncFIA(HI1), IncX1, IncY, Col8283	ISEsa1, ISSen13, ISSba14, MITEEc1, ISKpn21
ECP8	Pietermaritzburg	SXT	0.934	ST602	*sul*3, *dfr*A12, *tet*(A), *cml*A1, *aad*A1, *aad*A2, *qnr*S1	Col(pHAD28), IncFIB(AP001918), IncFII	MITEEc1, MITEYpe1, MIEPlu5, ISRor2, ISRor3
ECP10	Pietermaritzburg	SXT	0.934	ST602	*sul*3, *dfr*A12, *tet*(A), *cml*A1, *aad*A1, *aad*A2, *qnr*S1	Col(pHAD28), IncFIB(AP001918), IncFII	MITEEc1, MITEYpe1, MIEPlu5, ISRor2, ISRor3

AMC-amoxicillin-clavulanic acid, TZP- piperacillin-tazobactam, CXM-cefuroxime, FEP- cefepime, CAZ- ceftazidime, SXT- cotrimoxazole

Table 4: Antibiotic resistance genes, sequence types, plasmids, and insertion sequences found in antibiotic-resistant *E. faecium* isolates

Isolate ID	Source	Resistance pattern	Pathogenicity scores	Sequence Type	Antibiotic resistance genes	Plasmid replicons	Insertion sequences
EFD2	Durban	ERY-NIT	0.572	ST2042	*msr*(C), *aac(6')*-Ii,	rep1, repUS15	ISEfm1, IS19, IS6770, IS1252, IS1062
EFD7	Durban	ERY-NIT	0.499	ST2013	*msr*(C), *aac(6')*-Ii,	rep1, repUS15	ISEfm1, IS19, ISPye7, ISShes9, ISSma15
EFD8	Durban	ERY-NIT	0.622	ST94	*msr(C), aac(6')*-Ii,	rep29, rep14B, repUS15	ISPye7, ISShes9, ISFnu2, ISFnu1, ISPlag1
EFP1	Pietermaritzburg	ERY-DOX-NIT	0.806	ST2431	*dfr*G, *tet*(M), *te*t(L), *erm*(B), *msr*(C), *ant(6)*-Ia, *aph(3')*-III, *aac(6')*-Ii	rep29, repUS15, repUS43	ISHce1, ISCig1, ISMae8, ISCbo10, ISBcy1
EFP4	Pietermaritzburg	ERY-DOX-TET-NIT	0.629	ST94	*msr*(C), *aac(6')*-Ii	rep1, repUS15	ISEfa10, ISLmo16, ISLmo11, ISLse1, ISEnfa3

EFP5	Pietermaritzburg	ERY-DOX-NIT	0.806	ST2431	*dfr*G, *tet(*M), *tet*(L), *erm*(B), *msr*(C), *ant(6)*-Ia, *aph(3')*-III, *aac(6')*-Ii	repUS43, rep29, repUS15	ISFa5, ISFa11, ISFa17, ISVlu1, TnDra1
EFP6	Pietermaritzburg	ERY-TET-NIT	0.658	ST361	*tet(*M), *msr*(C), *aac(6'*)-Ii,	repUS15, repUS43	ISSydi1, ISAba50, ISMesp5, ISSpe3, IS1554

ERY- erythromycin, NIT- nitrofurantoin, DOX- doxycycline, TET- tetracycline

3.5. Mobile genetic elements (Plasmids, insertion sequences, and integrons)

PlasmidFinder revealed a total of seven different plasmid replicons in *E. coli* isolates. All Durban *E. coli* genomes harboured the same plasmid replicons IncFIA, IncX, IncY, Col(pPHAD28) and Col8282, and Pietermaritzburg isolates ECP8 and ECP10 shared similar plasmid replicons, Col(pPHAD28), IncFIB(AP001918) and IncFII. The IncF group was the common replicon in all isolates regardless of the isolate source. ECD6 and ECD9 had common insertion sequences [ISEsa1, ISSen13, ISSba14, MITEEc1, and ISKpn21]. ECD3 had different insertion sequences [ISEc17, IS3, IS3F, IS3H, ISEc16 and ISEaII] compared with isolates (Table 3).

A total of five *E. faecium* plasmid replicons (rep1, repUS15, rep29, rep14B, and repUS43) were revealed by PlasmidFinder. Plasmid replicons rep1, repUS43 and rep29 were each found in at least two isolates, and repUS15 was found in all seven isolates. Plasmid replicon repUS43 was unique to the Pietermaritzburg isolates, and rep14B was found in only one isolate from Durban (Table 4). All *E. faecium* genomes had the In1212 integron with only gene cassette *aac*A7 synonymous to *aac(6')*-Ii (Table 5). Class 1 integrons In13, which had *sul*1, *qacE*▲1 and *dfr*A5 gene cassettes, were found in all *E. coli* Durban isolates. The Pietermaritzburg isolates ECP8 and ECP10, carried Class 1 integrons In27/117/4 with *dfr*A12- 28 *aadA*1a- *aad*A2-*cml*A1 gene cassettes (Table 5).

Table 5: Class 1 integrons with the associated gene cassettes

Isolate					**Cassette arrays**			
ID	**Species**	**Source**	**MLST**	**Integron**	**GC1**	**GC2**	**GC3**	**GC4**
ECD3	*E. coli*	Durban	ST2491	In13	*sul*1	*qac*E▲1	*dfr*A5	
ECD4	*E. coli*	Durban	ST2491	In13	*sul*1	*qac*E▲1	*dfr*A5	
ECD5	*E. coli*	Durban	ST2491	In13	*sul*1	*qac*E▲1	*dfr*A5	
ECD6	*E. coli*	Durban	ST2491	In13	*sul*1	*qac*E▲1	*dfr*A5	
ECD9	*E. coli*	Durban	ST2491	In13	*sul1*	*qacE▲1*	*dfrA5*	
ECP8	*E. coli*	Pietermaritzburg	ST602	In27/117/4	*dfr*A12	*aad*A1a	*aad*A2	*cml*A1
ECP10	*E. coli*	Pietermaritzburg	ST602	In27/117/4	*dfr*A12	*aad*A1a	*aad*A2	*cml*A1

EFD2	*E. faecium*	Durban	ST2042	In1212	*aac*A7
EFD7	*E. faecium*	Durban	ST2013	In1212	*aac*A7
EFD8	*E. faecium*	Durban	ST94	In1212	*aac*A7
EFP1	*E. faecium*	Pietermaritzburg	ST2431	In1212	aacA7
EFP4	*E. faecium*	Pietermaritzburg	ST94	In1212	*aac*A7
EFP5	*E. faecium*	Pietermaritzburg	ST2431	In1212	*aac*A7
EFP6	*E. faecium*	Pietermaritzburg	ST361	In1212	*aac*A7

aacA7 is synonymous to aac(6')-iI (Integrall &CARD)

Associations between the antibiotic resistance genes and mobile genetic elements were identified. All the Durban *E. coli* genomes, ECD3, ECD4, ECD5, ECD6 and ECD9, had similar genomic environments (Table 6). $bla_{\text{TEM-1}}$ was consistently associated with IS1 family transposase and a Tn3 transposon. The sulfonamide, trimethoprim and disinfectant gene *qacE*▲1 in Durban isolates were associated with an IS6 family transposase. Although *sul, dfr* and *qac*E form class 1 integron gene cassettes, the contig carrying these genes in the Durban isolates was not associated with an integron integrase gene. In contrast, the gene cassettes in the Pietermaritzburg isolates had a genetic context, *sul*3:IS256 family transposase:*qac*L:*aad*A1:*cml*A1*:aad*A:*dfr*A12:Class 1 integron integrase Int1. Furthermore, *tet*(A) was 40 associated with an ISB family transposase, and *qnr*S1 was associated with an ISKpn19 family transposase. ECP8 and ECP10 had similar genomic environments except for the manganese resistance genes, which were not associated with any mobile elements in ECP10. One notable difference between the Durban and the Pietermaritzburg isolates was that the fluoroquinolones resistance gene in the Durban isolates was not associated 44 with a mobile genetic element. All *E. coli* resistance genes associated with MGEs were carried on plasmids 45 (Table 6).

E. faecium ARGs were associated with chromosomal and mobile genetic environments (Table 7). Most ARGs in *E. faecium* were located on the chromosomes (11). EFP1 and EFP5 had a contig with a diverse genomic environment consisting of erythromycin resistance gene *erm*(B), insertion sequence IS1182-like element

IS1182 family transposase, acquired aminoglycoside resistance genes *ant(6)*-Ia, *sat*4 and *aph(3')*-IIIa, a recombinase and an integrase. The other ARGs *msr*(C), *dfr*G, *tet*(L) and *tet*(M) in EFP5 were not associated with mobile genetic elements. The plasmid-mediated tetracycline resistance genes *tet*(L) and *tet*(M) in EFP1 were co-carried with a conjugal transfer protein, which may promote the transfer of these genes. Amongst all identified ARGs, only the tetracycline resistance genes were carried on a plasmid in one isolate (Table 7).

Table 1: Antibiotic resistance genomic environments of *E. coli* isolates

Isolate ID	Source	MLST	Contig	Synteny of resistance genes and MGEs	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
ECD3	Durban	ST2491	37	IS1 family transposase:::Tn3-like element Tn3 family transposase:recombinase family protein:blaTEM-1:recombinase family protein:::::conjugative DNA transfer protein:cag pathogenicity island cag12 family protein:conjugal transfer protein::transposase	*Escherichia coli plasmid pOLA52, EU370913.1*
			79	IS6 family transposase:sul1:qacE▲1:dfrA5	*Escherichia coli strain AR_0114 plasmid unitig_2, CP021734.1*
ECD4	Durban	ST2491	38	IS1 family transposase:::Tn3-like element Tn3 family transposase:recombinase family protein:blaTEM-1:recombinase family protein: mobP1 family relaxase:conjugative transfer protein::conjugative transfer protein:cag pathogenicity island cag12 family protein::transposase	*Escherichia coli plasmid pOLA52, EU370913.1*
			80	IS6 family transposase:sul1:qacE▲1:dfrA5	*Escherichia coli strain AR_0114 plasmid unitig_2, CP021734.1*

ECD5	Durban	ST2491	38	IS1 family transposase:::Tn3-like element Tn3 family transposase:recombinase family protein:blaTEM-1:recombinase family protein:mobP1 family relaxase:conjugative transfer protein:::conjugative DNA transfer family protein:cag pathogenicity island cag12 family protein:conjugal transfer protein::	*Escherichia coli plasmid pOLA52, EU370913.1*
			81	IS6 family transposase:sul1:qacE▲1:dfrA5	*Escherichia coli strain AR_0114 plasmid unitig_2, CP021734.1*
ECD6	Durban	ST2491	37	IS1 family transposase:::Tn3-like element Tn3 family transposase:recombinase family protein:blaTEM-1:recombinase family protein:mobP1 family relaxase:::conjugative DNA transfer family protein:cag pathogenicity island cag12 family protein:conjugal transfer protein::transposase	*Escherichia coli plasmid pOLA52, EU370913.1*
			83	IS6 family transposase:sul1:qacE▲1:dfrA5	*Escherichia coli strain AR_0114 plasmid unitig_2, CP021734.1*
ECD9	Durban	ST2491	37	IS1 family transposase::Tn3-like element Tn3 family transposase:recombinase family protein:blaTEM-1:recombinase family protein::mobP1 family relaxase:conjugative transfer family protein::cag	*Escherichia coli plasmid pOLA52, EU370913.1*

				pathogenicity island cag12 family protein:conjugal transfer protein::transposase	
			80	IS6 family transposase:sul1:qacE▲1:dfrA5	*Escherichia coli strain B4.H1.17 plasmid pB4.H1.17.clo,CP146554.1*
ECP8	Pietermaritzburg	ST602	46	sul3:IS256 family transposase:qacL:aadA1:cmlA1:aadA:dfrA12:Class 1 integron integrase Int1:DDE-type integrase/recombinase/transposase	*Escherichia coli 2018-01-ICC plasmid p2018-01-ICC_1DNA, AP027870.1*
			49	IS1-like element IS1B family transposase:Class A beta-lactamase:recombinase family protein:relaxase:tetR(A):tet(A):Tn3 family transposase	*Escherichia coli PSS-04 plasmid pPSS-04_4DNA, AP027670.1*
			56	recombinase:ISKra4-like element ISKpn19 family transposase:recombinase:qnrS1:transposase	*Klebsiella oxytoca strain 45 plasmid unnamed2, CP138769.1*
			61	IS1 family transposase:Tn3 family transposase:sitD:sitC:sitB:sitA	*Escherichia coli strain CPSS_Schuan plasmid pCPSS_IncFIB, CP053732.1*
ECP10	Pietermaritzburg	ST602	47	sul3:IS256 family transposase:qacL:aadA1:cmlA1:aadA:dfrA12:Class 1 Integron integrase Int1:DDE-type integrase/recombinase/transposase	*Escherichia coli 2018-01-ICC plasmid p2018-01-ICC_1DNA, AP027870.1*
			51	IS1-like element IS1B family transposase:Class A beta-lactamase:recombinase family protein:relaxase:tetR(A):tet(A):Tn3 family transposase	*Escherichia coli PSS-04 plasmid pPSS-04_4DNA, CP027670.1*

	57	recombinase:ISKra4-like element ISKpn19 family transposase:recombinase:qnrS1:transposase	*Klebsiella oxytoca strain 45 plasmid unnamed2, CP138769.1*

Table 7: Antibiotic genomic environments of *E. faecium* isolates

Isolate ID	Source	MLST	Contig	Synteny of resistance genes and MGEs	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
EFD2	Durban	ST2042	3	msr(C): recombinase recA	*Enterococcus faecium strain CBA7134 chromosome (CP025685.1)*
EFD7	Durban	ST2013	3	recombinase recA:msr(C)	*Enterococcus faecium strain DMEA02 chromosome, CP043484.1*
			11	aac(6')-Ii:IS982 family transposase	*Enterococcus faecium strain WN-18 chromosome, CP099979.1*
			33	transposase:recombinase:clpL	*Enterococcus faecium strain HRH41 plasmid unnamed1, CP141091.1*
EFD8	Durban	ST94	3	recombinase recA:msr(C):relaxase	*Enterococcus faecium strain DMEA02 chromosome, CP043484.1*
			52	transposase:clpL	*Enterococcus faecium strain WN-18 plasmid Pa, CP099980.1*
EFP1	Pietermaritzburg	ST2431	7	erm(B):IS1182-like element IS1182 family transposase:ant(6)-Ia:sat4:aph(3')-Iia:recombinase/intergrase	*Enterococcus faecium strain FB-1 chromosome, CP040907.1*

			26	dfrG:IS110 family transposase	*Enterococcus faecium strain AALTL chromosome, CP025754.1*
			80	tet(L) :tet(M) :conjugal transfer protein	*Enterococcus faecalis S7316 plasmid pS7316optrA DNA, LC499744.1*
EFP4	Pietermaritzburg	ST94	8	recombinase recA:msr(C)	*Enterococcus lactis strain SU-B46 chromosome, CP129887.1*
			12	aac(6')-Ii:transposase	*Enterococcus lactis strain JDM1 chromosome, CP078094.1*
EFP5	Pietermaritzburg	ST2431	5	erm(B):IS1182-like element IS1182 family transposase:ant(6)-Ia:sat4:aph(3')-IIIa:recombinase/integrase:recombinase/integrase	*Enterococcus faecium strain FB-1 chromosome, CP040907.1*
EFP6	Pietermaritzburg	ST361	8	conjugal transfer protein :::tet(M) :relaxase	*Enterococcus faecium strain HB-1 chromosome, CP040878.1*
			24	aac(6')-Ii:IS982 family transposase	*Enterococcus faecium strain DMEA09 chromosome, CP115812.1*
			32	transposase:clpL	*Enterococcus faecium strain HB-1 plasmid punnamed, CP040877.1*

3.6. Multilocus sequence typing (MLST) and phylogenetic relationships

Escherichia coli isolates had only two sequence types (ST2491 and ST602), predicted as human pathogens, with a confidence of 0.933-0.934 (Table 3). ST2491 isolates were from Durban, and the Pietermaritzburg isolates belonged to ST602. ST2491 isolates had the same ARGs and MGE associations but different resistance patterns. Some isolates were more resistant compared to others. In contrast to the Durban findings, ST602 had similar resistance patterns, ARGs and MGEs. One difference in the ARG and MGE association between the two ST602 isolates was the manganese transporter protein genes *sit*ABCD association with an insertion sequence, IS, and a Tn3 transposon in one isolate. This association was not identified in the other isolate harbouring the same genes. The *E. coli* isolates from this study were more closely related to human and animal isolates. The isolates from Durban arose from the same node as human (ST34), animal (ST10, ST206 and ST3531) and environment (ST10, ST165, ST1141) isolates. The Pietermaritzburg isolates (ST602) were closely related to other ST602 isolates from irrigation water and clinical material.

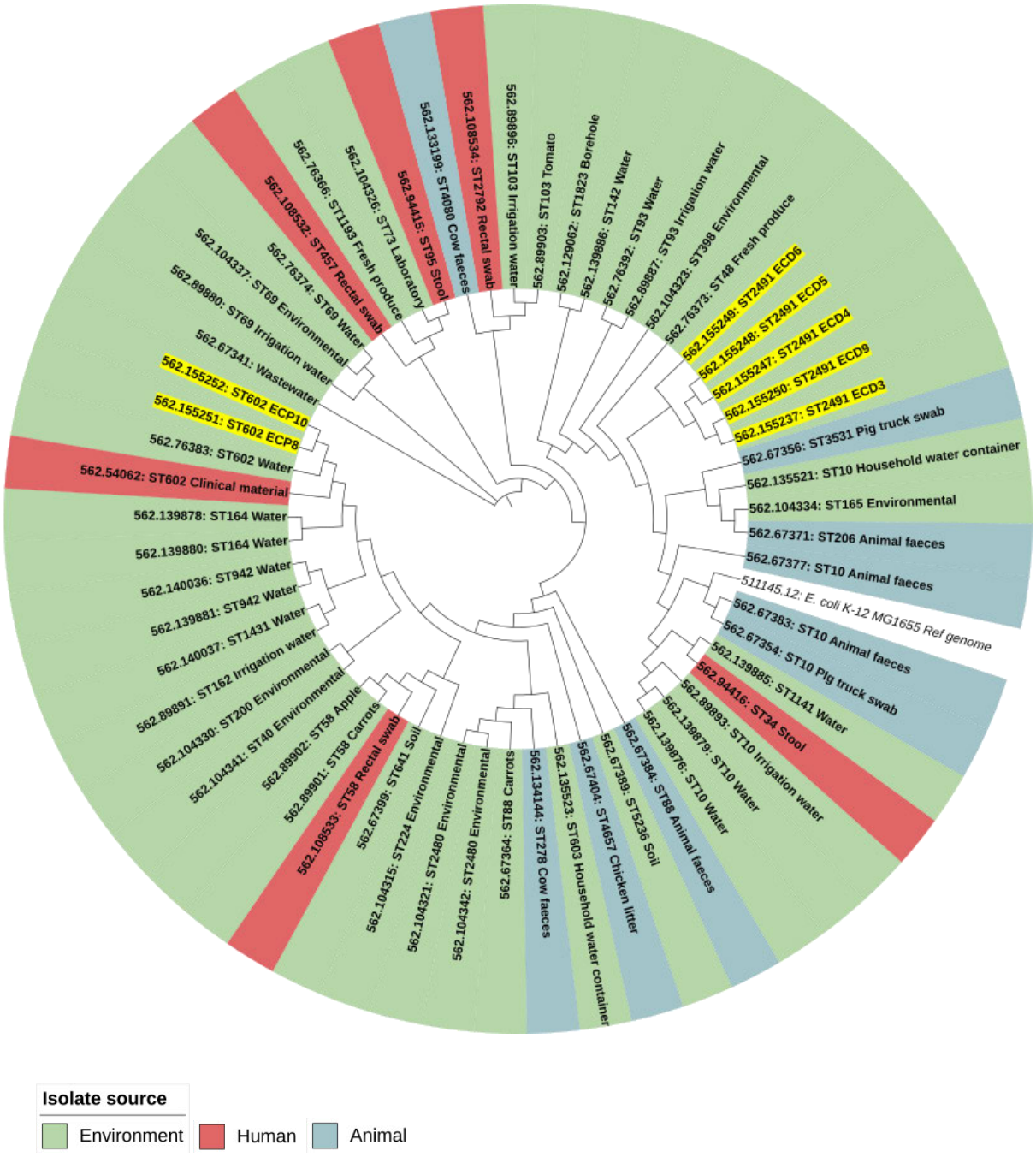


Fig 3. A phylogenetic tree showing the relationships of *E. coli* from this study (highlighted in yellow) with South African isolates from humans(red), animals (blue) and the environment (green) obtained from the BV-BRC website.

E. faecium isolates belonged to five sequence types (ST2043, ST2013, ST94, ST2431 and ST361). ST94 was found in Durban and Pietermaritzburg, and the rest of the sequence types were unique to their isolate source.

ST2042, ST2013 and ST94 from Durban had the same resistance pattern and ARGs. However, ST94 from Pietermaritzburg harboured an additional gene, *tet*(M). The Pietermaritzburg isolates belonging to ST2431 had the same resistance pattern, ARGs and the same plasmid replicons (rep29, repUS15 and repUS43). Only one ST2431 had a genetic environment on a plasmid, *tet*(L):*tet*(M):conjugal transfer protein. ST361 from Pietermaritzburg had a unique resistance pattern, ERY-TET-NIT, two plasmid replicons and only *tet*(M). However, the genetic environment on which *tet*(M) was found was located on a chromosome. Phylogenetic analysis of the *E. faecium* isolates revealed a closer relationship with human and environmental isolates from South Africa (Figure 5). ST94 from Durban was more closely related to ST2013 from Durban than ST94 from Pietermaritzburg. EFP6 (ST361) had a close relationship with other ST361 isolates from wastewater; it also arose from the same node as ST94 from wastewater. EFP1 and EFP5 (ST2431) arose from the same node isolates from urine (ST18), blood (ST761), hospital bed (ST80) and an ICU-blood pressure apparatus (ST907). EFP1 and EFP5 were predicted as human pathogens with a confidence of 0.806. This may indicate that ST2431 is of human origin or circulating in clinical environments. The BV-BRC has a limited availability of *E. faecium* genomes from South Africa.

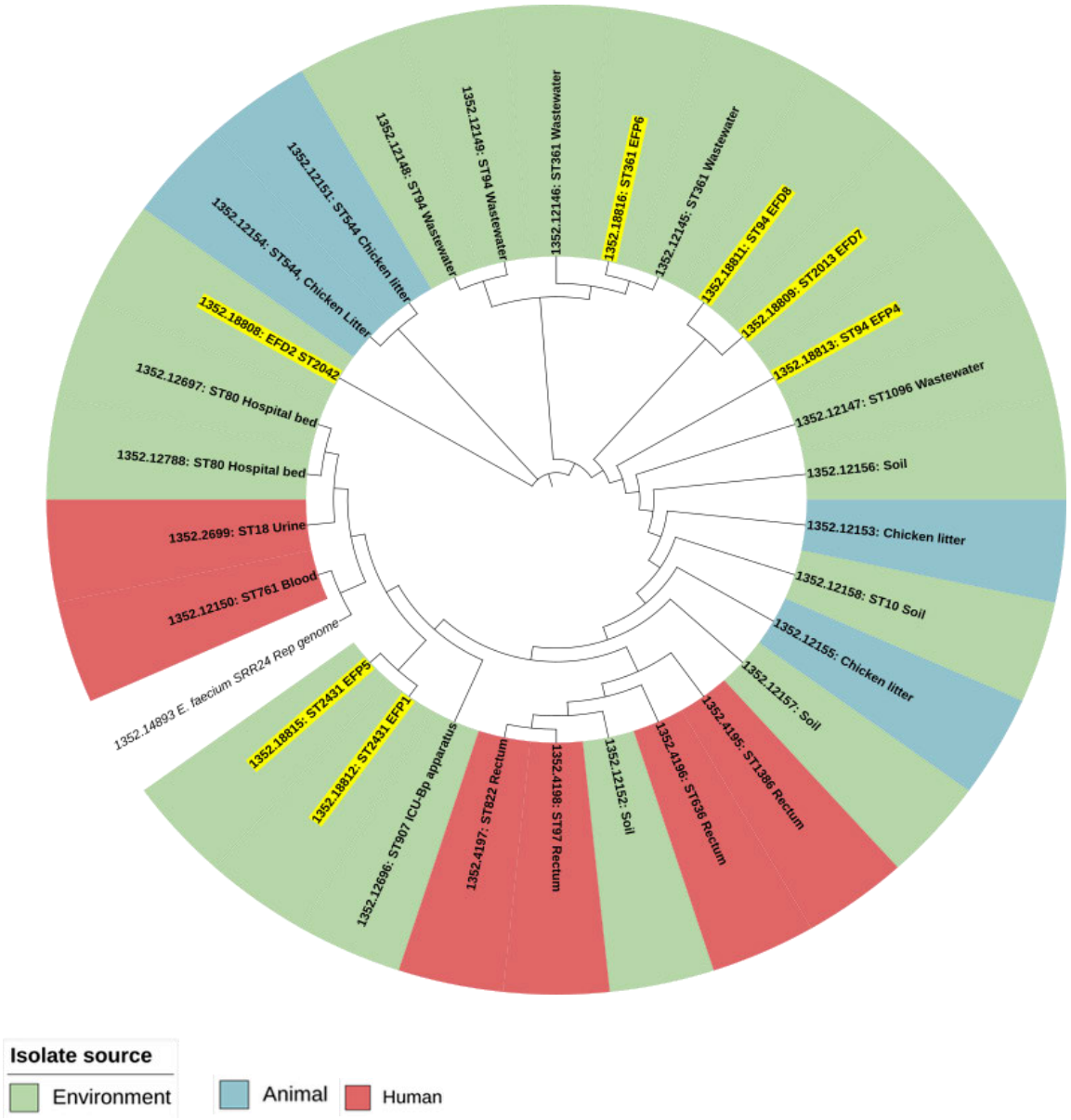


Figure 4: A phylogenetic tree showing the relationships of E. faecium from this study (highlighted in yellow) with South African isolates from humans(red), animals (blue) and the environment (green) obtained from the BV-BRC website.

4. Discussion

Surface water bodies are constantly polluted by antibiotic-resistant faecal coliform bacteria from various sources. Our study delineated the resistome, mobilome, multilocus sequence types and the phylogenetic relationships of drug-resistant *E. coli* and *E. faecium* from streams in Durban and Pietermaritzburg proximate to informal settlements. The faecal pollution indicators, *E. coli* and *E. faecium* were isolated and identified. Third and fourth-generation cephalosporin resistance in *E. coli* was attributed to efflux pumps. *E. faecium* isolates mostly carried the relevant resistance genes for the observed phenotype resistance.

4.1. Faecal coliform bacteria prevalence in surface water

The human and animal gastrointestinal tracts contain diverse microbial populations contributing to the host's physiological processes. Although most intestinal microbiota are not harmful, pathogenic *E. coli* and *E. faecium* strains can lead to infections. These species are introduced into the environment from various sources, such as open defaecation, untreated wastewater discharge, septic leakages, and agricultural and urban run-off (Islam, Sokolova & Hofstra, 2018). The presence of *E. coli* and enterococci bacteria in aquatic environments has also been used to indicate the presence of faecal pollution (Kubera, 2020), suggesting that the *E. coli* and *E. faecium* in our study were possibly of faecal origin.

Culture-dependent methods have been used to study the prevalence of *E. coli*, *E. faecium* and other bacteria (Milligan et al., 2023; Kumar et al., 2024; Mustafa et al., 2022; Jofré Bartholin, Barrera Vega & Berrocal Silva, 2023). In these methods, bacteria are grown under controlled laboratory conditions. Selective media are often used to select and optimise the growth of specific bacteria while inhibiting others (Al-Blooshi et al., 2021). Our study used selective media to isolate the bacteria from the sampled water. Despite their success and wide usage over time, culture-dependent methods still have major drawbacks. Some bacteria may present with the same morphology and colour characteristics due to similar biochemical processes, making it difficult to differentiate between them.

Supplemented *Enterococcus faecium* Chromoselect agar allows for the isolation of *Enterococcus faecium* based on its green colour appearance after incubation. This selective media is composed of ingredients serving various

purposes. The chromogenic substrate in the media is broken down by the β-glucosidase enzyme in the *Enterococcus* species, producing blue-coloured colonies (Al-Haj, 2024). Another ingredient, arabinose, is fermented by *E. faecium*, producing green-coloured colonies with a yellow colouration on the medium. *E. faecium* is differentiated from *Enterococcus faecalis* by its ability to ferment arabinose. Therefore, *E. faecalis* remains blue, while *E. faecium* colonies change to green (Soujanya & G S, 2021; Wigmore, Greenhill & Bean, 2024). Aztreonam in the agar base inhibits the growth of Gram-negative bacteria, and other Gram-positive bacteria are inhibited by cephalexin (Ramsey & MacGowan, 2016; Choi et al., 2023). Of the 20 putative *E. faecium* identified by selective media, 11 (55%) were confirmed by WGS. Other presumptive isolates were identified as *E. faecium* but with a lower species identity percentage (75-90%), i.e an *E. faecium* genome had 90% *E. faecium* and 10% *E. coli* identification indicating cross-contamination with *E. coli*. The cross-contamination may have been caused the low sensitivity of the media, contamination during processing, environmental factors or insufficient purification of the isolates. Therefore, future studies can reduce the chances of cross-contamination by increasing the purification cycles.

E. coli isolates were enumerated and isolated on Chromagar ECC, differentiating *E. coli* from other coliforms. Of the 20 presumptive isolates, 12 (60%) were molecularly identified, while others had cross-contamination with other isolates. A *Klebsiella* isolate was identified from one of the presumptive *E. coli* isolates. The medium selects for the growth of β-glucuronidase positive *E. coli*. This enzyme cleaves the chromogenic mix, giving the *E. coli* colonies a blue colour appearance while other coliforms appear mauve and no-coliforms appear white (Shabir et al., 2021; Kemper et al., 2023). However, this media has some limitations. There may be false negative results as some *E. coli* strains, particularly O157, may be β-glucuronidase negative. Also, this media has a 97% sensitivity for *E. coli* (Shabir et al., 2021). As this media allows the growth of other coliforms, false positives and misidentifications can occur.

Our sample sources in both Durban and Pietermaritzburg are exposed to human and industrial pollution, which may include faecal pollution, as these streams are proximate to industries and informal settlements lacking

WASH services. Moreover, The Durban stream is exposed to a Nature reserve upstream of the sample point. The nature reserve is a habitat for over 150 bird species and different animals. *E. coli* and *E. faecium* inhabit both human and animal guts, highlighting faecal contamination of the stream from both humans and animals

4.2. Antibiotic susceptibility profiles

Resistance to cotrimoxazole in *E. coli* (58%) was the highest in our study. Cotrimoxazole (sulfamethoxazole-trimethoprim) was first recommended for HIV-exposed infants in the 2000s as it was found to be effective against pneumocystis pneumonia (PCP) in HIV-positive adults (Daniels et al., 2022). Because of its wide clinical usage for both PCP and urinary tract infections, bacteria have developed resistance against this antibiotic (Poey et al., 2024), spreading from clinical settings, households, and wastewater to aquatic environments. A high prevalence (98%) of sulfamethoxazole-trimethoprim (SXT)-resistant *E. coli* was reported from Tsomo and Tyhume Rivers in the Eastern Cape (Fadare, Adefisoye & Okoh, 2020). Trimethoprim-sulfamethoxazole-resistant *E. coli* from hospital and municipal wastewater and surface water (river and lagoon) were found in a Ghanaian study. The river in that study was impacted by human activity, including industries, agriculture, open markets and health facilities; it was also prone to faecal pollution as it flowed past slums known for practising open defecation (Tettey et al., 2024a). The isolates in the Ghanaian study also were resistant to ciprofloxacin, ceftazidime, cefotaxime, and cefuroxime, amongst other antibiotics.

E. coli resistant to cefuroxime, ceftazidime and cefepime were identified in Durban in our study. Third-generation cephalosporin-resistant *E. coli* are critical pathogens in the WHO 2024 Bacterial Priority Pathogens List (World Health Organisation, 2024). These pathogens require continuous monitoring and surveillance of the resistance trends. Hanna et al. (2020) identified cefotaxime, ceftazidime and cefepime-resistant *E. coli* from river water in the second-largest state in India. These findings were consistent with the findings in our study. Like our sampling areas, the sampled river in the Indian study was prone to pollution from industrial, agricultural and domestic activities and was used for mass bathing. That study was conducted over three years, with sampling occurring once every season. An increase in resistance against cefotaxime, ceftazidime and cefepime was highlighted from the first to the third year, with the third year (2016) having the highest resistance rates (Hanna

et al., 2020). The increase in this resistance was attributed to a mass bathing event that had taken place before sampling time, underscoring a global problem of human anthropogenic activities contributing to the presence of antibiotic resistance in surface water. Moreover, the usage of these polluted surface waters by the communities poses a public health risk.

Our cephalosporin resistance findings were consistent with the findings of Pereira, Palmira and Ferreira (2023), who investigated the possible faecal contamination and antibiotic resistance in water streams from Portugal. The findings from this study highlighted the presence of cefotaxime, ceftazidime and cefepime-resistant *E. coli*. Resistance to amoxicillin-clavulanic acid and cotrimoxazole was also identified. Other observed resistances were against cefoxitin and ceftaroline, a fifth-generation cephalosporin (Pereira, Palmeira & Ferreira, 2023). The findings from these studies and our study align with the global prevalence and distribution of antibiotic-resistant *E. coli* in surface water.

The *E. faecium* isolates were mostly resistant to erythromycin. Erythromycin is a broad-spectrum antibiotic used in human and animal health, mainly against Gram-positive bacterial infections (Maina et al., 2024). Studies have reported erythromycin-resistant *E. faecium* from surface and wastewater. Ibekwe et al. (2024) reported a high prevalence of erythromycin-resistant *E. faecium* in Southern California. The isolates were collected from wastewater, a recreational area, and agricultural and urban run-off. The wastewater and recreational area isolates were mostly resistant to erythromycin, while the urban runoff isolates had the least resistance (Ibekwe, Obayiuwana, & Murinda, 2024). That study also found frequent resistance against tetracycline. Tetracycline and erythromycin are used in human and animal health and animal feed for growth promotion (Wu et al., 2023). We identified tetracycline resistance in our Pietermaritzburg isolates. Tetracycline is one of the most used antibiotics in veterinary medicine and animal production in South Africa (Selaledi et al., 2020), and Pietermaritzburg is home to several intensive food animal production systems for pigs and chickens. This may suggest that the stream in Pietermaritzburg was exposed to agricultural runoff in food and animal production systems. In contrast, we did not find tetracycline resistance in Durban, and the stream may not have been exposed to livestock farming.

Lee et al. (2023) reported a high prevalence of erythromycin resistance in *E. faecium* from four major rivers exposed to livestock farming, agriculture and urban areas in South Korea. The least resistance was against tetracycline (Lee et al., 2023). A study from the Eastern Cape isolated *E. faecium* from Kidd's Beach exposed to river discharge and municipal wastewater. The isolates were more resistant to erythromycin than tetracycline (Adeniji, Sibanda & Okoh, 2020). The differences in these resistance levels correlate with our findings; we had more resistance to erythromycin than to tetracycline in our Durban isolates. Therefore, these findings may reflect the antibiotic usage in the area or the main source of pollution in the surface waters.

4.3. **Detected antibiotic resistance genes**

Bioinformatics tools were used to determine the genotype resistance of the observed phenotype resistance. Our study identified the corresponding genotypic resistance in all isolates resistant against cotrimoxazole. The ARGs *sul* and *dfr* have been associated with cotrimoxazole resistance in aquatic isolates. Various studies have highlighted the high prevalence and possible dissemination of *sul* and *dfr* ARG-carrying strains in the aquatic environment (Wu et al., 2020; Mbanga et al., 2021c; Magaña-Lizárraga et al., 2022; Haenelt et al., 2023; Felis et al., 2024; Tettey et al., 2024b;). For example, Magaña-Lizárraga et al. (2022) found SXT-resistant *E. coli* carrying the *sul*1, *sul*2, *sul*3, *dfr*A12 and *dfr*A17 genes in surface water in Mexico. The isolate susceptible to SXT did not harbour any of these genes, associating the presence of these genes with the observed phenotypic resistance.

Efflux pumps mediate the uptake of nutrients and extrude toxic substances across the cytoplasmic membrane. In addition, the Gram-negative bacteria possess the outer membrane. This way, efflux pumps can occur in tripartite systems, conferring multidrug resistance. Gram-positive bacteria lack the outer membrane; therefore, single efflux pumps are only present in their cytoplasmic membrane (Auda, Ali Salman & Odah, 2020; Prajapati, Kleinekathöfer & Winterhalter, 2021). Drugs enter the bacterial cell through outer membrane porins in *E. coli* and diffuse directly into the cytoplasmic membrane in *E. faecium*. They are diffused into the inner membrane as they enter the periplasmic area. When overexpressed, efflux transporters, existing as single-component pumps and multiple or tripartite efflux systems, can extrude antibiotics (Li, Plésiat & Nikaido, 2015). This facilitates

the removal of multiple classes of antibiotics from the bacterial cells, leaving lower ineffective concentrations. The most clinically significant efflux pump associated with MDR resistance in *E. coli* belongs to the RND family, AcrAB-TolC (Weston et al., 2018). β-lactams can be extruded via this efflux pump (Ganjo et al., 2024). Our study identified an AcrAB-TolC efflux pump in all *E. coli* isolates.

Aquatic environments are constantly exposed to multiple pollutants such as heavy metals, pesticides, disinfectants and microplastics that exert selection pressure on bacteria and promote overexpression of the efflux pumps, aiding in co-resistance to antibiotics (Zhao, Yu & Zhang, 2023; Revol-Tissot, Boyer & Alibert, 2024). Previous studies have detected the AcrAB-TolC efflux pump in *E. coli*. Yuan et al. (2024) investigated the genetic traits of cephalosporin-resistant *E. coli* from tropical aquatic environments (community sewage, beaches, marine niches) in Singapore to report the transmission mechanisms of cephalosporin resistance. Resistance against cephalosporins in some isolates was attributed to the presence of ESBL genes (CTX-M). The efflux pump AcrAB-TolC was identified in all isolates, which is consistent with our findings. (Yuan et al., 2024).

Erythromycin resistance in *Enterococcus* is usually associated with the *erm* and *msr* genes. *erm*(B) is one of the erythromycin resistance determinants associated with pathogenic bacteria. Erythromycin antibiotics are target enzymes that inhibit protein synthesis (Adeniji, Sibanda & Okoh, 2020). The *erm*(B) gene encodes rRNA methylases, enzymes that modify nucleotides in the 23S rRNA, blocking macrolide binding (Mbanga et al., 2021a). Only two of our isolates carried *erm*(B) while the macrolide efflux pump gene, *msr*(C), was identified in all isolates, which may explain the observed erythromycin resistance. Cho et al. (2020) identified the *erm*(B) gene in erythromycin-resistant *E. faecium*. *E. faecium* was isolated from watershed samples in the USA to demonstrate and characterise the antibiotic resistance and mechanisms in enterococci from freshwater. Erythromycin resistance was not observed in isolates that lacked the *erm*(B) gene (Cho et al., 2020).

Farkas et al. (2022) investigated the impact of anthropogenic activities on the spread of antibiotic-resistant enterococci from aquatic environments (wastewater, hospital effluent, surface water and groundwater) in Romania. Almost all isolates resistant to erythromycin carried the *erm*(B) gene. One isolate did not have any relevant gene, while another carried the macrolide efflux pump gene, *mef*A (Farkas et al., 2022). We identified

the *efm*A efflux pump determinant that confers resistance to multidrug, including macrolides. Our study identified two tetracycline resistance genes, *tet*(M) and *tet*(L). These genes function differently to confer tetracycline resistance; *tet*(M) is associated with ribosomal protection, while *tet*(L) encodes the efflux pump. Other studies have associated the presence of these genes with tetracycline phenotypic resistance. Cho et al. (2020) correlated tetracycline resistance with the presence of *tet*(L) and *tet*(M) genes in surface water *E. faecium* from a watershed (Cho et al., 2020). In the study from the Eastern Cape, isolated *E. faecium* from Kidd's Beach was more resistant to tetracycline (Adeniji, Sibanda & Okoh, 2020). Adenijii, Sibanda and Okoh (2020) also identified *tet*(L) and *tet*(M) in tetracycline-resistant *E. faecium*.

4.4. Mobile genetic elements (Plasmids, insertion sequences, integrons)

Mobile genetic elements mediate the inter- and intra-species horizontal gene transfer of antibiotic resistance. The acquired antibiotic-resistance genes in our study were frequently associated with MGEs, integrons, insertion sequences, and transposons, and most of them were carried on plasmids. Transposable elements promote the movement of ARGs between genomic locations. Amongst the MGEs, plasmids are the most enriched with ARGs and facilitate horizontal gene transfer of ARGs (Dimitriu, 2022). Plasmids carrying ARGs associated with insertion sequences, transposons and integrons were mostly identified in our *E. coli* isolates, which signifies the role played by plasmids in the transfer of ARGs in *E. coli*.

The most common resistance plasmids in the Incompatibility (Inc) group were IncF (FIA, FIB, and FII). ESBL-producing epidemic strain ST131 contains the FII and FIA replicons (Citterio et al., 2020). Our Durban *E. coli* isolates contained only FIA replicon, and the Pietermaritzburg isolates carried both FIB and FII. The occurrence of the same Inc groups in bacterial strains from different sources can indicate horizontal transmission of closely related resistance-carrying plasmids (Puangseree et al., 2022). In addition to plasmids, insertion sequences, transposons, and integrons are necessary for the effective and extensive transfer of ARGs within various bacterial populations (Che et al., 2021). In our study, insertion sequences IS1, IS6 and the Tn3 family transposon were associated with ARGs in the *E. coli* isolates from Durban. The ARGs Pietermaritzburg isolates were associated with IS1, IS1B, IS526, ISKpn19 and the Tn3 family transposase. Insertion sequences and transposons play a

critical role in genomic evolution via activation or deletion of genes leading to structural mutations. Moreover, these transposable elements contribute to the plasmid-mediated horizontal transfer of resistance genes (Kanai, Tsuru & Furusawa, 2023)

Integrons comprise the integron integrase gene (*int*I), recombination site (*att*I), promoter and gene cassettes. The gene cassettes contain the sulfonamide resistance gene (*sul*) and a quaternary ammonium compound resistance gene (*qac*E). The integron structure allows for the accommodation and capturing of genes by the integron integrase (Zhang et al., 2020). Our study identified plasmid-carried Class 1 integron with a *dfr*A12-*aad*A1a-*aad*A2-*cml*A1 gene cassette profile in the Pietermaritzburg isolates. The co-carriage of these genes with the disinfectant resistance gene *qac*L suggests the co-transfer of these genes, enabling the co-selection of resistance to antibiotics and disinfectants in these isolates. The genetic context identified in our study, *su*l3:IS256 family transposase:qacL:aadA1:cmlA1:aadA:dfrA12:Class 1 Integron integrase Int1 has previously been identified in surface water *E. coli*. In the study by Yuan et al, (2024) mentioned above, two *E. coli* isolates with the gene cassettes, Int1-*dfr*A12-*aad*A2-*cml*A1-*aad*A1-*qac*L-IS256-*sul*3 were identified. Another isolate in that study had an integron with gene cassettes, Int1-*dfr*A12-*aad*A2-*cml*A1-*aad*A1-*qac*L-IS256-tet(M). These two integrons indicate the accommodation of different genes in the gene cassettes and their role in disseminating these genes across species.

The *E. faecium* ARGs were mostly chromosome-borne. Only the tetracycline resistance genes *tet*(M) and *tet*(L) were plasmid-borne and associated with a conjugate transfer protein. These genes were found in a genetic environment with a conjugal transfer protein required for the conjugative transfer of the genes while mediating tetracycline resistance (Mbanga et al., 2021a). Conjugation is the most widespread form of HGT, where genes are transferred when a donor and a recipient species form a mating pair complex. Moreover, the tetracycline resistance genetic environment was located on a plasmid, which is the main conjugation mediator (Popa, Barbu & Chifiriuc, 2018).

4.5. Multilocus sequence typing (MLST) and phylogenetic relationships

We identified two *E. coli* sequence types, ST602 from Pietermaritzburg and ST2491 from Durban. *E. coli* ST602 is more reported than ST2491 but not as prevalent as the epidemic ST131. A South African study reported the

presence of cefotaxime, ceftazidime, and cefepime-resistant *E. coli* isolate belonging to ST602 from irrigation water (Richter et al., 2024). Like our study, the ST602 from Richter et al. (2024) did not harbour any β-lactam resistance genes and carried the IncFIB and IncFII plasmid replicons. There are minimal, if any, reports on ST602 in surface water and wastewater; other studies have identified this sequence type in animals, especially poultry and wild birds (Belmahdi et al., 2022; Carhuaricra et al., 2022; Dalazen et al., 2023; Zhang et al., 2023; Liao et al., 2015). Most of these studies consistently reported ST602 as *bla*CTX-M carrying, which may highlight this sequence type as an important ESBL producer. Reports from human and environmental sources are not as prevalent, and ST602 is not a highly reported sequence type in South Africa.

From Figure 2, ST10, one of the clinically relevant and epidemiologically important sequence types (Islam et al., 2023), was found in various animal and environmental settings. Studies have reported ESBL-producing *E. coli* belonging to this sequence type isolated from a cattle farm and nearby river water in South Africa (Ramaite, Ekwanzala & Momba, 2022), poultry farms in Ghana (Falgenhauer et al., 2019), beef cattle and abattoirs in Nigeria (Aworh et al., 2022), a patient in Ghana (Asare Yeboah et al., 2024) from sheep in China (Zhao et al., 2022), and rivers in China (Liu et al., 2018). The prevalence of this sequence type in food-producing animals has implications for both humans and the environment. The transmission of antibiotic-resistant bacteria into the aquatic environments via agriculture run-off is heightened. Also, as a last resort, reserve antibiotics are used to treat the ABR bacteria; the antibiotics are disseminated in the surface water, leading to selection pressure, the development of antibiotic resistance and the spread of this resistance in the aquatic environment.

The *E. faecium* sequence type ST2431 (curated in 2023 on PubMLST) still lacks reports of its prevalence across human, animal and environmental sources. However, the close relationships between this and other sequence types from humans and hospital apparatus could indicate its human origin.

5. Conclusion

Whole genome sequencing and bioinformatics analysis revealed the identified isolates' resistome, mobilome, and phylogenetic relationships. We highlighted the presence of antibiotic-resistant *E. coli* and *E. faecium* pathogens in surface water and the associated antibiotic-resistance genes. Moreover, the associations of these

ARGs with mobile genetic elements indicate the possible transmission of these resistance genes from species to species, making these surface waters a possible reservoir for antibiotic resistance transmission.

CRediT authorship contribution statement

Fulufhelo. N. Mukwevho: Writing – original draft, Formal analysis, Data curation, methodology, sampling and laboratory analysis. **Joshua Mbanga:** Writing – review & editing, Data curation. **Linda Bester:** Writing – review & editing, Formal analysis. **Akebe L.K. Abia:** Writing – review & editing, Validation, Formal analysis, Methodology Supervision. **Arshad Ismail:** Writing – review & editing, Resources, Methodology. **Sabiha Y. Essack:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Data Availability Statement

All analysed data have been included in the manuscript.

Declaration of competing interest

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

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References

Adeniji, O.O., Sibanda, T. & Okoh, A.I. (2020) Molecular detection of antibiotic resistance and virulence gene determinants of Enterococcus species isolated from coastal water in the Eastern Cape Province, South Africa. *International Journal of Environmental Studies*. 78 (2), 1–20. doi:10.1080/00207233.2020.1785759.

Al-blooshi, S.Y., Latif, M.A.A., Sabaneh, N.K., Mgaogao, M. & Hossain, A. (2021) Development of a novel selective medium for culture of Gram-negative bacteria. *BMC Research Notes*. 14, 211. doi:10.1186/s13104-021-05628-2.

Al-Haj, R.I.A. (2024) Chromogenic agar detection of urinary tract infection. *International Journal for Sciences and Technology*. 19 (2), 31–39.

Asare Yeboah, E.E., Agyepong, N., Mbanga, J., Amoako, D.G., Abia, A.L.K., Ismail, A., Owusu-Ofori, A. & Essack, S.Y. (2024) Genomic characterization of multi drug resistant ESBL-producing Escherichia coli isolates from patients and patient environments in a teaching hospital in Ghana. *BMC Microbiology*. 24 (1). doi:10.1186/s12866-024-03406-1.

Aslam, B., Khurshid, M., Arshad, M.I., Muzammil, S., Rasool, M., Yasmeen, N., Shah, T., Chaudhry, T.H., Rasool, M.H., Shahid, A., Xueshan, X. & Baloch, Z. (2021) Antibiotic Resistance: One Health One World Outlook. *Frontiers in Cellular and Infection Microbiology*. 11 (November), 1–20. doi:10.3389/fcimb.2021.771510.

Athanasakopoulou, Z., Reinicke, M., Diezel, C., Sofia, M., Chatzopoulos, D.C., Braun, S.D., Reissig, A., Spyrou, V., Monecke, S., Ehricht, R., Tsilipounidaki, K., Giannakopoulos, A., Petinaki, E. & Billinis, C. (2021) Antimicrobial resistance genes in ESBL-producing Escherichia coli isolates from animals in Greece. *Antibiotics*. 10 (4), 1–15. doi:10.3390/antibiotics10040389.

Auda, I.G., Ali Salman, I.M. & Odah, J.G. (2020) Efflux pumps of Gram-negative bacteria in brief. *Gene Reports*. 20 (April), 100666. doi:10.1016/j.genrep.2020.100666.

Aworh, M.K., Ekeng, E., Nilsson, P., Egyir, B., Owusu-Nyantakyi, C. & Hendriksen, R.S. (2022) Extended-Spectrum ß-Lactamase-Producing Escherichia coli Among Humans, Beef Cattle, and Abattoir Environments in Nigeria. *Frontiers in Cellular and Infection Microbiology*. 12 (2022), 1–11. doi:10.3389/fcimb.2022.869314.

Belmahdi, M., Chenouf, N.S., Ait Belkacem, A., Martinez-Alvarez, S., Pino-Hurtado, M.S., Benkhechiba, Z., Lahrech, S., Hakem, A. & Torres, C. (2022) Extended Spectrum β-Lactamase-Producing Escherichia coli from Poultry and Wild Birds (Sparrow) in Djelfa (Algeria), with Frequent Detection of CTX-M-14 in Sparrow. *Antibiotics*. 11 (1814), 1–10. doi:10.3390/antibiotics11121814.

Bezabih, Y.M., Sabiiti, W., Alamneh, E., Bezabih, A., Peterson, G.M., Bezabhe, W.M. & Roujeinikova, A. (2021) The global prevalence and trend of human intestinal carriage of ESBL-producing Escherichia coli in the community. *Journal of Antimicrobial Chemotherapy*. 76 (1), 22–29. doi:10.1093/JAC/DKAA399.

Carhuaricra, D., Duran Gonzales, C.G., Rodríguez Cueva, C.L., Ignacion León, Y., Silvestre Espejo, T., Marcelo Monge, G., Rosadio Alcántara, R.H., Lincopan, N., Espinoza, L.L. & Maturrano Hernández, L. (2022) Occurrence and Genomic Characterization of mcr-1-Harboring Escherichia coli Isolates from Chicken and Pig Farms in Lima, Peru. *Antibiotics*. 11 (12), 1781. doi:10.3390/antibiotics11121781.

Catalano, A., Iacopetta, D., Ceramella, J., Scumaci, D., Giuzio, F., Saturnino, C., Aquaro, S., Rosano, C. & Sinicropi, M.S. (2022) Multidrug Resistance (MDR): A Widespread Phenomenon in Pharmacological Therapies. *Molecules*. 27 (3), 1–18. doi:10.3390/molecules27030616.

Che, Y., Yang, Y., Xu, X., Brinda, K., Polz, M.F., Hanage, W.P. & Zhang, T. (2021) Conjugative plasmids interact with insertion sequences to shape the horizontal transfer of antimicrobial resistance genes. *Proceedings of the National Academy of Sciences of the United States of America*. 118 (6), e200873118. doi:10.1073/pnas.2008731118.

Cho, S., Barrett, J.B., Frye, J.G. & Jackson, C.R. (2020) Antimicrobial resistance gene detection and plasmid typing among multidrug resistant enterococci isolated from freshwater environment. *Microorganisms*. 8 (9), 1–15. doi:10.3390/microorganisms8091338.

Choi, H.J., Lee, J.H., Choi, D.Y., Lee, J.E., Hori, S., Cho, K.S., Kim, J.H., Choi, S.H. & Kang, Y.J. (2023)

Pharmacokinetic-Pharmacodynamic Analysis of Cephalexin against Streptococcus parauberis in Olive Flounder (Paralichthys olivaceus: Temminck and Schlegel). *Aquaculture Research*. 2023. doi:10.1155/2023/5718802.

Citterio, B., Andreoni, F., Simoni, S., Carloni, E., Magnani, M., Mangiaterra, G., Cedraro, N., Biavasco, F. & Vignaroli, C. (2020) Plasmid Replicon Typing of Antibiotic-Resistant Escherichia coli From Clams and Marine Sediments. *Frontiers in Microbiology*. 11, 1101. doi:10.3389/fmicb.2020.01101.

Clinical and Laboratory Standards Institute. (2023). 2023 Breakpoint Implementation Toolkit (2023 BIT). https://clsi.org/meetings/ast/breakpoints-in-use-toolkit/ Date of access: 16 August 2023.

da Costa Filho, B.M., Duarte, A.C. & Santos, T.A.P.R. (2022) Environmental monitoring approaches for the detection of organic contaminants in marine environments: A critical review. *Trends in Environmental Analytical Chemistry*. 33 (September 2021), e00154. doi:10.1016/j.teac.2022.e00154.

Dalazen, G., Fuentes-Castillo, D., Pedroso, L.G., Fontana, H., Sano, E., Cardoso, B., Esposito, F., Moura, Q., Matinata, B.S., Silveira, L.F., Mohsin, M., Matushima, E.R. & Lincopan, N. (2023) CTX-M-producing Escherichia coli ST602 carrying a wide resistome in South American wild birds: Another pandemic clone of One Health concern. *One Health*. 17 (2023), 100586. doi:10.1016/J.ONEHLT.2023.100586.

Daniels, B., Kuhn, L., Spooner, E., Mulol, H., Goga, A., Feucht, U., Essack, S.Y. & Coutsoudis, A. (2022) Cotrimoxazole guidelines for infants who are HIV-exposed but uninfected: a call for a public health and ethics approach to the evidence. *The Lancet Global Health*. 10 (8), e1198–e1203. doi:10.1016/S2214-109X(22)00120-6.

Dimitriu, T. (2022) Evolution of horizontal transmission in antimicrobial resistance plasmids. *Microbiology (United Kingdom)*.168 (7) p.001214. doi:10.1099/mic.0.001214.

Fadare, F.T., Adefisoye, M.A. & Okoh, A.I. (2020) Occurrence, identification, and antibiogram signatures of selected Enterobacteriaceae from Tsomo and Tyhume rivers in the Eastern Cape Province, Republic of South Africa. *PLoS ONE*. 15 (12 December). doi:10.1371/journal.pone.0238084.

Falgenhauer, L., Imirzalioglu, C., Oppong, K., Akenten, C.W., Hogan, B., Krumkamp, R., Poppert, S., Levermann, V., Schwengers, O., Sarpong, N., Owusu-Dabo, E., May, J. & Eibach, D. (2019) Detection and characterization of ESBL-producing Escherichia coli from humans and poultry in Ghana. *Frontiers in Microbiology*. 10 (2019), 1–8. doi:10.3389/fmicb.2018.03358.

Farkas, A., Coman, C., Szekeres, E., Teban-Man, A., Carpa, R. & Butiuc-Keul, A. (2022) Molecular Typing Reveals Environmental Dispersion of Antibiotic-Resistant Enterococci under Anthropogenic Pressure. *Antibiotics*. 11 (9). doi:10.3390/antibiotics11091213.

Felis, E., Sochacki, A., Bajkacz, S., Łuczkiewicz, A., Jóźwiakowski, K., García, J. & Vymazal, J. (2024) Removal of selected sulfonamides and sulfonamide resistance genes from wastewater in full-scale constructed wetlands. *Science of the Total Environment*. 912 (2024), 169195. doi:10.1016/j.scitotenv.2023.169195.

Ganjo, A.R., Balaky, S.T.J., Mawlood, A.H., Smail, S.B. & Shabila, N.P. (2024) Characterization of genes related to the efflux pump and porin in multidrug-resistant Escherichia coli strains isolated from patients with COVID-19 after secondary infection. *BMC Microbiology*. 24 (1). doi:10.1186/s12866-024-03283-8.

Haenelt, S., Wang, G., Kasmanas, J.C., Musat, F., Richnow, H.H., da Rocha, U.N., Müller, J.A. & Musat, N. (2023) The fate of sulfonamide resistance genes and anthropogenic pollution marker intI1 after discharge of wastewater into a pristine river stream. *Frontiers in Microbiology*. 14, 1058350. doi:10.3389/fmicb.2023.1058350.

Hanna, N., Purohit, M., Diwan, V., Chandran, S.P., Riggi, E., Parashar, V., Tamhankar, A.J. & Lundborg, C.S. (2020) Monitoring of water quality, antibiotic residues, and antibiotic-resistant escherichia coli in the kshipra river in india over a 3-year period. *International Journal of Environmental Research and Public Health*. 17 (21), 1–22. doi:10.3390/ijerph17217706.

Ibekwe, A.M., Obayiuwana, A.C. & Murinda, S.E. (2024) Enterococcus Species and Their Antimicrobial Resistance in an Urban Watershed Affected by Different Anthropogenic Sources. *Water (Switzerland)*. 16 (1), 1–15. doi:10.3390/w16010116.

Islam, M.M.M., Sokolova, E. & Hofstra, N. (2018) Modelling of river faecal indicator bacteria dynamics as a basis for faecal contamination reduction. *Journal of Hydrology*. 563, 1000–1008. doi:10.1016/j.jhydrol.2018.06.077.

Islam, M.S., Rahman, A.M.M.T., Hassan, J. & Rahman, M.T. (2023) Extended-spectrum beta-lactamase in Escherichia coli isolated from humans, animals, and environments in Bangladesh: A One Health perspective systematic review and meta-analysis. *One Health*. 16, 100526. doi:10.1016/J.ONEHLT.2023.100526.

Iwu, C.D., Korsten, L. & Okoh, A.I. (2020) The incidence of antibiotic resistance within and beyond the agricultural ecosystem: A concern for public health. *MicrobiologyOpen*. 9 (9), 1–28. doi:10.1002/mbo3.1035.

Jofré Bartholin, M., Barrera Vega, B. & Berrocal Silva, L. (2023) Antibiotic-Resistant Bacteria in Environmental Water Sources from Southern Chile: A Potential Threat to Human Health. *Microbiology Research*. 14 (4), 1764–1773. doi:10.3390/microbiolres14040121.

Kanai, Y., Tsuru, S. & Furusawa, C. (2023) Insertion sequences: Simple mobile elements with rich ecological and evolutionary structures. *Current Opinion in Systems Biology*. 36, 100481. doi:10.1016/j.coisb.2023.100481.

Kemper, M.A., Veenman, C., Blaak, H. & Schets, F.M. (2023) A membrane filtration method for the enumeration of Escherichia coli in bathing water and other waters with high levels of background bacteria. *Journal of Water and Health*. 21 (8), 995–1003. doi:10.2166/wh.2023.004.

Kubera, Ł. (2020) Spread Patterns of Antibiotic Resistance in Faecal Indicator Bacteria Contaminating an Urbanized Section of the Brda River. *Microbial Ecology*. 81, 592–600. doi:10.1007/s00248-020-01624-4/Published.

Kumar, G., Balakrishna, K., Mukhopadhyay, C. & Eshwara, V.K. (2024) Characterization and comparative analysis of antimicrobial resistance in Escherichia coli from hospital and municipal wastewater treatment plants. *Journal of Water and Health*. 00 (0), 1–13. doi:10.2166/wh.2024.126.

KZN-Human Settlements (2020) *MEASURES TO ADDRESS HUMAN SETTLEMENTS CHALLENGES AND*

RESPOND TO COVID-19 NCOP PRESENTATION. (July), 1–16.
Lee, J.B., Kim, S.K., Lee, H.T., Chung, Y.S., Song, J.W. & Yoon, J.W. (2023) Epidemiological characterization of the antimicrobial resistant Enterococcus isolates from four major rivers in South Korea. *Journal of the Preventive Veterinary Medicine*. 47 (1), 24–32. doi:10.13041/jpvm.2023.47.1.24.

Li, X.Z., Plésiat, P. & Nikaido, H. (2015) The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clinical Microbiology Reviews*. 28 (2), 337–418. doi:10.1128/CMR.00117-14.

Liao, X.P., Xia, J., Yang, L., Li, L., Sun, J., Liu, Y.H. & Jiang, H.X. (2015) Characterization of CTX-M-14-producing Escherichia coli from food-producing animals. *Frontiers in Microbiology*. 6, 1136. doi:10.3389/fmicb.2015.01136.

Liu, H., Zhou, H., Li, Q., Peng, Q., Zhao, Q., Wang, J. & Liu, X. (2018) Molecular characteristics of extended-spectrum β-lactamase-producing Escherichia coli isolated from the rivers and lakes in Northwest China. *BMC Microbiology*. 18 (1), 1–12. doi:10.1186/s12866-018-1270-0.

Maina, U., Adaweren, E., Ngulde, S. & Umaru, B. (2024) Assessment of the Concentrations of Active Principle Content in Some Brands of Erythromycin Products Available for Veterinary Use in Maiduguri, Northeastern Nigeria. *Journal of Pharmacological and Pharmaceutical Research*. 1 (2), 53–59. doi:10.5455/jppr.20240612033337.

Mbanga, J., Abia, A.L.K., Amoako, D.G. & Essack, S.Y. (2020) Quantitative microbial risk assessment for waterborne pathogens in a wastewater treatment plant and its receiving surface water body. *BMC Microbiology*. 20 (1). doi:10.1186/s12866-020-02036-7.

Mbanga, J., Amoako, D.G., Abia, A.L.K., Allam, M., Ismail, A. & Essack, S.Y. (2021a) Genomic Analysis of Enterococcus spp. Isolated From a Wastewater Treatment Plant and Its Associated Waters in Umgungundlovu District, South Africa. *Frontiers in Microbiology*. 12, 648454. doi:10.3389/fmicb.2021.648454.

Mbanga, J., Amoako, D.G., Abia, A.L.K., Allam, M., Ismail, A. & Essack, S.Y. (2021b) Genomic Insights of Multidrug-Resistant Escherichia coli From Wastewater Sources and Their Association With Clinical

Pathogens in South Africa. *Frontiers in Veterinary Science*. 8, 636715. doi:10.3389/fvets.2021.636715.

Mcdonald, K.L., Garland, S., Carson, C.A., Gibbens, K., Parmley, E.J., Finley, R. & Mackinnon, M.C. (2021) Measures used to assess the burden of ESBL-producing *Escherichia coli* infections in humans: a scoping review. JAC-Antimicrobial Resistance. 3 (1). doi:10.1093/jacamr/dlaa104.

Milligan, E.G., Calarco, J., Davis, B.C., Keenum, I.M., Liguori, K., Pruden, A. & Harwood, V.J. (2023) A Systematic Review of Culture-Based Methods for Monitoring Antibiotic-Resistant Acinetobacter, Aeromonas, and Pseudomonas as Environmentally Relevant Pathogens in Wastewater and Surface Water. *Current Environmental Health Reports*. 10 (2), 154–171. doi:10.1007/s40572-023-00393-9.

Mustafa, S.S., Batool, R., Kamran, M., Javed, H. & Jamil, N. (2022) Evaluating the Role of Wastewaters as Reservoirs of Antibiotic-Resistant ESKAPEE Bacteria Using Phenotypic and Molecular Methods. *Infection and Drug Resistance*. 15 (1), 5715–5728. doi:10.2147/IDR.S368886.

Nabirye, C., Denyer Willis, L., Nayiga, S., Kayendeke, M., Staedke, S.G. & Chandler, C.I.R. (2023) Antibiotic 'entanglements': health, labour and everyday life in an urban informal settlement in Kampala, Uganda. *Critical Public Health*. 33 (1), 95–104. doi:10.1080/09581596.2021.1994526.

Nadimpalli, M.L., Marks, S.J., Montealegre, M.C., Gilman, R.H., Pajuelo, M.J., Saito, M., Tsukayama, P., Njenga, S.M., Kiiru, J. & Swarthout, J. (2020) Urban informal settlements as hotspots of antimicrobial resistance and the need to curb environmental transmission. *Nature Microbiology*. 5 (6), 787–795.

O'Toole, R.F., Leong, K.W.C., Cumming, V. & van Hal, S.J. (2023) Vancomycin-Resistant Enterococcus faecium and the emergence of new Sequence Types associated with Hospital Infection. *Research in Microbiology*. 104046. doi:10.1016/j.resmic.2023.104046.

Pereira, M.A., Palmeira, J.D. & Ferreira, H. (2023) Contamination of a Water Stream and Water Drainage Reaching Matosinhos Beach by Antibiotic-Resistant Bacteria. *Microorganisms*. 11 (12). doi:10.3390/microorganisms11122833.

Poey, M.E., de Los Santos, E., Aznarez, D., García-Laviña, C.X. and Laviña, M. (2024). Genetics of resistance to trimethoprim in cotrimoxazole resistant uropathogenic Escherichia coli: integrons, transposons, and single gene cassettes. *Frontiers in Microbiology*, *15*, 1395953. doi.org/10.3389/fmicb.2024.1395953.

Popa, L.I., Barbu, I.C. & Chifiriuc, M. (2018) Mobile Genetic Elements involved in the Horizontal Transfer of Antibiotic Resistance Genes. *Romanian Archives of Microbiology and Immunology*. 77 (4), 263–271. https://www.researchgate.net/publication/343180701.

Prajapati, J.D., Kleinekathöfer, U. & Winterhalter, M. (2021) How to Enter a Bacterium: Bacterial Porins and the Permeation of Antibiotics. *Chemical Reviews*. 121 (9), 5158–5192. doi:10.1021/acs.chemrev.0c01213.

Puangseree, J., Prathan, R., Srisanga, S., Angkittitrakul, S. & Chuanchuen, R. (2022) Plasmid profile analysis of Escherichia coli and Salmonella enterica isolated from pigs, pork and humans. *Epidemiology and Infection*. 150 (e110), 1–14. doi:10.1017/S0950268822000814.

Ramaite, K., Ekwanzala, M.D. & Momba, M.N.B. (2022) Prevalence and Molecular Characterisation of Extended-Spectrum Beta-Lactamase-Producing Shiga Toxin-Producing Escherichia coli, from Cattle Farm to Aquatic Environments. *Pathogens*. 11 (674), 1–17. doi:10.3390/pathogens11060674.

Ramsey, C. & MacGowan, A.P. (2016) A review of the pharmacokinetics and pharmacodynamics of aztreonam. *Journal of Antimicrobial Chemotherapy*. 71 (10), 2704–2712. doi:10.1093/jac/dkw231.

Revol-Tissot, J., Boyer, G. & Alibert, S. (2024) Molecular determinant deciphering of MIC-guided RND efflux substrates in E. coli. *Frontiers in Drug Discovery*. 4, 1326121. doi:10.3389/fddsv.2024.1326121.

Richter, L., Duvenage, S., du Plessis, E.M., Msimango, T., Dlangalala, M., Mathavha, M.T., Molelekoa, T., Kgoale, D.M. & Korsten, L. (2024) Genomic Evaluation of Multidrug-Resistant Extended-Spectrum β-Lactamase (ESBL)-Producing Escherichia coli from Irrigation Water and Fresh Produce in South Africa: A Cross-Sectional Analysis. *Environmental Science and Technology*. 58 (32), 14421–14438. doi:10.1021/acs.est.4c02431.

Selaledi, L.A., Hassan, Z.M., Manyelo, T.G. & Mabelebele, M. (2020) The current status of the alternative use to antibiotics in poultry production: An African perspective. *Antibiotics*. 9 (9), 1–18. doi:10.3390/antibiotics9090594.

Shabir, S., Khursheed, U., Sarwar, M., Ahmad, F., Shabir, R. & Saleem, S. (2021) Diagnostic Accuracy of Chromagartm Ecc for Detection and Enumeration of Escherichia Coli and Coliforms in Water Samples. *Pakistan Journal of Pathology*. 32 (2), 56–60.

Soni, K., Jyoti, K., Kumar, A. & Chandra, R. (2024) Coexistence of multidrug resistance and ESBL encoding genes - blaTEM, blaSHV, and blaCTX-M; its amplification and dispersion in the environment via municipal wastewater treatment plant. *Chemosphere*. 362 (2024), 142829. doi:10.1016/j.chemosphere.2024.142829.

Soujanya, B.R. & G S, B. (2021) Utility of Chromogenic Medium in Characterization of Enterococci in Urinary Tract Infection and Phenotypic Detection of Their Virulence Factors. *International Journal of Health Sciences and Research*. 11 (5), 278–283. doi:10.52403/ijhsr.20210544.

Tettey, R., Egyir, B., Tettey, P., Arko-Mensah, J., Ofori Addo, S., Owusu-Nyantakyi, C., Boateng, W. & Fobil, J. (2024a) Genomic analysis of multidrug-resistant Escherichia coli from Urban Environmental water sources in Accra, Ghana, Provides Insights into public health implications. *PLoS ONE*. 19 (5 May), 1–29. doi:10.1371/journal.pone.0301531.

Tettey, R., Egyir, B., Tettey, P., Arko-Mensah, J., Ofori Addo, S., Owusu-Nyantakyi, C., Boateng, W. & Fobil, J. (2024b) Genomic analysis of multidrug-resistant Escherichia coli from Urban Environmental water sources in Accra, Ghana, Provides Insights into public health implications. *PLOS ONE*. 19 (5), e0301531. doi:10.1371/JOURNAL.PONE.0301531.

Tian, X., Zheng, X., Sun, Y., Fang, R., Zhang, S., Zhang, X., Lin, J., Cao, J. & Zhou, T. (2020) Molecular mechanisms and epidemiology of carbapenem-resistant Escherichia coli isolated from Chinese patients during 2002–2017. *Infection and Drug Resistance*. 13, 501–512. doi:10.2147/IDR.S232010.

Weston, N., Sharma, P., Ricci, V. & Piddock, L.J.V. (2018) Regulation of the AcrAB-TolC efflux pump in Enterobacteriaceae. *Research in Microbiology*. 169 (7–8), 425–431. doi:10.1016/j.resmic.2017.10.005.

Wigmore, S.M., Greenhill, A.R. & Bean, D.C. (2024) Isolation and characterization of enterococci from poultry reveals high incidence of Enterococcus thailandicus in Victoria, Australia. *Journal of Applied Microbiology*. 135 (8), Ixae194. doi:10.1093/jambio/lxae194.

Williams, D.S., Costa, M.M., Celliers, L. & Sutherland, C. (2018) Informal settlements and flooding: Identifying strengths andweaknesses in local governance for water management. *Water*. 10 (7), 1–21. doi:10.3390/w10070871.

World Health Organisation (2024) *WHO bacterial priority pathogens list, 2024*. https://www.who.int/publications/i/item/9789240093461.

Wu, D.L., Zhang, M., He, L.X., Zou, H.Y., Liu, Y.S., Li, B.B., Yang, Y.Y., Liu, C., He, L.Y. & Ying, G.G. (2020) Contamination profile of antibiotic resistance genes in ground water in comparison with surface water. *Science of the Total Environment*. 715, 136975. doi:10.1016/j.scitotenv.2020.136975.

Wu, M., Yang, H., Wu, Q., Yang, Y. & He, Z. (2023) Adsorption and competition mechanism of tetracycline and erythromycin on montmorillonite: experimental and theoretical investigation. *Journal of Molecular Liquids*. 370 (2023), 121037. doi:10.1016/j.molliq.2022.121037.

Yuan, Q., Li, W., Goh, S.G., Chen, S.L., Ng, O.T., He, Y. & Gin, K.Y.H. (2024) Genetic traits and transmission of antimicrobial resistance characteristics of cephalosporin resistant Escherichia coli in tropical aquatic environments. *Journal of Hazardous Materials*. 479, 135707. doi:10.1016/j.jhazmat.2024.135707.

Zhang, S., Abbas, M., Rehman, M.U., Huang, Y., Zhou, R., Gong, S., Yang, H., Chen, S., Wang, M. & Cheng, A. (2020) Dissemination of antibiotic resistance genes (ARGs) via integrons in Escherichia coli: A risk to human health. *Environmental Pollution*.266 p.115260. doi:10.1016/j.envpol.2020.115260.

Zhang, S., Shu, Y., Wang, Y., Zhong, Z., Wang, M., et al. (2023) High rate of multidrug resistance and integrons in Escherichia coli isolates from diseased ducks in select regions of China. *Poultry Science*. 102 (10), 1–13. doi:10.1016/j.psj.2023.102956.

Zhao, F., Yu, Q. & Zhang, X. (2023) A Mini-Review of Antibiotic Resistance Drivers in Urban. *Water*. 15 (3165), 1–15.

Zhao, X., Zhao, H., Zhou, Z., Miao, Y., Li, R., Yang, B., Cao, C., Xiao, S., Wang, X., Liu, H., Wang, J. & Yang, Z. (2022) Characterization of Extended-Spectrum β-Lactamase-Producing Escherichia coli Isolates That Cause Diarrhea in Sheep in Northwest China. *Microbiology Spectrum*. 10 (4), 1–12. doi:10.1128/spectrum.01595-22.

Supplementary Material

Table S1: Genomic characteristics of the molecularly identified *E. coli* and *E. faecium* isolates

Isolate ID	Species	Accession numbers	Sequence length	No of contigs	GC content (%)	Longest contig size (bp)	N50 value (bp)	L50 value
ECD3	*E. coli*	SAMN43012351	5048814	104	50.7	343629	143921	13
ECD4	*E. coli*	SAMN43012352	5045685	110	50.6	342437	143921	13
ECD5	*E. coli*	SAMN43012353	5048008	110	50.6	342437	143922	13
ECD6	*E. coli*	SAMN43012354	5047417	110	50.6	262108	137348	14
ECD7	*E. coli*	SAMN43012355	4727814	67	50.8	462276	182821	8
ECD8	*E. coli*	SAMN43012356	4730387	73	50.8	482374	193791	8
ECD9	*E. coli*	SAMN43012357	5046306	112	50.6	262108	146743	13
ECD10	*E. coli*	SAMN43012358	4727398	76	50.8	461597	149653	9
ECP4	*E. coli*	SAMN43012359	4562287	127	50.9	164916	72002	23
ECP5	*E. coli*	SAMN43012360	5046380	110	50.6	262108	137348	14
ECP8	*E. coli*	SAMN43012362	4834810	88	50.8	547222	163351	9
ECP10	*E. coli*	SAMN43012363	4815303	90	50.8	545778	153285	11
EFD2	*E. faecium*	SAMN43041485	2741270	120	38.1	195998	86569	12
EFD3	*E. faecium*	SAMN43041486	2869965	95	38.3	503179	161062	6
EFD5	*E. faecium*	SAMN43041488	2634497	63	37.9	267768	168785	6
EFD7	*E. faecium*	SAMN43041489	2682887	78	38.2	257511	96094	9
EFD8	*E. faecium*	SAMN43041490	2712004	91	38.1	221350	106560	10

EFD9	*E. faecium*	SAMN43041491	2686652	25	38.2	700144	210980	4
EFD10	*E. faecium*	SAMN43041492	2670883	186	38.2	351176	100285	7
EFP1	*E. faecium*	SAMN44006568	2810216	196	37.9	125260	30343	24
EFP4	*E. faecium*	SAMN43012366	2779980	58	38.1	285103	120832	8
EFP5	*E. faecium*	SAMN43012367	2805380	193	37.9	110532	30604	25
EFP6	*E. faecium*	SAMN43012368	2693571	60	38.2	221404	104805	8

CHAPTER THREE

Conclusions, limitations and recommendations

This study compares and contrasts the molecular epidemiology of antibiotic-resistant ESKAPEE pathogens in surface water in proximity to two informal settlements in two cities in KwaZulu-Natal.

3.1. Conclusions

The following conclusions are drawn with reference to the study objectives:

To determine the contamination with and prevalence of ESKAPEE pathogens in the surface water using selective media Selective media were used to isolate and phenotypically identify the ESKAPEE pathogens. *E. faecium* and *E. coli* were the most prevalent bacterial species after molecular confirmation by WGS. The identified isolates were mostly prevalent in Durban samples. Other species identified were *Aeromonas, Exiguobacterium, Pseudomonas putida, Mammalicoccus scuiri, Klebsiella varicolla, klebsiella quasipneumoniae* and *Providencia alcalifaciens* indicating the importance of confirming presumptive identification by molecular methods.

- **Determine the antibiotic resistance profiles of the ESKAPEE pathogens using Clinical and Laboratory Standards Institute (CLSI) guidelines.**

I. The VITEK® 2 platform was used, and the CLSI guidelines were followed for results interpretation.

II. The *E. coli* isolates were non-susceptible (resistant and intermediate) to cotrimoxazole (50%), piperacillin/tazobactam (25%), amoxicillin/clavulanic acid (17%), cefuroxime (17%), ceftazidime (17%), and cefepime (17%). Isolates from Durban were more resistant than Pietermaritzburg isolates. Four antibiograms were identified in the resistant *E. coli* isolates

III. *E. faecium* isolates were non-susceptible (resistant and intermediate) to erythromycin (100%); nitrofurantoin (82%), fosfomycin (45%), doxycycline (27%), tetracycline (18%). More resistance was observed in the Pietermaritzburg isolates compared to the Durban isolates. Four antibiograms were identified in the MDR *E. faecium* isolates

IV. Tigecycline resistance was identified in all three antibiotic-resistant *K. pneumoniae* isolates. Two isolates were multidrug resistant against β-lactams, aminoglycosides, tetracycline, fluoroquinolones, and sulfonamides. Three antibiograms were identified. V. *Enterobacter kobei* was only resistant piperacillin-tazobactam

VI. Resistance to the β-lactams was the most prevalent in the Enterobacterales.

- **Determine the ARGs, and MGEs associated with the antibiotic-resistant ESKAPEE pathogens using whole genome sequencing and bioinformatics.**

I. The ARGs identified in *E. coli* isolates were, $bla_{\text{TEM-1}}$ (5), *sul*1 (5) and *dfr*A5 (5), *sul*3(2), *dfr*A12(2), conferring resistance to amoxicillin-clavulanic acid, piperacillin-tazobactam and cotrimoxazole, respectively. Other silent resistance genes were *qnr*B19 (5), *qnr*S1(2), *tet*(A)(2), *cml*A1(2), *aad*A1(2) and *aad*A2(2).

II. The ARGs identified in the *E. faecium* isolates were, *msr*(C)(5), *erm*(B)(2), *tet*(M)(2) and *tet*(L)(2), conferring resistance to erythromycin and tetracycline respectively**.** The silent ARGs were *aac(6)*-Ii (5), *ant(6)*-Ia(2), *aph(3")*-III(2), and *dfr*G(2). The ARGs *aac(6)*-Ii, and *msr*(C) were common in both cities.

III. The ARGs identified in the *K. pneumoniae* isolates was; $bla_{\text{SHV-75}}$(2), $bla_{\text{SHV-110}}$(1), $bla_{\text{SHV-81}}$(1), $bla_{\text{TEM-1B}}$(2), $bla_{\text{CTX-M-14}}$(1), $bla_{\text{CTX-M-15}}$(1), $bla_{\text{OXA- 1}}$(2), *sul*2(2), *dfr*A14(2), *tet*(A) (2), *oqx*A (3) and *oqx*B(3) conferring resistance amoxicillin-clavulanic acid, piperacillin-tazobactam, cefuroxime, cotrimoxazole, tigecycline, and ciprofloxacin respectively. *cat*B3 and *fos*A were not associated with phenotypic resistance. The ARGs bla_{SHV}, *fos*A and the multidrug efflux pump determinants *oqx*A and *oqx*B were common in all isolates. bla_{CTXM}, bla_{TEM}, bla_{OXA}, *sul*2, *dfr*A14, *tet*(A), and *cat*B3 were unique to the Durban isolates.

IV. The beta-lactamase gene bla_{ACT} conferring resistance to piperacillin-tazobactam and *tet*(A) were the only identified ARGs in *Enterobacter kobei.*

V. The identified insertion sequences and transposons in *E. coli* isolates were IS1, IS1B, IS6, IS526, ISKpn19 and Tn3. Class 1 integrons In13, and In27/117/4 were identified. The plasmid replicons IncF were most common in the Durban and Pietermaritzburg isolates. IncX and IncY were unique to the Durban isolates.

VI. IS982 was the only insertion sequence associated with resistance genes in the Durban *E. faecium* isolates. The Pietermaritzburg isolates carried IS1182 and IS110 associated with the resistance genes. Class 1 integrons In1212 were identified in all *E. faecium* isolates. Five plasmid replicons were identified; rep14B was unique to the Durban isolates, while repUS43 was unique to the Pietermaritzburg isolates.

VII. Insertion sequences associated with the ARGs in *K. pneumoniae* isolates were IS3, ISKpn25, ISL3, IS1, IS5, IS5075, IS91, ISEcP1, IS6100. Transposons were Tn3 and Tn5043. Class 1 integrons were In191 and In30. The Pietermaritzburg isolate did not possess a Class 1 integron associated with resistance genes. The IncF group was common in all isolates.

VIII. TnA51 and IncFII were the only MGEs associated with an antibiotic resistance gene in *E. kobei*. Class 1 integrons were not identified.

3. Ascertain the clonality and phylogenies of the isolates using whole genome sequencing and bioinformatics. Bioinformatics analysis revealed two distinct *E. coli* sequence types, ST2491 from Durban and ST602 from Pietermaritzburg. The *E. faecium* isolates belonged to five sequence types; ST94, ST361, ST2013, ST2042, and ST2431. ST94 was found in both Pietermaritzburg and Durban. ST2013 and ST2042 were unique to Durban while ST2431 and ST361 were unique to Pietermaritzburg isolates. *K. pneumoniae* isolates belonged to two sequence types ST133 from Durban and ST4268 from Pietermaritzburg. *E. kobei* belonged to ST691. Close relationships of two different sequence types (ST2013 and ST91) were detected in the *E. faecium* isolates. Phylogenetic relationships with other South African isolates from humans, animals and the environment were identified.

3.2. Limitations

- The study had a small sample size (n=140), and ten of each species from the two sample sites, which may not have represented an accurate picture of the overall resistant population. Only 43% of the sequenced 65 isolates were correctly identified. Of these, 57% were misidentified on selective media, further affecting representation.
- More isolates were identified from one city than the other: this limited antibiotic resistance comparison between the two cities.
- There is currently no selective media specific to *Enterobacter*, necessitating the use of selective media that also allows growth of other Gram-negative bacteria.

- Other bacterial species, with the same colour and morphology as the target species, grew on the selective media, leading to the misidentification of many of our species.

3.3. Recommendations

Future studies should:

- Include a larger sample size with longer sampling times to not only give insight into the current antibiotic resistance but also into the progression of antibiotic resistance over time at the sample sites.
- Species-specific selective media with the highest sensitivity, specificity and post-predictive value should be used.
- Undertake molecular identification via the PCR of housekeeping genes unique to each bacterial species followed by WGS.
- Investigate potentially “novel” antibiotic resistance mechanisms in resistant isolates lacking phenotype-genotype match.

APPENDICES

Appendix 1: Antibiotic-resistant *Klebsiella pneumoniae* isolates

The antibiotic resistance profiles, resistance genes, and mobile genetic elements for the identified *K. pneumoniae* isolates are presented in the following tables.

Table A1: The antibiotic resistance profiles of *K. pneumoniae* isolates with the associated resistance genes and mobile genetic elements

Isolate ID	Accession numbers	Source	Resistance pattern	Sequence Type	Antibiotic Resistance genes	Plasmid replicons	Insertion sequences
KPD5	SAMN44006569	Durban	AMC-TZP-CXM-TOB-COT-CIP-TGC	ST133	*bla*$_{SHV-75}$, *bla*$_{CTXM-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$,*sul*2, *dfr*A14, *tet*(A), *cat*B3, *oqx*A, *oqx*B, *aph(6)*-Id, *aph(3'')*-Ib, *aac(6')*-Ib-cr, qnrB1,fosA6	IncFIB(K), IncFII(K)	ISSty2, IS1230B, ISeaI1, ISPeat2, ISEc17
KPD9	SAMN43041495	Durban	TZP-CXM-TOB-COT-CIP-TGC	ST133	*bla*$_{SHV-75}$, *bla*$_{CTX-M-14}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, *sul*2, *dfr*A14 *tet*(A), *cat*B3, *oqx*B, *oqx*A, *aph(6)*-Id, *aph(3'')*-Ib, *aac(6')*-Ib-cr, *qnr*B1, *fos*A6	Col(pHAD28), Col440I, IncFIB(K), IncFII(K)	ISSty2, S1230B, ISEal1, ISPeat2, ISEc17
KPP4	SAMN43041498	Pietermaritzburg	AMC-TGC	ST4268	*bla*$_{SHV-110}$, *bla*$_{SHV-8}$1, *oqx*A, *oqx*B, *fos*A	Col(pHAD28), Col440I, IncFIA(HI1) IncFIB(K), IncFIB(pQil) IncFII(K), IncR, repB(R1701)	ISKpn21, ISSty2, IS1230B, ISPeat2, ISEaI1

β-lactamase genes (*bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{OXA-1}$) were the most prevalent. Other detected ARGs included aminoglycosides ARGs [*aph(6)*-Id*, aph(3'')*- Ib) and *aac(6')*-Ib], phenicol ARGs [*cat*B3, *oqx*A and *oqx*B], sulfonamide [*sul*2], a tetracycline ARG *[tet*(A)], a quinolone ARG [*qnr*B1] and a trimethoprim ARG [*dfr*A14]. A total of seven different plasmid replicons were found in the *K. pneumoniae* isolates [IncFIB(K), IncFII(K), Col(pHAD28), Col1440I,

IncFIA(HI1), IncFIB(pQil) and Inc(K)] (Table 1). The Durban isolates had the same insertion sequences [ISSty2, IS1230B, ISeaI1, ISPeat2 and ISEc17]. The Pietermaritzburg isolate had almost the same but ISKpn21 replaced ISEc17. Only the Durban isolates had Class 1 integrons, i.e., these were In191 with a *dfr*A14 gene cassette in KPD5 and KPD9 and the latter also contained In30 with *aac*A7 and *cat*B3 gene cassettes (Table 2).

Table 2: Class 1 integrons and the associated gene cassettes

			Cassette arrays			
Isolate ID	MLST	Integron	GC1	GC2	GC3	GC4
KPD5	ST133	In191	*dfr*A14			
KPD9	ST133	In191	*dfr*A14			
		In30	*aac*A7	*cat*B3		
KPP4	-	-	-	-	-	-

K. pneumoniae isolates KPD5 and KPD9 had a high ARG and MGE association. The ARGs were associated with recombinase, transposase, insertion sequences and Class 1 integrons (Table 3).

Table 3: Antibiotic resistance genetic environments of *K. pneumoniae* isolates

Isolate ID	MLST	Contig	Synteny of resistance genes and MGEs	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
KPD5	ST133	1	recombinase/integrase:*bla*-SHV-75:IS3 family transposase	*Klebsiella pneumoniae* strain KP_NORM_BLD_115332 chromosome, CP153786.1
		4	*oqx*A:*oqx*B14:recombination regulator recX:recombinase recA:*sit*C	*Klebsiella pneumoniae* strain NK_H25_013 chromosome, CP152646.1
		6	recombination-promoting nuclease/putative transposase:*fos*A5	*Klebsiella pneumoniae* strain BM343 chromosome, CP063939.1
		19	ISL3-like element ISKpn25 family transposase::::::ISL3 family transposase:IS3 family transpoase:transposase:IS1 family transposase:transposase:IS5 family transposase:IS110-like element IS5075 family transposase:*sul*2:*aph(3'')*-Ib:*aph(6)*-Id:IS91 family transposase:*bla*TEM-1:recombinase family protein:IS1380-like element ISEcp1 family transposase:*bla*CTX-M-15:Tn3-like element Tn3 family transpoase	*Klebsiella pneumoniae* strain F17KP0040 plasmid pF17KP0040-2, CP052140.1
		29	*qnr*B1:Tn3-like element IS3 family transposase:Tn3-like element Tn5043 family transposase:recombinase family protein	*Klebsiella pneumoniae* strain NK_H12_086 plasmid pNK_H12_086.1, CP152918.1
		30	IS6-like element IS6100 family transposase:plasmid mobilization relaxasome protein *mob*C:*dfr*A14: Class 1 integron integrase intI1	*Klebsiella pneumoniae* strain NK_H12_086 plasmid pNK_H12_086.1, CP152918.1
		31	*tet*(A):t*etR*(A):relaxase:Tn3 family transposase	*Klebsiella pneumoniae* strain NK_H12_086 plasmid pNK_H12_086.1, CP152918.1
KPD9	ST133	1	recombinase:*bla*SHV-75:IS3 family transposase	*Klebsiella pneumoniae* strain BA13643 chromosome, CP102490.1
		4	*oqx*A:o*qx*B14:::recombinase:*sit*C	*Klebsiella pneumoniae* strain SB612 chromosome, CP084830.1

		6	recombination-promoting nuclease/putative transposase::*fos*A5	*Klebsiella pneumoniae* strain BM343 chromosome, CP063939.1
		18	IS3-like element ISKpn25::ISL3- family transposase:ISNCY family transposase:IS3 family transposase:transposase:IS1 family transposase:IS5 family transposase:IS110-like element IS5075 family transposase:*sul*2:*aph(3'')*-Ib:*aph(6)*-Id:IS91 family transposase:*bla*TEM-1:recombinase family protein:IS1380-like element ISEcp1 family transposase:*bla*CTX-M-15::Tn3-like element Tn3 family transposase	*Klebsiella pneumoniae* strain F17KP0040 plasmid pF17KP0040-2, CP052140.1
		25	*qnr*B1:Tn3-like element IS3000 family transposase:Tn3-like element Tn5403:recombinase family protein	*Klebsiella pneumoniae* subsp. *pneumoniae* strain N3041884 plasmid p1-N3041884, CP165872.1
		26	IS6-like element IS6100 family transposase:plasmid mobilization relaxasome protein *mob*C:*dfr*A14: Class 1 integron integrase intI1	*Klebsiella pneumoniae* subsp. *pneumoniae* strain N3041884 plasmid p1-N3041884, CP165872.1
		27	*tet(*A):t*etR*(A):relaxase:Tn3 family transposase	*Klebsiella pneumoniae* subsp. *pneumoniae* strain N3041884 plasmid p1-N3041884, CP165872.1
KPP4	ST4268	2	*Amp*E:transposase:transposase:*fos*A5::	*Klebsiella pneumoniae* strain RHBSTW-00636 chromosome, CP056372.1
		3	*bla*SHV-11:IS3 family transposase	*Klebsiella pneumoniae* strain Kpn37 chromosome, CP153628.1
		11	*oqx*A:*oqx*B5::recombination regulator *rec*X:recombinase *rec*A:*sit*C	*Klebsiella pneumoniae* strain LH102-A chromosome, CP035194.1

Appendix 2: Antibiotic Resistant *Enterobacter* spp

The antibiotic resistance profiles, resistance genes, and mobile genetic elements for the identified *Enterobacter spp* isolates are presented in the following tables.

Table 1: Antibiotic resistance profile and the associated resistance genes and mobile genetic elements

Isolate ID	Accession numbers	Source	Resistance pattern	species	Sequence Type	Antibiotic Resistance genes	Plasmid replicons	Insertion sequences
ABD10	SAMN43041485	Durban	TZP	*E. kobei*	ST691	*bla*ACT-9*, bla*ACT-14 *tet*(A)	Col(pHAD28). IncFIA(hi1), IncFIB(K), repB(R1701)	MITEKpn1, IS1511, ISPa107, ISHar4, ISRop3

Only three resistance genes were identified in *E. kobei*, *bla*ACT-9, *bla*ACT-14 and *tet*(A) (Table 1). Four different plasmid replicons were identified Col(pHAD28), IncFIX(hil), IncFIB(K), and repB(R1701). The identified insertion sequence included, MITEKpn1, IS1511, ISPa107, ISHar4 and ISRop3. Class 1 integrons were not identified.

Table 2: Antibiotic resistance genomic environments of *E. kobei*

Isolate ID	MLST	Contig	Synteny of resistance genes and MGEs	Plasmid/chromosomal sequence with closest nucleotide homology (accession number)
ABD10	ST691	41	relaxase:tetR(A):tet(A):Tn3-like element TnA51 family transposase:IncFII family plasmid initiation repA:::::mobF family relaxase:::conjugal transfer protein	*Klebsiella pneumoniae* strain T7-180 plasmid pT7-180.1

A genetic environment of tetracycline resistance gene tet(A) was found in *E. kobei*. The genetic context relaxase:tetR(A):*tet*(A):Tn3-like element TnA51 family transposase:IncFII family plasmid initiation repA:::::mobF family relaxase:::conjugal transfer protein was carried on a plasmid (Table 2).

Appendix 3: Research Ethics Evaluation 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

Fulufhelo Naomi Mukwevho

a complété avec succès - has successfully completed

Module 2 (2023) - Research Ethics Evaluation

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

[redacted]

Professeur Dominique Sprumont
Coordinateur TRREE Coordinator

Release Date: 2024/02/22

CID : OY7dBQ8m1m

APPROVED BY SIWF ISFM

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[REV : 20220217]

Appendix 4: Approval from the Biomedical Research Ethics Committee

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
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