Antimicrobial resistance mechanisms of linezolid resistant staphylococci and enterococci collected in Gauteng, South Africa

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Antimicrobial resistance mechanisms of linezolid resistant staphylococci and enterococci collected in Gauteng, South Africa

by

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Submitted in fulfilment of the requirements for the degree

MAGISTER SCIENTIAE

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I, the undersigned, declare that the dissertation hereby submitted to the University of Pretoria for the degree MSc (Medical Microbiology) and the work contained therein is my original work and has not previously, in its entirety or in part, been submitted to any university for a degree. I further declare that all sources cited are acknowledged using a list of references.

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DEDICATION

This dissertation is dedicated to my husband, Dean Addison, whose constant support and encouragement during the challenges of graduate school enabled me to stay on course. You kept me fed and watered with chocolate and tea while ensuring the (your) cats cuddled with me during the endlessly long nights of writing as the world around me slept. I am truly thankful for having you in my life.

"Anyone who has never made a mistake has never tried anything new."

Albert Einstein

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOLS

%	percentage
α	alpha
β	beta
δ	delta
γ	gamma
±	plus-minus
®	registered
°C	degree Celsius
g	grams
h	hours
L	litre
M	molar
min	minutes
mL	millilitre
sec	seconds
™	trademark
V	Volt
x g	gravitational force
µg	microgram
µL	microlitre
µM	micromolar

ABBREVIATIONS

A site	:	Aminoacyl site
A	:	Adenine
AIDS	:	Acquired Immunodeficiency Syndrome
ATCC	:	American Type Culture Collection
BC	:	Blood culture
BHI	:	Brain heart infusion
bp	:	Base pair
C	:	Cytosine
CC	:	Clonal complex
CDC	:	Centres for Disease Control and Prevention
cfr	:	Chloramphenicol-florfenicol resistance gene
CLSI	:	Clinical and Laboratory Standards Institute
CoNS	:	Coagulase-negative staphylococci
CVC	:	Central venous catheter
ddl	:	D-alanine:D-alanine ligase gene
dH_2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
EDTA	:	Ethylenediaminetetraacetic acid
FISH	:	Fluorescent *in situ* hybridisation
G	:	Guanine
HGT	:	Horizontal gene transfer
HIV	:	Human Immunodeficiency Virus
I	:	Intermediate
ICU	:	Intensive care unit
IDSA	:	Infectious Diseases Society of America
kb	:	Kilo base
L22	:	Ribosomal protein L22/*rplV*

L3	:	Ribosomal protein L3/*rplC*
L4	:	Ribosomal protein L4/*rplD*
LE	:	Low electroendosmosis
LEADER	:	Linezolid Experience and Accurate Determination of Resistance
MALDI-TOF	:	Matrix-assisted laser desorption ionisation time of flight
MAO	:	Monoamine oxidase
Mb	:	Mega base
MDR	:	Multidrug resistance/resistant
MGE	:	Mobile genetic element
MIC	:	Minimum inhibitory concentration
MLST	:	Multilocus sequence typing
M-PCR	:	Multiplex polymerase chain reaction
mRNA	:	Messenger ribonucleic acid
MRSA	:	Methicillin-resistant *Staphylococcus aureus*
MS	:	Mass spectrometry
NCBI	:	National Center for Biotechnology Information
NHGRI	:	National Human Genome Research Institute
NHLS	:	National Health Laboratory Service
NRF	:	National Research Foundation
nuc	:	Thermonuclease gene
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PFGE	:	Pulsed-field gel electrophoresis
PTC	:	Peptide translocation centre
PVL	:	Panton-Valentine leucocidin
R	:	Resistant
RNA	:	Ribonucleic acid
rRNA	:	Ribosomal ribonucleic acid
S	:	Sensitive/susceptible
SCC*mec*	:	Staphylococcal Cassette Chromosome penicillin binding protein (*mec*)
SNP	:	Single nucleotide polymorphisms
sodA	:	Superoxide dismutase gene
spp.	:	Species
ST	:	Sequence type
T	:	Thymine
TAD	:	Tshwane Academic Division
TB	:	Tuberculosis
TBE	:	Tris-borate ethylenediaminetetraacetic acid
TDM	:	Therapeutic drug monitoring
TE	:	Tris-ethylenediaminetetraacetic acid
tRNA	:	Transfer ribonucleic acid
U	:	Uracil
UID	:	Unique identification number
UK	:	United Kingdom
UPGMA	:	Unweighted Pair Group Method with Arithmetic
USA	:	United States of America
UV	:	Ultraviolet
VRE	:	Vancomycin-resistant enterococci
WGS	:	Whole genome sequencing
WHO	:	World Health Organization
XDR	:	Extensively drug-resistant
ZAAPS	:	Zyvox annual appraisal of potency and spectrum
ZR	:	ZymoResearch

LIST OF MICROBIAL SPECIES

A. baumanni	:	*Acinetobacter baumannii*
E. coli	:	*Escherichia coli*
E. faecalis	:	*Enterococcus faecalis*
E. faecium	:	*Enterococcus faecium*
K. pneumoniae	:	*Klebsiella pneumoniae*
M. tuberculosis	:	*Mycobacterium tuberculosis*
P. aeruginosa	:	*Pseudomonas aeruginosa*
S. aureus	:	*Staphylococcus aureus*
S. auricularis	:	*Staphylococcus auricularis*
S. capitis	:	*Staphylococcus capitis*
S. delphini	:	*Staphylococcus delphini*
S. epidermidis	:	*Staphylococcus epidermidis*
S. haemolyticus	:	*Staphylococcus haemolyticus*
S. hominis	:	*Staphylococcus hominis*
S. hyicus	:	*Staphylococcus hyicus*
S. intermedius	:	*Staphylococcus intermedius*
S. lugdunensis	:	*Staphylococcus lugdunensis*
S. lutrae	:	*Staphylococcus lutrae*
S. pseudintermedius	:	*Staphylococcus pseudintermedius*
S. saprophyticus	:	*Staphylococcus saprophyticus*
S. schleiferi	:	*Staphylococcus schleiferi*

LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

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ANTIMICROBIAL RESISTANCE MECHANISMS OF LINEZOLID RESISTANT STAPHYLOCOCCI AND ENTEROCOCCI COLLECTED IN GAUTENG, SOUTH AFRICA

by

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SUMMARY

Staphylococci and enterococci are key human pathogens responsible for infections associated with healthcare settings. Linezolid is crucial for managing multidrug-resistant (MDR) infections. The monitoring of resistance to antimicrobial agents is a global effort. Linezolid has two surveillance programs which endeavour to monitor linezolid resistance, namely: Zyvox® Annual Appraisal of Potency and Spectrum (ZAAPS) and Linezolid Experience and Accurate Determination of Resistance (LEADER). In contrast to the ZAAPS and LEADER surveillance programs, linezolid resistance data is lacking in South Africa. This study aimed to determine linezolid resistance 23S ribosomal ribonucleic acid (rRNA) gene mutations and the acquired chloramphenicol-florfenicol resistance (*cfr*) gene of staphylococci and enterococci obtained from public and private hospitals in Gauteng, South Africa.

A total of 79 staphylococcal isolates (43 *Staphylococcus capitis*, 27 *Staphylococcus epidermidis* and nine *Staphylococcus haemolyticus*) and 32 enterococcal isolates (28 *Enterococcus faecalis* and four *Enterococcus faecium*) were obtained for investigation. Initial linezolid susceptibility was evaluated using the VITEK® 2 automated system (bioMérieux, France). Staphylococcal

and enterococcal isolates showing intermediate resistant and resistant according to the 2019 Clinical and Laboratory Standards Institute (CLSI) guidelines were selected for this study. The minimum inhibitory concentration (MIC) values were confirmed using the ETEST® (bioMérieux, France). Confirmatory identification multiplex polymerase chain reaction (M-PCR) assays and *cfr* gene detection by polymerase chain reaction (PCR) was used, followed by evaluation of relatedness using pulsed-field gel electrophoresis (PFGE). Whole-genome sequencing (WGS) of seven *S. epidermidis* isolates and three *S. capitis* isolates determined the 23S rRNA gene mutations and confirmed the presence of the *cfr* gene.

The ETEST® (bioMérieux, France) MIC values of the staphylococcal isolates ranged between 8 µg/mL and > 256 µg/mL and the enterococcal isolates MIC values ranged between 2 µg/mL and 4 µg/mL. All the staphylococcal isolates were resistant to linezolid and the enterococcal isolates showed susceptibility and intermediate resistance, according to the 2019 CLSI guidelines. The *cfr* gene was found in eight *S. epidermidis* isolates. The *S. capitis* isolates and *S. haemolyticus* isolates were all *cfr* negative. The dominant sequence type (ST) among the *S. epidermidis* isolates was ST23 (n = 4), followed by ST2 (n = 2) and ST22 (n = 1), all of which are clinically relevant STs having been extensively reported among nosocomial infections.

Several 23S rRNA gene mutations were observed in this study among the *S. epidermidis* isolates and the *S. capitis* isolates. Known and previously reported mutations found in this study were C2190T, G2603T and C2561T among *S. epidermidis* and *S. capitis*. However, several unknown and previously unreported 23S rRNA gene mutations were observed among *S. capitis*, namely: T2157A, T2346C, C2287G, A2295G, A2296G, C2302G, A2305G, C2308G and A2314C. The *S. capitis* isolate that showed all previously unreported 23S rRNA mutations had a significantly higher MIC value, thus indicating that 23S rRNA gene mutations are a significant contributing factor in linezolid resistance. These novel 23S rRNA gene mutations are not previously reported in the literature and are therefore important for future research. The PFGE results showed diversity among the staphylococcal isolates between hospitals, suggesting a wide spread of the strains. Linezolid resistance is concerning for antimicrobial management efforts and the data generated from this study provides valuable information regarding the prevalence of linezolid resistant strains circulating in the Gauteng region of South Africa.

Keywords: Linezolid, Resistance, *cfr*, 23S rRNA mutations, MDR

CHAPTER 1

Introduction

1.1 Background

Staphylococci and enterococci are Gram-positive bacteria that cause opportunistic healthcare-associated and community-associated infections (Santajit and Indrawattana, 2016). Staphylococci and enterococci are recognised as commensal human flora, therefore creating the problem for ongoing opportunistic infection (Santajit and Indrawattana, 2016). Staphylococcal infections of clinical importance are mostly caused by *Staphylococcus aureus*, *Staphylococcus capitis*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* (Czekaj *et al.*, 2015; Tevell *et al.*, 2017). Enterococcal infections are mostly caused by *Enterococcus faecalis* and *Enterococcus faecium*, both of which are considered important pathogens globally (Bender *et al.*, 2018; Bi *et al.*, 2018).

The treatment of infections caused by *S. aureus* is complicated by methicillin-resistant *S. aureus* (MRSA) since these infections are often multidrug-resistant (MDR) (Zahedi Bialvaei *et al.*, 2017; Fiore *et al.*, 2018). *Staphylococcus epidermidis*, *S. capitis* and *S. haemolyticus* are coagulase-negative staphylococci (CoNS) that may cause severe infections in the compromised host and account for about 30% of bloodstream-associated infections as well as sepsis in immunocompromised patients and neonates (Pinheiro *et al.*, 2016; Tevell *et al.*, 2017). Further examples of staphylococcal infections include bacteraemia, complicated skin and soft-tissue infections, device-related infections, infective endocarditis, nosocomial pneumonia and osteoarticular infections (Tong *et al.*, 2015). *Enterococcus faecalis* and *E. faecium* form part of the natural human gastrointestinal tract and faecal microbiota and may cause a range of infections such as bacteraemia, surgical site infections, endocarditis and urinary tract infections in nosocomial settings (Bender *et al.*, 2016). Enterococcal infections are often a result of faecal contamination (van Harten *et al.*, 2017). In most cases, *E. faecalis* remains susceptible to ampicillin; however, *E. faecium* is mostly MDR presenting treatment challenges due to the lack of available treatment options for these infections (Doernberg *et al.*, 2017).

Linezolid is from the oxazolidinone class of antimicrobials, introduced in 2000, that is used to treat Gram-positive infections caused by methicillin-resistant staphylococci and vancomycin-resistant enterococci (VRE) (Ishiwada *et al.*, 2016). A systematic review by An and colleagues (2013), compared the effectiveness and safety of oral linezolid to vancomycin for staphylococci

and enterococci related infections. Since vancomycin is administered intravenously, it requires patients to either be hospitalised or have intravenous access at the point of care (An *et al.*, 2013). Linezolid is advantageous because it is 100% bioavailable in the oral form which allows patients to be given oral administration rather than intravenous administration, thereby reducing the length and costs of hospital stay (Hashemian *et al.*, 2018). Linezolid is only suitable for the treatment of Gram-positive bacteria due to Gram-negative bacteria being intrinsically resistant (Long and Vester, 2012). The intrinsic nature of Gram-negative bacteria is due to efflux pumps that prevent linezolid accumulating in the cell (Long and Vester, 2012).

Antimicrobial resistance in staphylococcal and enterococcal isolates is identified by using phenotypic and genotypic methods (Bard and Lee, 2018). Phenotypic antimicrobial susceptibility testing is conducted using dilution methods such as broth microdilution or diffusion methods such as disk diffusion and ETEST® (bioMérieux, France) (Doern *et al.*, 2016). Automated platforms such as the VITEK® 2 automated system (bioMérieux, France) are also routinely employed in phenotypic antimicrobial susceptibility testing (Doern, 2018). The minimum inhibitory concentration (MIC) is a standardised antimicrobial testing method used to measure the lowest concentration of an antimicrobial agent to inhibit the growth of a specific bacterium (Scheerans *et al.*, 2015). The MIC value is essential to identify appropriate antimicrobial agents and the concentrations sufficient to effectively treat the infection (Scheerans *et al.*, 2015). According to the 2019 Clinical and Laboratory Standards Institute (CLSI) breakpoint tables, *Staphylococcus* spp. MIC values ≤ 4 µg/mL indicate susceptibility and ≥ 8 µg/mL indicate resistance (CLSI, 2019). *Enterococcus* spp. MIC values ≤ 2 µg/mL indicate susceptibility, 4 µg/mL indicate intermediate susceptibility and ≥ 8 µg/mL indicate resistance (CLSI, 2019). Genotypic screening is a supplement to phenotypic testing and uses methods such as polymerase chain reaction (PCR) for the detection of antimicrobial resistance and virulence genes (Bard and Lee, 2018). Molecular characterisation and clonal relatedness of isolates are performed by genotyping methods such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and whole-genome sequencing (WGS) (Werner *et al.*, 2007). These genotyping methods are used as epidemiological typing tools for tracing MDR bacterial pathogens that are difficult to treat or during outbreak investigations (Wang *et al.*, 2014).

The mode of action of linezolid is the inhibition of the initiation process of bacterial protein synthesis of Gram-positive bacteria, by preventing the formation of the 70S ribosomal initiation

complex (Doern *et al.*, 2016). The 70S ribosomal initiation complex is a vital component of the bacterial translation process, formed by binding together the large 50S ribosomal subunit with the small 30S subunit (Mendes *et al.*, 2014). The large 50S ribosomal subunit contains the peptide translocation centre (PTC), which is the site of catalysis for the formation of peptide bonds for protein synthesis (Beringer and Rodnina, 2007). Linezolid binds to the PTC resulting in the inhibition of protein synthesis by inhibiting transition of the incoming aminoacyl transfer ribonucleic acid (tRNA) to the aminoacyl site (A site), therefore halting the growth of bacteria (Long and Vester, 2012; Bender *et al.*, 2015). Linezolid has a unique mode of action because there is no cross-resistance between oxazolidinones and other protein-synthesis inhibitors (Mendes *et al.*, 2014).

The main mechanisms of linezolid resistance reported in staphylococci and enterococci are namely: (i) point mutations of the 23S ribosomal ribonucleic acid (rRNA) gene, (ii) mutations of the 50S ribosomal protein L3 (L3/*rplC* gene), ribosomal protein L4 (L4/*rplD* gene) and ribosomal protein L22 (L22/*rplV* gene) and (iii) the acquisition of the chloramphenicol-florfenicol resistance (*cfr*) gene which is a plasmid-mediated mechanism (Gupta, 2016). These mechanisms of resistance are unique to linezolid, therefore reducing the possibility of cross-resistance among Gram-positive bacteria that have developed resistance to antimicrobial agents that inhibit protein synthesis such as chloramphenicol, clindamycin and the macrolides (Doern *et al.*, 2016).

The most frequent mechanism that results in the development of linezolid resistance is the point mutations in the 23S rRNA gene that causes modification of the antimicrobial target binding A site (Tian *et al.*, 2014). Staphylococci and enterococci have four to six copies (alleles) of the genes encoding the 23S rRNA gene and when more than one allele is mutated, clinically significant resistance occurs (Hollenbeck and Rice, 2012; Doern *et al.*, 2016). However, the mutation of only one allele is adequate to confer linezolid resistance, but the extent of resistance may depend on the number of mutated alleles (Werner *et al.*, 2007). The most common mutations in the 23S rRNA gene are: guanine-to-thymine at nucleotide 2576 (G2576T), thymine-to-adenine at nucleotide 2500 (T2500A) and guanine-to-adenine at nucleotide 2234 (G2234A) (Doern *et al.*, 2016). Various other 23S rRNA gene mutations have been characterised such as A2058G, A2059G, A2503G, C2192T, C2207A, C2461T, G2061T, G2083T, G2234A, G2341A, G2345A, G2505A, G2766T, T2219C, T2326C and T2504C (Doern *et al.*, 2016; Zahedi Bialvaei *et al.*, 2017). Linezolid resistance can also develop when

the 50S ribosomal L3, L4 and L22 ("L" indicates the protein is a component of the large subunit of the ribosome) proteins mutate, which are located close to the PTC (Song *et al.*, 2017). Mutations in the conserved regions of ribosomal proteins L3, L4 and L22, which are encoded by the genes *rplC*, *rplD* and *rplV*, respectively, are associated with elevated MICs for linezolid (Song *et al.*, 2017).

The linezolid resistance mechanism of the most significant concern involves the *cfr* gene, which is plasmid-mediated (Zahedi Bialvaei *et al.*, 2017). The *cfr*-mediated resistance mechanism encodes an rRNA methyltransferase that adds a methyl group at the C-8 position of the adenine at nucleotide 2503 (A2503) in the 23S rRNA gene of the 50S large ribosomal subunit (Arias *et al.*, 2008). The acquisition of the *cfr* gene is a non-mutational mechanism of resistance (Doern *et al.*, 2016). The *cfr* gene is a MDR gene because the result of methylation by the *cfr* gene product confers resistance to five classes of antimicrobials, namely: phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A (the PhLOPSA phenotype) (Tian *et al.*, 2014). The *cfr* gene is carried on a mobile genetic element (MGE) that can be transmitted between different species of bacteria and has been detected in *S. aureus*, CoNS, enterococci and streptococci (Doern *et al.*, 2016). Due to the horizontal transfer capability and its association with the PhLOPSA phenotype, the *cfr*-mediated resistance is of grave concern to treatment outcomes (Becker *et al.*, 2014). Determining the risk of the *cfr* gene distribution among clinical staphylococci and enterococci isolates is vital to avoid the spread of linezolid resistance (Cafini *et al.*, 2016).

Linezolid resistance has increased worldwide since 2001, when the first linezolid-resistant staphylococci were reported (Tewhey *et al.*, 2014; Song *et al.*, 2017). Bacteria can be intrinsically resistant to antimicrobial agents, meaning that the bacterial species have natural resistance abilities against a particular antimicrobial agent through its inherent structural or functional characteristics (Blair *et al.*, 2015). However, bacteria can acquire antimicrobial resistance *via* a MGE such as plasmids, bacteriophages and transposons that can introduce additional genes (Blair *et al.*, 2015).

Resistance to antimicrobials is a global challenge that risks placing humanity into a post-antimicrobial age where many improvements in the treatment and prevention of infections become negated through the risk of untreatable infection. The identification, monitoring and management of the mechanisms of linezolid resistance are critical to equip medical facilities with the information required to manage resistant bacteria and retain a working arsenal of

antimicrobial agents. The management of staphylococci and enterococci infections relies not only on fast, accurate detection but effective treatment to reduce morbidity and mortality of patients. The purpose of this study was to determine the MIC values, point mutations associated with the 23S rRNA gene and the prevalence of the *cfr* gene in *S. epidermidis* isolates, *S. capitis* isolates and *S. haemolyticus* isolates (Chapter 3), including the determination of the genetic relatedness of these strains circulating in the Gauteng province. The *E. faecalis* and *E. faecium* isolates are covered in Chapter 4.

1.2 Aim

This study aimed to determine the intrinsic and acquired resistance mechanisms of linezolid resistant staphylococci and enterococci isolates and to determine the genetic relatedness of circulating isolates obtained from public and private hospitals in Gauteng, South Africa.

1.3 Objectives

The objectives of this research study were:

- To determine the intrinsic and acquired resistance mechanisms (23S rRNA gene and *cfr* gene) of linezolid resistant staphylococci and enterococci using phenotypic and genotypic methods.
- To determine the molecular epidemiology of linezolid resistant staphylococci and enterococci using molecular techniques.
- To compare the phenotypic and genotypic antimicrobial resistance results.

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CHAPTER 2

Literature review

2.1 Introduction

In the late 20th century, the antimicrobial resistance crisis began and was observed most notably among nosocomial infections (Safdar and Maki, 2002). Nosocomial infections that are resistant to at least three antimicrobial classes are known as multidrug-resistant (MDR) strains which place a substantial drain on healthcare systems by incurring costs owing to prolonged treatment and hospitalisation (Santajit and Indrawattana, 2016; Fiore *et al.*, 2018). The discovery and development of antimicrobial agents are a significant development of modern medicine that has positively changed the outcome of healthcare-associated and community-associated infections, therefore improving quality of life and increasing lifespan (Rossolini *et al.*, 2014). However, years of antimicrobial misuse has applied selective pressure resulting in the emergence and survival of MDR strains (Bell *et al.*, 2014). Antimicrobial resistance develops into a clinical problem when the incidence of resistance compromises the efficacy of empirical antimicrobial therapy (Andersson and Hughes, 2010). The misuse of antimicrobial agents continues to contribute to the ineffective treatment of infectious diseases, causing higher rates of morbidity and mortality (Bell *et al.*, 2014). However, antimicrobial resistance is inevitable and understanding the evolution of bacterial resistance mechanisms and the spread of resistant strains will provide vital information in the development of new antimicrobials (Wright, 2010).

Staphylococcus species (spp.) and *Enterococcus* spp. are Gram-positive pathogens that currently pose significant challenges in terms of antimicrobial resistance (Rossolini *et al.*, 2014). Staphylococci and enterococci form part of the ESKAPE pathogens, namely: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. (van Harten *et al.*, 2017). The Infectious Diseases Society of America (IDSA) formulated the ESKAPE acronym to describe this group of pathogens due to the ability of the bacterium to effectively ‘escape’ the effects of antimicrobial agents (Pogue *et al.*, 2015). The ESKAPE pathogens display an increase in resistance to antimicrobial agents, which can lead to devastating outcomes (Pogue *et al.*, 2015). Multidrug-resistant *Staphylococcus* spp. and *Enterococcus* spp. continually contribute to the burden of nosocomial infections leading to the requirement for newer antimicrobial agents to treat the emergence of new resistance mechanisms (Pogue *et al.*, 2015; Sader *et al.*, 2019). Linezolid is an oxazolidinone antimicrobial agent that was clinically approved for use in 2000

for the treatment of severe infections caused by MDR Gram-positive bacteria (Takada *et al.*, 2017). Linezolid demonstrates effectiveness against clinically significant Gram-positive bacteria such as vancomycin-resistant enterococci (VRE) and methicillin-resistant *S. aureus* (MRSA) (Zahedi Bialvaei *et al.*, 2017).

2.2 Overview of *Staphylococcus* and *Enterococcus*

Staphylococci and enterococci are frequently involved in causing healthcare-associated and community-associated infections such as bacteraemia, bone and joint infections, central nervous system infections, infections associated with medical devices, neonatal sepsis, intestinal infections, skin infections, respiratory tract infections and urinary tract infections (Fisher and Phillips, 2009; Rossolini *et al.*, 2010). Staphylococci and enterococci present significant challenges regarding antimicrobial resistance and are relevant pathogens of clinical interest (Rossolini *et al.*, 2014).

2.2.1 Taxonomy of *Staphylococcus* and *Enterococcus*

Bacteria are classified into genera and species according to a binomial Linnean scheme (Pottinger *et al.*, 2014b). Up until the 1960s, the taxonomic classification of *Staphylococcus* was grouped with *Micrococcus* because both *Staphylococcus* and *Micrococcus* consisted of cluster forming cocci (Mathema *et al.*, 2009). During the 1960s, the differences in the genetic makeup revealed a significant distinction between *Staphylococcus* and *Micrococcus* due to base-pair content (Mathema *et al.*, 2009). All deoxyribonucleic acid (DNA) is double-stranded and composed of four nucleotides, namely: adenine (A), cytosine (C), guanine (G) and thymine (T) (NHGRI, 2019). Ribonucleic acid (RNA) is similar to DNA but is single-stranded and contains uracil (U) instead of thymine (NHGRI, 2019). Three types of RNA exist in the cell: messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) (NHGRI, 2019). Analysis of DNA has enabled classification based on the overall ratio of AT to GC base pairs within DNA (Pottinger *et al.*, 2014b). Differences were found in the GC content between *Staphylococcus* and *Micrococcus* that revealed a low GC content (30% to 39%) among *Staphylococcus* and a higher GC content (66% to 75%) was found in *Micrococcus* (Mathema *et al.*, 2009). The staphylococci now fall under the Kingdom of *Eubacteria*, extending to the *Bacilli* class (Becker *et al.*, 2014).

The *Enterococcus* spp. were initially classified as Lancefield Group D streptococci, developed by Rebecca Lancefield, due to the Group D cell wall antigen (glycerol teichoic acid in the cell wall) (Vu and Carvalho, 2011; Hollenbeck and Rice, 2012). The enterococci were reclassified

in 1984 into the *Enterococcus* genus when DNA testing confirmed significant differences between enterococci and streptococci (Vu and Carvalho, 2011). Table 2.1 shows the current scientific classification of the *Staphylococcus* and *Enterococcus* spp. observed in this study (Euzéby, 2019a; Euzéby, 2019b).

Table 2.1: Taxonomic classification of *Staphylococcus* spp. and *Enterococcus* spp.

Staphylococcus	**Scientific Classification**			
	Domain	*Bacteria*		
	Kingdom	*Eubacteria*		
	Phylum	*Firmicutes*		
	Class	*Bacilli*		
	Order	*Bacillales*		
	Family	*Staphylococcaceae*		
	Genus	*Staphylococcus*		
	Species	*capitis*	*epidermidis*	*haemolyticus*
	Binomial name	*Staphylococcus capitis*	*Staphylococcus epidermidis*	*Staphylococcus haemolyticus*
Enterococcus	**Scientific Classification**			
	Domain	*Bacteria*		
	Kingdom	*Eubacteria*		
	Phylum	*Firmicutes*		
	Class	*Bacilli*		
	Order	*Lactobacillales*		
	Family	*Enterococcaceae*		
	Genus	*Enterococcus*		
	Species	*faecalis*	*faecium*	
	Binomial name	*Enterococcus faecalis*	*Enterococcus faecium*	

Currently, there are 53 *Staphylococcus* spp. and 28 subspecies recognised in the List of Prokaryotic Names with Standing in Nomenclature (Euzéby, 2019b). *Enterococcus* spp. consist of 58 species and two subspecies as recognised in the List of Prokaryotic Names with Standing in Nomenclature (Euzéby, 2019a). The most critical clinically significant staphylococcal species is *S. aureus*, followed by *S. capitis*, *S. epidermidis* and *S. haemolyticus* (Becker *et al.*, 2015; Rupp and Fey, 2015). The *Enterococcus* spp. that are clinically significant are *E. faecalis* and *E. faecium* (Arias and Murray, 2015).

2.2.2 Characteristics of *Staphylococcus* and *Enterococcus*

The *Staphylococcus* genus is catalase-positive, aerobic and facultatively anaerobic, nonmotile and non-spore-forming cocci (Becker *et al.*, 2014). Staphylococci have non-fastidious growth requirements, forming irregular “grape-like” clusters that are visible under the microscope (Becker *et al.*, 2014; Santajit and Indrawattana, 2016). Staphylococci have a diameter of 0.7 µm to 1.2 µm and proliferate in aerobic conditions withstanding pH levels from pH 4.8 to pH 9.4 (optimal at pH 7) (Somerville and Proctor, 2009). Additionally, staphylococci can resist drying while surviving at extreme temperatures as high as 60°C (optimal at 37°C) and tolerate

high salt concentrations (10% NaCl) (Somerville and Proctor, 2009). Staphylococci are divided into two groups: the coagulase-positive and the coagulase-negative staphylococci (CoNS) (David and Daum, 2010). Coagulase is a protein enzyme, produced by coagulase-positive staphylococci, that enables the conversion of soluble fibrinogen in plasma to insoluble fibrin (von Eiff *et al.*, 2002). *Staphylococcus aureus* produces coagulase and is the only clinically relevant coagulase-positive staphylococcal species and all other staphylococcal species are CoNS due to lack of coagulase production (Gu *et al.*, 2013).

The *Enterococcus* genus occurs as single cocci, pairs or short and long chains (Arias and Murray, 2015; Santajit and Indrawattana, 2016). Enterococci are catalase-negative, produce lactic acid and are non-spore forming, facultatively anaerobic bacteria (Fisher and Phillips, 2009). Due to being facultatively anaerobic, enterococci are able to proliferate in aerobic and anaerobic environments, readily switching from respiration to fermentation (Vu and Carvalho, 2011). Enterococci can withstand extreme conditions such as a broad pH range (pH 4.8 to pH 9.6, optimal at pH 7.5), temperature extremes (5°C to 50°C, optimal at 37°C) and high salt concentrations (6.5% NaCl) (Vu and Carvalho, 2011; Hollenbeck and Rice, 2012; Arias and Murray, 2015). Enterococci are also able to withstand the presence of bile salts, therefore allowing colonisation within the intestinal tract (Fisher and Phillips, 2009; Zhang *et al.*, 2013).

2.3 Clinical significance and pathogenesis of *Staphylococcus* and *Enterococcus*

The *Staphylococcus* spp. cause a wide range of superficial and invasive nosocomial infections such as boils, furuncles, bacteraemia, device-related infections, infective endocarditis, pleuropulmonary infections, pneumonia, osteomyelitis as well as skin and soft tissue infections (Que and Moreillon, 2015; Tong *et al.*, 2015). Immunocompromised patients are at a higher risk of CoNS infections (Becker *et al.*, 2015). The CoNS species such as *S. capitis*, *S. epidermidis* and *S. haemolyticus* represent the majority of nosocomial staphylococcal infections (Becker *et al.*, 2014).

Staphylococcus capitis is a constant coloniser of the scalp and is often reported in cases of neonatal sepsis and adult infections such as bacteraemia, endocarditis and bone infections (Butin *et al.*, 2017; Tevell *et al.*, 2017). *Staphylococcus capitis* has been shown to increase its range of habitats beyond the scalp during antimicrobial therapy (Tevell *et al.*, 2017). *Staphylococcus epidermidis* is a common cause of bacteraemia, catheter-related infections and infections of a variety of medical devices, such as prosthetic joints, artificial heart valves, and cerebrospinal fluid shunts (Rupp and Fey, 2015; Pinheiro *et al.*, 2016). Strains of *S. epidermidis*

can establish predominance in hospital environments, surviving on fabrics and surfaces resulting in the potential for the rapid spread between wards and hospitals (Rupp and Fey, 2015). *Staphylococcus haemolyticus* is involved in bacteraemia and sepsis, particularly in immunocompromised individuals and neonates (Pinheiro *et al.*, 2016). Next to *S. epidermidis*, *S. haemolyticus* is the second most frequently isolated CoNS from clinical cases, notably from bacteremic infections (Czekaj *et al.*, 2015).

The enterococci are commensals among the healthy flora in the gastrointestinal tract and have emerged as significant nosocomial pathogens (Daniel *et al.*, 2017). *Enterococcus faecalis*, along with the closely related *Enterococcus faecium* are responsible for the majority of enterococcal infections and are opportunistic in immunocompromised individuals (Daniel *et al.*, 2017; van Harten *et al.*, 2017). *Enterococcus faecalis* and *E. faecium* cause infections such as urinary tract infections, surgical site infections, burn wound infections, bacteraemia, sepsis, endocarditis, peritonitis and neonatal meningitis (Strateva *et al.*, 2016). Enterococcal infections are challenging to treat due to intrinsic resistance, acquisition of plasmid-mediated resistance mechanisms and the ability of enterococci to proliferate in the nosocomial environment (Bi *et al.*, 2018; Chen *et al.*, 2018). As a result, *E. faecalis* and *E. faecium* have emerged as leading therapeutic challenges associated with nosocomial infections (Daniel *et al.*, 2017).

The pathogenesis of staphylococci and enterococci is enhanced by virulence factors that mediate adhesion and invasion of host tissues, modulate immunity and increase the severity of infection (Strateva *et al.*, 2016). The *Staphylococcus* spp. exfoliative toxins A and B virulence factors mediate infections such as scalded skin syndrome, impetigo, skin infections and atopic dermatitis (Mertz *et al.*, 2007). Other staphylococcal virulence factors such as enterotoxins A and B mediate food poisoning and the Panton-Valentine leucocidin (PVL) gene mediates necrotising pneumonia and toxic shock syndrome (Mertz *et al.*, 2007). Additionally, staphylococci harbour antimicrobial resistance genes acquired *via* staphylococcal cassette chromosome (SCC) *mec* elements (Soumya *et al.*, 2017).

The *Enterococcus* spp. harbour several virulence factors that can cause or exacerbate disease such as aggregation substance (*agg*) that increases adherence of cells and increases biofilm formation; gelatinase (*gel*E) that hydrolyses collagen, gelatin and haemoglobin and cytolysin (*cyl*A), also called haemolysin that lyses cells (Anderson *et al.*, 2015). Additional virulence factors are enterococcal surface proteins (*esp*) that are associated with urinary tract colonisation and biofilm formation and hyaluronidase (*hyl*) that facilitates intestinal colonisation (Fisher and

Phillips, 2009; Anderson *et al.*, 2015; Heidari *et al.*, 2016; Marchi *et al.*, 2018).

2.3.1 Biofilm formation of *Staphylococcus* and *Enterococcus*

Staphylococci and enterococci are naturally present on the skin and are environmentally ubiquitous resulting in the ability to form biofilms on implanted medical devices such as central venous catheters, pacemakers and prosthetic materials (Hitzenbichler *et al.*, 2017; Soumya *et al.*, 2017). Biofilms develop in four separate stages, namely stage one: (i) attachment involving the adhesion of planktonic bacteria to an implanted medical device or tissue; stage two: (ii) accumulation of a microcolony that forms multilayers of extracellular material; stage three: (iii) maturation of the biofilm and stage four: (iv) detachment where the biofilms disperses (Fey, 2010; Soumya *et al.*, 2017).

A biofilm consists of microbial communities enclosed in a matrix of layers, primarily extracellular polysaccharide substances, enabling bacterial proliferation and preventing antimicrobial agents reaching the target (Santajit and Indrawattana, 2016). Biofilms are considered a key virulence factor in the pathogenesis of staphylococci and enterococci that leads to infection progression due to facilitating the restricted penetration of antimicrobial agents and restriction of the host immune system (Strateva *et al.*, 2016; Soumya *et al.*, 2017). Medical device implants contribute to the MDR infection rate, limiting therapeutic options when the inserted devices become colonised (Becker *et al.*, 2014). The consequence of medical implants becoming infected may result in the overall failure of the clinical procedure due to device failure and limited treatment options as antimicrobial agents are unable to penetrate the biofilm, leading to increased morbidity, mortality and healthcare cost (Becker *et al.*, 2014). In some cases of bacteraemia, a biofilm form on the heart valves resulting in endocarditis (van Harten *et al.*, 2017).

Biofilm-related infections are complicated to treat as biofilms severely decrease the efficacy of antimicrobial agents (van Harten *et al.*, 2017). Biofilm growth on implants is impervious to phagocytosis making infection eradication extremely difficult (Archer *et al.*, 2011; Hashem *et al.*, 2017). The biofilm matrix effectively provides a barrier to maintain the conditions needed to reduce the activity of antimicrobial agents such as low oxygen, low pH, high carbon dioxide and low water availability (Santajit and Indrawattana, 2016). Bacterial cells within a mature biofilm can withstand antimicrobial agents at concentrations 10 to 1000 times higher than the normal recommended dose (Hashem *et al.*, 2017). Therefore, under these conditions, it is difficult to eliminate bacteria using conventional antimicrobials (Santajit and Indrawattana,

2016). Biofilms often require surgical intervention due to the nature of antimicrobial resistance within the biofilm (Archer *et al.*, 2011).

2.4 Antimicrobial resistance and treatment of *Staphylococcus* and *Enterococcus* infections

Antimicrobial resistance is classified as either intrinsic or acquired (Martinez *et al.*, 2017). Intrinsic resistance is inherent and acquired resistance results from DNA mutations or the acquisition of new genetic determinants transferred on plasmids or transposons (Patel and Richter, 2015). The choice of antimicrobial therapy depends on the site of infection and the antimicrobial susceptibility profile of the isolate (Faron *et al.*, 2016). Antimicrobial agents are often used together to create a synergistic mode of action such as in the case of infective endocarditis, even though this form of treatment may include antimicrobial agents to which the bacterium is considered intrinsically resistant when used as a standalone therapy (Faron *et al.*, 2016). Newer antimicrobial therapies such as linezolid are last-line agents for the treatment of resistant staphylococci and enterococci, including infections caused by MRSA and VRE (Shen *et al.*, 2013). Linezolid is an effective and generally well-tolerated alternative therapy because it reaches high concentrations in musculoskeletal tissues such as skin, synovial fluid and bone (Soriano *et al.*, 2007; Morata *et al.*, 2014). Linezolid is 100% bioavailable in the oral form, therefore allowing patients to be switched from intravenous to oral antimicrobial treatment enabling the patient to be sent home, thereby reducing hospitals costs and complications (Zahedi Bialvaei *et al.*, 2017).

2.4.1 Antimicrobial stewardship in limiting antimicrobial resistance

Antimicrobial stewardship programmes have been introduced worldwide in response to the increase in antimicrobial resistance, but the available data in Southern Africa is limited (Brink *et al.*, 2016). Antimicrobial stewardship aims to improve patient outcomes, controlling the spread of MDR infections and ultimately to reduce antimicrobial resistance (Hulscher and Prins, 2017). Antimicrobial stewardship not only reduces resistance but also improves the incidence of adverse side effects and reduces hospital readmissions due to improved guidelines in the use of treatment agents (Cakmakci, 2015). To achieve an effective antimicrobial stewardship programme requires a sustained, coordinated effort between clinicians and other medical care providers such as hospital personnel, laboratory personnel and pharmacists to optimise the appropriate use of antimicrobial agents (Hulscher and Prins, 2017). The current antimicrobial resistance crisis is due to the decisions of many role players such as farmers who

use antimicrobials prophylactically for livestock, politicians who set the regulations in public healthcare, medical insurance companies that dictate access to certain medications due to cost implications, pharmaceutical companies that promote specific agents for profitable gain and healthcare providers who prescribe (Pottinger *et al.*, 2014a). However, the barriers limiting the implementation of antimicrobial stewardship programmes in South Africa across all public and private hospitals is due to a lack of infectious disease expertise and resources alongside the vast geographical distribution of healthcare facilities (Brink *et al.*, 2016).

2.4.2 Antibiograms in antimicrobial stewardship

An antibiogram reports the susceptibility of pathogens to a wide range of antimicrobial agents (Halstead *et al.*, 2004). Antibiograms provide antimicrobial resistance data in antimicrobial stewardship programmes contributing to the monitoring and evaluation of resistance trends to aid empiric treatment guidelines for prescribing clinicians (Diekema and Saubolle, 2011). Empiric therapy is used for the initial treatment of critically ill patients because healthcare providers need to make a 'best guess' and start treatment while waiting for a conclusive diagnosis from the laboratory (Pottinger *et al.*, 2014a; Brink *et al.*, 2016). Once a conclusive diagnosis is confirmed, specifically directed therapy can take over from empiric therapy because the patient-specific culture susceptibility tests provide final guidance (Pottinger *et al.*, 2014a). However, antibiograms need to be updated regularly for clinicians to better guide empiric treatment at the patient bedside (Diekema and Saubolle, 2011).

2.4.3 Antimicrobial agent innovation void

Pathogenic bacteria have the ability to adapt and resist antimicrobial agents resulting in the discovery of resistance mechanisms faster than the discovery of new antimicrobial classes and agents (WHO, 2014; Ribeiro da Cunha *et al.*, 2019). Since the initial discovery of antimicrobial agents in the early 20th century, new classes of antimicrobial agents have been discovered and approved for use (Talkington *et al.*, 2016; Ribeiro da Cunha *et al.*, 2019). The discovery trend continued but slowed in the 1980s (WHO, 2014). However, since the discovery of antimicrobial agents has slowed, a void has now emerged with no new classes of antimicrobial agents being approved for clinical use (WHO, 2014; Talkington *et al.*, 2016; Ribeiro da Cunha *et al.*, 2019). The decline in the discovery of antimicrobial agents is shown in Figure 2.1 (Duval *et al.*, 2019). This decline in the discovery of new antimicrobial classes has resulted in pathogenic bacteria being exposed to the same set of antimicrobial agents, mechanisms of actions and protocols (WHO, 2014; Talkington *et al.*, 2016; Ribeiro da Cunha *et al.*, 2019).

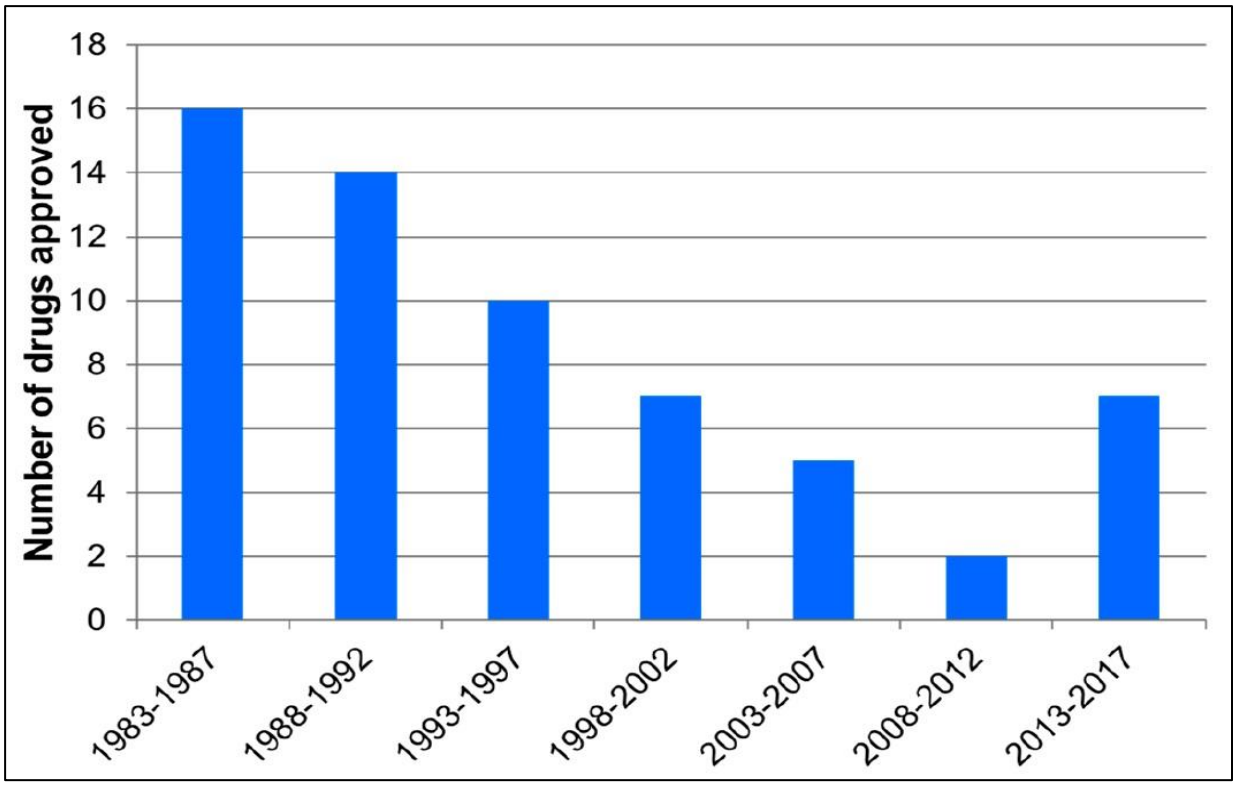

Figure 2.1: The evolution of antimicrobial agents approved since 1983 (Duval *et al.*, 2019)

However, new antimicrobial agents have been developed, although not necessarily with unique mechanisms of action but rather modifications of established antimicrobial classes such as tedizolid, oritavancin, dalbavancin and ceftaroline (Duval *et al.*, 2019; Talbot *et al.*, 2019). Antimicrobial stewardship has increased over the past decades, but the lack of central schemes and the narrow focus in research have contributed to increased resistance globally (Ribeiro da Cunha *et al.*, 2019).

2.4.4 Infection prevention and control

The leading cause of morbidity and mortality worldwide is due to infectious diseases that are problematic to treat due to the rapid spread of MDR (Tang *et al.*, 2017). Infection prevention and control procedures are vital in controlling the spread of infectious pathogens among staff and patients in healthcare facilities (Storr *et al.*, 2017). The adherence to infection control measures by healthcare workers is vital and the education of healthcare workers in infection control measures are essential (Smiddy *et al.*, 2019). However, infection control practices need to continually evolve to effectively reduce the spread of infectious diseases (Tang *et al.*, 2017; Smiddy *et al.*, 2019).

2.5 Linezolid: the first oxazolidinone antimicrobial

Linezolid is a bacteriostatic synthetic agent of the oxazolidinone class of antimicrobials (Doern

et al., 2016). Linezolid (trade name Zyvox®) was the first of the oxazolidinone antimicrobial agents, introduced into therapy in 2000, that have demonstrated clinical effectiveness against severe Gram-positive infections such as MRSA and VRE (Pfaller *et al.*, 2017; Bi *et al.*, 2018). Due to the prevalence of MDR and the limited treatment options for infections caused by Gram-positive cocci, the oxazolidinones are likely to remain vital as part of treatment protocols (Sader *et al.*, 2019). However, since the approval of linezolid for clinical use in 2000, linezolid resistance has progressively been identified, which significantly challenges successful treatment (Bi *et al.*, 2018).

2.5.1 Linezolid mode of action

The mode of action of linezolid is bacteriostatic and inhibits protein synthesis before it begins (Figure 2.2) (Douros *et al.*, 2015; Zahedi Bialvaei *et al.*, 2017). The bacterial ribosome has two subunits, the 30S and the 50S (Bozdogan and Appelbaum, 2004). The 30S subunit is composed of 16S rRNA and 21 proteins (S1 to S21) and the 50S subunit is composed of 5S and 23S rRNAs and 36 proteins (L1 to L36) (Bozdogan and Appelbaum, 2004). The two subunits combine to form a 70S ribosome which initiates protein synthesis and separates when synthesis is complete (Bozdogan and Appelbaum, 2004).

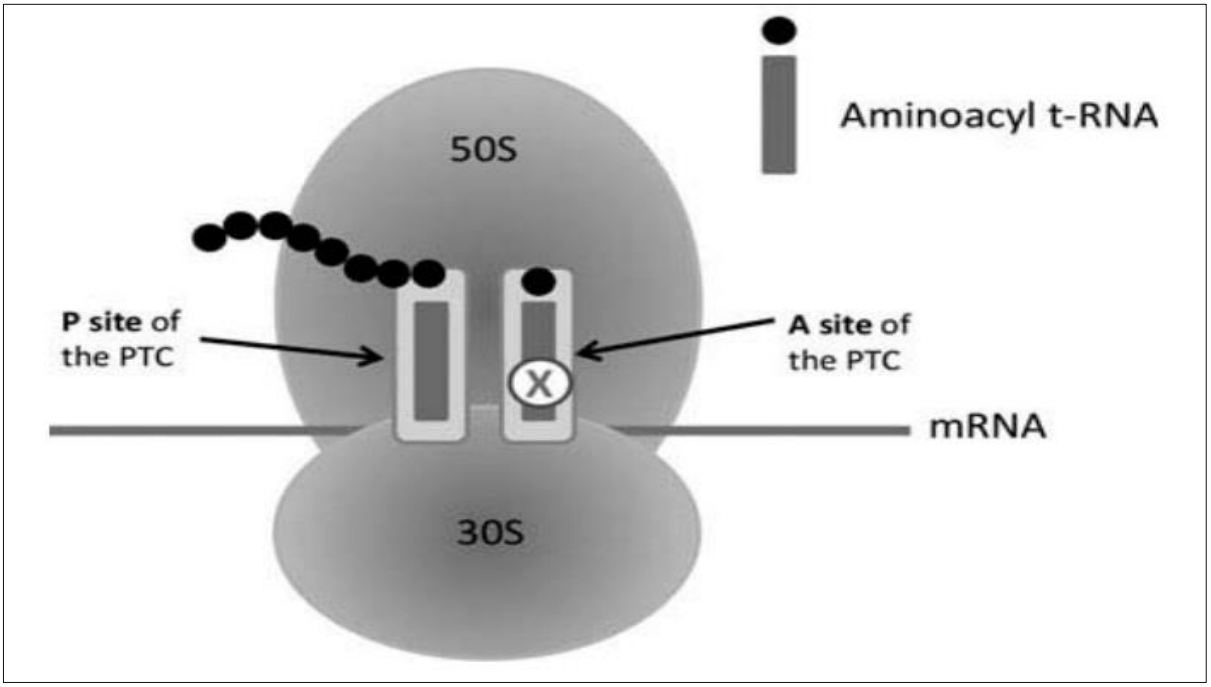

Figure 2.2: Linezolid (X) exerts antimicrobial action by inhibiting bacterial protein synthesis (Douros *et al.*, 2015)

Linezolid inhibits protein synthesis by binding to the aminoacyl site (A site) of the peptide

translocation centre (PTC) of the 50S ribosomal subunit, consequently preventing the formation of the larger 70S ribosomal complex that initiates protein synthesis (Gupta, 2016). Treatment with linezolid has become extensive for staphylococci and enterococci infections due to the unique mode of action, which prevents cross-resistance with other antimicrobial agents (Zahedi Bialvaei *et al.*, 2017).

2.5.2 Linezolid surveillance programs

The global Zyvox® Annual Appraisal of Potency and Spectrum (ZAAPS) program and the Linezolid Experience and Accurate Determination of Resistance (LEADER) program are two linezolid surveillance programs that monitor linezolid susceptibility (Bi *et al.*, 2018). The ZAAPS program has been conducted since 2002 and surveys the spectrum and activity of linezolid against Gram-positive pathogens outside of the United States of America (USA) (Mendes *et al.*, 2014b). The LEADER surveillance program, introduced in 2004, monitors and tracks the activity, spectrum and resistance of linezolid within the USA (Pfaller *et al.*, 2017). Both ZAAPS and LEADER continually monitor linezolid activity and resistance mechanisms, including the identification of emerging mechanisms involving the Gram-positive agents, staphylococci, enterococci and streptococci (Flamm *et al.*, 2016). The ZAAPS and LEADER linezolid resistance surveillance programs report that the overall incidence of linezolid resistance has remained low at < 1% (Pfaller *et al.*, 2017). However, antimicrobial therapy with linezolid must be used with caution and monitored for side effects and resistance development, especially during prolonged treatment of greater than two weeks (Mendes *et al.*, 2014a).

2.5.3 Linezolid use in *Mycobacterium tuberculosis*

About a quarter of the global populace is infected with *Mycobacterium tuberculosis* with the risk of developing tuberculosis (TB) (WHO, 2014). The latest 2019 TB report from the World Health Organization (WHO) states that TB remains amongst the top ten causes of mortality worldwide and the foremost cause of mortality from a single infectious agent, ranking above Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS) (WHO, 2014). South Africa is one of 14 countries which appear on the WHO list of high burden countries for TB, MDR-TB and TB/HIV infections (WHO, 2014). Linezolid is effective in treating resistant TB as an off-label treatment and is becoming part of new combination treatment regimens for MDR-TB and extensively drug resistant (XDR) TB (Song *et al.*, 2015; Kamp *et al.*, 2017). The complications of the high burden of HIV infections in South Africa combined with the high incidence rate of TB increases the necessity of linezolid in the treatment

of MDR-TB and XDR-TB (WHO, 2014; Song *et al.*, 2015; Kamp *et al.*, 2017).

2.5.4 Tedizolid: a next-generation oxazolidinone agent

Tedizolid, a next-generation oxazolidinone agent, is a prodrug with activity against Gram-positive pathogens, including mycobacteria (Burdette and Trotman, 2015; Ruiz *et al.*, 2019). A prodrug is an inactive agent that is converted chemically in the serum into the active agent (Ruiz *et al.*, 2019). Tedizolid is similar to linezolid in chemical structure, but the number of binding sites is increased due to a para-oriented ring structure (D-ring), which demonstrates enhanced antimicrobial action against bacteria that have reduced susceptibility to linezolid (Bassetti *et al.*, 2019). Most notably, tedizolid is not affected by the presence of the chloramphenicol-florfenicol resistance (*cfr*) gene and this is due to a chemical difference from linezolid, where tedizolid has a hydroxymethyl group in the lateral chain (Bassetti *et al.*, 2019). Therefore, antimicrobial cross-resistance to tedizolid is likely associated with chromosomal resistance rather than plasmid-mediated resistance (Hasannejad-Bibalan *et al.*, 2019). However, the activity of tedizolid against linezolid resistant strains requires further testing and research before recommendations can be made for treatment (Hasannejad-Bibalan *et al.*, 2019).

2.6 Adverse events in linezolid treatment

Linezolid causes several severe adverse effects, especially during prolonged use over periods of greater than 14 days (Douros *et al.*, 2015; Lee and Caffrey, 2018; Ruiz *et al.*, 2019). The common adverse effects of linezolid therapy affect the gastrointestinal tract and the central nervous system causing diarrhoea, nausea, vomiting and headache (Douros *et al.*, 2015; Santini *et al.*, 2018). Prolonged linezolid therapy has the potential to cause mitochondrial toxicity and dysfunction due to the secondary inhibition of mitochondrial protein synthesis, causing interference with mitochondrial function (Garrabou *et al.*, 2017; Santini *et al.*, 2017). Mitochondrial toxicities and dysfunction include peripheral neuropathy, optic neuropathy, lactic acidosis and myelosuppression (Song *et al.*, 2015). Other linezolid related toxicities include hematotoxicity, hepatotoxicity, hypoglycaemia, neurotoxicity and thrombocytopenia (Douros *et al.*, 2015; Santini *et al.*, 2018). Linezolid is metabolised in the liver and elimination takes place *via* non-renal (70%) and renal (30%) routes; however, hepatotoxicity is rare (Douros *et al.*, 2015; Santini *et al.*, 2018). Patients undergoing prolonged linezolid therapy should have lactate levels monitored weekly along with complete blood counts (Santini *et al.*, 2017). Linezolid toxicity effects are clinically relevant and must be taken into account as part of the benefit-risk evaluation before the commencement of linezolid therapy and the immediate

discontinuation of linezolid therapy is advised when toxicities begin to show (Douros *et al.*, 2015).

2.6.1 Monoamine oxidase inhibitors

The oxazolidinones are nonselective monoamine oxidase (MAO) inhibitors resulting in treatment challenges during concomitant use with serotonergic or adrenergic agents (Douros *et al.*, 2015). Administration of linezolid with serotonergic agents is not advised without observation for serotonin syndrome (Ramsey *et al.*, 2013). For patients receiving linezolid treatment alongside adrenergic agents such as imipramine, blood pressure monitoring is advised (Ramsey *et al.*, 2013). However, serotonergic interactions are more frequent than adrenergic interactions (Ramsey *et al.*, 2013).

2.7 Linezolid resistance mechanisms

Linezolid resistance is characterised by several mechanisms involving mutational and non-mutational resistance mechanisms (Jian *et al.*, 2018). Mutational resistance mechanisms are point mutations in the domain V region of the 23s rRNA gene and mutations in the *rplC*, *rplD* and *rplV* genes that encode the 50S ribosomal proteins L3, L4 and L22, respectively (Bender *et al.*, 2015). Non-mutational resistance mechanisms involve the acquisition of the *cfr*, *optr*A and *poxt*A genes that are plasmid-mediated and transferable between Gram-positive bacteria (Bender *et al.*, 2015; Bender *et al.*, 2018).

2.7.1 Point mutations of the 23S rRNA gene

Point mutations (a change in one nucleotide) of the 23S rRNA lead to resistance in enterococci and staphylococci (Bi *et al.*, 2018). The 23S rRNA gene mutation, which causes modification of the ribosome at the PTC, is the most common mechanism of linezolid resistance (Bi *et al.*, 2018). The PTC binding pocket comprises highly conserved nucleotides in staphylococci and enterococci that interact directly with linezolid (Mendes *et al.*, 2014a). Mutations of the 23S rRNA are frequently detected among staphylococci and enterococci isolates and mutation at the G2576U position is the most common (Takaya *et al.*, 2015). Various other 23S rRNA mutations within domain V have been reported such as G2447U, C2461U, U2500A, G2534U, G2603U and U2504A (Tewhey *et al.*, 2014). The various 23S rRNA mutational changes alter the function of the 23S rRNA proteins, resulting in decreased susceptibility to linezolid (Stefani *et al.*, 2010). As multiple copies of 23S rRNA genes exist in bacteria, resistance usually requires mutations in two or more copies (Bi *et al.*, 2018). The number of mutations of the 23S rRNA is directly related to the linezolid minimum inhibitory concentration (MIC) (Tewhey *et al.*,

2014). The number of 23S rRNA genes mutated depends on the duration and dosage of treatment with linezolid (Stefani *et al.*, 2010).

2.7.2 Mutations in 50S ribosomal proteins

Linezolid resistance has also been associated with mutations in the ribosomal L3, L4 and L22 proteins, which border the PTC where linezolid binds (Miller *et al.*, 2014; Tewhey *et al.*, 2014; Tian *et al.*, 2014). The L3, L4 and L22 mutations are often found together with or without mutations of the 23S rRNA gene and the presence of the *cfr* gene (Mendes *et al.*, 2014a). Mutations of the ribosomal L3, L4 and L22 proteins are associated with a four-fold increase in the linezolid MIC in staphylococci and enterococci (Wolter *et al.*, 2005; Miller *et al.*, 2014).

The L3, L4 and L22 proteins are encoded by the *rplC*, *rplD* and *rplV* genes, respectively (Gabriel *et al.*, 2015). The *rplC* gene encoding the L3 ribosomal protein is situated in the S10 operon, which is regulated by the L4 ribosomal protein, the product of the *rplD* gene, placed just downstream of the *rplC* gene (Klitgaard *et al.*, 2015). The *rplD* gene is crucial and is considered as one of the minimal set of genes essential for bacterial life (Wolter *et al.*, 2005). Growth studies by Wolter and colleagues (2005) found that the diminished growth rates of the transformants indicated that the L4 mutations are related to fitness cost. Mutations may inhibit antimicrobial binding, but the consequence of this is that protein synthesis may be affected (Wolter *et al.*, 2005). Bacteria adapt to a decline in fitness as a consequence of resistance mutations by cultivating compensatory mutations that restore their fitness without affecting resistance (Wolter *et al.*, 2005). However, few studies have confirmed the relationship of each of the ribosomal protein mutations with the levels of linezolid resistance (Mendes *et al.*, 2014a).

2.7.3 Acquisition of plasmid-mediated resistance genes

The *cfr* gene is a plasmid-mediated non-mutational resistance mechanism in staphylococci and enterococci (Jian *et al.*, 2018). Methicillin-resistant *S. aureus* was originally detected in 1997 in a *Staphylococcus* spp. isolate of animal origin, however, the first report of clinical linezolid-resistant MRSA with an acquired *cfr* gene originated from Colombia in 2007 (Bender *et al.*, 2015). The *cfr* gene encodes an RNA methyltransferase that targets and alters the adenine nucleotide of the 23S rRNA at position A2503, specifically by adding a methyl group at the C-8 position (Long and Vester, 2012; Bender *et al.*, 2015). The 23S rRNA nucleotide A2503 is located in a functionally critical region of the ribosome (LaMarre *et al.*, 2011). The modification of A2503 in domain V of the 23S rRNA confers resistance, thus hindering the binding of linezolid (Tewhey *et al.*, 2014). Additionally, the modification of the adenine nucleotide

confers MDR to the following antimicrobial classes: phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A (known as the PhLOPSA phenotype) (Jian *et al.*, 2018).

The *cfr* gene is increasingly being detected in Gram-positive cocci (Pfaller *et al.*, 2017). The spread of the *cfr* gene is directly related to mobile elements and low fitness cost associated with the gene expression (LaMarre *et al.*, 2011; Long and Vester, 2012). Any genes that significantly decrease cell fitness are lost in the absence of selection pressure, while even in the absence of exposure to antimicrobial agents, those that occur at a low cost persevere in the cells (LaMarre *et al.*, 2011). Competition experiments involving wild-type and inactivated *cfr* reveal that the small fitness cost relates to expressing the protein rather than the C-8 methylation (Long and Vester, 2012). The low fitness cost of *cfr* is disconcerting as cells are likely to be inclined to maintaining the gene even in the absence of antimicrobial selection (Long and Vester, 2012). Predicting the rate of expansion of a resistance mechanism is driven by understanding its maintenance efficiency and its fitness cost (LaMarre *et al.*, 2011).

In addition to the *cfr* gene, other plasmid-mediated resistance genes have recently emerged, namely: *cfr*(B), *optr*A and *poxt*A (Sadowy, 2018; Sassi *et al.*, 2019). The *cfr*(B) gene, recently found in *E. faecium*, is a variant of the *cfr* gene sharing 75% nucleotide identity with the *cfr* gene (Sassi *et al.*, 2019). The *optr*A gene confers resistance to oxazolidinones and phenicols (Klupp *et al.*, 2016; Sassi *et al.*, 2019). The *poxt*A gene confers a decrease in susceptibility to phenicols, oxazolidinones and tetracyclines (Antonelli *et al.*, 2018). However, the prevalence of the *poxt*A gene has yet to be defined (Sassi *et al.*, 2019).

2.8 Antimicrobial susceptibility testing of *Staphylococcus* and *Enterococcus*

Antimicrobial susceptibility testing is essential to confirm the level of antimicrobial resistance in bacterial isolates (Jorgensen and Ferraro, 2009). Antimicrobial susceptibility testing is performed by disk diffusion, broth microdilution and ETEST® (bioMérieux, France) methods to determine the susceptibility of a bacterium against various antibiotics (Jorgensen and Ferraro, 2009; Bard and Lee, 2018). Additionally, automated platforms are also routinely employed such as the VITEK® 2 automated system (bioMérieux, France), MicroScan (Beckman Coulter, USA) and Sensititre (Thermo Scientific, USA) (Humphries and Hindler, 2016; Doern, 2018). The MIC is the smallest amount of an antimicrobial agent required to inhibit the visible growth of a bacterium (Bard and Lee, 2018). Antimicrobial susceptibility results are reported as susceptible, intermediate or resistant (Rodloff *et al.*, 2008). Disk zones and MIC values are interpreted using clinical breakpoints as set out by the Clinical and Laboratory Standards

Institute (CLSI), which set consensus standards followed by laboratories worldwide (CLSI, 2019). Susceptible indicates that the bacterium is adequately responsive to the antimicrobial agent; therefore there is a high likelihood of therapeutic success using recommended doses (Rodloff *et al.*, 2008; Bard and Lee, 2018). Intermediate indicates that the bacterial strain is inhibited *in vitro* by a concentration of the antimicrobial agent; therefore clinical efficacy might still be achieved depending on the dosage or site of infection (Rodloff *et al.*, 2008; Bard and Lee, 2018). Resistant indicates that the antimicrobial being tested is likely to fail if used for treatment (Rodloff *et al.*, 2008; Bard and Lee, 2018). Determining antimicrobial susceptibility of clinical isolates is required for providing optimum antimicrobial therapy for patients but also for monitoring the spread of resistance throughout the hospital and the community (Foxman *et al.*, 2005).

2.9 Identification and characterisation of *Staphylococcus* and *Enterococcus*

The identification of isolates to species level is essential in understanding the pathogenic potential of the bacterium, determine clinical significance and to guide antimicrobial treatment (Pottinger *et al.*, 2014b; Stratton, 2018). Species identification methods for staphylococci and enterococci consist of phenotypic-based methods, proteomics-based methods and nucleic acid-based methods (Cherkaoui *et al.*, 2010; Becker *et al.*, 2014).

2.9.1 Phenotypic identification of *Staphylococcus* and *Enterococcus*

Phenotypic identification methods have been conventionally used in diagnostic laboratories to identify bacteria isolated from clinical specimens (Manuselis and Mahon, 2015). Conventional identification procedures include tube-based biochemical reactions and observation of physical characteristics such as colony morphology, odour, Gram-staining and agglutination tests (Carroll and Patel, 2015). Staphylococci and enterococci are non-fastidious; therefore blood agar and nutrient agar can routinely be used for culture (Manuselis and Mahon, 2015). Selective or differential media, such as mannitol salt agar and bile esculin azide agar can be used for the identification of staphylococci and enterococci, respectively (Mahlen and Kumar, 2015). Mannitol salt agar and bile esculin azide agar contain chemical substances to enhance the growth of selected species of staphylococci and enterococci while inhibiting other species (Mahlen and Kumar, 2015).

Phenotypic identification methods of staphylococci and enterococci are popular due to affordability, yet the time involved and possible failure in culturing are disadvantageous (Manuselis and Mahon, 2015). Due to the need for faster and simpler methods, several

automated systems for the identification of bacterial isolates have been developed such as the VITEK® 2 automated system (bioMérieux, France), BD Phoenix™ (BD Life Sciences, USA), API Staph ID 32 (bioMérieux, France) and the MicroScan WalkAway (Beckman Coulter, USA) (Carroll and Patel, 2015).

2.9.2 Proteomic identification of *Staphylococcus* and *Enterococcus*

The automated proteomic matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) system provides rapid and accurate species-level identification of bacteria by examining the ribosomal protein profiles of intact cells direct from culture (Cherkaoui *et al.*, 2010). A laser ionises the biomolecules in an electric field which enter a flight tube (Mahlen and Kumar, 2015). Mass-to-charge ratios separate the biomolecules which generate a specific signal creating a molecular signature (Mahlen and Kumar, 2015). Final species-level identification is based on matching MS patterns from the isolate, which are matched with mass spectra contained in a reference library (Chun *et al.*, 2015). Newer MALDI-TOF MS instruments have the ability to perform combined automated antimicrobial susceptibility testing and species identification; therefore, MALDI-TOF MS has the potential to replace phenotypic and genotypic methods in routine diagnostics (Rupp and Fey, 2015).

2.9.3 Nucleic acid identification of *Staphylococcus* and *Enterococcus*

The identification and characterisation of *Staphylococcus* and *Enterococcus* through nucleic acid-based approaches is more sensitive and specific than the traditional phenotypic culture-based approaches (Roberts, 2014). Nucleic acid-based assays are more commonly utilised in reference and research laboratories but have a limited application in routine diagnostic laboratories due to the high cost associated with molecular methods (Hirotaki *et al.*, 2011; Becker *et al.*, 2014). Polymerase chain reaction (PCR) is a method that allows the amplification of pre-determined genes (DNA regions) using specifically targeted primers (Al-Talib *et al.*, 2009; Becker *et al.*, 2014). Identification of *Staphylococcus* and *Enterococcus* using PCR, multiplex polymerase chain reaction (M-PCR) and real-time PCR assays are the most common nucleic acid-based approaches used to identify the species (Becker *et al.*, 2014; Roberts, 2014). Additionally, various PCR assays have been developed to identify genus, species, virulence factors and antimicrobial resistance genes (Al-Talib *et al.*, 2009). Other nucleic acid-based techniques such as Gene Xpert (Cepheid, USA), fluorescent *in situ* hybridisation (FISH) and microarrays are not commonly used to identify staphylococcal and enterococcal isolates (Wilson *et al.*, 2015).

2.9.3.1 Molecular detection of the *cfr* resistance gene

The presence of the *cfr* gene is detected using specific PCR assays after DNA extraction (Silva-Del Toro *et al.*, 2016). Specific primers and PCR running conditions target the *cfr* gene in a singleplex PCR assay; therefore, the detection of the *cfr* gene is a cost-effective and straightforward process (Doern *et al.*, 2016). The *cfr* gene amplicons are 746 bp in size and are detected using conventional gel electrophoresis (Doern *et al.*, 2016). Additionally, the *cfr* gene can be present with or without 23S rRNA gene mutations both of which can be confirmed using whole-genome sequencing (WGS) (Bi *et al.*, 2018).

2.9.4 Molecular typing of *Staphylococcus* and *Enterococcus*

Molecular typing aims to uncover the genetic diversity among different isolates of the same bacterial species of which genetically related strains have the same characteristics and epidemiologically unrelated strains have different characteristics (Tenover *et al.*, 1997; Li *et al.*, 2009). The mechanisms of transmission and pathogenesis of a disease can be determined by molecular typing which improves prevention efforts (Foxman *et al.*, 2005; Li *et al.*, 2009). Molecular strain typing is useful in surveillance, outbreak investigations, determination of relatedness and to follow the course of an infection (Foxman *et al.*, 2005; Li *et al.*, 2009). Molecular typing assays involve nonamplified methods such as pulsed-field gel electrophoresis (PFGE) and amplified methods such as multilocus sequence typing (MLST) and WGS (Mahlen and Kumar, 2015).

2.9.4.1 Pulsed-field gel electrophoresis of *Staphylococcus* and *Enterococcus*

Pulsed-field gel electrophoresis is a nonamplified DNA fingerprinting/strain typing technique based on the analysis of enzymatic restriction fragments of DNA (Mahlen and Kumar, 2015). Pulsed-field gel electrophoresis is a useful molecular typing technique for typing many different bacterial species (Tenover *et al.*, 1997). The DNA is digested with a restriction enzyme, an endonuclease such as *Sma*I for Gram-positive bacteria (Becker *et al.*, 2015). The restriction enzyme generates DNA fragments that can be resolved as a pattern of distinct bands by PFGE (Tenover *et al.*, 1997). Pipetting during DNA extraction mechanically shears the DNA resulting in unacceptable quality for PFGE separations; therefore, in PFGE, bacterial cells are embedded directly into agarose (Lonza, USA) plugs with the restriction enzyme (Sharma-Kuinkel *et al.*, 2016). Lysis of the entire cell takes place inside the agarose (Lonza, USA) plugs allowing purification of DNA without shearing, yielding DNA fragments at different lengths, providing a fingerprint of the whole genome (Sharma-Kuinkel *et al.*, 2016). Standard DNA gel

electrophoresis only resolves fragments up to 50 kb in size, but PFGE separates large genomic DNA fragments (entire genomic DNA) up to 10 Megabase (Mb) in size (Herschleb *et al.*, 2007; Sharma-Kuinkel *et al.*, 2016). The restricted fragments are separated by time-associated size-dependent reorientation of DNA migration on agarose (Lonza, USA) gel electrophoresis in an alternating voltage gradient in which the orientation of the electrical field switches direction (Herschleb *et al.*, 2007; Ranjbar *et al.*, 2014). Resultant PFGE banding patterns serve as virtual barcodes that compare different patterns and identifies similarities, providing typing of the strains and determines the relatedness of the isolates (Sharma-Kuinkel *et al.*, 2016). Strain typing data is used in the investigation of infection outbreaks (Foxman *et al.*, 2005). The comparison of the banding patterns shows isolates belonging to the same strain, therefore showing that isolates of the same strain are clonally related (Foxman *et al.*, 2005). Additionally, strain data indicates the source of the isolate such as hospital-associated or community-associated (Lakhundi and Zhang, 2018).

Visual analysis of the banding patterns group strains as 'related' (i.e. identical banding patterns), 'closely related' (i.e. three or fewer band differences), 'possibly related' (i.e. four to six band differences) and 'different' (i.e. ≥ seven-band differences) (Tenover *et al.*, 1995; Widerstrom *et al.*, 2012). Comparison and analysis of PFGE band patterns is done using computer software such as the GelCompar II (Applied Maths, Belgium) program that uses the Dice coefficient and the "unweighted pair group method with arithmetic mean" (UPGMA) to construct a dendrogram (Widerstrom *et al.*, 2012; Goering and Fey, 2014). Isolates are considered genetically related if the banding pattern of the cluster shows ≥ 80% similarity (Applied Maths, Belgium), which corresponds to the Tenover criteria (Tenover *et al.*, 1995). The cataloguing of clusters are based on the number of isolates grouped together at the ≥ 80% similarity cut off, with five or more isolates in one group indicating a major cluster, less than five isolates in a group indicate minor clusters and single isolates are described as singletons (Tenover *et al.*, 1995). Pulsed-field gel electrophoresis, being highly discriminatory, has been considered the "gold standard" of the molecular typing methods because of the high degree of isolate differentiation (Li *et al.*, 2016). However, PFGE is being challenged by WGS, which can identify genetic variation, distinguishing strains from one another at the base sequence level (Goering and Fey, 2014).

2.9.4.2 Multilocus sequence typing of *Staphylococcus* and *Enterococcus*

Multilocus sequence typing is a highly discriminatory amplified DNA fingerprinting method

that uses PCR amplification of seven housekeeping genes to characterise bacterial species and the genes are compared with the sequences of other isolates collected in a central database (https://pubmlst.org) (Szabó, 2014; Que and Moreillon, 2015). Housekeeping genes are excellent controls in molecular methods due to the constitutive natures of the genes, meaning they are transcribed continually and are always being expressed (Mahlen and Kumar, 2015). Each isolate is characterised by the alleles (a variant of the similar DNA sequence located at a given locus) at each of the seven loci (fixed positions on a chromosome) and the profile of all seven alleles for the isolate is defined a sequence type (ST) (Perez-Losada *et al.*, 2013). Bacteria that share all seven alleles are defined as clones, sharing five identical alleles is a clonal complex (CC) and sharing less than five alleles is defined as unrelated (Que and Moreillon, 2015). There are limitations for MLST as routine infection control, or outbreak investigations are not well suited for MLST due to the high cost and labour intensity involved in the sequencing of the PCR amplicons (Szabó, 2014). Another limitation of MLST is that only approximately 0.1% of the genome is examined (Tang *et al.*, 2017). However, since the introduction of DNA sequencing technology, the sequencing of entire genomes is now possible and affordable (Tang *et al.*, 2017). Therefore, WGS offers higher discriminatory power than current reference standard typing tools such as PFGE and MLST (Rossen *et al.*, 2018).

2.9.4.3 Whole-genome sequencing to investigate linezolid resistance mechanisms

Whole-genome sequencing is a powerful molecular typing method used to identify pathogens and discriminate between closely related isolates (Lindsay, 2014; Gilchrist *et al.*, 2015). Whole-genome sequencing can be used to study genetic changes such as horizontal gene transfer (HGT), recombination, gene insertions and gene deletions (Li *et al.*, 2009). Additionally, WGS can be used to identify single nucleotide polymorphisms (SNPs) which are nucleotide variations occurring within a specific part of a DNA sequence between members of a species (Li *et al.*, 2009). Typing approaches such as MLST and SNP analysis are becoming redundant in favour of WGS (Ranjbar *et al.*, 2014).

Resultant data from WGS can rapidly and accurately identify outbreak isolates, identify virulence factors and generate information on the presence of antimicrobial resistance genes - the ‘resistome’ (Torok and Peacock, 2012; Gilchrist *et al.*, 2015). The utilisation of WGS in outbreak analysis can be employed to map the route of disease transmission within a population and present information on the likely source, with the potential to reduce further infection, morbidity and cost (Harris *et al.*, 2013; Gilchrist *et al.*, 2015). The increase in data volumes

from WGS will challenge laboratories to become proficient in processing genome data (Tang *et al.*, 2017). The augmentation of existing epidemiological and genotyping methods will contribute to infection prevention and control as successful interventions in disease outbreaks can be more targeted (Tang *et al.*, 2017). Typing data generated from WGS is vast and in-depth, which contributes to the increasing redundancy of traditional molecular typing approaches (Tyson *et al.*, 2018). Therefore, the advancement of WGS technologies has the potential to become the sole diagnostic, molecular characterisation and epidemiological tool (Ranjbar *et al.*, 2014).

2.10 Summary

The growing prevalence of antimicrobial resistance in Gram-positive pathogens, such as staphylococci and enterococci, is a significant public health concern that complicates efforts to prevent and treat infectious diseases (Gould, 2012; Doernberg *et al.*, 2017). The spread of antimicrobial resistance represents one of the most grave perils to human health and if left unchecked (Koser *et al.*, 2014). Resistance to antimicrobials is a global challenge that risks placing humanity into a post-antimicrobial era where many advances in the treatment and prevention of diseases and conditions become negated through the risk of untreatable infection (van Harten *et al.*, 2017). Estimates show that at least 700 000 people die per year due to infections that are due to MDR bacteria and this is projected to escalate to 10 million deaths per year by 2050 if the current trajectory is not altered (Crofts *et al.*, 2017). The management of staphylococci and enterococci infections relies not only on fast, accurate detection but effective treatment to reduce morbidity and mortality of patients and to prevent potential outbreaks; therefore, it is imperative for laboratories to accurately identify resistance mechanisms (Doern *et al.*, 2016).

Linezolid is crucial to the treatment of infections caused by Gram-positive bacteria, including staphylococci and enterococci, but irrational treatment with linezolid could elevate the MIC value resulting in resistance (Bi *et al.*, 2018). Healthcare providers need to evaluate the antimicrobial susceptibility of bacterial pathogens using surveillance such as ZAAPS and LEADER that collect relevant data for tracking linezolid resistance (Bi *et al.*, 2018). With the decreasing efficacy of antimicrobials, healthcare facilities around the world are experiencing growing numbers of MDR infections that are increasingly difficult to effectively treat, resulting in an upturn in patient morbidity, mortality and growing healthcare costs (van Harten *et al.*, 2017). Precautions need to be in place to take into account risk factors such as long-term

hospitalisation, prior exposure to antimicrobials and underlying diseases in the prophylaxis of linezolid resistance (Bi *et al.*, 2018).

Data regarding linezolid resistance in staphylococci and enterococci in South Africa is limited. The purpose of this study was to detect and characterise clinical linezolid resistant staphylococci and enterococci isolates, as well as to determine the genetic relatedness of these strains circulating in public and private sectors in Gauteng, South Africa. The study also aimed to determine the prevalence of linezolid resistance among these pathogens and to determine the linezolid resistance mechanism present. Phenotypic methods employed in the study were ETEST® (bioMérieux, France) and molecular methods employed in the study were PCR assays, PFGE and WGS.

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CHAPTER 3

Linezolid resistance mechanisms in multidrug-resistant staphylococcal isolates collected from a private laboratory in Gauteng, South Africa

The editorial style of the International Journal of Antimicrobial Agents was followed in this chapter

3.1 Abstract

Linezolid resistance is emerging among multidrug-resistant (MDR) staphylococcal infections due to common resistance mechanisms such as 23S ribosomal ribonucleic acid (rRNA) gene mutations and acquisition of the plasmid-mediated chloramphenicol-florfenicol resistance (*cfr*) gene. Seventy-nine linezolid resistant staphylococcal isolates cultured from blood were obtained from private hospitals. Blood cultures were processed using the BACT/ALERT® 3D (bioMérieux, France) followed by identification using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Bruker, USA). Speciation was confirmed using multiplex polymerase chain reaction (M-PCR) assays. Linezolid susceptibility results were determined using the VITEK® 2 automated system (bioMérieux, France) and re-evaluated using the ETEST® (bioMérieux, France). Pulsed-field gel electrophoresis (PFGE) determined genetic relatedness. Seven representative *S. epidermidis* isolates and three *S. capitis* isolates were selected for whole-genome sequencing (WGS) to ascertain 23S rRNA gene mutations. The M-PCR assays identified *S. capitis*, n = 43; *S. epidermidis*, n = 27 and *S. haemolyticus*, n = 9. The ETEST® (bioMérieux, France) minimum inhibitory concentration (MIC) values for linezolid ranged between 8 µg/mL and > 256 µg/mL. Using a polymerase chain reaction (PCR) assay, the *cfr* gene was only detected in eight (29.6%) of the 27 *S. epidermidis* isolates and none of the other staphylococcal species. The PFGE showed clonal relatedness within clusters and genetic variability among single isolates. The sequence types (ST) among the *S. epidermidis* isolates included ST23 (57.1%, n = 4/7), ST2 (28.6%, n = 2/7) and ST22 (14.3%, n = 1/7). Various 23S rRNA gene mutations were found in the *S. epidermidis* isolates and the *S. capitis* isolates. The presence of these resistance mechanisms confirms that antimicrobial stewardship efforts need to be intensified in South Africa.

Keywords: *Staphylococcus*, Linezolid resistance, Antimicrobial treatment, *cfr*, 23S rRNA, MDR

3.2 Introduction

Linezolid is an oxazolidinone antimicrobial agent indicated for Gram-positive infections and approved for the treatment of multidrug-resistant (MDR) staphylococcal infections caused by *Staphylococcus capitis*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* [1, 2]. Multidrug-resistance indicates resistance to three or more antimicrobial classes of antimicrobial agents [3]. Linezolid was introduced for clinical use in the year 2000 and has been used successfully in the treatment of MDR infections including methicillin-resistant *Staphylococcus aureus* (MRSA) [4, 5]. Advantages of linezolid use include 100% bioavailability in the oral form and effective tissue penetration [6]. Disadvantages include myelosuppression, lactic acidosis, serotonin syndrome and optic neuropathy [6]. The use of linezolid in the treatment of staphylococcal infections is increasing due to MDR among the coagulase-negative staphylococci (CoNS) and nephrotoxicity caused by vancomycin [2, 7]. Globally, approximately 2% of CoNS are resistant to linezolid, but the rates of resistance may be higher due to under-reporting and high rates of linezolid usage [8].

The mode of action of linezolid is unique to the oxazolidinone class of antibiotics, preventing cross-resistance to other protein synthesis inhibitors such as tetracycline, erythromycin and clindamycin [9]. Bacterial protein synthesis is inhibited by linezolid by targeting the peptide translocation centre (PTC) and hindering the assembly of the ribosome [4]. The mechanisms that mediate linezolid resistance are facilitated by chromosomal- and plasmid-mediated mechanisms [10]. Chromosomally-mediated mechanisms involve mutations in the 23S ribosomal ribonucleic acid (rRNA) gene that alters the linezolid binding site [10, 11]. The plasmid-mediated resistance mechanism is less common and involves the acquisition of the chloramphenicol-florfenicol resistance (*cfr*) gene that encodes a methyltransferase [5, 11]. The *cfr* gene is of great concern due to facilitating interspecies spread identified among staphylococcal spp. [5]. The result of the acquisition of the *cfr* gene confers phenotypic drug resistance to the following antimicrobial classes: phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A (known as the PhLOPSA phenotype) [12].

Linezolid is considered one of the few remaining available treatment options for the management of MDR staphylococcal infections; therefore laboratory methods should first correctly identify resistant infections treated with linezolid, taking into account the serious nature of such infections [5, 11]. The focus of this study was to determine the presence of two linezolid resistance mechanisms involving mutations in the 23S rRNA gene and the acquired

cfr gene among MDR staphylococcal isolates recovered from blood cultures displaying phenotypic resistance to linezolid.

3.3 Methods

3.3.1 Study setting and isolate collection

This study was a retrospective, observational study. A total of 79 linezolid resistant staphylococcal isolates were collected from clinical blood cultures. Isolates were collected by Ampath Laboratories from 2016 to 2018 across 26 private hospitals in Gauteng, South Africa. Among the 79 isolates, 75 were blood cultures and four were cultures from central venous catheters. Blood cultures were processed using the BACT/ALERT® 3D (bioMérieux, France). The isolates were initially identified to species level by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) (Bruker, USA). Initial antimicrobial susceptibility testing was performed using the VITEK® 2 automated system (bioMérieux, France). The inclusion criterion was any staphylococcal isolate that was resistant to linezolid with a minimum inhibitory concentration (MIC) value ≥ 8 µg/mL, according to the 2019 Clinical and Laboratory Standards Institute (CLSI) staphylococcal guidelines. Only one isolate per patient was included to avoid duplication. Unfortunately, patient data regarding underlying diseases and prior antimicrobial treatment were not available.

3.3.2 Processing and storage of staphylococcal isolates

The staphylococcal isolates were collected from Ampath Laboratories and streaked onto blood agar (Oxoid Ltd, UK) and incubated (Vacutec, South Africa) overnight at 37°C. Following the incubation period, the blood agar (Oxoid Ltd, UK) plates were visually inspected for colony purity and morphology. Gram-staining was carried out to ensure culture purity. A single pure colony from each blood agar (Oxoid Ltd, UK) plate was inoculated into 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA) containing brain heart infusion (BHI) broth (Merck, Germany) and incubated (Stuart, UK) with constant shaking at 220 rpm overnight at 37°C. A negative BHI broth (Merck, Germany) control was included to ensure contamination had not taken place. The BHI broth (Merck, Germany) cultures were prepared in a 50% glycerol (Merck, Germany) solution at a 1:1 ratio and stored at -20°C for future analysis.

3.3.3 Linezolid antimicrobial susceptibility testing

All linezolid resistant staphylococcal isolates underwent repeat linezolid antimicrobial susceptibility testing using ETEST® (bioMérieux, France) to confirm linezolid resistance found with the VITEK® 2 automated system (bioMérieux, France). Briefly, stored isolates were

streaked onto blood agar (Oxoid Ltd, UK) and incubated (Vacutec, South Africa) at 37°C for 24 hours. Single colonies from each isolate were used to make saline cell suspensions and adjusted to match the 0.5 McFarland standard using a Densichek (bioMérieux, France). The cell suspensions were inoculated onto Mueller-Hinton agar (Oxoid Ltd, UK) within 15 minutes of preparation. The linezolid ETEST® (bioMérieux, France) strip was aseptically placed onto the inoculated Mueller-Hinton agar (Oxoid Ltd, UK) and incubated (Vacutec, South Africa) at 37°C for 18 hours. *Staphylococcus aureus* American Type Culture Collection (ATCC) 29213 was used for quality control. The interpretation of the MIC values was based on the 2019 CLSI guidelines. The 2019 CLSI staphylococcal breakpoint guidelines specify that linezolid MIC values of ≤ 4 µg/mL indicate susceptibility and ≥ 8 µg/mL indicate resistance [13].

3.3.4 Total genomic DNA extraction

The genomic deoxyribonucleic acid (DNA) of the staphylococcal isolates was extracted using a commercial kit, the ZymoResearch (ZR) Fungal/Bacterial DNA MiniPrep™ kit [14]. Briefly, a single pure colony from each isolate was inoculated into 2 mL of BHI broth (Merck, Germany) and incubated (Stuart, UK) with constant shaking at 220 rpm overnight at 37°C. The DNA extraction was carried out using the cultured BHI broth (Merck, Germany) as per manufacturer instructions and the extracted pure DNA underwent quantitation using a NanoPhotometer (Implen, Germany). The pure DNA was stored at -20°C for all downstream polymerase chain reaction (PCR) applications.

3.3.5 Identification of staphylococcal isolates

Identification and species confirmation of the staphylococcal isolates were conducted using multiplex polymerase chain reaction (M-PCR) assays using previously described primers shown in Table 3.1 [15-17]. The genes targeted in the M-PCR assays included the 16S rRNA gene for the *Staphylococcus* genus (597 bp), the superoxide dismutase (*sodA*) gene of *S. capitis* (103 bp), the nuclease (*nuc*) gene of *S. epidermidis* (251 bp) and the nuclease (*nuc*) gene of *S. haemolyticus* (434 bp). Positive controls used were reference strains *S. capitis* ATCC 35661, *S. epidermidis* ATCC 12228 and *S. haemolyticus* ATCC 29970 and nuclease-free water (QIAGEN, Netherlands) was used for the negative control. The reaction composition used for the identification of staphylococcal isolates included the following: 7.5 µL of MyTaq™ Red Mix (Bioline, UK), 0.15 µL of forward primer (0.2 µM), 0.15 µL of reverse primer (0.2 µM), 6.7 µL of nuclease-free water (QIAGEN, Netherlands) and 0.5 µL of template DNA to make up a total reaction volume of 15 µL. The PCR thermocycler (BioRad, UK) conditions were as

follows: 95°C for 5 min; 35 cycles of 95°C for 30 sec, 55°C for 3 min, 72°C for 90 sec and a final cycle of 72°C for 10 min. The visualisation of PCR amplicons is detailed in section 3.3.7.

3.3.6 Molecular detection of the *cfr* gene

All staphylococcal isolates were screened for the presence of the *cfr* gene (746 bp) using a PCR assay with primers previously described by Doern *et al*. (2016) as shown in Table 3.2. The reaction composition used to detect the *cfr* gene was as follows: 7.5 µL of MyTaq™ Red Mix (Bioline, UK), 0.15 µL of forward *cfr* gene primer (0.2 µM), 0.15 µL of reverse *cfr* gene primer (0.2 µM), 6.2 µL of nuclease-free water (QIAGEN, Netherlands) and 1 µL of template DNA to make up a reaction volume of 15 µL. The PCR thermocycler (BioRad, UK) conditions were as follows: 94°C for 10 min; 35 cycles of 94°C for 30 sec, 57°C for 30 sec, 74°C for 30 sec and a final cycle of 72°C for 10 min. The PCR amplicons were visualised as described in section 3.3.7.

3.3.7 Visualisation of PCR amplicons

The DNA amplicons of all M-PCR and PCR assays were visualised using agarose (Lonza, USA) gel electrophoresis on a 2% agarose (Lonza, USA) gel stained with 5 µL of ethidium bromide (10 µg/mL) (Sigma-Aldrich, USA) which was added to the gel before casting. The agarose (Lonza, USA) gels were run in a 1x Tris-Borate-Ethylenediaminetetraacetic acid (TBE) (Sigma-Aldrich, USA) buffer at 100 V for 100 min. A 50 bp DNA ladder (ThermoScientific, USA) was included as a molecular weight marker to determine the sizes of the amplicons. The amplicons within the agarose (Lonza, USA) gel were visualised under ultraviolet (UV) light using the Gel Doc EZ System (BioRad, UK) and the bands were compared to the 50 bp DNA ladder (ThermoScientific, USA).

3.3.8 Molecular typing using pulsed-field gel electrophoresis

The genetic relatedness of the staphylococcal isolates was determined using pulsed-field gel electrophoresis (PFGE). The PFGE protocol was conducted as per the Unified PFGE protocol for Gram-positive bacteria, defined by the Division of Healthcare Quality Promotion, Centers for Disease Control and Prevention [18]. A minor modification was added to the protocol which included an overnight lysis step of the plugs at 51°C (Stuart, UK) to ensure complete lysis. The genomic DNA of the isolates was digested in agarose (Lonza, USA) plugs using the *Sma*I restriction enzyme (New England Biolabs, UK) and separated on a 1.2% SeaKem low electroendosmosis (LE) agarose (Lonza, USA) gel. The gel was run in a 0.25x TBE (Sigma-Aldrich, USA) buffer using the Rotaphor PFGE System 6.0 (Biometra, Germany). The

S. aureus reference strain ATCC 12600 was used as a molecular size standard for normalisation [19]. The PFGE running parameters were set at 220 V linear to 200 V at a constant angle of 120°. The interval inverse was off and the switch time was 5 sec linear to 40 sec. The gel ran for 25 hours at 13°C. After 25 hours, the gel was stained with ethidium bromide (0.25 µg/mL) (Sigma-Aldrich, USA) for 30 min, followed by destaining for 30 min by submerging the gel in ultrapure water. During staining and destaining, the gel was kept protected from ambient light to prevent photobleaching. After destaining, the gel was viewed and photographed under UV light using the Gel Doc XR+ System (BioRad, UK).

The resulting PFGE banding patterns were analysed using BioNumerics Seven (Applied Maths, Belgium) with a similarity cut-off of 80% to define relatedness [19]. A major cluster was defined, consisting of ≥ 5 isolates and a minor cluster defined consisted of < 5 isolates [19]. Cluster analysis was determined using the Dice Coefficient (with 0.5% tolerance and 0.5% optimisation), which is a band-based similarity coefficient measuring the similarity of common and different bands.

3.3.9 Whole-genome sequencing of representative isolates

Ten isolates were further investigated using whole-genome sequencing (WGS). Seven representative *S. epidermidis* isolates and three representative *S. capitis* isolates were selected based on ETEST® (bioMérieux, France) results and diversity according to the PFGE dendrogram data. The seven *S. epidermidis* isolates consisted of four *cfr* positive isolates and three *cfr* negative isolates with MIC values ranging between 8 µg/mL and > 256 µg/mL. The three *S. capitis* isolates were all *cfr* negative and were chosen from two major PFGE clusters for WGS due to variation in MIC values which ranged between 24 µg/mL and 128 µg/mL. The *S. haemolyticus* isolates were excluded from WGS due to the small sample size of the isolates. Whole-genome sequencing was performed using the Illumina MiSeq instrument with 100x coverage and sequenced as paired-end read lengths of 2 x 300 base pair (bp). The generated read data from Illumina MiSeq sequencing was quality checked using FastQC V0.11.8 to determine the quality and depth of the raw reads [20]. The sequences were trimmed with Trimmomatic V0.39 [21]. The output from Trimmomatic V0.39 was confirmed using FastQC V0.11.8, followed by *de novo* assembly using SPAdes V3.13.0 [22]. The assembled *S. epidermidis* genomes were between 2.5 Mb and 2.7 Mb in length with an average GC content of 31.9% and the assembled *S. capitis* genomes were approximately 2.5 Mb in length with an average GC content of 32.7%. The *de novo* assemblies from SPAdes V3.13.0 were confirmed

against the National Center for Biotechnology Information (NCBI) reference genomes using QUAST [23]. Once the assembly mapping to the reference genomes was confirmed using QUAST, the contigs were run against the ResFinder database to identify point mutations within the domain V of the 23S rRNA gene [24, 25].

3.4 Results

3.4.1 Linezolid antimicrobial susceptibility

Linezolid resistance was confirmed using the ETEST® (bioMérieux, France) and compared against the initial antimicrobial susceptibility data obtained from the VITEK® 2 automated system (bioMérieux, France) (Table 3.3 and Figure 3.1). Only 68 isolates (26 *S. epidermidis* isolates, 37 *S. capitis* isolates and five *S. haemolyticus* isolates) were subjected to the ETEST® (bioMérieux, France), since 11 isolates (one *S. epidermidis* isolate, six *S. capitis* isolates and four *S. haemolyticus* isolates) were not successfully recovered from cryo-storage (Table 3.7). The 2019 CLSI breakpoint tables state that linezolid MIC values of ≤ 4 µg/mL indicate susceptibility and ≥ 8 µg/mL indicates resistance for staphylococci [13]. The successfully recovered staphylococcal isolates remained resistant to linezolid (8 µg/mL to > 256 µg/mL) on ETEST® (bioMérieux, France). The antibiogram data of the *S. capitis* isolates, the *S. epidermidis* isolates and the *S. haemolyticus* isolates were obtained from laboratory records and used to determine the extent of MDR among the isolates as shown in Table 3.4.

3.4.2 Identification of staphylococcal isolates

Multiplex-PCR assays confirmed the identification of 43 *S. capitis*, 27 *S. epidermidis* and nine *S. haemolyticus* linezolid resistance isolates in agreement with the initial identification results of the MALDI-TOF MS (Bruker, USA). The visualisation of PCR amplicons using gel electrophoresis is shown in Figure 3.2.

3.4.3 Screening of the *cfr* gene

The PCR screening for the *cfr* gene showed that eight (29.6%) of the 27 *S. epidermidis* isolates were positive (Table 3.3). The *S. capitis* isolates and the *S. haemolyticus* isolates were *cfr* gene negative. The *cfr* positive *S. epidermidis* isolates showed an amplification band of the expected size (746 bp), shown in Figure 3.3, which was compatible with the *cfr* fragment previously reported by Doern *et al*. (2016). Linezolid MIC values ranged between 8 µg/mL and > 256 µg/mL among the *cfr* positive *S. epidermidis* isolates.

3.4.4 Molecular typing of staphylococcal isolates

Molecular typing of the isolates (24 *S. epidermidis* isolates, 40 *S. capitis* isolates and six *S. haemolyticus*) was carried out using PFGE; however, nine isolates (three *S. epidermidis* isolates, three *S. capitis* isolates and three *S. haemolyticus* isolates) were non-typable due to the failure of cryo-storage recovery (Table 3.7). The Dice Coefficient (with 0.5% tolerance and 0.5% optimisation) was used for the band-based similarity coefficient with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) for cluster analysis. The dendrogram of the 24 *S. epidermidis* isolates defined one major cluster containing ten isolates, four minor clusters and five singletons as shown in Figure 3.4. The 24 *S. epidermidis* isolates that were typed using PFGE were from nine different hospitals. Among the 40 *S. capitis* isolates, three major clusters and four minor clusters were identified per the dendrogram shown in Figure 3.5. The spread of the 40 *S. capitis* isolates was diverse across 20 different hospitals. The dendrogram of the six *S. haemolyticus* isolates from four different hospitals are shown in Figure 3.6 and showed two minor clusters and one singleton. A cut-off value of ≥ 80% was used for all isolates.

3.4.5 Whole-genome sequencing data from representative isolates

The WGS data confirmed the presence of the *cfr* gene in the four *cfr* gene positive *S. epidermidis* isolates that tested positive on the PCR assay. The *S. epidermidis* isolates were assigned sequence type (ST) numbers as determined by *in silico* analysis from the WGS data using the *S. epidermidis* multilocus sequence typing (MLST) scheme. The dominant ST among the *S. epidermidis* isolates was ST23 (57.1%, n = 4/7), followed by ST2 (28.6%, n = 2/7) and ST22 (14.3%, n = 1/7). The *S. capitis* isolates were not assigned a ST because the MLST scheme is not available to date. The *S. epidermidis* isolates and the *S. capitis* isolates displayed various mutations in domain V of the 23S rRNA gene, shown in Table 3.5 and Table 3.6, respectively.

3.5 Discussion

This study reports linezolid resistance among staphylococcal isolates and the dissemination of strains among private hospitals in Gauteng, South Africa. Linezolid resistant staphylococcal infections are becoming more prevalent due to the increasing use of linezolid in the treatment of these infections. To our knowledge, this study presents the first data on linezolid resistance relating to the 23S rRNA gene mutations and the acquisition of the *cfr* gene, among staphylococcal isolates, circulating in Gauteng, South Africa. The results of this study indicate that *S. capitis*, *S. epidermidis* and *S. haemolyticus* related infections with varying levels of

linezolid resistance are emerging and spreading in Gauteng, South Africa.

The ETEST® (bioMérieux, France) susceptibility testing results exhibited varying levels of linezolid resistance with MIC values ranging from 8 µg/mL and > 256 µg/mL, in agreement with reporting by Nordmann *et al*. (2019). However, the presence of additional linezolid resistance mechanisms, such as the *optr*A gene or *poxt*A gene may contribute to the variation in MIC values. Additionally, reading of the ETEST® (bioMérieux, France) strips requires visual interpretation often resulting in subjectivity when being manually interpreted [26, 27]. Previous studies have noted how observations between experienced users varied significantly in the interpretation of ETEST® (bioMérieux, France) MIC values [28, 29]. Therefore, the subjective interpretation of ETEST® (bioMérieux, France) MIC values may contribute to the under-reporting of linezolid resistance and may contribute to the difference in the MIC values reported in this study [30].

The successfully typed PFGE isolates show the dissemination of linezolid resistant *S. epidermidis* isolates, *S. capitis* isolates and *S. haemolyticus* isolates among private hospitals in the Gauteng region of South Africa. The *S. epidermidis* isolates contained in Cluster A of the dendrogram were from five different hospitals. However, five of these isolates were from the same hospital, indicating spread within the hospital environment. The five *S. epidermidis* singletons were distantly related and show spread among various hospitals (Figure 3.4). The *S. capitis* isolates and *S. haemolyticus* isolates showed dissemination across various hospitals in all clusters. The *S. capitis* isolates formed three major clusters and four minor clusters, showing clonal relatedness between the isolates (Figure 3.5). However, the *S. capitis* isolates were spread over 20 different hospitals displaying multiclonal spread, which suggests possible dissemination by staff between hospitals. The six *S. haemolyticus* isolates were spread across 4 hospitals (Figure 3.6). Further investigation is required to determine the extent and cause of such diverse dissemination between hospitals.

Sequence typing of the *S. epidermidis* isolates consisted of ST23 (n = 4), followed by ST2 (n = 2) and ST22 (n = 1), which are reported widely among linezolid resistant isolates in clinical settings, suggesting hospital adaptation [31, 32]. The four *S. epidermidis* isolates in ST23 were from two hospitals (three isolates from one hospital and one isolate from another hospital), indicating predominant spread within the hospital of ST23 (Table 3.5). Similar to findings reported by Martínez-Meléndez *et al*. (2016), the *S. epidermidis* ST23 isolates in this study were the most prevalent with half of the ST23 strains being *cfr* positive. Reports show that

isolates belonging to ST23 have been found in various countries including Argentina, Germany, Greece, Hungary, Iceland, Poland, Portugal, United States and Uruguay [33]. Linezolid resistance in ST2 strains have been previously described worldwide [33]. The two *S. epidermidis* ST2 isolates in this study came from different hospitals and were *cfr* positive with only one mutation (Table 3.5). The MIC values of these two isolates were both 8 µg/mL, suggesting that the *cfr* gene has less impact on linezolid resistance than that of multiple 23S rRNA gene mutations. Sequence type 22 is known to be common; however this study found it to be the least common [31].

The most common linezolid resistance 23S rRNA gene mutation is G2576T; however, it was not detected in this study. The *S. epidermidis* isolates carried known mutations of the 23S rRNA gene (C2190T, C2561T and G2603T) (Table 3.5). The double mutations (C2190T and G2603T) in the ST22 *S. epidermidis* isolate and ST23 *S. epidermidis* isolates have also been reported by Cidral *et al*. (2015). The C2561T mutation was found in one ST23 *S. epidermidis* isolate and was previously reported in a ST2 *S. epidermidis* isolate; however, very little research reports the C2561T mutation [34]. Lincopan *et al*. (2009) reported the first finding of the G2603T mutation in *S. epidermidis* and research carried out by Zhou *et al*. (2015) reports the first G2603T mutation in *S. capitis*. Studies by Sorlozano *et al*. (2010) and Cidral *et al*. (2015) report findings of the C2190T mutation in the 23S rRNA gene to be associated with resistance to linezolid in *S. epidermidis*. However, the C2190T mutation has also been reported in *S. hominis* isolates [35].

The *S. capitis* isolates showed two known 23S rRNA gene mutations: C2190T and G2603T. Additionally, the *S. capitis* isolates showed various unknown 23S rRNA gene mutations, namely: T2157A, T2346C, C2287G, A2295G, A2296G, C2302G, A2305G, C2308G and A2314C (Table 3.6). One *S. capitis* isolate displayed a combination of the known and unknown 23S rRNA gene mutations found in this study, possibly due to selective pressure of prior linezolid exposure. Unfortunately, prior patient treatment data was not available to confirm this finding. This *S. capitis* isolate had an MIC value of 128 µg/mL suggesting that these unknown 23S rRNA gene mutations, which have not yet been reported in the literature, play a significant role in increased linezolid resistance (Table 3.6). The *S. epidermidis* isolates containing a combination of 23S rRNA gene mutations and the presence of the *cfr* gene showed significantly higher (> 256 µg/mL) linezolid resistance than isolates with only one of these resistance mechanisms (Table 3.5). The *cfr* gene was detected in only eight *S. epidermidis* isolates from

all 79 staphylococcal isolates, which may suggest that *cfr* mediated resistance is still rare among staphylococci.

Linezolid resistance is a global issue with reports of emerging resistance mechanisms spanning many countries including North America, South America, Europe and Asia [36, 37]. The global dissemination of MDR Gram-positive bacterial infections has resulted in linezolid being increasingly used in patient treatment; however, the increased use of linezolid creates considerable selection pressure that requires continual careful monitoring of linezolid use [37].

3.6 Conclusion

This study aimed to establish the presence of the most common linezolid resistance mechanisms (23S rRNA gene mutations and *cfr* gene presence) among staphylococcal isolates in Gauteng, South Africa. We were successful in confirming the presence of both resistance mechanisms across various private hospitals. Additionally, some novel 23S rRNA gene mutations were observed. These findings provide perspective on the linezolid mechanisms present in Gauteng, South Africa. However, there is a need for future investigations to be carried out to determine the impact of linezolid resistance mechanism. Future investigations need to cover aspects such as: (i) research over time to establish the rate of the increase in linezolid resistance, (ii) expand the geographic area to include areas outside of Gauteng, South Africa to increase the applicability of findings and (iii) include other linezolid resistance mechanisms to accurately determine the impact each mechanism has on the overall resistance landscape.

The findings in this study highlight the need for continual surveillance of linezolid resistance among staphylococcal infections within South Africa. The clinical use of linezolid is favourable due to the spectrum of activity and oral bioavailability. However, maintaining judicious use of linezolid is important and the prescription of linezolid needs to be controlled and optimised as extended use often creates substantial selection pressure for resistance.

Ethics statement

Ethical approval was obtained for the study from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (Ethics reference number: 477/2017). Individual patient consent was waivered since the study was observational and patient care was not influenced at any stage.

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Tables

Table 3.1: Primer sequences used for the identification of staphylococcal isolates

Target gene	Primer sequence (5'→3')*	Amplicon size (bp)	Reference
Staphylococcus species (16S rRNA)	GCAAGCGTTATCCGGATTT	597	[15]
	CTTAATGATGGCAACTAAGC		
S. capitis Superoxide dismutase (*sodA*)	TCAGATATTCAAACTGCAGTACG	103	[17]
	CTACTTCACCTTTTTCTTCAGA		
S. epidermidis Thermonuclease (*nuc*)	TTGTAAACCATTCTGGACCG	251	[16]
	ATGCGTGAGATACTTCTTCG		
S. haemolyticus Thermonuclease (*nuc*)	TAGTGGTAGGCGTATTAGCC	434	
	ACGATATTTGCCATTCGGTG		

* All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa)

Table 3.2: The *cfr* gene primers used for staphylococcal isolates

Target gene	Primer sequence (5'→3')*	Amplicon size (bp)	Reference
Chloramphenicol-florfenicol resistance (*cfr*)	TGAAGTATAAAGCAGGTTGGGAG	746	[5]
	ACCATATAATTGACCACAAGCAGC		

* All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa)

Table 3.3: Staphylococcal linezolid antimicrobial susceptibility and *cfr* gene screening

Isolate	Specimen type	Species identification	ETEST® MIC (µL/mL) (interpretation)		VITEK® 2 MIC (µL/mL) (interpretation)		*cfr*
1	BC	*S. epidermidis*	24	R	16	R	Neg
2	BC	*S. epidermidis*	8	R	NT	NT	Neg
3*	BC	*S. epidermidis*	8	R	8	R	Pos
4	BC	*S. epidermidis*	8	R	8	R	Neg
5*	BC	*S. epidermidis*	16	R	16	R	Neg
6	BC	*S. epidermidis*	8	R	32	R	Neg
7*	BC	*S. epidermidis*	> 256	R	16	R	Pos
8	BC	*S. epidermidis*	> 256	R	64	R	Neg
9	BC	*S. epidermidis*	NG	NG	16	R	Neg
10	BC	*S. epidermidis*	16	R	64	R	Neg
11	BC	*S. epidermidis*	12	R	8	R	Neg
12	BC	*S. epidermidis*	16	R	32	R	Neg
13	CVC	*S. epidermidis*	12	R	32	R	Neg
14*	BC	*S. epidermidis*	8	R	> 256	R	Pos
15*	BC	*S. epidermidis*	16	R	> 256	R	Neg
16	BC	*S. epidermidis*	8	R	8	R	Pos
17	CVC	*S. epidermidis*	32	R	> 256	R	Neg
18	BC	*S. epidermidis*	16	R	64	R	Pos
19	BC	*S. epidermidis*	128	R	8	R	Neg
20	BC	*S. epidermidis*	8	R	8	R	Pos
21	BC	*S. epidermidis*	24	R	16	R	Neg
22*	BC	*S. epidermidis*	128	R	> 256	R	Neg
23	BC	*S. epidermidis*	128	R	32	R	Neg
24	BC	*S. epidermidis*	16	R	64	R	Neg
25*	BC	*S. epidermidis*	> 256	R	64	R	Pos
26	BC	*S. epidermidis*	8	R	8	R	Pos
27	BC	*S. epidermidis*	12	R	32	R	Neg
28	BC	*S. capitis*	32	R	16	R	Neg
29	BC	*S. capitis*	NG	NG	32	R	Neg
30	BC	*S. capitis*	16	R	16	R	Neg
31	BC	*S. capitis*	24	R	16	R	Neg
32	BC	*S. capitis*	32	R	32	R	Neg
33	BC	*S. capitis*	NG	NG	64	R	Neg
34	BC	*S. capitis*	32	R	16	R	Neg
35	CVC	*S. capitis*	NG	NG	32	R	Neg
36*	CVC	*S. capitis*	24	R	> 256	R	Neg
37	BC	*S. capitis*	NG	NG	16	R	Neg
38	BC	*S. capitis*	48	R	16	R	Neg
39	BC	*S. capitis*	32	R	32	R	Neg
40	BC	*S. capitis*	32	R	> 256	R	Neg

* = shading indicates the isolate was sent for WGS; BC = blood culture; *cfr* = chloramphenicol-florfenicol resistance; CVC = central venous catheter; MIC = minimum inhibitory concentration; Neg = negative; NG = no growth; NT = not tested; Pos = positive; R = resistant

Table 3.3: Staphylococcal linezolid antimicrobial susceptibility and *cfr* gene screening (continued)

Isolate	Specimen type	Species identification	ETEST® MIC (µL/mL) (interpretation)		VITEK® 2 MIC (µL/mL) (interpretation)		*cfr*
41	BC	*S. capitis*	24	R	128	R	Neg
42	BC	*S. capitis*	24	R	32	R	Neg
43	BC	*S. capitis*	32	R	64	R	Neg
44	BC	*S. capitis*	32	R	128	R	Neg
45	BC	*S. capitis*	NG	NG	64	R	Neg
46	BC	*S. capitis*	32	R	8	R	Neg
47	BC	*S. capitis*	NG	NG	8	R	Neg
48	BC	*S. capitis*	24	R	16	R	Neg
49	BC	*S. capitis*	24	R	16	R	Neg
50	BC	*S. capitis*	48	R	8	R	Neg
51	BC	*S. capitis*	24	R	48	R	Neg
52*	BC	*S. capitis*	128	R	32	R	Neg
53	BC	*S. capitis*	24	R	16	R	Neg
54	BC	*S. capitis*	16	R	16	R	Neg
55	BC	*S. capitis*	24	R	32	R	Neg
56	BC	*S. capitis*	24	R	32	R	Neg
57	BC	*S. capitis*	32	R	32	R	Neg
58	BC	*S. capitis*	24	R	64	R	Neg
59*	BC	*S. capitis*	24	R	64	R	Neg
60	BC	*S. capitis*	16	R	64	R	Neg
61	BC	*S. capitis*	24	R	32	R	Neg
62	BC	*S. capitis*	16	R	128	R	Neg
63	BC	*S. capitis*	12	R	64	R	Neg
64	BC	*S. capitis*	16	R	16	R	Neg
65	BC	*S. capitis*	64	R	16	R	Neg
66	BC	*S. capitis*	16	R	32	R	Neg
67	BC	*S. capitis*	32	R	8	R	Neg
68	BC	*S. capitis*	24	R	16	R	Neg
69	BC	*S. capitis*	48	R	16	R	Neg
70	BC	*S. capitis*	32	R	16	R	Neg
71	BC	*S. haemolyticus*	NG	NG	8	R	Neg
72	BC	*S. haemolyticus*	32	R	64	R	Neg
73	BC	*S. haemolyticus*	32	R	16	R	Neg
74	BC	*S. haemolyticus*	NG	NG	16	R	Neg
75	BC	*S. haemolyticus*	NG	NG	128	R	Neg
76	BC	*S. haemolyticus*	64	R	64	R	Neg
77	BC	*S. haemolyticus*	8	R	16	R	Neg
78	BC	*S. haemolyticus*	NG	NG	32	R	Neg
79	BC	*S. haemolyticus*	16	R	128	R	Neg

* = shading indicates the isolate was sent for WGS; BC = blood culture; *cfr* = chloramphenicol-florfenicol resistance; CVC = central venous catheter; MIC = minimum inhibitory concentration; Neg = negative; NG = no growth; NT = not tested; Pos = positive; R = resistant

Table 3.4: Antibiogram data indicating the extent of multidrug-resistance in linezolid resistant isolates

Isolate	Species identification	Antimicrobial agent											
		Cloxacillin	Gentamicin	Clindamycin	Erythromycin	Co-trimoxazole	Fusidic acid	Rifampicin	Tetracycline	Teicoplanin	Vancomycin	Daptomycin	Linezolid
1	*S. epidermidis*	R	R	R	R	R	S	R	NT	S	S	NT	R
2	*S. epidermidis*	R	R	R	R	R	S	R	NT	S	S	NT	R
3	*S. epidermidis*	R	R	R	R	R	R	R	NT	S	S	S	R
4	*S. epidermidis*	R	R	R	R	R	R	S	NT	S	S	S	R
5	*S. epidermidis*	R	R	R	R	R	S	R	R	S	S	S	R
6	*S. epidermidis*	R	R	R	R	R	R	S	NT	S	S	NT	R
7	*S. epidermidis*	R	R	R	R	R	S	R	NT	S	S	S	R
8	*S. epidermidis*	R	R	R	R	R	R	R	NT	S	S	S	R
9	*S. epidermidis*	R	R	R	R	R	S	R	NT	S	S	S	R
10	*S. epidermidis*	R	S	R	R	R	S	R	NT	S	S	S	R
11	*S. epidermidis*	R	R	R	R	R	S	R	NT	S	S	S	R
12	*S. epidermidis*	R	S	R	R	R	R	S	NT	I	S	NT	R
13	*S. epidermidis*	R	R	R	R	R	S	R	R	S	S	S	R
14	*S. epidermidis*	R	R	R	R	R	R	R	NT	I	S	S	R
15	*S. epidermidis*	R	R	R	R	S	R	S	NT	S	S	S	R
16	*S. epidermidis*	R	R	R	R	R	R	R	NT	S	S	S	R
17	*S. epidermidis*	R	R	R	R	R	R	S	R	S	S	S	R
18	*S. epidermidis*	R	R	R	R	R	S	R	NT	S	S	S	R
19	*S. epidermidis*	R	S	R	R	R	S	R	NT	S	S	S	R
20	*S. epidermidis*	R	R	R	R	R	R	R	NT	S	S	NT	R
21	*S. epidermidis*	R	R	R	R	R	R	R	NT	S	S	S	R
22	*S. epidermidis*	R	R	R	R	R	S	R	R	S	S	S	R
23	*S. epidermidis*	R	R	R	R	R	S	R	R	S	S	S	R
24	*S. epidermidis*	R	R	R	R	R	R	S	S	I	S	S	R
25	*S. epidermidis*	R	S	R	R	S	S	R	NT	S	S	S	R
26	*S. epidermidis*	R	R	R	R	R	R	R	NT	S	S	S	R
27	*S. epidermidis*	R	R	R	R	R	R	R	R	S	S	S	R
28	*S. capitis*	R	R	R	R	R	R	R	NT	S	S	S	R
29	*S. capitis*	R	S	R	R	S	R	S	NT	S	S	NT	R
30	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	NT	R
31	*S. capitis*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	R
32	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	NT	R
33	*S. capitis*	R	R	R	R	S	R	S	R	S	S	S	R
34	*S. capitis*	R	S	R	R	S	R	S	NT	S	S	S	R
35	*S. capitis*	R	S	R	R	S	I	S	S	S	S	S	R
36	*S. capitis*	R	R	R	R	S	R	S	R	S	S	S	R

I = intermediate; NT = not tested; R = resistant; S = susceptible

Table 3.4: Antibiogram data indicating the extent of multidrug-resistance in linezolid resistant isolates (continued)

Isolate	Species identification	Antimicrobial agent											
		Cloxacillin	Gentamicin	Clindamycin	Erythromycin	Co-trimoxazole	Fusidic acid	Rifampicin	Tetracycline	Teicoplanin	Vancomycin	Daptomycin	Linezolid
37	*S. capitis*	R	R	R	R	S	R	R	NT	S	S	NT	R
38	*S. capitis*	R	R	R	R	R	R	S	NT	S	S	NT	R
39	*S. capitis*	R	R	R	R	S	R	S	R	S	S	NT	R
40	*S. capitis*	R	R	R	R	R	R	S	NT	S	S	NT	R
41	*S. capitis*	R	R	R	R	R	S	R	NT	S	S	S	R
42	*S. capitis*	R	R	R	R	R	R	S	NT	S	S	NT	R
43	*S. capitis*	R	R	R	R	S	NT	S	NT	NT	NT	NT	R
44	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	NT	R
45	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	NT	R
46	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	NT	R
47	*S. capitis*	R	R	R	R	S	R	S	R	S	S	NT	R
48	*S. capitis*	R	S	R	R	R	R	R	NT	S	S	S	R
49	*S. capitis*	R	R	R	R	S	R	S	R	S	S	NT	R
50	*S. capitis*	R	R	R	R	R	R	S	NT	S	S	NT	R
51	*S. capitis*	R	R	R	R	S	R	S	NT	R	S	NT	R
52	*S. capitis*	R	R	R	R	R	R	R	NT	S	S	NT	R
53	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	NT	R
54	*S. capitis*	R	R	R	R	S	R	S	R	S	S	S	R
55	*S. capitis*	R	R	R	R	S	R	R	NT	S	S	NT	R
56	*S. capitis*	R	R	R	R	S	NT	S	NT	S	S	NT	R
57	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	S	R
58	*S. capitis*	R	R	R	R	S	R	S	R	S	S	NT	R
59	*S. capitis*	R	R	R	R	S	NT	S	NT	S	S	NT	R
60	*S. capitis*	R	R	R	R	R	R	S	NT	S	S	NT	R
61	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	S	R
62	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	S	R
63	*S. capitis*	R	S	R	R	S	R	S	R	S	S	NT	R
64	*S. capitis*	R	R	R	R	S	R	S	R	S	S	S	R
65	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	NT	R
66	*S. capitis*	R	R	R	R	R	R	R	NT	S	S	NT	R
67	*S. capitis*	R	R	R	R	S	R	S	R	S	S	S	R
68	*S. capitis*	R	R	R	R	S	NT	S	R	S	S	NT	R
69	*S. capitis*	R	S	R	R	S	R	S	NT	S	S	S	R
70	*S. capitis*	R	R	R	R	S	R	S	NT	S	S	S	R
71	*S. haemolyticus*	R	R	R	R	R	R	S	NT	S	S	NT	R
72	*S. haemolyticus*	R	R	R	R	R	R	S	NT	S	S	S	R

I = intermediate; NT = not tested; R = resistant; S = susceptible

Table 3.4: Antibiogram data indicating the extent of multidrug-resistance in linezolid resistant isolates (continued)

Isolate	Species identification	Antimicrobial agent											
		Cloxacillin	Gentamicin	Clindamycin	Erythromycin	Co-trimoxazole	Fusidic acid	Rifampicin	Tetracycline	Teicoplanin	Vancomycin	Daptomycin	Linezolid
73	*S. haemolyticus*	R	R	R	R	R	R	R	S	S	S	S	R
74	*S. haemolyticus*	R	R	R	R	R	R	S	NT	S	S	S	R
75	*S. haemolyticus*	R	R	R	R	R	R	R	NT	S	S	S	R
76	*S. haemolyticus*	R	R	R	R	R	R	R	NT	S	S	NT	R
77	*S. haemolyticus*	R	R	R	R	R	R	R	NT	S	S	S	R
78	*S. haemolyticus*	R	R	R	R	R	R	R	R	S	S	S	R
79	*S. haemolyticus*	R	R	R	R	R	R	S	S	S	S	S	R

I = intermediate; NT = not tested; R = resistant; S = susceptible

Table 3.5: Mutations of the 23S rRNA gene in *S. epidermidis* isolates with MIC values

Isolate	14	3	15	5	22	7	25
Hospital	Z	R	CC	U	Z	U	U
ST	ST2	ST2	ST22	ST23	ST23	ST23	ST23
cfr	Pos	Pos	Neg	Neg	Neg	Pos*	Pos*
23S rRNA gene mutations	2190 C→T	2190 C→T	2190 C→T 2603 G→T	2190 C→T 2603 G→T	2190 C→T 2561 C→T 2603 G→T	2190 C→T 2603 G→T	2190 C→T 2603 G→T
ETEST® MIC (µL/mL)	8	8	16	16	128	> 256	> 256

* = shading indicates isolates with double 23S rRNA gene mutations and the *cfr* gene resulting in significantly higher MIC values; C = cytosine; *cfr* = chloramphenicol-florfenicol resistance; G = guanine; MIC = minimum inhibitory concentration; Neg = negative; Pos = positive; ST = sequence type; T = thymine

Table 3.6: Mutations of the 23S rRNA gene in *S. capitis* isolates with MIC values

Isolate	36	59	52
Hospital	CC	AA	B
cfr	Neg	Neg	Neg
23S rRNA gene mutations*	2190 C→T 2603 G→T	2190 C→T 2603 G→T	2157 T→A 2190 C→T 2346 T→C 2603 G→T
	2157 T→A 2346 T→C	2157 T→A 2346 T→C	2287 C→G 2295 A→G 2296 A→G 2302 C→G 2305 A→G 2308 C→G 2314 A→C
ETEST® MIC (µL/mL)	24	24	128

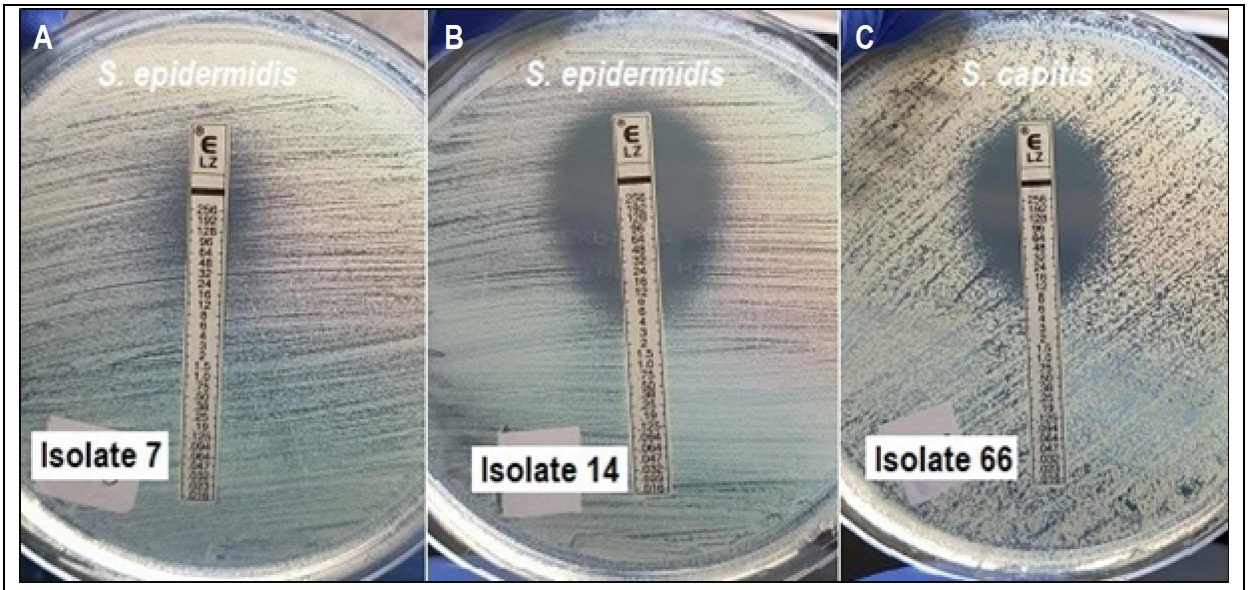
* = shading shows previously unreported 23S rRNA gene mutations; A = adenine; C = cytosine; *cfr* = chloramphenicol-florfenicol resistance; G = guanine; MIC = minimum inhibitory concentration; Neg = negative; T = thymine

Table 3.7: Detailed breakdown of isolate recovery or failure from cryo-storage

Species identification	Count	ETEST®	PFGE
S. epidermidis	2	Successful	Recovery failure
	1	Recovery failure	Recovery failure
	24	Successful	Successful
S. capitis	1	Successful	Recovery failure
	2	Recovery failure	Recovery failure
	4	Recovery failure	Successful
	36	Successful	Successful
S. haemolyticus	1	Successful	Recovery failure
	2	Recovery failure	Recovery failure
	2	Recovery failure	Successful
	4	Successful	Successful

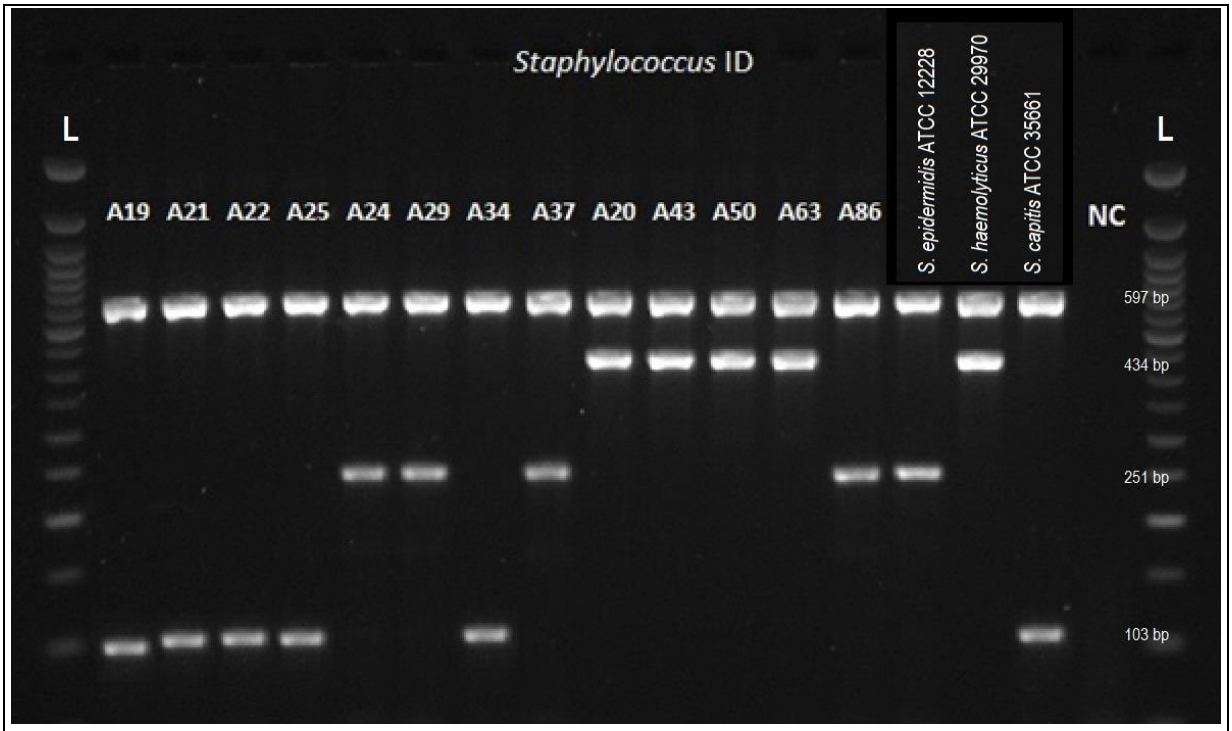
Count = number of isolates

Figures



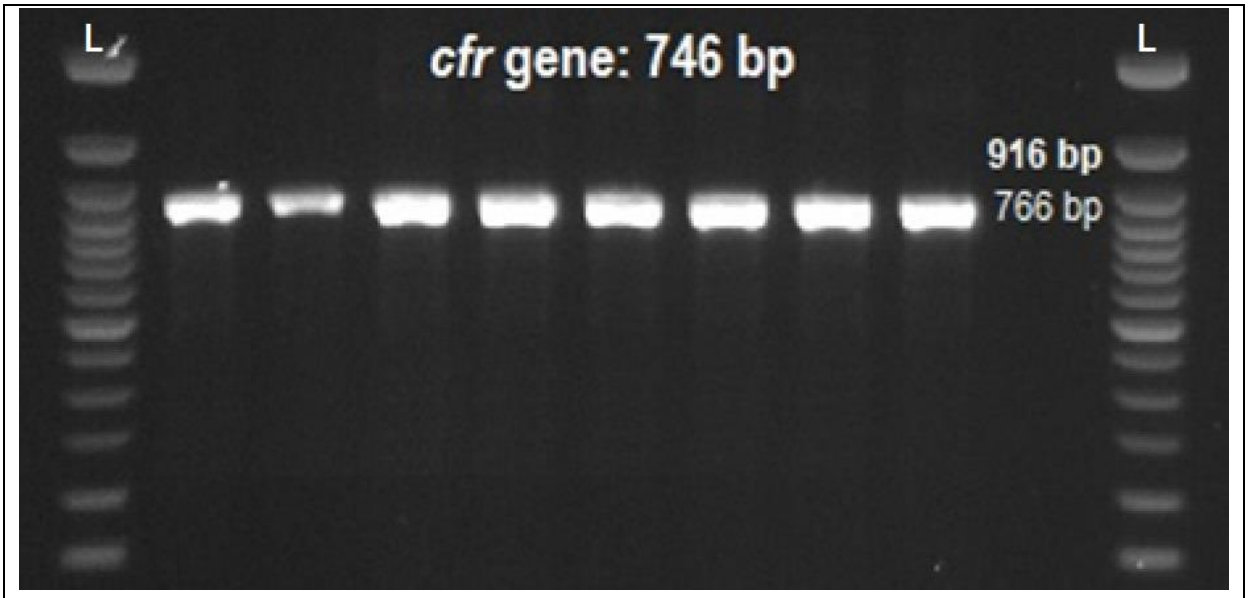
A = *S. epidermidis*, MIC > 256 µL/mL; B = *S. epidermidis*, MIC 8 µL/mL; C = *S. capitis*, MIC 16 µL/mL

Figure 3.1: Linezolid ETEST® (bioMérieux, France) susceptibility of staphylococcal isolates performed on Mueller-Hinton agar (Oxoid Ltd, UK)



ATCC 12228 = *S. epidermidis* positive control; ATCC 29970 = *S. haemolyticus* positive control; ATCC 35661 = *S. capitis* positive control; L = molecular marker; NC = negative control

Figure 3.2: Multiplex PCR assay for the simultaneous identification of staphylococcal isolates



766 bp = *cfr* gene; L = molecular marker; NC = negative control

Figure 3.3: Detection of the *cfr* gene in staphylococcal isolates

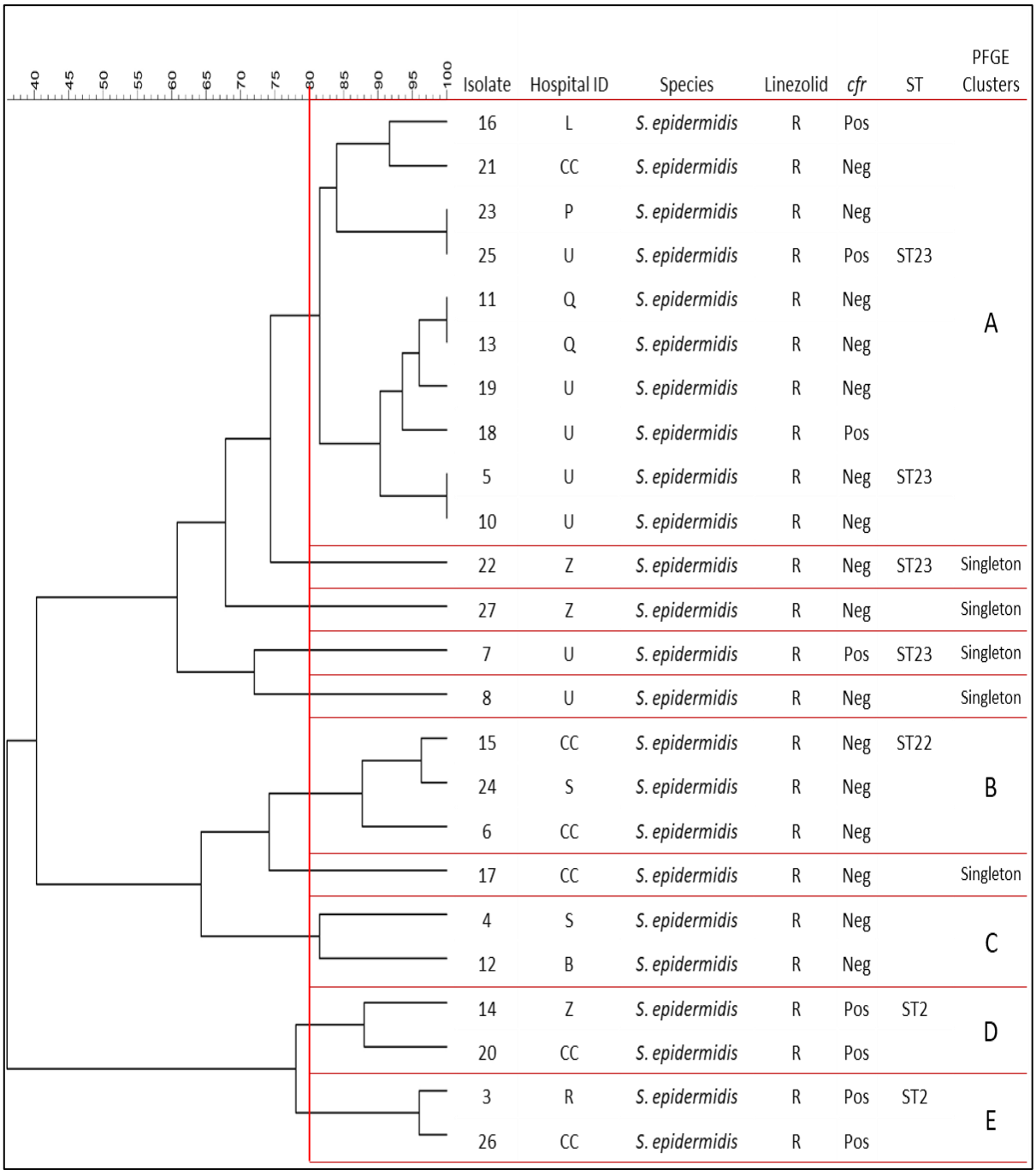


Figure 3.4: **Dendrogram constructed after PFGE analysis of 24 *S. epidermidis* isolates showing one major cluster, four minor clusters and five singletons using a cut-off value of ≥ 80%**

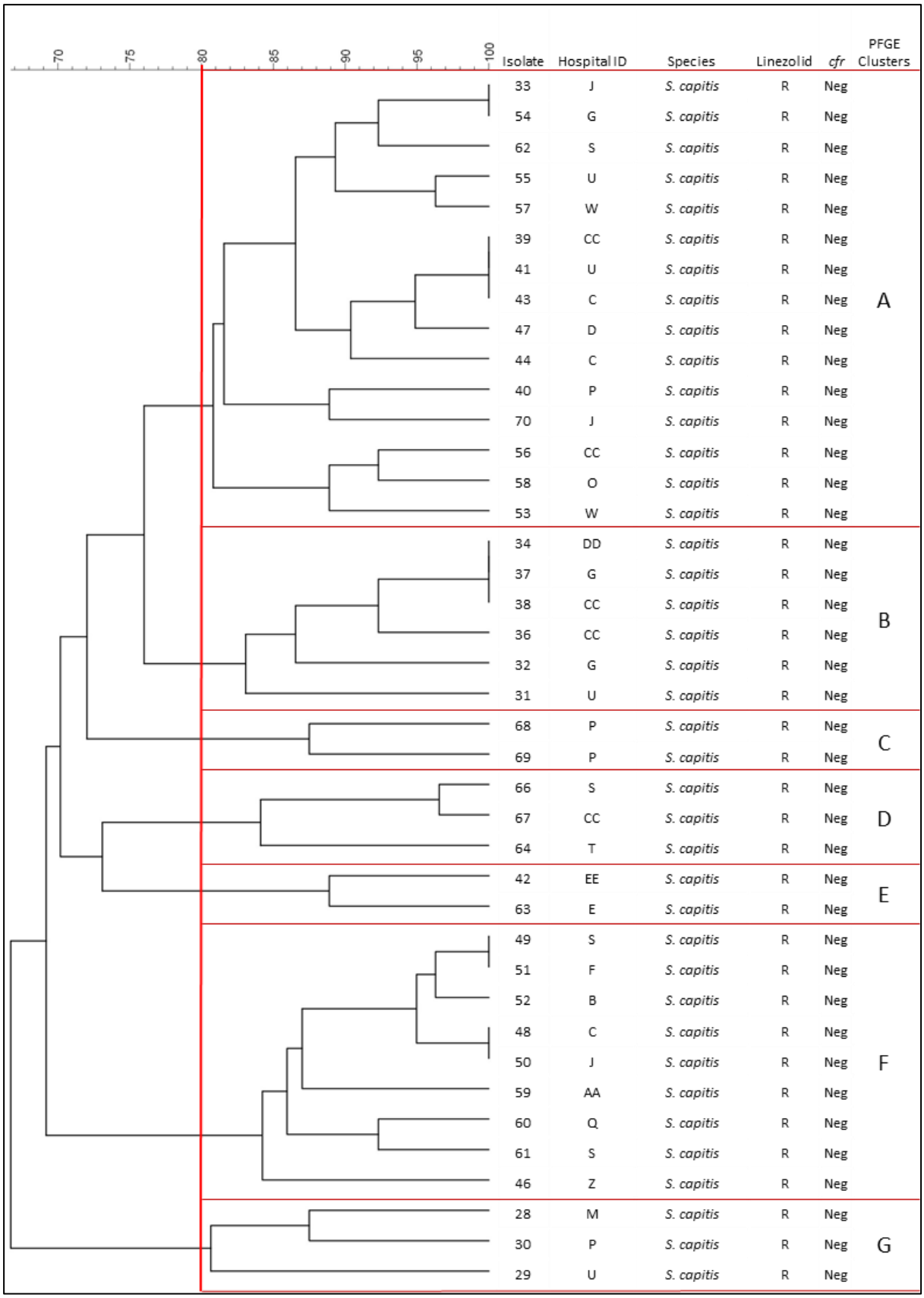
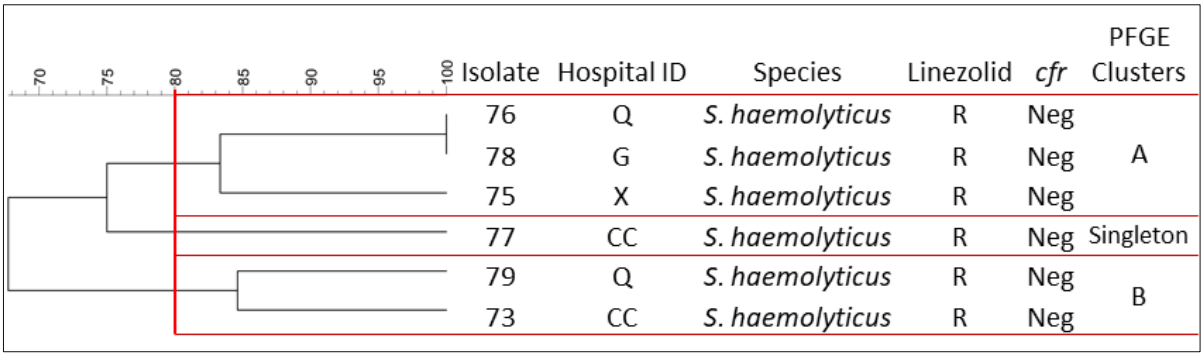


Figure 3.5: **Dendrogram constructed after PFGE analysis of 40 *S. capitis* isolates showing three major clusters and four minor clusters using a cut-off value of ≥ 80%**



Isolate	Hospital ID	Species	Linezolid	*cfr*	PFGE Clusters
76	Q	*S. haemolyticus*	R	Neg	A
78	G	*S. haemolyticus*	R	Neg	
75	X	*S. haemolyticus*	R	Neg	
77	CC	*S. haemolyticus*	R	Neg	Singleton
79	Q	*S. haemolyticus*	R	Neg	B
73	CC	*S. haemolyticus*	R	Neg	

Figure 3.6: Dendrogram constructed after PFGE analysis of six *S. haemolyticus* isolates showing two minor clusters and one singleton using a cut-off value of ≥ 80%

CHAPTER 4

Screening for linezolid resistance and the *cfr* gene among *Enterococcus faecalis* and *Enterococcus faecium* isolates collected in Gauteng, South Africa

The editorial style of BMC Infectious Diseases was followed in this chapter

4.1 Abstract

Background: Linezolid is an oxazolidinone class of antimicrobial agent used for the treatment of severe infections caused by Gram-positive bacterium, such as vancomycin-resistant enterococci (VRE). However, linezolid resistance is increasing due to chromosomal- and plasmid-mediated mechanisms such as mutations of the 23S ribosomal ribonucleic acid (rRNA) gene and the acquisition of the chloramphenicol-florfenicol resistance (*cfr*) gene, respectively. This study attempted to define 23S rRNA gene mutations and detect the *cfr* gene among enterococcal isolates circulating in Gauteng, South Africa.

Methods: *Enterococcus faecalis* isolates (n = 28) and *Enterococcus faecium* isolates (n = 4) were collected from public and private hospitals from 2013 to 2015. Selection criteria included *E. faecalis* isolates and *E. faecium* isolates that were linezolid intermediate or resistant from antimicrobial susceptibility testing results using the VITEK® 2 automated system (bioMérieux, France), according to the 2019 Clinical and Laboratory Standards Institute (CLSI) guidelines. Linezolid resistance was re-evaluated from the minimum inhibitory concentration (MIC) values using the ETEST® (bioMérieux, France). Multiplex polymerase chain reaction (M-PCR) assays confirmed the species of the enterococcal isolates and the presence of the *cfr* gene was screened for using a singleplex polymerase chain reaction (PCR) assay.

Results: The PCR assays confirmed the identity of 28 *E. faecalis* and four *E. faecium* isolates. The ETEST® (bioMérieux, France) showed that the MIC values of the isolates ranged between 2 μg/mL and 4 μg/mL, testing susceptible and intermediate resistance according to the 2019 CLSI guidelines. The presence of the *cfr* gene was not detected in any of the isolates.

Conclusions: This study did not detect the presence of the *cfr* gene, nor resistance among the *E. faecalis* isolates and *E. faecium* isolates using the ETEST® (bioMérieux, France). However, globally the emergence of linezolid resistance among enterococcal isolates diminishes treatment options, raising the need for better antimicrobial surveillance and stewardship efforts.

Keywords: Linezolid, *Enterococcus*, Resistance, *cfr* gene

4.2 Background

Enterococcal infections are commonly caused by *Enterococcus faecalis* and *Enterococcus faecium* which are natural intestinal commensals, therefore putting the immunocompromised host at higher risk of infection [1,2]. Infections associated with *E. faecalis* and *E. faecium* include severe hospital- and community-associated infections such as endocarditis, bacteraemia, urinary tract infections, meningitis, pelvic and wound infections [1,2]. *Enterococcus faecium* forms part of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), which are leading causes of multidrug-resistant (MDR) nosocomial infections, known to "ESKAPE" treatment [3]. The treatment of MDR enterococcal infections remains challenging and alternative antimicrobial agents such as linezolid are required; however, linezolid resistance among enterococcal infections is increasingly detected worldwide [4-6].

Linezolid resistance is most commonly mediated by chromosomal- and plasmid-mediated mechanisms such as mutations of the 23S ribosomal ribonucleic acid (rRNA) gene and acquisition of the chloramphenicol-florfenicol resistance (*cfr*) gene [7]. The most common 23S rRNA gene mutation is at position G2576T of domain V; however, many other mutations have been reported [8]. Additionally, mutations of the L3, L4 and L22 ribosomal proteins are reported in enterococcus [9,10]. The *cfr* gene is less common but more worrisome due to interspecies spread of this mechanism [7,11]. The result of acquisition of the *cfr* gene confers a MDR phenotype that includes the antimicrobial classes: phenicol, lincosamide, oxazolidinone, pleuromutilin and streptogramin A (known as the PhLOPSA phenotype) [12]. The *cfr* gene can mediate resistance in the absence of other resistance mechanisms [13]. Another plasmid-mediated linezolid resistance mechanisms is the *optr*A gene that has been identified in clinical *E. faecalis* isolates and *E. faecium* isolates [6,14-16].

To effectively utilise linezolid as a therapeutic agent, it is vital that antimicrobial susceptibility testing outcomes are considered, particularly when treating enterococcal MDR infections because linezolid-resistant enterococci is related to previous linezolid exposure [9,17]. The focus of this study was to investigate the incidence of linezolid resistance and to screen for the presence of the *cfr* gene among *E. faecalis* and *E. faecium* isolates obtained from the public and private sectors.

4.3 Methods

4.3.1 Study setting and isolate collection

This study was a laboratory based retrospective observational study. A total of 32 clinical *E. faecalis* isolates (n = 28) and *E. faecium* isolates (n = 4) were collected from 2013 to 2015, across five public hospitals and three private hospitals in Gauteng, South Africa. The isolates were cultured from abscess (superficial) (n = 2), blood culture (n = 4), catheter urine (n = 3), fluid/aspirate (n = 3), intravenous catheter tip (n = 2), midstream urine (n = 4), swab (superficial) (n = 5), tissue (n = 1) and urine (n = 8). The initial identification of the public sector isolates was established using the VITEK® 2 automated system (bioMérieux, France). The private sector isolates were initially identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Bruker, USA). Antimicrobial susceptibility testing of all isolates was performed using the VITEK® 2 automated system (bioMérieux, France) in accordance to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The 2019 CLSI breakpoint tables state that *E. faecalis* and *E. faecium* minimum inhibitory concentration (MIC) values ≤ 2 µg/mL indicate susceptibility, 4 µg/mL indicate intermediate susceptibility and ≥ 8 µg/mL indicate resistance [18]. Inclusion criteria for the study was *E. faecalis* isolates and *E. faecium* isolates that tested as intermediate resistant or resistant to linezolid with an MIC value > 4 µg/mL, according to the 2019 CLSI enterococcal guidelines [18].

4.3.2 Isolate processing and storage of enterococcal isolates

The *E. faecalis* and *E. faecium* isolates were cultured on blood agar (Oxoid Ltd, UK) by incubating (Vacutec, South Africa) overnight at 37°C. To ensure culture purity, the blood agar (Oxoid Ltd, UK) plates were visually inspected followed by Gram-staining. A single pure colony from each isolate was inoculated into 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA) containing brain heart infusion (BHI) broth (Merck, Germany) and incubated (Stuart, UK) with constant shaking at 220 rpm overnight at 37°C. An extra microcentrifuge tube (Scientific Specialities Inc., USA) containing BHI broth (Merck, Germany) was included but not inoculated to serve as the control to ensure contamination had not taken place. The BHI broth (Merck, Germany) cultures were stored in a 50% glycerol (Merck, Germany) solution at -20°C for future analysis.

4.3.3 Antimicrobial susceptibility testing of enterococcal isolates

Linezolid ETEST® (bioMérieux, France) was used to confirm the initial antimicrobial

susceptibility data of the *E. faecalis* isolates and *E. faecium* isolates obtained from the VITEK® 2 automated system (bioMérieux, France). Briefly, the stored *E. faecalis* and *E. faecium* isolates were cultured using blood agar (Oxoid Ltd, UK) and incubated (Vacutec, South Africa) at 37°C for 24 hours. Saline suspensions were prepared using a Densichek (bioMérieux, France) with a single colony from each isolate and adjusted to the 0.5 McFarland standard. Within 15 minutes of preparation, the cell suspensions were inoculated onto Mueller-Hinton agar (Oxoid Ltd, UK) followed by aseptically placing the linezolid ETEST® (bioMérieux, France) strip onto the inoculated Mueller-Hinton agar (Oxoid Ltd, UK). The ETEST® (bioMérieux, France) plates were incubated (Vacutec, South Africa) at 37°C for 18 hours. Validation of the MIC values was performed by concurrent testing using the *E. faecalis* reference strain, American Type Culture Collection (ATCC) 29212, per the CLSI guidelines [18]. The *E. faecalis* and *E. faecium* 2019 CLSI breakpoints specify that a linezolid MIC value of ≤ 2 µg/mL indicates susceptibility, 4 µg/mL indicates intermediate susceptibility and ≥ 8 µg/mL indicates resistance [18].

4.3.4 Total genomic DNA extraction of enterococcal isolates

The deoxyribonucleic acid (DNA) of the *E. faecalis* and *E. faecium* isolates was extracted using the ZymoResearch (ZR) Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, USA). Briefly, using 2 mL of BHI broth (Merck, Germany) per isolate, a single pure colony from each isolate was incubated (Stuart, UK) with constant shaking at 220 rpm overnight at 37°C. A negative control was included as quality control. After the isolates were successfully cultured in BHI broth (Merck, Germany), DNA extraction was carried out per manufacturer instructions. A NanoPhotometer (Implen, Germany) and agarose (Lonza, USA) gel electrophoresis were used to quantify the extracted DNA. The extracted DNA was stored at -20°C for all downstream polymerase chain reaction (PCR) applications.

4.3.5 Molecular methods to confirm *Enterococcus* species

Multiplex PCR (M-PCR) assays were used to confirm speciation of the isolates using previously described primers, shown in Table 4.1 [19]. Targeted genes included the 16S rRNA gene of the *Enterococcus* genus (733 bp), the D-alanine:D-alanine ligase (*ddl*) gene of *E. faecalis* (940 bp) and the *ddl* gene of *E. faecium* (658 bp). Positive and negative quality controls were included in the PCR assays. The *E. faecalis* reference strain ATCC 29212 and *E. faecium* reference strain ATCC 700221 were used as the positive controls and nuclease-free water (QIAGEN, Netherlands) as the negative control. Each enterococcal M-PCR assay consisted of the following composition: 7.5 µL of MyTaq™ Red Mix (Bioline, UK), 0.15 µL

of forward primer (0.2 µM), 0.15 µL of reverse primer (0.2 µM), 6.7 µL of nuclease-free water (QIAGEN, Netherlands) and 0.5 µL of template DNA to make up a total reaction volume of 15 µL. The PCR thermocycler (BioRad, UK) conditions were as follows: initial activation and denaturation (95°C for 5 min), followed by 35 cycles of denaturation (95°C for 30 sec), annealing (55°C for 90 sec), extension (74°C for 30 sec) and a final elongation step (72°C for 5 min). The visualisation of PCR amplicons is detailed in section 4.3.7.

4.3.6 Screening for the *cfr* gene

A singleplex PCR assay was used to screen for the presence of the *cfr* gene (746 bp) among the enterococcal isolates. A *cfr* gene positive *Staphylococcus epidermidis* isolate was used as a positive control and nuclease free water was used as a negative control. Previously described primers were used as described by Doern *et al.* (2016), shown in Table 4.2. The *cfr* gene PCR assay consisted of the following reaction composition per isolate: 7.5 µL of MyTaq™ Red Mix (Bioline, UK), 0.15 µL of forward *cfr* gene primer (0.2 µM), 0.15 µL of reverse *cfr* gene primer (0.2 µM), 6.2 µL of nuclease-free water (QIAGEN, Netherlands) and 1 µL of template DNA to make up a reaction volume of 15 µL. The PCR thermocycler (BioRad, UK) conditions were as follows: initial activation and denaturation (94°C for 10 min), followed by 35 cycles of denaturation (94°C for 30 sec), annealing (57°C for 30 sec), extension (74°C for 30 sec) and a final elongation step (72°C for 10 min). An agarose (Lonza, USA) gel electrophoresis was used to detect any positive amplicons as described in section 4.3.7.

4.3.7 Visualisation of PCR amplicons

The investigation of PCR amplicons from all M-PCR and PCR assays was carried out using conventional gel electrophoresis on a 2% agarose (Lonza, USA) gel. During the casting of the gel, 5 µL of ethidium bromide (10 µg/mL) (Sigma-Aldrich, USA) was added in order to visualise the PCR products under UV light. A 1x Tris-Borate-Ethylenediaminetetraacetic acid (TBE) (Sigma-Aldrich, USA) buffer was used to run the gels at 100 V for 100 min. A 50 bp DNA ladder (ThermoScientific, USA) was loaded at each end of every gel to determine the sizes of the amplicons. Visualisation of the gels was done using ultraviolet (UV) light with the Gel Doc EZ System (BioRad, UK).

4.4 Results

4.4.1 Antimicrobial susceptibility testing of linezolid

All *E. faecalis* isolates (n = 28) and *E. faecium* isolates (n = 4) underwent ETEST® (bioMérieux, France) antimicrobial susceptibility testing after being successfully recovered from cryo-

storage (Figure 4.1). The 2019 CLSI *E. faecalis* and *E. faecium* breakpoint tables state that MIC values ≤ 2 µg/mL indicate susceptibility, 4 µg/mL indicate intermediate susceptibility and ≥ 8 µg/mL indicate resistance [18]. The *E. faecalis* isolates and *E. faecium* isolates MIC values from ETEST® (bioMérieux, France) ranged between 2 µg/mL to 4 µg/mL as indicated in Table 4.3. Therefore, none of the *E. faecalis* isolates and *E. faecium* isolates were resistant to linezolid.

4.4.2 Isolate identification and *cfr* gene screening

The identification of 28 *E. faecalis* isolates and four *E. faecium* isolates was confirmed using M-PCR assays. The band sizes of the targeted genes for identification are shown in Figure 4.2. The *E. faecalis* isolates and *E. faecium* isolates were negative for the *cfr* gene.

4.5 Discussion

The *E. faecalis* and *E. faecium* isolates in this study were not linezolid resistant according to the ETEST® (bioMérieux, France) antimicrobial susceptibility testing results. The linezolid susceptibility results showed categorical agreement between the ETEST® (bioMérieux, France) and the VITEK® 2 automated system (bioMérieux, France) within one doubling dilution. The low MIC values found in this study align to research carried out by Hua *et al*. (2019). Only one isolate was not in agreement with an MIC value of 2 µg/mL (susceptible) from the ETEST® (bioMérieux, France) and 8 µg/mL (resistant) from the VITEK® 2 automated system (bioMérieux, France). The differences seen in the MIC values between the ETEST® (bioMérieux, France) and the VITEK® 2 automated system (bioMérieux, France) are subjective which may account for the lack of agreement observed between the two methods [20].

The most common mechanisms of linezolid resistance include 23S rRNA gene mutations and the acquisition of plasmid-mediated genes such as the *cfr* gene [5,21]. Hua *et al*. (2019) report that the *cfr* gene is responsible for higher (> 16 µg/mL) levels of linezolid resistance due to the antimicrobial target site being altered. However, the *cfr* gene was not detected in any isolates. Additionally, none of the isolates were specifically linezolid resistant (8 µg/mL) on ETEST® (bioMérieux, France). Therefore, the possibility remains that the *optr*A gene or mutations of the ribosomal proteins L3, L4 and L22 may be the contributing factors to the low MIC values that were observed, as described by Klupp *et al*. (2016) and Hua *et al*. (2019). However, the *optr*A gene and ribosomal proteins L3, L4 and L22 were outside the scope of this study, therefore further testing was suspended. Furthermore, a broader investigation of enterococcal isolates that are above an MIC value of 8 µg/mL would be better suited for future studies.

4.6 Conclusion

To our knowledge, this study is the first linezolid specific resistance investigation pertaining to enterococcal isolates circulating in Gauteng, South Africa. The scope of this study was limited to what were determined to be the most likely causes of resistance based on extensive previous research regarding linezolid resistance among enterococci [9,22-24]. The *E. faecalis* isolates and *E. faecium* isolates in this study were not linezolid resistant according to the ETEST® (bioMérieux, France) antimicrobial susceptibility testing results. The absence of the *cfr* gene among the isolates was not surprising given the low levels of resistance detected in the initial testing of the isolates. Therefore, given that the MIC values were low and that the *cfr* gene was absent, there is probability that the enterococcus isolates in this study are all wild type [8,25]. The continual observation and surveillance of linezolid resistance is vital because linezolid plays an important role in the treatment of Gram-positive infections which are becoming increasingly resistant to front line antimicrobial agents. However, *E. faecalis* and *E. faecium* remain a significant cause of infection, particularly in nosocomial infections and further studies are needed to determine the spread of linezolid resistance among clinical *E. faecalis* and *E. faecium* isolates. The application and prescription of linezolid needs to be controlled and optimised in order to preserve the use of last-line antimicrobial agents.

Ethics statement

The Research Ethics Committee, Faculty of Health Sciences, University of Pretoria approved this study (Ethics reference number: 477/2017). The study was observational and patient care was not influenced at any stage, therefore informed individual patient consent was waivered.

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Tables

Table 4.1: Primers used for the identification of enterococcal isolates

Target gene	Primer sequence (5'→3')*	Amplicon size (bp)	Reference
Enterococcus species (16S rRNA)	TCAACCGGGGAGGGT	733	19
	ATTACTAGCGATTCCGG		
E. faecalis D-alanine:D-alanine ligase (*ddl*)	TCAAGTACAGTTAGTCTTTATTAG	941	
	ACGATTCAAAGCTAACTGAATCAGT		
E. faecium D-alanine:D-alanine ligase (*ddl*)	TTGAGGCAGACCAGATTGACG	658	
	TATGACAGCGACTCCGATTCC		

* All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa)

Table 4.2: The *cfr* gene primers used for enterococcal isolates

Target gene	Primer sequence (5'→3')*	Amplicon size (bp)	Reference
Chloramphenicol-florfenicol resistance (*cfr*)	TGAAGTATAAAGCAGGTTGGGAG	746	9
	ACCATATAATTGACCACAAGCAGC		

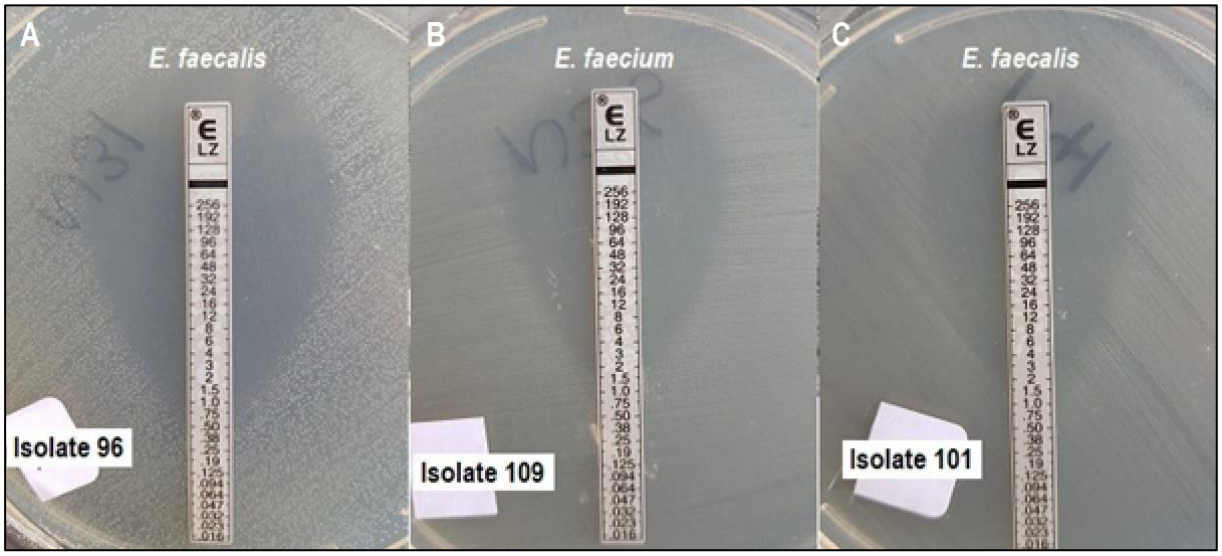
* All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa)

Table 4.3: Enterococcal linezolid antimicrobial susceptibility and *cfr* gene screening

Isolate	Specimen type	Organism identification	ETEST® MIC (µL/mL) (interpretation)		VITEK® 2 MIC (µL/mL) (interpretation)		*cfr*
80	AB	*E. faecalis*	4	I	4	I	Neg
81	UR	*E. faecalis*	4	I	4	I	Neg
82	CU	*E. faecalis*	4	I	4	I	Neg
83	ICT	*E. faecalis*	2	S	4	I	Neg
84	MU	*E. faecalis*	4	I	4	I	Neg
85	UR	*E. faecalis*	4	I	4	I	Neg
86	UR	*E. faecalis*	2	S	4	I	Neg
87	FA	*E. faecalis*	4	I	4	I	Neg
88	TI	*E. faecalis*	4	I	4	I	Neg
89	CU	*E. faecalis*	4	I	4	I	Neg
90	UR	*E. faecalis*	4	I	4	I	Neg
91	SS	*E. faecalis*	4	I	4	I	Neg
92	MU	*E. faecalis*	2	S	4	I	Neg
93	FA	*E. faecalis*	2	S	NT	NT	Neg
94	SS	*E. faecalis*	2	S	4	I	Neg
95	MU	*E. faecalis*	2	S	4	I	Neg
96	AB	*E. faecalis*	2	S	4	I	Neg
97	UR	*E. faecalis*	4	I	4	I	Neg
98	UR	*E. faecalis*	2	S	4	I	Neg
99	UR	*E. faecalis*	2	S	4	I	Neg
100	SS	*E. faecalis*	2	S	4	I	Neg
101	MU	*E. faecalis*	4	I	4	I	Neg
102	SS	*E. faecalis*	4	I	4	I	Neg
103	SS	*E. faecalis*	4	I	4	I	Neg
104	ICT	*E. faecalis*	2	S	4	I	Neg
105	UR	*E. faecalis*	2	S	4	I	Neg
106	BC	*E. faecalis*	2	S	4	I	Neg
107	BC	*E. faecalis*	4	I	4	I	Neg
108	CU	*E. faecium*	4	I	4	I	Neg
109	BC	*E. faecium*	2	S	4	I	Neg
110	FA	*E. faecium*	2	S	8	R	Neg
111	BC	*E. faecium*	2	S	4	I	Neg

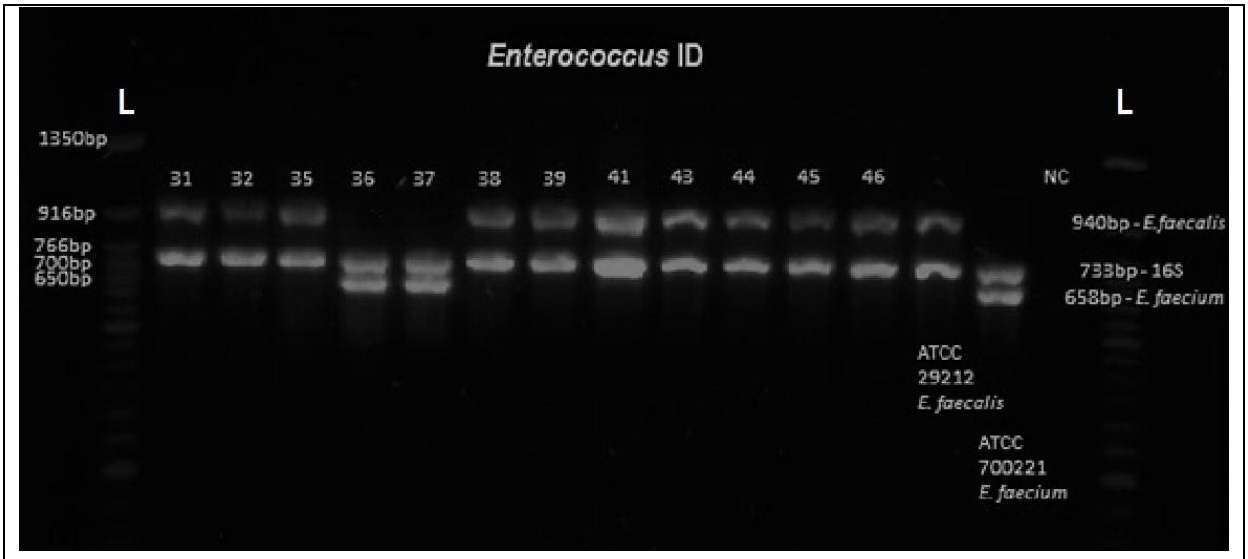
AB = abscess; BC = blood culture; *cfr* = chloramphenicol-florfenicol resistance; CU = catheter urine; FA = fluid/aspirate; I = intermediate; ICT = intravenous catheter tip; MIC = minimum inhibitory concentration; MU = midstream urine; Neg = negative; NT = not tested; R = resistant; S = susceptible; SS = swab (superficial); TI = tissue; UR = urine

Figures



A = *E. faecalis*, MIC 2 µL/mL; B = *E. faecium*, MIC 2 µL/mL; C = *E. faecalis*, MIC 4 µL/mL

Figure 4.1: Linezolid ETEST® (bioMérieux, France) susceptibility of enterococcal isolates performed on Mueller-Hinton agar (Oxoid Ltd, UK)



ATCC 29212 = *E. faecalis* positive control; ATCC 700221 = *E. faecium* positive control; L = molecular marker; NC = negative control

Figure 4.2: Multiplex PCR assay for the simultaneous identification of enterococcal isolates

CHAPTER 5

Conclusion

5.1 Concluding remarks

Antimicrobial resistance is increasing globally in spite of increased funding from the pharmaceutical industry and expanding scientific research. Antimicrobial resistance has become a major global health priority, creating a heavy burden of disease due to the challenging issues surrounding the spread and dissemination of resistance (WHO, 2014). Staphylococci and enterococci are commensal bacterium and opportunistic pathogens responsible for important antimicrobial resistant infections such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE). Linezolid remains one of the critical antimicrobial agents in providing effective treatment against multidrug-resistant (MDR) infections. The identification of linezolid antimicrobial resistance mechanisms is essential to implement successful control and prevention strategies. However, information on these mechanisms has not been available for South Africa.

There is great concern regarding antimicrobial resistance in staphylococci and enterococci in regard to linezolid resistance (Pfaller *et al.*, 2017). To our knowledge, this study presents the first insight into the mechanisms involved in linezolid resistance within one geographical area in South Africa. In order to remain within the scope and budget of this study, two specific targets were identified for investigation, namely: 23S ribosomal ribonucleic acid (rRNA) gene mutations and chloramphenicol-florfenicol resistance (*cfr*) gene acquisition. These two targets represent the most common linezolid resistance mechanisms reported in existing literature and are thus the best starting point for the study of linezolid resistance in Gauteng, South Africa.

Phenotypic antimicrobial susceptibility testing, molecular typing and whole-genome sequencing (WGS) were deemed to be the most suitable methods for this study in terms of cost, speed, ease of use, interpretation and standardisation. Due to a time lapse between isolate collection and this study, the isolate minimum inhibitory concentration (MIC) values were reconfirmed as part of the selection criteria screening. The use of ETEST® (bioMérieux, France) confirmed the antimicrobial susceptibility among the isolates after initial antimicrobial susceptibility testing with the VITEK® 2 automated system (bioMérieux, France). However, some isolates were not successfully recovered from storage and were not able to be tested.

The *cfr* gene poses a great threat of dissemination, enabling the easy spread of resistance, due

to it being a plasmid-mediated mechanism of resistance (Sadowy, 2018). The *cfr* gene mediates resistance among various Gram-positive bacteria and it is well tolerated due to low fitness cost (Vester, 2018). In this study, all staphylococcal and enterococcal isolates were screened for the *cfr* gene and only eight *S. epidermidis* isolates were confirmed to be positive. However, knowledge regarding the extent and involvement regarding the *cfr* gene remains limited in South Africa. Therefore, further research needs to be carried out to establish the spread and threat that the *cfr* gene poses. Genotyping methods are important to monitor the circulation of specific pathogenic strains which can carry important antimicrobial resistance genes. The pulsed-field gel electrophoresis (PFGE) analysis of the *S. capitis*, *S. epidermidis* and *S. haemolyticus* isolates showed genetic variation in each species with some isolates clustering together at the ≥ 80% similarity cut off point. Three *S. capitis* isolates, three *S. epidermidis* isolates and three *S. haemolyticus* isolates were untypable using PFGE following extensive attempts to recover the isolates from storage.

The characterisation of the staphylococcal isolates showed that 23S rRNA gene mutations occurred in all isolates that were sent for WGS. The known and previously reported mutations among the *S. epidermidis* isolates included C2190T, C2561T and G2603T. However, the *S. capitis* isolates harboured unknown and unreported 23S rRNA mutations, such as T2157A, T2346C, C2287G, A2295G, A2296G, C2302G, A2305G, C2308G and A2314C. One *S. capitis* isolate was observed to have all of these novel mutations and showed an increased MIC value. This finding warrants further investigation to determine the extent of 23S rRNA mutations on linezolid resistance.

All staphylococcal isolates were confirmed resistant to linezolid, suggesting that these isolates all carried at least one mutation which was confirmed with the detection of 23S rRNA gene mutations among the isolates which underwent WGS. Seven *S. epidermidis* isolates were sequenced using WGS. Three sequence types (STs) were found *in silico*, namely: ST23, ST22 and ST2. The isolates from ST23 and ST2 are almost solely nosocomial and meaningfully augmented for antimicrobial resistance and biofilm creation, indicating hospital adaptation (Lee *et al.*, 2018). The ST22 isolate is a pandemic ST which has previously been shown to exhibit dependence on linezolid, providing selective advantage under linezolid pressure (Karavasilis *et al.*, 2015). The STs confirmed in this study are of significant clinical relevance and represent a worrying reflection of the STs circulating in hospitals in Gauteng, South Africa.

The Zyvox® Annual Appraisal of Potency and Spectrum (ZAAPS) and the Linezolid

Experience and Accurate Determination of Resistance (LEADER) surveillance programs provide critical clinical insights on the spread and mechanisms of linezolid resistance for much of the world but exclude South Africa, which has no participating laboratories in these surveillance programs. The information provided by ZAAPS and LEADER has little practical use in its applicability in South Africa as there has been no corroborating research, therefore the results from this study attempt to begin to bridge the information gap. The monitoring of linezolid susceptibility is essential for the review and advancement of antimicrobial guidelines. Although the lack of data on linezolid tolerability or efficacy is a core limitation in this study, the results obtained will serve as a source for future comparison with other studies over time relating to linezolid resistance. As additional limitations, it cannot be excluded that these findings might have been biased owing to possibly unreported clinical conditions and mortality rates. The threat of antimicrobial resistance remains a grave concern and the impacts of the various resistance determinants are difficult to predict.

5.2 Future research

The findings of this study serve as a good point of reference for future studies directed towards antimicrobial stewardship and the continuous surveillance of linezolid resistance. A better understanding of linezolid resistant epidemiology is required in South Africa to ensure effective infection control procedures are in place and appropriate antimicrobial regimens for linezolid resistant infections. Novel 23S rRNA gene mutations were observed in this study, requiring further investigation. Based on this information, efficient infection control procedures and appropriate antimicrobial regimens should be maintained. The education of healthcare providers and public awareness of hygiene is essential to control the spread of infection. Attention should be paid to hand hygiene compliance to keep healthcare workers and patients safe from preventable harm. Future research should be directed towards: (i) detection of other linezolid resistance mechanisms such as the L3, L4, L22 mutations and the *optr*A gene and *poxt*A gene, (ii) infection control measures between healthcare workers, (iii) antimicrobial stewardship of linezolid and (iv) the continuous surveillance of antimicrobial resistance patterns. Staphylococci and enterococci remain important pathogens and continual research is required to monitor the genetic evolution of resistance and to obtain insights into the various interactions between the host and the pathogen. Further research is required to fully understand the magnitude of linezolid resistance among public and private hospitals in South Africa.

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ANNEXURE A

Buffers, gels, enzymes and reagents used in experimental procedures

1. Brain Heart Infusion (BHI) broth (500 mL)

BHI powder (Merck, Germany)	18.5	g*
Distilled water (dH_2O)	300	mL

*500 mL ÷ 1000 × 37 = 18.5 g in 500 mL

Dissolve 18.5 g of BHI broth (Merck, Germany) powder in 300 mL of dH_2O and bring the volume to 500 mL. Sterilise the solution by autoclaving (121°C for 15 min at 15 Barr). Allow to cool and store at 4°C.

2. 50% Glycerol (1:1) solution (500 mL)

Glycerol (Merck, Germany)	250	mL
dH_2O	150	mL

Add 250 mL of glycerol (Merck, Germany) to 150 mL of dH_2O and bring the volume to 500 mL. Sterilise by autoclaving (121°C for 15 min at 15 Barr).

3. Stock solution of Ethylenediaminetetraacetic acid (EDTA) (0.5 M; pH 8.0) (1 L)

0.5 M of EDTA (Sigma-Aldrich, USA)	186.1	g*
Sodium hydroxide (NaOH) pellets (Merck, Germany)	20	g
dH_2O	800	mL

* 0.5 M × 1 L × 372.24 g/mol = 186.1 g

Weigh and measure the components and add to 800 mL dH_2O. Bring the volume to 1 000 mL, adding pellets of NaOH to adjust the pH to 8.0. Sterilise by autoclaving (121°C for 15 min at 15 Barr).

4. Stock solution of Tris-Boric EDTA (TBE) buffer (5X; pH 8.3) (1 L)

0.45 M Trizma base (Sigma-Aldrich, USA)	54	g
0.44 M Boric acid (Sigma-Aldrich, USA)	27.5	g
0.5 M EDTA (Sigma-Aldrich, USA) (pH 8.0)	20	mL
dH_2O	800	mL

Weigh and measure the components and add to 800 mL dH_2O. Bring the volume to 1 000 mL, adjusting the pH to 8.3. Sterilise by autoclaving (121°C for 15 min at 15 Barr).

5. Working solution of Tris-Boric EDTA (TBE) buffer (1X; pH 8.0) (1 L)

5X TBE (Sigma-Aldrich, USA) buffer (pH 8.3)	200	mL*
dH_2O	800	mL

* (1X × 1 000 mL) ÷ 5X = 200 mL

Measure the components and add to 800 mL dH_2O. Bring the volume to 1 000 mL. Store at room temperature (± 25°C).

6. SeaKem® LE agarose gel: conventional gel electrophoresis (2%) (100 mL)

SeaKem® low electroendosmosis (LE) agarose (Lonza, USA)	2.0	g
1X TBE (Sigma-Aldrich, USA) buffer (pH 8.0)	100	mL
Ethidium bromide (10 mg/mL) (Sigma-Aldrich, USA)	5	µL

Weigh the agarose (Lonza, USA) gel powder and add to 100 mL of 1X TBE (Sigma-Aldrich, USA) buffer. Heat the solution until the agarose (Lonza, USA) powder is completely dissolved. Prior to casting the gel, add 5 µL of Ethidium bromide (Sigma-Aldrich, USA) to allow UV-light visualisation. Cast the gel in an assembled casting tray (BioRad, UK) with the appropriate comb (BioRad, UK) and allow to set for approximately one hour.

7. Stock solution of Tris-Hydrochloric acid (HCl) (1.0 M; pH 8.0) (500 mL)

Trizma base (Sigma-Aldrich, USA)	60.57	g
Hydrochloric acid (HCl) (Merck, Germany)	21	mL
Ultrapure water	300	mL

Dissolve 60.57 g of Tris (Amresco, USA) in 300 mL of ultrapure water, add the HCl and mix by swirling. Adjust the pH to 8.0 with HCl. Bring the volume to 500 mL. Sterilise by autoclaving (121°C for 15 min at 15 Barr).

8. Sodium acetate (NaOAc) (pH 4.5) (10 mL)

20 mM Sodium acetate (Merck, Germany)	164	mg
Ultrapure water	6	mL

Weigh NaOAc out and dissolve in 6 mL of ultrapure water. Adjust the pH to 4.5 using glacial acetic acid (Merck, Germany) and bring the volume to 10 mL. Sterilise by autoclaving (121°C for 15 min at 15 Barr).

9. Tris EDTA (TE) buffer (10 mM Tris: 1 mM EDTA) (pH 8.0) (1 000 mL)

1.0 M Tris (Amresco, USA) (pH 8.0)	10	mL

0.5 M EDTA (Sigma-Aldrich, USA) (pH 8.0)	2	mL
Ultrapure water	800	mL

Measure the components and add to 800 mL ultrapure water. Bring the volume to 1 000 mL. Sterilise by autoclaving (121°C for 15 min at 15 Barr). Use in the pulsed-field gel electrophoresis (PFGE) plug preparation of Gram-positive bacteria, the washing and storage of all plugs and acts as the casting agarose (Lonza, USA) solvent during plug preparation.

10. Cell lysis buffer (50 mM Tris: 50 mM EDTA: 1% sodium sarcosine) (500 mL)

1.0 M Tris (Amresco, USA) (pH 8.0)	25	mL
0.5 M EDTA (Sigma-Aldrich, USA) (pH 8.0)	50	mL
N-lauroylsarcosine sodium salt (Sigma-Aldrich, USA)	5	g
Sterile ultrapure water	300	mL

Add 25 mL of 1 M Tris (Amresco, USA) and 50 mL of EDTA (Sigma-Aldrich, USA) to 300 mL of sterile ultrapure water. Dissolve 5 g of sarcosine in the solution by warming it to 50°C for 30 min. The solution should not be autoclaved and can be stored for up to a year.

11. Working solution of 0.25X TBE buffer (pH 8.0) (2 730 mL)

5X TBE (Sigma-Aldrich, USA) buffer	136.5 mL
Ultrapure water	2 500 mL

Measure the components and add to the ultrapure water. Bring to a total volume of 2 730 mL. Use 2 400 mL for the PFGE running buffer, 325 mL in the PFGE agarose (Lonza, USA) gel, 5 mL during the restriction enzyme digest inactivation and 25 mL in the sealing of the plug slices.

12. SeaKem® LE agarose gel: pulsed-field gel electrophoresis (1.2%) (325 mL)

SeaKem® low electroendosmosis (LE) agarose (Lonza, USA)	3.9	g
0.25X TBE (Sigma-Aldrich, USA) buffer (pH 8.0)	325	mL

Weigh the agarose (Lonza, USA) gel powder and add to 325 mL of 0.25X TBE (Sigma-Aldrich, USA) buffer. Heat the solution until the agarose (Lonza, USA) powder is completely dissolved. No ethidium bromide (Sigma-Aldrich, USA) is added to the molten agarose (Lonza, USA) for PFGE. Cast the gel in an assembled casting tray (Biometra, Germany) with the appropriate comb (Biometra, Germany) and allow to set for approximately one hour.

13. Ethidium bromide (EtBr) solution (0.25 µg/mL) (1 000 mL)

Ethidium bromide solution (10 mg/mL) (Sigma-Aldrich, USA)	250	µL
Sterile ultrapure water	1 000	mL

Add 250 µL of ethidium bromide (Sigma-Aldrich, USA) to 1 000 mL of sterile ultrapure water and mix by gently inverting the closed container. Cover the container with aluminium foil to limit light exposure and prevent photobleaching. This solution can be re-used up to 10 times.

14. Proteinase K (20 mg/mL) (12.5 mL)

Proteinase K (Roche Applied Science, Germany)	250	mg
Nuclease-free water (QIAGEN, Germany)	12.5	mL

Add 12.5 mL of nuclease-free water (QIAGEN, Netherlands) to the pre-packaged 250 mg Proteinase K (Roche Applied Science, Germany). Allow it to dissolve completely. Divide the stock solution into small aliquots (500 µL) and store at -20°C. Only thaw the number of aliquots required and discard any unused Proteinase K (Roche Applied Science, Germany). Do not allow more than one freeze-thaw cycle after the initial dissolving. Require 45 µL per isolate per PFGE run.

15. Lysozyme (20 mg/mL) (5 mL)

Lysozyme (Sigma-Aldrich, USA)	100	mg
TE buffer (pH 8)	4	mL

Dissolve 100 mg of lysozyme (Sigma-Aldrich, USA) in 4 mL of TE buffer. Bring to a total volume of 5 mL. Swirl the solution to mix it. Divide the stock solution into small aliquots (100 µL) and store at -20°C. Only thaw the number of aliquots required and discard any unused lysozyme (Sigma-Aldrich, USA). Do not allow more than one freeze-thaw cycle after the initial dissolving. Require 20 µL per isolate per PFGE run.

16. Lysostaphin (1 mg/mL) (5 mL)

Lysostaphin (Sigma-Aldrich, USA)	5	mg
20 mM Sodium acetate (NaOAc) (pH 4.5)	5	mL

Add 5 mL of NaOAc to the pre-packaged 5 mg lysostaphin (Sigma-Aldrich, USA). Allow it to dissolve completely. Divide the stock solution into small aliquots (50 µL) and store at -20°C. Only thaw the number of phials required and discard any unused lysostaphin (Sigma-Aldrich, USA). Do not allow more than one freeze-thaw cycle after the initial dissolving. Require 5 µL per isolate per PFGE run.

17. *Sma*I restriction enzyme digestion per plug slice (50 U/plug slice)

Nuclease-free water (QIAGEN, Netherlands)	173	µL
10x CutSmart™ buffer (ThermoScientific, USA)	22	µL
10 U/µL *Sma*I (New England Biolabs, UK)	5	µL
Incubation temperature	37°C	
Incubation time	2 h	

Prepare a restriction enzyme master mixture for the number of plug slices to be digested. For a single plug slice in the following order, add 173 µL of nuclease-free water (QIAGEN, Netherlands), followed by 22 µL of 10x CutSmart™ buffer and 5 µL of the *Sma*I restriction enzyme (New England Biolabs, UK), to prevent adherence of the enzyme to the microcentrifuge tube (Scientific Specialities Inc., USA). If more than one plug slice is to be digested, use 200 µL of restriction enzyme master mixture per plug slice.

18. The American Type Culture Collection (ATCC) control strains

Staphylococcus aureus ATCC 12600
Staphylococcus aureus ATCC 29213
Staphylococcus capitis ATCC 35661
Staphylococcus epidermidis ATCC 12228
Staphylococcus haemolyticus ATCC 29970
Enterococcus faecium ATCC 700221
Enterococcus faecalis ATCC 29212

ANNEXURE B

Detailed description of experimental procedures

1. Storage of linezolid resistant staphylococcal and enterococcal isolates

a) Staphylococcal and enterococcal isolates collected from the NHLS/TAD and Ampath Laboratories were streaked onto blood agar (Oxoid Ltd, UK) plates and incubated (Vacutec, South Africa) at 37°C for 24 hours.

b) After incubation the blood agar (Oxoid Ltd, UK) plates were visually examined to check for contamination and confirm morphology, followed by Gram-staining to further ensure culture purity.

c) A pure colony from each isolate culture was inoculated into BHI broth (Merck, Germany) and incubated (Stuart, UK) with constant shaking at 220 rpm overnight at 37°C with a negative control to ensure contamination had not taken place.

d) Following the incubation period, the negative control was visually confirmed as negative.

e) The BHI broth (Merck, Germany) cultures were prepared for freeze storage by adding 900 µL of the BHI broth (Merck, Germany) culture to 900 µL of 50% sterile glycerol (Merck, Germany) in 2 mL sterile cryotubes (Greiner Bio-One, Germany).

f) The cryotubes (Greiner Bio-One, Germany) were stored overnight at -20°C and moved the next day to -80°C for storage until future analysis.

2. ETEST® (bioMérieux, France) of linezolid resistant staphylococcal and enterococcal isolates

a) The stored staphylococcal and enterococcal isolates were streaked onto blood agar (Oxoid Ltd, UK) plates and incubated (Vacutec, South Africa) at 37°C for 24 hours.

b) Following incubation, a single colony was picked up with a sterile cotton swab (Davies Diagnostics, South Africa) and a cell suspension was made in 1 mL sterile saline.

c) The bacterial cell suspension of each isolate was adjusted to match the 0.5 McFarland standard using a Densichek (bioMérieux, France). If the bacterial cell suspension was too dense, the suspension was diluted by adding sterile saline, until comparable with the 0.5 McFarland standard. If the bacterial cell suspension was too dilute, additional colonies were picked up and added until comparable with the 0.5 McFarland standard.

d) The cell suspension was inoculated onto Mueller-Hinton agar (Oxoid Ltd, UK) within 15 min of preparing the adjusted inoculum and the linezolid ETEST® (bioMérieux,

France) strip was placed aseptically onto the Mueller-Hinton agar (Oxoid Ltd, UK) using sterile forceps.

e) The inoculated Mueller-Hinton agar (Oxoid Ltd, UK) plates were incubated (Vacutec, South Africa) at 37°C for 24 hours.

f) Staphylococcal and enterococcal control strains were included: *S. aureus* American Type Culture Collection (ATCC) 29213 and *E. faecalis* ATCC 29212, respectively.

g) Following incubation, the MIC value was read from the scale where the ellipse edge intersects the ETEST® (bioMérieux, France) strip at complete inhibition of all growth. The linezolid MIC values were interpreted as susceptible, intermediate or resistant in accordance to the 2019 CLSI breakpoint tables (CLSI, 2019).

3. Total DNA extraction of linezolid resistant staphylococcal and enterococcal isolates (ZymoResearch, 2019)

a) Freeze stored staphylococcal and enterococcal isolates were thawed on ice and aseptically streaked onto blood agar (Oxoid Ltd, UK) plates for incubation (Vacutec, South Africa) at 37°C for 24 hours.

b) After incubation, one colony from each isolate was inoculated into 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA) containing sterile BHI broth (Merck, Germany) for further incubation (Stuart, UK) with constant shaking at 220 rpm overnight at 37°C. A negative control was included to ensure contamination had not taken place.

c) Following shaking incubation, the negative control was examined to ensure that contamination was not present and the microcentrifuge tubes (Scientific Specialities Inc., USA) containing the cultures were centrifuged (Labnet, USA) for 5 min at 5 000 *x g*.

d) The ZymoResearch (ZR) *Quick*-DNA™ Fungal/Bacterial Miniprep Kit was prepared for use (ZymoResearch, 2019). A volume of 500 µL of β-mercaptoethanol (Merck, Germany) was added to the 100 mL Genomic Lysis Buffer for a final dilution of 0.5 %.

e) The supernatant was discarded and the pellet resuspended in 200 µL of phosphate buffered saline (PBS) (Gibco™, NZ).

f) The 200 µL PBS (Gibco™, NZ) containing the resuspended pellet was transferred to a ZR BashingBead™ Lysis Tube.

g) A volume of 750 µL of BashingBead™ Buffer was added to the ZR BashingBead™ Lysis Tube containing the resuspended pellet.

h) The ZR BashingBead™ Lysis Tube was secured in a Disruptor Genie (Scientific Industries, USA) and processed at maximum speed for 5 min.

i) The ZR BashingBead™ Lysis Tube was centrifuged (Labnet, USA) at 10 000 *x g* for 1 min.

j) A Zymo-Spin™ III-F Filter was placed in a Collection Tube and 400 µL of the supernatant from the ZR BashingBead™ Lysis Tube was transferred and centrifuged (Labnet, USA) at 8 000 *x g* for 1 min.

k) A total of 1 200 µL of Genomic Lysis Buffer with β-mercaptoethanol (Merck, Germany) was added to the filtrate in the Collection Tube from Step J.

l) A Zymo-Spin™ IICR Column was placed in a Collection Tube and 800 µL of the mixture from step K was transferred and centrifuged (Labnet, USA) at 10 000 *x g* for 1 min. The flow through in the Collection Tube was discarded and this step was repeated.

m) A volume of 200 µL of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuged (Labnet, USA) at 10 000 *x g* for 1 min.

n) A volume of 500 µL DNA Wash Buffer was added to the Zymo-Spin™ IICR Column and centrifuged (Labnet, USA) at 10 000 *x g* for 1 min.

o) The Zymo-Spin™ IICR Column was transferred to a sterile 2 mL microcentrifuge tube (Scientific Specialities Inc., USA) and 100 µL of DNA Elution Buffer was directly added to the column matrix and centrifuged (Labnet, USA) at 10 000 *x g* for 30 sec to elute the pure DNA.

p) The eluted ultrapure DNA was quantified using a NanoPhotometer (Implen, Germany).

q) The ultrapure DNA was stored at -20°C for all downstream PCR applications.

4. Multiplex-PCR assays to identify staphylococcal and enterococcal isolates

a) Staphylococcal isolates, *S. capitis*, *S. epidermidis* and *S. haemolyticus*, were identified using previously described primers (Al-Talib *et al.*, 2009; Hirotaki *et al.*, 2011; Kim *et al.*, 2018). Enterococcal isolates, *E. faecalis* and *E. faecium*, were identified using previously described primers (Graham *et al.*, 2009).

b) The primer sequences, genes detected and the amplicon sizes for the identification assays are detailed in Annexure C.

c) The primers and MyTaq™ Red Mix (Bioline, UK) were thawed on ice and briefly spun down.

d) A primer mixture consisting of forward and reverse primers for each gene was prepared

from 20 µM primer stock solutions. The final primer concentration of each primer was 0.2 µM in a reaction volume of 15 µL.

e) Specific volumes of MyTaq™ Red Mix (Bioline, UK) and nuclease-free water (QIAGEN, Netherlands) were added to the primer mix to make up a reaction composition as detailed in Table B1.

f) A volume of 14.5 µL of the reaction composition was aliquoted into 0.2 mL PCR tubes (Lasec, South Africa), followed by adding 0.5 µL of thawed template DNA into the respective PCR tube (Lasec, South Africa).

g) The following staphylococcal ATCC strains were included as positive controls: *S. capitis* ATCC 35661, *S. epidermidis* ATCC 12228 and *S. haemolyticus* ATCC 29970. The enterococcal positive controls used were *E. faecium* ATCC 700221 and *E. faecalis* ATCC 29212. A negative control was included using nuclease-free water (QIAGEN, Netherlands) replacing template DNA to ensure cross contamination had not occurred.

h) The 0.2 mL PCR tubes (Lasec, South Africa) were briefly spun down before being placed into the thermocycler. The PCR thermocycling (BioRad, UK) conditions used for staphylococcal and enterococcal identification are detailed in Table B2 and Table B3, respectively.

Table B1: Reaction composition used for the identification of staphylococci and enterococci

Contents	Volume per reaction (µL)
MyTaq™ Red Mix (Bioline, UK)	7.5
Forward primer (0.2 µM)	0.15
Reverse primer (0.2 µM)	0.15
Nuclease-free water (QIAGEN, Netherlands)	6.7
Template DNA	0.5
Total volume	**15**

Table B2: The PCR conditions used for the identification of staphylococci

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	35
Annealing	55	3 min	
Extension	72	90 sec	
Final elongation	72	10 min	1

Table B3: The PCR conditions used for the identification of enterococci

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	10 min	1
Denaturation	94	30 sec	35
Annealing	56	30 sec	
Extension	74	30 sec	
Final elongation	72	7 min	1

5. Detection of the *cfr* gene in staphylococcal and enterococcal isolates

a) A PCR assay using previously described primers was used to determine the presence of the *cfr* gene (Doern *et al.*, 2016). The primer sequences, details of the *cfr* gene detected and the amplicon sizes for the *cfr* gene are detailed in Annexure C.

b) The forward and reverse *cfr* primers from 20 µM stock solutions and MyTaq™ Red Mix (Bioline, UK) were thawed on ice and briefly spun down.

c) A reaction mixture consisting of forward and reverse *cfr* primers, nuclease-free water (QIAGEN, Netherlands) and MyTaq™ Red Mix (Bioline, UK) was calculated and mixed per Table B4.

d) A volume of 14 µL of the reaction composition was aliquoted into 0.2 mL PCR tubes (Lasec, South Africa), followed by adding 1 µL of thawed template DNA into the respective PCR tube (Lasec, South Africa).

e) A negative control was included using nuclease-free water (QIAGEN, Netherlands) replacing template DNA to ensure cross contamination had not occurred.

f) The 0.2 mL PCR tubes (Lasec, South Africa) were briefly spun down before being placed into the thermocycler. The PCR thermocycling (BioRad, UK) conditions used for the detection of the *cfr* gene are detailed in Table B5.

Table B4: Reaction composition used for the detection of the *cfr* gene in staphylococcal and enterococcal isolates

Contents	Volume per reaction (µL)
MyTaq™ Red Mix (Bioline, UK)	7.5
Forward primer (0.2 µM)	0.15
Reverse primer (0.2 µM)	0.15
Nuclease-free water (QIAGEN, Netherlands)	6.2
Template DNA	1.0
Total volume	**15**

Table B5: The PCR conditions used for the detection of the *cfr* gene

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	10 min	1
Denaturation	94	30 sec	35
Annealing	57	30 sec	
Extension	74	30 sec	
Final elongation	72	10 min	1

6. Analysis of the identification M-PCR products and the *cfr* gene detection PCR products by conventional gel electrophoresis of staphylococcal and enterococcal isolates

a) The DNA amplicons of all staphylococcal and enterococcal identification M-PCR assays and *cfr* gene PCR assays were detected using conventional gel electrophoresis on a 2% agarose (Lonza, USA) gel stained with 5 µL of ethidium bromide (10 µg/mL) (Sigma-Aldrich, USA) during casting of the gel.

b) The agarose (Lonza, USA) gels were run in a 1x TBE (Sigma-Aldrich, USA) buffer at 100 V for 100 min.

c) A 50 bp DNA ladder (ThermoScientific, USA) was included as a molecular weight marker to determine the sizes of the amplicons.

d) The amplicons within the agarose (Lonza, USA) gel were visualised under UV light using the Gel Doc EZ System (BioRad, UK) and the bands were compared to the 50 bp DNA ladder (ThermoScientific, USA).

7. Pulsed-field gel electrophoresis of linezolid resistant staphylococcal and enterococcal isolates (CDC, 2019)

Day 1: Culturing and buffer preparation

a) Stored linezolid resistant staphylococcal isolates and reference marker *S. aureus* ATCC 12600 were spread onto blood agar (Oxoid Ltd, UK) for confluent growth and incubated (Vacutec, South Africa) at 37°C for 18 hours.

b) All buffers and enzymes required for the PFGE run were prepared.

c) All microcentrifuge tubes (Scientific Specialities Inc., USA) and conical tubes (Greiner Bio-One, Germany) required for the PFGE run were labelled with the respective isolate numbers.

Day 2: Casting and lysis of plugs

a) A 1.2% SeaKem LE agarose (Lonza, USA) solution was prepared for the PFGE plugs

by dissolving 0.30 g of SeaKem LE agarose (Lonza, USA) in 25 mL of TE buffer.

b) The agarose (Lonza, USA) solution was kept at 54°C in a hybridisation oven to prevent solidification prior to casting.

c) The incubated blood agar (Oxoid Ltd, UK) plates were visually examined for contamination.

d) A volume of 1 000 µL of TE buffer was added to a 15 mL conical tube (Greiner Bio-One, Germany) for each isolate.

e) A sterile cotton swab (Davies Diagnostics, South Africa) was moistened in TE buffer and used to gently sweep bacteria from the respective incubated plate.

f) The bacterial cells were resuspended in the respective conical tubes (Greiner Bio-One, Germany) by gently spinning the sterile cotton swab (Davies Diagnostics, South Africa) in the TE buffer. The bacterial cell suspensions were kept on ice until the optical density could be measured.

g) A total of 200 µL of each cell suspension was added to a microtitre plate (ThermoScientific, USA) to measure the absorbance. A blank was prepared by adding 200 µL of uninoculated TE buffer to an empty well.

h) The ELx800 Absorbance Microplate Reader (BioTek Instruments, USA) was calibrated according to the manufacturer instructions.

i) The absorbance was measured at 630 nm by placing the microtitre plate (ThermoScientific, USA) into the ELx800 Absorbance Microplate Reader (BioTek Instruments, USA).

j) The optical density was established at 1.2 to 1.8 (after subtracting the blank reading from the measured reading of the isolate) for the isolates.

k) If the optical density of the isolate was too high, additional TE buffer was added to decrease the concentration of the bacterial cells in suspension. If the optical density of the isolate was too low, additional bacterial cells were picked up with a sterile cotton swab (Davies Diagnostics, South Africa) and added to the cell suspension to increase the concentration of the bacterial cells.

l) A total of 400 µL of the adjusted cell suspensions was transferred to sterile 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA).

m) A volume of 20 µL of thawed lysozyme (Sigma-Aldrich, USA) stock solution (20 mg/mL) was added to each microcentrifuge tube (Scientific Specialities Inc., USA) and incubated (Stuart, UK) at 56°C for 20 min. Any unused thawed lysozyme (Sigma-Aldrich, USA) solution was discarded.

n) After incubation, 20 µL of thawed proteinase K (20 mg/mL) (Roche Applied Science, Germany) and 5 µL of thawed lysostaphin (1 mg/mL) (Sigma-Aldrich, USA) were added, followed by gentle mixing with the pipette. Any unused thawed proteinase K (Roche Applied Science, Germany) and lysostaphin (Sigma-Aldrich, USA) were discarded.

o) A volume of 400 µL of the 1.2% melted agarose (Lonza, USA) was transferred to the cell suspension and gently mixed by pipetting up and down. The cell suspension agarose (Lonza, USA) mixture was immediately dispensed into the appropriate plug well mold.

p) After casting, the plugs were allowed to solidify in the mold at 4°C for 10 min.

q) While the plugs were solidifying, a cell lysis master mix was prepared. Proteinase K (Roche Applied Science, Germany) (25 µL per isolate) and cell lysis buffer (5 mL per isolate) were mixed in an appropriately sized flask and 5 mL of the mix was decanted into a set of fresh conical tubes (Greiner Bio-One, Germany). Any unused thawed Proteinase K (Roche Applied Science, Germany) was discarded.

r) The solidified plugs were prepared for lysis. Excess agarose (Lonza, USA) at the top of the plug mold was trimmed away with a scalpel. Each plug was gently pushed out of the plug mold directly into the conical tubes (Greiner Bio-One, Germany) containing the cell lysis master mix, ensuring the plug was fully immersed.

s) The plugs were incubated (Stuart, UK) at 51°C with constant shaking at 170 rpm for 18 hours.

Day 3: Washing and storage of plugs

a) A fresh set of conical tubes (Greiner Bio-One, Germany) was prepared and approximately 10 mL of preheated ultrapure water was transferred into each of the conical tubes (Greiner Bio-One, Germany).

b) The conical tubes (Greiner Bio-One, Germany) containing the plugs were removed from the shaking incubator (Stuart, UK) and the lysis buffer was poured off. Great care was taken not to discard or break the plugs while removing the lysis buffer.

c) The plugs were transferred to the conical tubes (Greiner Bio-One, Germany) containing preheated ultrapure water and incubated (Stuart, UK) at 51°C while shaking at 170 rpm for 15 min.

d) After incubation, the ultrapure water was poured off and 10 mL of fresh preheated ultrapure water was added and incubated (Stuart, UK) at 51°C while shaking at 170 rpm for 15 min.

e) Following incubation, the ultrapure water was poured off and 10 mL of preheated TE buffer was added, followed by further incubation (Stuart, UK) at 51°C while shaking at 170 rpm for 15 min. The TE buffer washes were repeated four times.

f) After the TE washes, the plugs were stored at 4°C in 1 500 µL of TE buffer in microcentrifuge tubes (Scientific Specialities Inc., USA).

Day 4: Restriction enzyme digestion (Part 1)

a) Only 15 isolates, accompanied (1 plug slice per isolate per run) by the appropriate reference size standard (three plug slices per run), were digested at a time for a single PFGE run because there were only 18 wells available on the PFGE comb (Biometra, Germany).

b) A fresh set of 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA) was prepared and labelled.

c) A restriction enzyme mix was prepared for the pre-restriction incubation step consisting of CutSmart™ restriction buffer (20 µL per plug slice) and nuclease-free water (QIAGEN, Netherlands) (180 µL per plug slice) to make a total of 200 µL per plug.

d) A total of 200 µL of the pre-restriction mix was aliquoted into the fresh set of 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA) with three extra 2 mL microcentrifuge tubes (Scientific Specialities Inc., USA) for the appropriate reference size standard. This mixture was kept on ice until the plug slices could be added.

e) The plugs were cut into 2 mm slices with a scalpel on a microscope slide and transferred to the respective microcentrifuge tube (Scientific Specialities Inc., USA) prepared previously.

f) Three plugs were cut for the appropriate reference size standard and a single plug was cut for each of the isolates. The remaining part of the plug was replaced into the TE buffer and stored at 4°C. The plug slices were incubated in a digital dry bath (Labnet, USA) at 37°C for 10 min.

g) While the plug slices were incubating, a new restriction enzyme master mix (to include the enzyme) was prepared in a conical tube (Greiner Bio-One, Germany) and kept on ice until used. The new restriction enzyme master mix consisted of nuclease-free water (QIAGEN, Netherlands) (173 µL per plug slice), CutSmart™ restriction buffer (22 µL per plug slice) and *Sma*I restriction enzyme (New England Biolabs, UK) (5 µL per plug slice).

h) After incubation, the buffer was removed from each plug slice using a pipette with care

to avoid disrupting the plug.

i) A volume of 200 µL of the restriction enzyme master mix was added to each microcentrifuge tube (Scientific Specialities Inc., USA) containing the plug slices and incubated in a digital dry bath (Labnet, USA) at 37°C for 2 hours.

Day 4: PFGE run (Part 2)

a) During the 2 hour restriction enzyme step, a volume of 2 730 mL of 0.25x TBE (Sigma-Aldrich, USA) buffer was prepared and divided into 325 mL for the agarose (Lonza, USA) gel (300 mL for the gel and 25 mL for sealing of the plug slices in the agarose (Lonza, USA) gel), 2 400 mL for the gel electrophoresis running buffer and 3 600 µL for inactivation of the restriction enzyme (200 µL per plug slice).

b) A 1.2% SeaKem LE agarose (Lonza, USA) gel was prepared.

c) The gel casting tray (Biometra, Germany) was placed on a level surface and the gel casting frame was secured with the four screws provided.

d) The edge of the gel casting frame was sealed with molten agarose (Lonza, USA), the 18-well gel comb (Biometra, Germany) was positioned and the gel was poured and allowed to solidify for 45 min at room temperature (± 23°C). Enough molten agarose (± 25 mL) (Lonza, USA) was kept aside to seal the plugs slices into the wells of the gel.

e) While the gel was solidifying, the cooling tank of the Rotaphor PFGE System 6.0 (Biometra, Germany) was filled with 2.7 L of ultrapure water and the electrophoresis chamber was filled with 2 400 mL of the 0.25x TBE (Sigma-Aldrich, USA) electrophoresis running buffer.

f) The cooling tank and the electrophoresis chamber was set at 5°C and 13°C, respectively. The pump was switched on to allow the electrophoresis running buffer to reach the correct temperature.

g) The comb (Biometra, Germany) was carefully removed from the solidified agarose (Lonza, USA) gel.

h) After restriction enzyme incubation, the restriction enzyme was removed from each microcentrifuge tube (Scientific Specialities Inc., USA) with care to avoid disrupting the plug slices.

i) A total of 200 µL of 0.25x TBE (Sigma-Aldrich, USA) buffer was added to each restricted plug slice to inactivate the restriction enzyme and stop further restriction.

j) After 5 min, the TBE (Sigma-Aldrich, USA) buffer was removed and the restricted plug slices were loaded into the wells of the agarose (Lonza, USA) gel. The *S. aureus* ATCC

12600 reference marker plugs were loaded into wells 1, 10 and 18. The wells were sealed with the molten agarose (Lonza, USA).

k) The casting frame was carefully removed and excess agarose (Lonza, USA) was removed using a scalpel. The wells were levelled to ensure free circulation around the gel during the PFGE run.

l) The four corner insulators were firmly mounted on the distance pillars of the gel casting tray (Biometra, Germany) and the tray assembly was lowered into the gel electrophoresis chamber (pump switched off).

m) The PFGE controller configuration parameters are detailed in Table B6.

n) After setting the parameters, the pump was switched on and the electrophoresis was started.

Table B6: Staphylococcal PFGE running parameters

Parameter	Details
Duration	25 h
Temperature	13°C
Interval	5 s linear to 40 s
Interval inverse	OFF
Angle	120° constant
Voltage	220 V linear to 200 V

Day 5: Staining and viewing the gel

a) At the end of the run, the gel was carefully removed from the casting plate and placed into a light proof container. Ethidium bromide (Sigma-Aldrich, USA) solution was poured over the gel, the lid replaced onto the container and left for 30 min to stain.

b) After 30 min, the ethidium bromide (Sigma-Aldrich, USA) solution was poured into a foil covered bottle and the gel was destained by submerging in ultrapure water for 30 min.

c) After destaining, the gel was viewed under UV light using the Gel Doc XR+ System (BioRad, UK) and subsequently discarded.

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ANNEXURE C

Primers utilised in PCR assays

Table C1: Primers used for identification of staphylococcal isolates

Target gene	Primer name*	Primer sequence (5'→3')**	Tm (°C) ***	Amplicon size (bp)	Reference
Staphylococcus species (16S rRNA)	16SRRNA-F	GCAAGCGTTATCCGGATTT	58	597	(Al-Talib *et al.*, 2009)
	16SRRNA-R	CTTAATGATGGCAACTAAGC	56		
S. capitis Superoxide dismutase (*sodA*)	CAPI-F	TCAGATATTCAAACTGCAGTACG	52	103	(Kim *et al.*, 2018)
	CAPI-R	CTACTTCACCTTTTTCTTCAGA	50		
S. epidermidis Thermonuclease (*nuc*)	EPI-F	TTGTAAACCATTCTGGACCG	58	251	(Hirotaki *et al.*, 2011)
	EPI-R	ATGCGTGAGATACTTCTTCG	58		
S. haemolyticus Thermonuclease (*nuc*)	HAE-F	TAGTGGTAGGCGTATTAGCC	60	434	
	HAE-R	ACGATATTTGCCATTCGGTG	58		

* All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa)

** A = adenine; T = thymine; C = cytosine; G = guanine

*** Tm = primer melting temperature

Table C2: Primers used for identification of enterococcal isolates

Target gene	Primer name*	Primer sequence (5'→3')**	Tm (°C) ***	Amplicon size (bp)	Reference
Enterococcus species (16S rRNA)	ENTE-F	TCAACCGGGGAGGGT	59	733	(Graham *et al.*, 2009)
	ENTE-R	ATTACTAGCGATTCCGG	55		
E. faecalis D-alanine:D-alanine ligase (*ddl*)	FAEC-F	TCAAGTACAGTTAGTCTTTATTAG	56	941	
	FAEC-R	ACGATTCAAAGCTAACTGAATCAGT	60		
E. faecium D-alanine:D-alanine ligase (*ddl*)	FACI-F	TTGAGGCAGACCAGATTGACG	63	658	
	FACI-R	TATGACAGCGACTCCGATTCC	63		

* All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa)

** A = adenine; T = thymine; C = cytosine; G = guanine

*** Tm = primer melting temperature

Table C3: *cfr* gene primers used for staphylococcal and enterococcal isolates

Target gene	Primer name*	Primer sequence (5'→3')**	Tm (°C) ***	Amplicon size (bp)	Reference
Chloramphenicol-florfenicol resistance (*cfr*)	*CFR*-F	TGAAGTATAAAGCAGGTTGGGAG	61	746	(Doern *et al.*, 2016)
	CFR-R	ACCATATAATTGACCACAAGCAGC	61		

* All primers were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa)

** A = adenine; T = thymine; C = cytosine; G = guanine

*** Tm = primer melting temperature

References

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ANNEXURE D

Captured data

Table D1: Details of *S. epidermidis* (n = 27) isolates, *S. capitis* (n = 43) isolates and *S. haemolyticus* (n = 9) isolates

Isolate	Isolate ID	Hospital ID	Specimen type	Organism identification	ETEST® MIC (µL/mL)		VITEK® 2 MIC (µL/mL)		*cfr*	WGS
1	A01	L	BC	*S. epidermidis*	24	R	16	R	Neg	
2	A03	K	BC	*S. epidermidis*	8	R	NT	NT	Neg	
3	A101	R	BC	*S. epidermidis*	8	R	8	R	Pos	ST2
4	A105	S	BC	*S. epidermidis*	8	R	8	R	Neg	
5	A106	U	BC	*S. epidermidis*	16	R	16	R	Neg	ST23
6	A11	CC	BC	*S. epidermidis*	8	R	32	R	Neg	
7	A110	U	BC	*S. epidermidis*	> 256	R	16	R	Pos	ST23
8	A112	U	BC	*S. epidermidis*	> 256	R	64	R	Neg	
9	A17	Q	BC	*S. epidermidis*	NG	NG	16	R	Neg	
10	A24	U	BC	*S. epidermidis*	16	R	64	R	Neg	
11	A29	Q	BC	*S. epidermidis*	12	R	8	R	Neg	
12	A37	B	BC	*S. epidermidis*	16	R	32	R	Neg	
13	A39	Q	CVC	*S. epidermidis*	12	R	32	R	Neg	
14	A40	Z	BC	*S. epidermidis*	8	R	> 256	R	Pos	ST2
15	A41	CC	BC	*S. epidermidis*	8	R	> 256	R	Neg	ST22
16	A46	L	BC	*S. epidermidis*	8	R	8	R	Pos	
17	A54	CC	CVC	*S. epidermidis*	32	R	> 256	R	Neg	
18	A60	U	BC	*S. epidermidis*	16	R	64	R	Pos	
19	A69	U	BC	*S. epidermidis*	128	R	8	R	Neg	
20	A73	CC	BC	*S. epidermidis*	8	R	8	R	Pos	
21	A78	CC	BC	*S. epidermidis*	24	R	16	R	Neg	
22	A79	Z	BC	*S. epidermidis*	96	R	> 256	R	Neg	ST23
23	A86	P	BC	*S. epidermidis*	128	R	32	R	Neg	
24	A88	S	BC	*S. epidermidis*	16	R	64	R	Neg	
25	A90	U	BC	*S. epidermidis*	128	R	64	R	Pos	ST23
26	A96	CC	BC	*S. epidermidis*	8	R	8	R	Pos	
27	A98	Z	BC	*S. epidermidis*	12	R	32	R	Neg	
28	A100	M	BC	*S. capitis*	32	R	16	R	Neg	
29	A104	U	BC	*S. capitis*	NG	NG	32	R	Neg	
30	A109	P	BC	*S. capitis*	16	R	16	R	Neg	
31	A111	U	BC	*S. capitis*	24	R	16	R	Neg	
32	A19	G	BC	*S. capitis*	32	R	32	R	Neg	
33	A21	J	BC	*S. capitis*	NG	NG	64	R	Neg	
34	A22	DD	BC	*S. capitis*	32	R	16	R	Neg	
35	A25	U	CVC	*S. capitis*	NG	NG	32	R	Neg	

- = sequence type unavailable (PubMLST, 2019); BC = Blood culture; *cfr* = chloramphenicol-florfenicol resistance; CVC = Central venous catheter; ID = identification; MIC = minimum inhibitory concentration; Neg = negative; NG = no growth; NT = not tested; Pos = positive; R = resistant; ST = sequence type; WGS = whole-genome sequencing

Table D1: Details of *S. epidermidis* (n = 27) isolates, *S. capitis* (n = 43) isolates and *S. haemolyticus* (n = 9) isolates (continued)

Isolate	Isolate ID	Hospital ID	Specimen type	Organism identification	ETEST® MIC (µL/mL)		VITEK® 2 MIC (µL/mL)		*cfr*	WGS
36	A26	CC	CVC	*S. capitis*	24	R	> 256	R	Neg	-
37	A28	G	BC	*S. capitis*	NG	NG	16	R	Neg	
38	A30	CC	BC	*S. capitis*	48	R	16	R	Neg	
39	A32	CC	BC	*S. capitis*	32	R	32	R	Neg	
40	A33	P	BC	*S. capitis*	32	R	> 256	R	Neg	
41	A34	U	BC	*S. capitis*	24	R	128	R	Neg	
42	A35	EE	BC	*S. capitis*	24	R	32	R	Neg	
43	A36	C	BC	*S. capitis*	32	R	64	R	Neg	
44	A38	C	BC	*S. capitis*	32	R	128	R	Neg	
45	A42	CC	BC	*S. capitis*	NG	NG	64	R	Neg	
46	A44	Z	BC	*S. capitis*	32	R	8	R	Neg	
47	A45	D	BC	*S. capitis*	NG	NG	8	R	Neg	
48	A48	C	BC	*S. capitis*	24	R	16	R	Neg	
49	A53	S	BC	*S. capitis*	24	R	16	R	Neg	
50	A55	J	BC	*S. capitis*	48	R	8	R	Neg	
51	A57	F	BC	*S. capitis*	24	R	48	R	Neg	
52	A58	B	BC	*S. capitis*	128	R	32	R	Neg	-
53	A59	W	BC	*S. capitis*	24	R	16	R	Neg	
54	A61	G	BC	*S. capitis*	16	R	16	R	Neg	
55	A65	U	BC	*S. capitis*	24	R	32	R	Neg	
56	A66	CC	BC	*S. capitis*	24	R	32	R	Neg	
57	A67	W	BC	*S. capitis*	32	R	32	R	Neg	
58	A68	O	BC	*S. capitis*	24	R	64	R	Neg	
59	A70	AA	BC	*S. capitis*	24	R	64	R	Neg	-
60	A71	Q	BC	*S. capitis*	16	R	64	R	Neg	
61	A75	S	BC	*S. capitis*	24	R	32	R	Neg	
62	A77	S	BC	*S. capitis*	16	R	128	R	Neg	
63	A80	E	BC	*S. capitis*	12	R	64	R	Neg	
64	A81	T	BC	*S. capitis*	16	R	16	R	Neg	
65	A82	H	BC	*S. capitis*	64	R	16	R	Neg	
66	A83	S	BC	*S. capitis*	16	R	32	R	Neg	
67	A89	CC	BC	*S. capitis*	32	R	8	R	Neg	
68	A92	P	BC	*S. capitis*	24	R	16	R	Neg	
69	A94	P	BC	*S. capitis*	48	R	16	R	Neg	
70	A99	J	BC	*S. capitis*	32	R	16	R	Neg	
71	A04	G	BC	*S. haemolyticus*	NG	NG	8	R	Neg	
72	A103	DD	BC	*S. haemolyticus*	32	R	64	R	Neg	
73	A107	CC	BC	*S. haemolyticus*	32	R	16	R	Neg	
74	A20	CC	BC	*S. haemolyticus*	NG	NG	16	R	Neg	

- = sequence type unavailable (PubMLST, 2019); BC = Blood culture; *cfr* = chloramphenicol-florfenicol resistance; CVC = Central venous catheter; ID = identification; MIC = minimum inhibitory concentration; Neg = negative; NG = no growth; NT = not tested; Pos = positive; R = resistant; ST = sequence type; WGS = whole-genome sequencing

Table D1: Details of *S. epidermidis* (n = 27) isolates, *S. capitis* (n = 43) isolates and *S. haemolyticus* (n = 9) isolates (continued)

Isolate	Isolate ID	Hospital ID	Specimen type	Organism identification	ETEST® MIC (µL/mL)		VITEK® 2 MIC (µL/mL)		*cfr*	WGS
75	A43	X	BC	*S. haemolyticus*	NG	NG	128	R	Neg	
76	A50	Q	BC	*S. haemolyticus*	64	R	64	R	Neg	
77	A63	CC	BC	*S. haemolyticus*	8	R	16	R	Neg	
78	A64	G	BC	*S. haemolyticus*	NG	NG	32	R	Neg	
79	A84	Q	BC	*S. haemolyticus*	16	R	128	R	Neg	

- = sequence type unavailable (PubMLST, 2019); BC = Blood culture; *cfr* = chloramphenicol-florfenicol resistance; CVC = Central venous catheter; ID = identification; MIC = minimum inhibitory concentration; Neg = negative; NG = no growth; NT = not tested; Pos = positive; R = resistant; ST = sequence type; WGS = whole-genome sequencing

Table D2: Details of *E. faecalis* (n = 28) and *E. faecium* (n = 4) isolates

Isolate	Isolate ID	Hospital ID	Specimen type	Organism identification	ETEST® MIC (µL/mL)		VITEK® 2 MIC (µL/mL)		*cfr*
80	1	Y	AB	*E. faecalis*	4	I	4	I	Neg
81	2	I	UR	*E. faecalis*	4	I	4	I	Neg
82	4	I	CU	*E. faecalis*	4	I	4	I	Neg
83	6	Y	ICT	*E. faecalis*	2	S	4	I	Neg
84	9	I	MU	*E. faecalis*	4	I	4	I	Neg
85	10	Y	UR	*E. faecalis*	4	I	4	I	Neg
86	11	I	UR	*E. faecalis*	2	S	4	I	Neg
87	14	I	FA	*E. faecalis*	4	I	4	I	Neg
88	15	I	TI	*E. faecalis*	4	I	4	I	Neg
89	16	Y	CU	*E. faecalis*	4	I	4	I	Neg
90	17	Y	UR	*E. faecalis*	4	I	4	I	Neg
91	18	Y	SS	*E. faecalis*	4	I	4	I	Neg
92	20	V	MU	*E. faecalis*	2	S	4	I	Neg
93	23	Y	FA	*E. faecalis*	2	S	NT	NT	Neg
94	25	I	SS	*E. faecalis*	2	S	4	I	Neg
95	28	N	MU	*E. faecalis*	2	S	4	I	Neg
96	31	Y	AB	*E. faecalis*	2	S	4	I	Neg
97	32	I	UR	*E. faecalis*	4	I	4	I	Neg
98	35	I	UR	*E. faecalis*	2	S	4	I	Neg
99	38	Y	UR	*E. faecalis*	2	S	4	I	Neg
100	39	Y	SS	*E. faecalis*	2	S	4	I	Neg
101	41	I	MU	*E. faecalis*	4	I	4	I	Neg
102	43	BB	SS	*E. faecalis*	4	I	4	I	Neg
103	44	I	SS	*E. faecalis*	4	I	4	I	Neg
104	45	Y	ICT	*E. faecalis*	2	S	4	I	Neg
105	46	I	UR	*E. faecalis*	2	S	4	I	Neg
106	A05	F	BC	*E. faecalis*	2	S	4	I	Neg
107	A07	A	BC	*E. faecalis*	4	I	4	I	Neg
108	13	Y	CU	*E. faecium*	4	I	4	I	Neg
109	36	Y	BC	*E. faecium*	2	S	4	I	Neg
110	37	I	FA	*E. faecium*	2	S	8	R	Neg
111	A09	C	BC	*E. faecium*	2	S	4	I	Neg

AB = abscess; BC = blood culture; *cfr* = chloramphenicol-florfenicol resistance; CU = catheter urine; FA = fluid/aspirate; I = intermediate; ICT = intravenous catheter tip; ID = identification; MIC = minimum inhibitory concentration; MU = midstream urine; Neg = negative; NT = not tested; R = resistant; S = susceptible; SS = swab (superficial); TI = tissue; UR = urine

References

PubMLST (2019) PubMLST - Public databases for molecular typing and microbial genome diversity. Databases hosted on PubMLST. Available online: https://pubmlst.org/databases [Accessed 28 October 2019].

ANNEXURE E

Research ethics approval certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

13 November 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 477/2017
Title: Antibiotic resistance mechanisms of linezolid resistant staphylococci and enterococci collected in Gauteng, South Africa

Dear Mrs KL Addison

The **Annual Renewal** as supported by documents received between 2019-10-14 and 2019-11-06 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-11-06.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-11-13.
- Please remember to use your protocol number (477/2017) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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